THE ROLE OF STAT1 IN CHLAMYDIA-INDUCED TYPE I INTERFERON RESPONSES IN OVIDUCT EPITHELIUM

Kristen L. Hosey

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Microbiology and Immunology,
Indiana University

May 2013

Accepted by the Faculty of Indiana University, in partial
fulfillment of the requirements for the degree of Doctor of Philosophy.

_____
Wilbert A. Derbigny, Ph.D., Chair

_____
Janice S. Blum, Ph.D.

Doctoral Committee

_____
Mark G. Goebl, Ph.D.

December 14, 2012

_____
Raymond M. Johnson, M.D., Ph.D.

_____
Mark H. Kaplan, Ph.D.

DEDICATION

This thesis is dedicated to my inspiration; my amazing son King A. Cole. To my parents, Reverend Terrell and Shirlene Allen, and Ricky and Lillie Jones, thank you for your unconditional love and support. To my grandmother, Braddis A. Hosey, thank you for being the glue that held us all together; without your faith, strength, and resilience our family would not be as successful as we are today. To those who inspired me early in life, but who are no longer here to witness this accomplishment, Darlene Hosey, Quitman Page, James Page, Willa M. Page, Elbert R. Page, and J.B. Page, I know that you have been with me through this journey and I know that you are smiling down on me now as I cross the finish line.

ACKNOWLEDGEMENTS

I would like to thank first and foremost my GOD, who has never left my side throughout this journey. I would like to thank my mentor, Dr. Wilbert A. Derbigny for his guidance and encouragement; not only in academics and research, but in life as well. He has always believed in my abilities even when I didn't believe in myself. I will forever be grateful for his unwavering support. I would also like to thank my research committee members: Dr. Janice Blum, Dr. Mark Kaplan, Dr. Raymond Johnson, and Dr. Mark Goebl. Thank you all for always providing great advice, guidance and suggestions on my research, and for your patience and support. The knowledge and skills I have gained from you all have helped me grow as a scientist. I would also like to thank Dr. Akira Moh for his suggestions and for providing an essential component of my research; the STAT1 knock-out mice. I would like to thank the Yang Lab, Kaplan Lab, Blum Lab, Harrington Lab, Bauer Lab, and the Van der Pool Lab (James Williams) for their technical assistance and invaluable guidance and help throughout this entire project. I would also like to thank the past and present members of the Derbingy Lab, especially Katherine Toomey, who helped me tremendously from the beginning and Dr. Sishun Hu, who has helped me tremendously towards the end.

To my peers and good friends, Dr. Joanna Walker, Dr. Nathalie Ahyi, Dr. Tabitha Hardy, Dr. Tammi Taylor, Dr. Jeremy Lott, Takeisha Farmer and Carla Mangum, thank you for your continued moral support and friendship throughout these trying times. Many of you have paved the way for me and I am forever grateful. To Donna "Cookie" Anderson and Frederick Hamilton, thank you for always lending a hand, listening ear, or a simple hug. Finally, to my family, thank you all for holding me down. Thank you for

the late night talks. Thank you for the celebrations when I would come home to visit; even though it made it harder to leave. But most of all, thank you for your prayers, love and support. To my fiancé, Adrian Cole, thank you for pushing me and loving and supporting me through it all. I thank all of you, and this would not have been possible without you all.

ABSTRACT

Kristen L. Hosey

THE ROLE OF STAT1 IN CHLAMYDIA-INDUCED TYPE I INTERFERON RESPONSES IN OVIDUCT EPITHELIUM

Progression of *Chlamydia* into upper reproductive tract epithelium and the induction of subsequent immune responses to infection are major contributors to *Chlamydia*-induced pathogenesis of the genital tract. We reported that *C. muridarum* infection of the oviduct epithelial cells (OEs) secrete IFN-β in a TLR3 dependent manner. However, we showed that the *C. muridarum* infected TLR3-deficient OEs were still able to secrete minimal amounts of IFN-β into the supernatants, which is suggestive that there are other signaling pathways that contribute to *Chlamydia*-induced IFN-β synthesis in these cells. Previous studies describing the activation of the JAK/STAT signaling pathway during *Chlamydia* infection of cervical epithelial cells proposes a putative role for STAT1 in the synthesis of type I IFNs during *Chlamydia* infection. The present study investigated the role of STAT1 in *Chlamydia*-induced IFN-β production in OEs. OEs were infected with *Chlamydia muridarum* and analyzed at 24 hours by RT-PCR and western blot to determine STAT1 expression. STAT (-/-) OEs were infected and IFN-β production measured by ELISA. Quantitative real-time PCR analyses were performed at 6 and 16 hour post-infection to elucidate the mechanisms involved in IFN-β production during infection. Fluorescent microscopy was used to observe changes in *Chlamydia* replication. STAT1 activation and expression were significantly increased in wild-type (WT) OEs upon infection. TLR3 (-/-) OEs showed diminished STAT1 protein activation and expression. Augmented STAT1 protein expression corresponded to STAT1 mRNA

levels. ELISA analyses revealed significantly less IFN-β production in infected STAT1 (-/-) OEs compared to WT OEs. Quantitative real-time PCR data showed that gene expression of IFN-β and of type I IFN signaling components were significantly increased during late stage *Chlamydia* infection, dependent on STAT1. Temporal regulation and increases in expression of IFN-α subtypes during infection were STAT1-dependent. Our results implicate STAT1-mediated signaling as a contributor to the *C. muridarum*-induced synthesis of IFN-β and other type I IFNs in OEs. We previously described a major role for TLR3 in the early-stage *Chlamydia*-induced synthesis of IFN-β in OEs; the results from this study suggest a role for STAT1 in the synthesis of type I IFNs that occurs during early and late stages of infection.

Wilbert A. Derbigny, Ph.D., Chair

TABLE OF CONTENTS

LIST OF TABLES xii

LIST OF FIGURES xiii

LIST OF ABBREVIATIONS xvii

INTRODUCTION 1

I. *Chlamydia trachomatis* 1

II. *C. trachomatis* Urogenital Serovars D-K 5

III. *C. trachomatis*-induced Host Immune Responses 7

IV. Type I Interferons 15

V. STAT1 23

VI. STAT1 and Pathogen-induced Immune Responses 27

VII. Present Studies 29

MATERIALS AND METHODS 31

I. Mice 31

II. Reagents 31

III. BMDM Isolation 32

IV. Cells and bacteria 33

V. RNA interference: generation of STAT1-knockdown OEs 34

VI. *Chlamydia muridarum* infections 37

VII. Enzyme-linked immunosorbent assay (ELISA) determination of cytokine production 37

VIII. RT-PCR 38

IX. Western blotting 38

X. Recombinant cytokine treatment 39

XI. Neutralization Experiments 40

XII. Quantitative real-time PCR 43

XIII. Immunofluorescent Staining 44

XIV. STAT1 Inhibitor Experiment 45

XV. Statistical Analysis 45

RESULTS 46

CHAPTER I. ESTABLISH A ROLE FOR STAT1 IN REGULATING *CHLAMYDIA*-INDUCED TYPE I IFN RESPONSES IN OE CELLS 46

A. *C. muridarum* induces increased STAT1 protein activation/expression and Stat1 gene expression 46

B. STAT1 is required for *C. muridarum*-induced IFN-β production during the late stage of infection 50

C. STAT1 is critical for regulating *in vitro C. muridarum* replication/growth in OE cells 52

D. STAT1 is critical for up-regulating Ifnb transcription during late-stage *C. muridarum* infection 55

E. *C. muridarum*-induced type I IFN production is highly dependent on STAT1, but is differentially regulated by the type I signaling pathway via the IFNAR in OE cells 64

F. Neutralization of IFN-β activity has no effect on Ifnb gene induction during *C. muridarum* infection in OE cells 83

CHAPTER II. ESTABLISHING THE TEMPORAL ACTIVATION OF STAT1 AND DETERMINING A ROLE FOR STAT1 IN REGULATING PRO-INFLAMMATORY CYTOKINE RESPONSES IN *C. MURIDARUM*-INFECTED OE CELLS 88

A. IRF3 protein activation is substantially increased during early-intermediate stages of *C. muridarum* infection in OE cells 88

B. STAT1 protein activation/expression and Stat1 gene expression are disrupted in *C. muridarum*-infected TLR3-deficient OE cells 92

C. Early-stage STAT1 activation may contribute to optimal IFN-β production in *C. muridarum*-infected OE cells 96

D. STAT1 plays a central role in IL-6 production in *C.muridarum*-infected OE cells 100

DISCUSSION 111

A. STAT1 is required for *C. muridarum*-induced IFN-β production during the late stage of infection 112

B. *C. muridarum*-induced type I IFN and proinflammatory cytokine production is highly dependent on STAT1, but is differentially regulated by the type I signaling pathway in OE cells 113

C. Temporal and differential upregulation of *C. muridarum*-induced Ifna gene subtypes in OEs requires STAT1, type I IFN-mediated signaling via IFNAR, and is attenuated by TLR3 116

CONCLUSION 125

FUTURE DIRECTIONS 126

REFERENCES 131

CURRICULUM VITAE

LIST OF TABLES

Table 1. Primers used in quantitative real-time PCR experiments 44

LIST OF FIGURES

Figure 1 The *Chlamydia* replication cycle....4

Figure 2 Pathogen-induced TLR signaling: *Chlamydia*-induced innate immune responses in epithelial cells....13

Figure 3 Type I IFN signaling....19

Figure 4 Ifnb gene enhanceosome....21

Figure 5 Type I IFN positive-feedback amplification loop....22

Figure 6 STAT1 protein structure....27

Figure 7 Basic plasmid map of psimSTAT1....36

Figure 8 *C. muridarum* infection increases Stat1 mRNA message relative to mock -infected controls in WT oviduct epithelial cells....48

Figure 9 *C. muridarum* infection increases STAT1 protein expression and activation relative to mock-infected controls in WT OEs....49

Figure 10 *C. muridarum*-induced IFN-β production is diminished in STAT1 -deficient OEs....51

Figure 11 *C. muridarum*-infected STAT1-deficient OE cells display aberrantly shaped *Chlamydia* inclusion-forming units; implicating uncontrolled *C. muridarm* replication....54

Figure 12 *C. muridarum*-induced Ifnb gene expression is induced to similar levels early in infection in both WT and TLR3-deficient OEs....57

Figure 13 *C. muridarum*-induced Ifnb gene expression substantially reduced in TLR3-deficient OEs at 16 hr post infection....58

Figure 14 STAT1 is indispensable for effective *Chlamydia*-induced Ifnb gene induction in OE cells 59
Figure 15 *C. muridarum*-induced upregulation of components of the type I IFN signaling pathway substantially occur during the late stage of infection and is STAT1 dependent 62
Figure 16 IFNα-2 gene induction occurs preferentially during early-stage *Chlamydia* infection, is IFNAR1-dependent, and is attenuated by TLR3 in OE cells 67
Figure 17 IFNα-4 gene induction occurs preferentially during late-stage *Chlamydia* infection, is IFNAR1-dependent, and is attenuated by TLR3 in OE cells 69
Figure 18 STAT1 is required for Ifna2 and Ifna4 gene induction during *Chlamydia* infections in OE cells 71
Figure 19 Late-stage *C. muridarum*-induced Ifnb gene induction occurs independently of type I IFN signaling via the IFNAR in OE cells 75
Figure 20 Exogenous IFN-β induces early-stage Ifna2 gene expression through the type I IFN signaling pathway via the IFNAR in OE cells 76
Figure 21 Exogenous IFN-β induces minimal Ifna4 gene expression independent of the type I IFN signaling pathway via the IFNAR in OE cells 78
Figure 22 Exogenous IFN-β upregulates Ifnb gene induction independently of type I IFN signaling via the IFNAR in OE cells 80

Figure 23 IFN-α subtypes induced with exogenous IFN-β are differentially regulated by STAT1 and induction is independent of type I IFN signaling via the IFNAR in STAT1-deficient OE cells....81
Figure 24 Neutralization of functional IFN-β does not affect *C. muridarum*-induced Ifnb gene induction in OE cells....85
Figure 25 Neutralization of IFN-β substantially reduces exogenous IFN-β-induced Ifnb gene expression in OE cells....86
Figure 26 *C. muridarum* infection does not induce Irf3 gene transcription in OE cells....90
Figure 27 IRF3 activation is substantially increased at early-intermediate stages of *C. muridarum* infection in OE cells....91
Figure 28 *C. muridarum*-induced Stat1 mRNA message is severely reduced in TLR3-deficient OE cells....94
Figure 29 STAT1 protein expression and activation are diminished in *C. muridarum*-induced TLR3-deficient OE cells....95
Figure 30 Changes in STAT1 activation during *C. muridarum* infection in OE cells....98
Figure 31 Early inhibition of STAT1 activation substantially reduces *C. muridarum*-induced IFN-β production in OE cells....99
Figure 32 *C. muridarum*-induced IL-6 production is significantly reduced in STAT1-deficient BMDMs....102
Figure 33 *C.muridarum*-induced IL-6 production is significantly decreased in STAT1-deficient oviduct epithelial cells....103

Figure 34 STAT1-knockdown oviduct epithelial cells display significantly reduced *C.muridarum*-induced IL-6 production at 24 hours 104
Figure 35 *C.muridarum*-induced TLR3 stimulation and STAT1 activation are required for late-stage IFN-β secretion in OE cells 106
Figure 36 IFN-α subtypes require STAT1 and the IFNAR for induction, and are attenuated by TLR3 during *C.muridarum* infection in OE cells.....107
Figure 37 IFN-β requires STAT1, but not the IFNAR1 for induction during late-stage *C. muridarum* infection in OE cells.....108
Figure 38 Early-stage STAT1 activation may contribute to optimal "late-stage" IFN-β production during *C. muridarum* infection in OE cells 109
Figure 39 STAT1 is required for IL-6 secretion during *C. muridarum* infection 110
Figure 40 Possible mechanism of STAT1-mediated IFNAR1-independent IFN-β production by intracellular transcription factors in OE cells during *C. muridarum* infection.....118
Figure 41 Possible mechanism of early STAT1 activation and optimal IFN-β production through *C. muridarum*-induced IL-6 production in OE cells.....123

LIST OF ABBREVIATIONS

Ag-Ab	Antigen-antibody
AP-1	Activator protein-1
BMDDCs	Bone marrow-derived dendritic cells
BMDMs	Bone marrow-derived macrophages
BSA	Bovine serum albumin
CBP	CREB-binding protein
CCL5	CC-chemokine ligand 5
cHSP60	*Chlamydia* heat shock protein 60
CMV	Cytomegalovirus
CpG	Cytidine phosphateguanosine
cPLA2	Cytosolic phospholipase A2
CXCL1	CXC-chemokine ligand 1
CXCL10	CXC-chemokine ligand 10
CXCL16	CXC-chemokine ligand 16
DBD	DNA-binding domain
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EB	Elementary body
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum

FC3	Fetal clone serum
FITC	Fluorescein isothiocyanate-conjugated
GFP	Green fluorescent protein
GM-CSF	Granulocyte/monocyte colony-stimulating factor
HATs	Histone acyteltransferases
HMG I	High mobility group I
IB	Inclusion body
IFN	Interferon
IFN-α	Interferon-alpha
Ifna	Interferon-alpha gene
IFN-β	Interferon-beta
Ifnb	Interferon-beta gene
IFN-γ	Interferon-gamma
IFNAR	Interferon alpha receptor
IFNAR1	Interferon alpha receptor 1
IFNAR 2	Interferon alpha receptor 2
IFNGR	Interferon gamma receptor
IFU	Inclusion forming unit
IKKs	Inhibitor of kappa B kinases
IL-1	Interleukin 1
IL-6	Interleukin 6
Il6	Interleukin 6 gene
IL-8	Interleukin 8

IL-10	Interleukin 10
IL-12	Interleukin 12
IL-18	Interleukin 18
Inc	Inclusion
IRAKs	IL-1 receptor–associated kinases
IRF 1	Interferon regulatory factor 1
IRF 3	Interferon regulatory factor 3
IRF 7	Interferon regulatory factor 7
IRF 9	Interferon regulatory factor 9
ISGs	Interferon-stimulated genes
ISGF 3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK	Janus-activated kinase
LGV	Lymphogranuloma venereum
LP	Lipoprotein
LPS	Lipopolysaccharide
MAPKs	Mitogen-activated protein kinases
MAVS	Mitochondrial antiviral signaling protein
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MOMP	Major outer-membrane protein
MoPn	Mouse pneumonitis
MyD88	Myeloid differentiation primary response gene 88

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKs	Natural killer cells
NLRs	NOD-like receptors
NOD	Nucleotide oligomerization domain
OE	Oviduct epithelial cells
Omc	Outer-membrane complex
Omp	Outer-membrane protein
PAMPs	Pathogen-associated molecular patterns
pDCs	Plasmacytoid dendritic cells
PGN	Peptidoglycan
PI	Post-infection
PID	Pelvic inflammatory disease
PRD	Positive regulation domain
PRR	Pattern recognition receptor
RAS	Rat sarcoma
RB	Reticulate body
RIG-1	Retinoic acid-inducible gene 1
RLRs	RIG-1-like receptors
SH2	Src-homology 2
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription

STAT1	Signal transducer and activator of transcription 1protein
Stat1	Signal transducer and activator of transcription 1 gene
STAT2	Signal transducer and activator of transcription 2 protein
STAT3	Signal transducer and activator of transcription 3 protein
STD	Sexual transmitted disease
STING	Stimulator of IFN gene
TAD	Transactivation domain
TAK1	Transforming growth factor-beta–activated kinase 1
TANK	TRAF-associated NF-κB activator kinase
TBK1	TANK-binding kinase 1
T_H-1	T helper 1
TBK1	TANK-binding kinase 1
TIR	Toll-IL-1 receptor
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TLR3	Toll-like receptor 3
TNF-α	Tumor necrosis factor alpha
TRAFs	TNF receptor associated factors
TRAM	TRIP-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon
TTSS	Type III secretion system
TYK2	Tyrosine kinase 2
VSV	Vesicular stomatitis virus

WT	Wild-type
Y	Tyrosine
Φ	Macrophages

INTRODUCTION

I. *Chlamydia trachomatis*

Chlamydiaceae are obligate intracellular parasites that were once considered to be viruses because they are small enough to pass through 45 μm filters. Historically, the family Chlamydiaceae consisted of one genus, *Chlamydia*, in which all species were grouped [1-4]. However, based on genomic studies, the family was divided into two genera, *Chlamydia* and *Chlamydophila* [5-8]. There are three species within the Chlamydiaceae family that exist as pathogenic organisms capable of causing human disease including: *Chlamydia pneumonia* (respiratory illnesses and cardiovascular disease), *Chlamydia psittaci* (zoonotic pneumonia), and *Chlamydia trachomatis* (genital tract infections and trachoma) [9]. The *Chlamydia* genera consist of other pathogenic members that cause clinical disease in animals including the mouse homolog *Chlamydia muridarum* (*C. muridarum;* formally known as *C. trachomatis* mouse pneumonitis (MoPn)); and *Chlamydia suis* the swine homolog.

C. trachomatis is a gram-negative, obligate intracellular bacterium [2, 10]. Though *Chlamyida trachomatis* is capable of infecting classical immune cells such as macrophages (Φ), dendritic cells (DCs) [11-13], as well as fibroblasts [14, 15]; *C. trachomatis* infections are restricted to humans, primarily to the non-ciliated epithelial cells of the respiratory tract, conjunctivae, mucosa, and urogenital tract [16, 17]. Based on specific host restrictions, the *C. trachomatis* species were further divided into two biovars: (i) the trachoma biovar that is associated with trachoma or primary infection of the conjunctiva (eye infections), and (ii) the lymphogranuloma venereum (LGV) biovar which are urogenital infections that primarily affect the inguinal lymph nodes. Antigenic

variations in the major outer membrane proteins (MOMP) of these biovars called for furtherdivision into serovars that were associated with a specific disease [18, 19]. Serovars Ab, B, Ba, and C are associated with trachoma. Serovars L1-L3 are associated with LGV. Serovars D-K are associated with primary urogenital tract infections and Chlamydia-associated reactive arthritis [20].

Unlike other bacteria, the Chlamydiaceae have a unique developmental cycle whereby it can exist in either of two forms: an infectious, non-metabolically active elementary body (EB) and the non-infectious, metabolically active reticulate body (RB) [21, 22]. These EBs and RBs comprise the biphasic replication cycle part of all *Chlamydia* species (Figure 1). A typical *Chlamydia* developmental cycle begins by rapid attachment and entry of the EB into the host cell via a poorly understood receptor-mediated endocytosis pathway, followed by the recruitment of components of the host cell cytoskeleton that results in the formation of an intracellular vesicle containing the infectious EB known as the inclusion body. *Chlamydia* EBs express cysteine-rich outer membrane proteins and complexes (i.e. OmcA, OmcB) that reversibly interact with host surface glycosaminoglycans [23, 24], followed by an irreversible attachment to components of host estrogen receptors [25, 26]. EBs also presumably express preformed Type III Secretion Systems (TTSS) that export *Chlamydial* signaling molecules to the host cell as a means to recruit actin that aids in EB entry and ultimately, trafficking of the inclusion body to the peri-Golgi region of the host cell [27, 28]. The EBs undergo primary differentiation to the metabolically active RB; a process initiated by the disruption of deoxyribonucleic acid (DNA)-histone interactions, presumably by *Chlamydia* metabolites [29]. Primary differentiation involves modification of the

inclusion body through a process regulated by *Chlamydia* inclusion membrane (Inc) proteins, and the interception of vacuoles containing sphingomyelin, cholesterol, and endocytic GTPases from endocytic/exocytic pathways [30-34]. Rapid replication of the RBs to identical progeny occurs and continues via binary fission from 6- 24 hours, and the number of progeny is normally directly proportional to the growth of the inclusion body [22]. The secondary differentiation involves the redifferentiation of the newly synthesized RBs to EBs. This stage is marked by increases in Omc genes and histone-like proteins and by down-regulation of components of the TTSS system [35, 36]. At 48-72 hours the EBs are subsequently released from the host cell via cell lysis, where they are free to re-infect neighboring cells and thereby repeat the replication cycle.

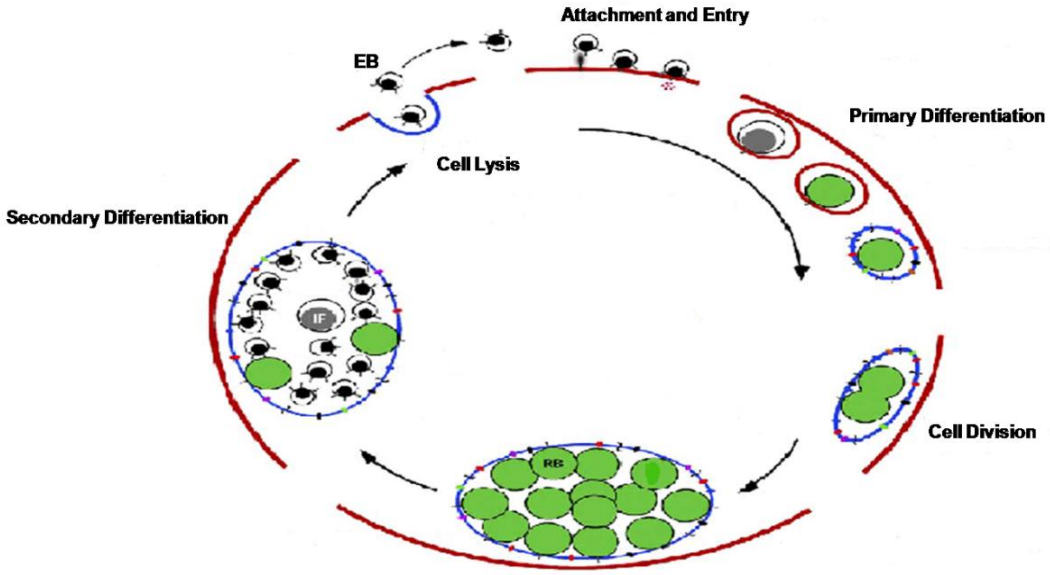


Figure 1. The *Chlamydia* replication cycle

Chlamydia undergoes a unique, biphasic replication cycle upon infection. The infectious EB attaches to and enters the host cell via receptor-mediated endocytosis. Once inside the cell the EB remains in an intracellular inclusion forming unit and begins to enter a primary differentiation stage where the EB differentiates to the metabolically active, noninfectious RB. The RB divides via binary fission and rapidly produces identical progeny RBs. Note that the structure containing the newly-formed RBs is now termed an inclusion body (IB). Near the end of the infection cycle newly synthesized RBs undergo a secondary differentiation stage where they redifferentiate to the infectious EBs. The host cell is lysed and the infectious EBs released; reinitiating the infectious cycle in neighboring cells. Figure adapted from [22].

II. *C. trachomatis* Urogenital Serovars D-K

C. trachomatis is the most common bacterial sexually transmitted disease in the United States [37]. It is estimated by the World Health Organization that 90 million of the 500 million new cases of sexually transmitted diseases (STDs) per year, are caused by *C. trachomatis* serovars D to K [38]; with the highest prevalence among women from ages 15-25 [39]. Progression of *C. trachomatis* infection into the upper reproductive tract epithelium causes significant inflammation [40-44]. In women, chronic infections with urogenital serovars of *C. trachomatis* and progression of *Chlamydia* into the upper reproductive tract epithelium can cause pelvic inflammatory disease (PID), scarring, and infertility [42, 43, 45, 46], all of which are irreversible. Estimated incidences of *Chlamydia*-associated ectopic pregnancies and tubal infertility ranges from 5-25%, and 10-20%, respectively [47]. The annual costs of treating sexually transmitted diseases in the United States is approximately 15.9 billion dollars; with *Chlamydia*-related costs estimated to almost 2.4 billion dollars and steadily increasing [48, 49].

Chlamydia urogenital infections are curable via oral administration of antibiotics. A single-dose regimen of the macrolid antibiotic azithromycin, or a multi-dose regimen of the tetracycline antibiotic doxycycline are equally efficacious in treating *Chlamydia* urogenital infections [50-54]. However, *C. trachomatis* genital infections are clinically asymptomatic and are thus a major public health concern [55]. Reports indicate that approximately 75% of women and up to 50% of men show no clinical signs or symptoms upon *Chlamydia* infection [37, 56-58], and up to 40% of asymptomatic women will develop PID due to lack of treatment and progression of *C. trachomatis* into upper reproductive tract epithelium [37, 58, 59]. The inability to detect clinical manifestations

of *C. trachomatis* infections contributes, significantly, to the deleterious post-infection sequealae and increased transmission between sexual partners. The development of an immunization regimen that efficiently combats *C. trachomatis* is warranted and has the potential of decreasing *Chlamydia* prevalence worldwide.

Early trials of candidate *Chlamydia* vaccines revealed a short-lived protective immune response with increased post-infection sequealae. These early candidate vaccines included both live attenuated, and heat-inactivated whole organisms of *Chlamydia* which were capable of inducing host immune responses similar to natural infections, albeit for a limited time and at suboptimal levels. However, the vaccines exposed the host to antigens or virulent factors that contributed to host pathology [60-64]. In later *Chlamydia* vaccine trials a subunit-approach was used to generate candidate vaccines. The subunit vaccines were proposed to be advantageous due to lack of host exposure to the virulent factors that induced immunopathology; however these vaccines proved to be incapable of inducing a strong cell-mediated response, which is a major requirement for protective immunity against *Chlamydia* [63, 65-72]. More recently DNA-based vaccines have been generated and current studies using a combinational approach (i.e. DNA-based and subunit-based vaccines) show that this strategy for *Chlamydia* vaccination evoked both humoral and cell-mediated protective immune responses without significantly increasing the immunopathology as seen with earlier vaccine candidates [63, 73-77]. Thus, the development of an efficacious vaccine requires in depth understanding of the immunologic mechanisms that evoke both protective and pathological responses during *Chlamydia* infection; particularly in reproductive epithelium.

III. *C. trachomatis*-induced Host Immune Responses

The host immunological responses to *C. trachomatis* infections vary among biovars. While trachoma serovars predominantly induce cell-mediated immune responses contributing to delayed hypersensitivity (a possible evasion mechanism), the LGV serovars induce initial cellular immune responses, followed by a strong cell-mediated and humoral adaptive immune response [78-82]. Furthermore, Dessus-Babus, et al, in a study comparing LGV serovar L2 and urogenital serovar E, showed that HeLa 229 cells induced with these serovars displayed different innate immune responses [83]. Most of the details of *Chlamydia*-induced immune responses and the mechanisms that are implicated in both clearance and pathology have been unveiled in mouse models of *Chlamydia* genital infections using *C. muridarum* [84-87]. Though there have been some major differences cited in *C. muridarum* genital infections in mice versus *C. trachomatis* genital infections in humans, including: differences in infection duration/clearance rates, differences in immune evasion mechanisms, and differences in allelelic variation of the MOMP antigen [84, 86, 88-91], the immune responses in experimental mouse models of *C.muridarum* vaginal infections closely mimics those of natural acute *C. trachomatis* vaginal infections in humans [85, 87, 92, 93]; and thus serve as a useful tool in elucidating the immunobiology of *C. trachomatis* gential tract infections.

Though mucosal barriers of the genital tract provide the first line of defense, epithelial cells lining the genital tract act as sentinels for invading *Chlamydia trachomatis* infection. Classically, epithelial cells were not thought to be critical players in innate immunity. However, the resulting immune responses to Chlamydia infection in epithelial cells included the production and secretion of several cytokines and chemokines required

for several host adaptive functions such as restricting the pathogen and recruiting/activating innate immune cells {natural killer cells (NKs), Φ, DCs}, and adaptive immune cells {naïve T-lymphocytes (T-cells) and B-lymphocytes (B-cells)} to the sites of infection [17, 94-100]. *Chlamydia*-infected epithelial cells have been shown to secrete acute-phase pro-inflammatory cytokines and chemokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), CXC-chemokine ligand 1(CXCL1), CXC-chemokine ligand 16 (CXCL16), CC-chemokine ligand 5 (CCL5 or Rantes), and granulocyte/monocyte colony-stimulating factor (GM-CSF) [17, 44, 84, 95, 99]. It has also been demonstrated during *C. trachomatis* infection, that immunomodulatory cytokines such as the type I IFNs, IFN-α and IFN-β, interleukin-10 (IL-10), interleukin-12 (IL-12) and tumor necrosis factor-alpha (TNF-α), are all up-regulated in both the cervix and the fallopian tubes [44, 101]. Studies have also shown increased secretion of mature interleukin-18 (IL-18), a potent inducer of the type II IFN interferon-gamma (IFN-γ) in infected human epithelial cells, via a caspase-1-dependent mechanism [102]. The ultimate clearance and control of *Chlamydia* infections are dominated by IFN-γ [85, 103-105]. IFN-γ is a cytokine produced by classical innate immune cells and adaptive immune cells that is induced and regulated by acute-phase or innate chemokines and cytokines including IFN-α and IFN-β [44, 84, 106, 107]. The majority of these chemokines and cytokines promote T helper 1 (T_H-1) protective immunity, resulting predominately in $CD4^+$ T-cell-mediated resolution of *Chlamydia* infection [84, 108, 109]; although $CD8^+$ T-cells have been implicated [110, 111]. These findings implicate the epithelial cell cytokine response as a major contributor to host protective immunity during *Chlamydia* infections.

In mammalian cells, the invasion of microorganisms is sensed via a specialized family of membrane bound PRRs called Toll-like receptors (TLRs) [112, 113]. The TLRs are stimulated by the recognition of pathogen-associated molecular patterns (PAMPs) that result in the synthesis of acute inflammatory cytokines and chemokines including CCL5, IL-6, GM-CSF, IL-1β and type I IFNs [17, 112, 114, 115]. TLRs transduce signals initiated by binding of a distinct PAMP to the leucine-rich repeat-containing ectodomains, followed by propagation via the transmembrane domains, and ultimately via the intracellular Toll-IL-1 receptor (TIR) domain for efficient downstream signal transduction [112, 113, 116, 117]. In mammals there are at least 13 TLRs that recognize specific PAMPs; of which TLRs 1-9 are conserved among human and murine species, with 10 functional TLRs in humans (1-10) and 12 functional TLRs in mice (1-9, 11-13) [113, 114, 118]. PAMPs recognized by TLRs are derived from various microbes including: bacteria, viruses, fungi and parasites and are recognized based partly on the cellular location of the TLR [112, 113, 117]. Cell surface TLRs, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 recognize microbial components such as lipoproteins (LP), petidoglycan (PGN), lipopolysaccharide (LPS), flagellin and uropathogenic components; respectively. TLRs 3, 7, 8, and 9 are located intracellularly within endosomal vesicles and recognize nucleic acids such as double-stranded ribonucleic acid (dsRNA), single-stranded RNA (ssRNA), and unmethylated 2′-deoxyribo-cytidine-phosphateguanosine (CpG) DNA; respectively [113, 116, 119-126]. Distinct immune responses (i.e. inflammatory cytokine production, type I IFN production) induced by individual TLRs are also attributed to the recruitment of distinct TIR-domain containing adaptor molecules such as TIR-domain-containing adaptor protein (TIRAP), myeloid

differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), TRIP-related adaptor molecule (TRAM), TNF receptor associated factors (TRAFs); the subsequent activation of specific kinases such as IL-1 receptor–associated kinases (IRAKs), transforming growth factor-beta–activated kinase 1 (TAK1), TRAF-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), inhibitor of kappa B kinases (IKKs), mitogen-activated protein kinases (MAPKs); and transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), and interferon regulatory factor 3 (IRF3) [116, 122, 127-132]. In addition, other non-TLR PRRs such as retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), inflammasomes and recently identified double-stranded DNA (dsDNA) sensors localized throughout the cytosol are also activated by PAMPs derived from viruses and intracellular bacteria, to initiate the appropriate immune responses [113, 133-1°].

Studies in DCs have led investigators to theorize that differential expression of TLRs, both at the cell surface and in endosomal compartments, heavily influences the type of immune responses induced by microbial pathogens [113, 138]. In macrophages activated by bacteria, both TLR2 and TLR4 mediate the secretion of proinflammatory cytokines as demonstrated by the ability of heat-killed *Staphylococcus aureus* to induce NF-κB activation in human monocytes, and the inability to activate NF-κB in TLR4-deficient macrophages upon induction, respectively [139-142]. Applequist et al, demonstrated that many of the classical innate immune cells of myeloid lineage express a wide variety of TLRs, whereas the cells involved in adaptive immune responses and of lymphoid lineage selectively expressed TLRs based on cellular location [143]. TLRs are

also differentially expressed on nonimmune cells that contribute to inflammatory and immunoregulatory responses. Previous studies in humans have shown that both primary and transformed uterine epithelial cells express TLRs 1-9 [144, 145], while primary and immortalized cervical epithelial cells express TLRs 1-6 lacking TLR4 expression [146], and Fallopian tube tissue homogenates to express TLR2 and TLR4 [147]. Intestinal epithelial cells express TLR4 at relatively low levels [148]. It is important to note that the expression of TLRs can be modulated directly by microbial PAMPs or indirectly by autocrine signaling of secreted chemokines and cytokines [149-152].

The role of TLRs in *Chlamydia*-induced host immune responses have been extensively studied. *C. trachomatis*-induced IL-8 production in cervical carcinomas and kidney epithelial cells has been shown to require functional MyD88 and TLR2 [153]. In *Chlamydia pneumoniae*-induced DCs, TLR2 has been shown to be involved in initiating the production of innate chemokines and cytokines [154]. TLR2 has also been shown to mediate proinflammatory cytokine production in *C. muridarum*-induced oviduct epithelial cells via a MyD88-dependent mechanism [155]; and is implicated in *C. muridarum*-induced fallopian tube pathology [156]. Though TLR2 is predominantly implicated in *Chlamydia*-induced innate immune responses, *Chlamydia* heat shock protein 60 (cHSP60) has been shown to induce NF-κB activation via TLR4 in vascular endothelial cells [157, 158]. Additionally, *C. pnuemoniae*-induced bone marrow-derived dendritic cells (BMDDCs) have been shown to secrete IL-12p40 dependent on TLR2 and TLR4 [154]. Furthermore, *Chlamydia* infections induce a robust IFN response via stimulation of TLRs. In bone marrow-derived macrophages (BMDMs) *C. pneumoniae* was shown to induce IFN-β, IFN-α, and IFN-γ genes in a MyD88/TRAF6/IRAK-4-

dependent manner [159]. Nagarajan et al, showed an approximate 80% decrease in IFN-β mRNA from *C. muridarum*-induced MyD88-deficient peritoneal Φ compared to wild-type peritoneal Φ [160]. Human vascular endothelial cells have also shown a dependence on mitochondrial antiviral signaling (MAVS) protein, an adaptor molecule involved in RLRs signaling; and IRF3 for IFN-β protein and gene expression in a *C. pneumoniae* infection model. A *C. muridarum* rat model utilizing prostate epithelial cells was shown to upregulate TLR2 and TLR4 gene expression, as well as, to recruit these TLRs to the inclusion body and to significantly induce CXCL10 (IP-10) protein expression; a type I IFN-dependent gene that can serve as an indirect marker for IFN-β/α production [161]. Because the host immune response to *Chlamydia* infection results in the TLR-dependent synthesis of acute innate cytokines and chemokines (Figure 2) that are directly and/or indirectly involved in leukocyte recruitment, activation, and polarization [44, 155, 162], these findings highlight the importance of TLRs in the primary innate inflammatory response to *Chlamydia* infection, and in the initiation of adaptive immune responses required for protective immunity.

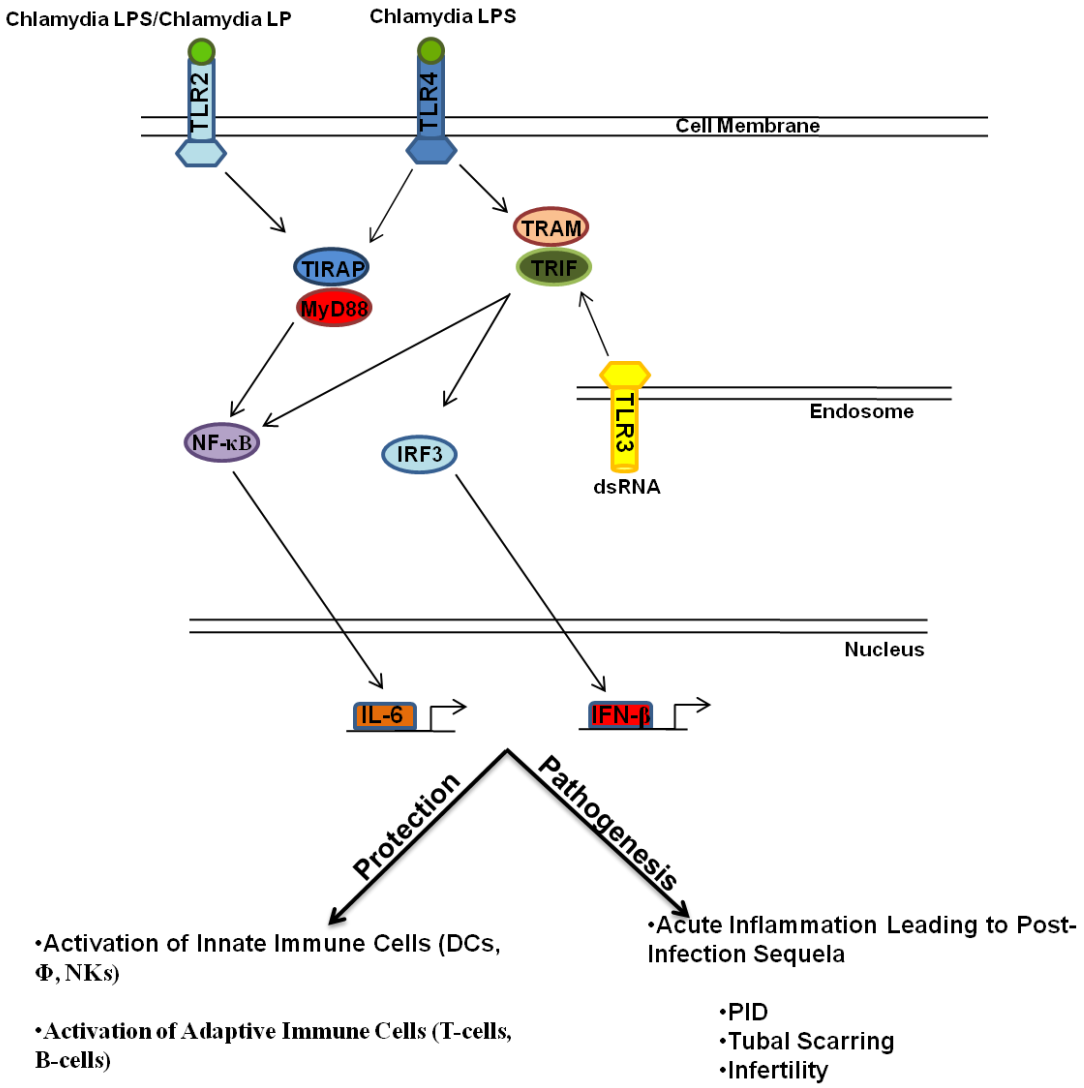


Figure 2. Pathogen-induced TLR signaling: *Chlamydia*-induced innate immune responses in epithelial cells

Upon infection of epithelial cells *Chlamydia* PAMPs (i.e. lipoproteins, LPS) or, possibly, nucleic acids generated upon intracellular invasion bind to cell surface TLR2/TLR4 or endosomal TLR3; respectively. TLR stimulation leads to the recruitment and activation of cytosolic TIR domain-containing adaptor molecules, namely MyD88 (specific to TLR2 and TLR4) and TRIF (specific to TLR3 and TLR4). These adaptors

and co-adapters activate specific kinases that ultimately phosphorylate/activate specific transcription factors (i.e. NF-κB and IRF3). Phosphorylated NF-κB and IRF3 translocate to the nucleus where they bind to specific promoter sequences of target genes such as Il6 and Ifnb; respectively, and initiate transcription. In *Chlamydia* infections these cytokines have been implicated in both clearance and the associated pathologies [17, 85, 163-166].

IV. Type I Interferons

Interferons were first identified as factors responsible for viral-induced resistance to infections with dissimilar viruses, termed viral interference [167, 168]. Interferons have since been identified as cytokines that act as antimicrobial, antiviral, and immunomodulatory agents during viral and intracellular bacterial infections; primarily functioning to restrict viral and/or bacterial replication [169-171]. In vitro and in vivo studies show the ability of interferons to modulate the cytotoxic activity of a variety of immune cells including T-cells, macrophages, NKs; and the subsequent activation of these cells by modulating the expression of surface molecules such as the major histocomapatibility complex (MHC) class I antigens [172-174]. These properties implicate interferons in innate and adaptive immune responses to pathogens.

The IFN family consists mainly of three classes: type I (namely IFN-α/IFN-β), type II (IFN-γ), and type III (IFN-λs) [174-176]. The type III IFNs have been closely compared to the type I IFN family based on their mode of signaling, however due to their restricted tissue distributions they were rarely studied until recently. The type II IFNs consist of one gene product, IFN-γ, which is induced primarily in macrophages, NKs, and T-cells. In contrast, type I IFNs consist of a single gene product known as IFN-β, and several partially homologous gene products that encode IFN-α subtypes that are ubiquitously produced in various cell types in response to viral and/or bacterial infections [177, 178]. The classes were divided accordingly based on the ability of specific interferons to bind to a common receptor; IFN-β and IFN-α bound to the interferon alpha receptor 1 and 2 (IFNAR1, IFNAR2) or type I IFN receptor, and IFN-γ bound to the interferon gamma receptor 1 and 2 (IFNGR1, IFNGR2) or type II IFN receptor [179-

181]. Both classes of IFNs and the corresponding receptor upon stimulation, employ the use of permanently associated Janus protein tyrosine kinases. The JAKs phosphorylate and activate a specific set of cytoplasmic signal tranducers and activators of transcription (STAT) proteins that translocate to the nucleus, and act as transcriptional machinery inducing the transcription of IFN-stimulated genes (ISGs) [168, 178, 182].

Type I IFNs stimulate the activation of the Janus Kinases (JAK1), tyrosine kinase 2 (TYK2), and the subsequent phosphorylation of the cytoplasmic tail of the heterodimeric IFNAR1 and IFNAR2 receptors. This phophorylated cytoplasmic tail of the receptor serves as a docking site or recruitment site for STAT1 and/STAT2, upon which they become phosphorylated or activated by the JAKs and form two distinct transcription factors consisting of STAT1 homodimers or STAT1-STAT2 heterodimers complexed with interferon regulatory factor 9 (IRF9); known as the interferon-stimulated gene factor 3 (ISGF3). The ISGF3 complex can then translocate to the nucleus where it binds to a DNA sequence in the promoter region of ISGs known as the IFN-stimulated response element (ISRE) to initiate gene transcription [168, 169, 178, 183]. A typical type I IFN signaling cascade is represented in Figure 3.

Type I IFNs are secreted by a vast array of cell types upon pathogen recognition. A key requirement for the induction of type I IFNs (noteably IFN-β and IFN-α4) is the activation of IRF3 and interferon regulatory factor 7 (IRF7); which make up part of the enhanceosome, a transcription factor complex in which each component binds to specific promoter sequences of the IFN-β gene (Figure 4) stimulating transcripition [184-187]. PRRs that recognize viral or other nucleic acids are stimulated to produce type I IFNs [113, 116, 188]. In macrophages and some dendritic cells, TLR 3 and 4 have been

implicated in viral and bacterial-induced type I interferon production, respectively [116, 189, 190]. In contrast, plasmacytoid dendritic cells (pDCs) are potent interferon producers, and have been shown to produce large amounts of type I IFNs during viral infections; however, this production is dependent on endosomal TLRs 7 and 9, and signaling is mediated through MyD88 and constitutively expressed IRF7 [113, 129, 168, 191]. Additionally, cytosolic PRRs such as RLRs have also been implicated in IRF3 mediated type I IFN production during viral infection [178, 192]. In addition, the NLRs NOD1 and NOD2 can induce the production of type I IFNs in epithelial cells and macrophages, respectively, via novel mechanisms leading up to the activation of IRF7 and IRF5 during bacterial infections [193, 194].

Type I interferons have been shown to aid in their own production by an autocrine/paracrine positive feedback mechanism in which they bind to the type I interferon receptor of the same cell or neighboring cells, stimulating the JAK/STAT signaling pathway (Figure 5). The binding of type I IFNs (namely IFN-β and IFN-α4 subtype) to the IFNAR heterodimer leads to the production or amplification of IFN-responsive genes, including type I interferon genes via JAK/STAT signaling [168, 184]. The increased expression of the IRF7 protein via JAK/STAT signaling has been directly correlated to increased amplification of all type I IFN genes and is thought to be the major requirement for production or amplification of type I IFNs through this mechanism [195].

Resting cells express a basal level of IRF7 that has been shown to be involved in the early stage IFN-β induction upon initial viral or intracellular bacterial infection; however, further expression of IRF7 was required for subsequent induction of IFN-α [184, 196-198]. Furthermore, in vitro studies show a requirement for early stage IFN-β in the production of IFN-α in *Listeria*-induced macrophages [199].

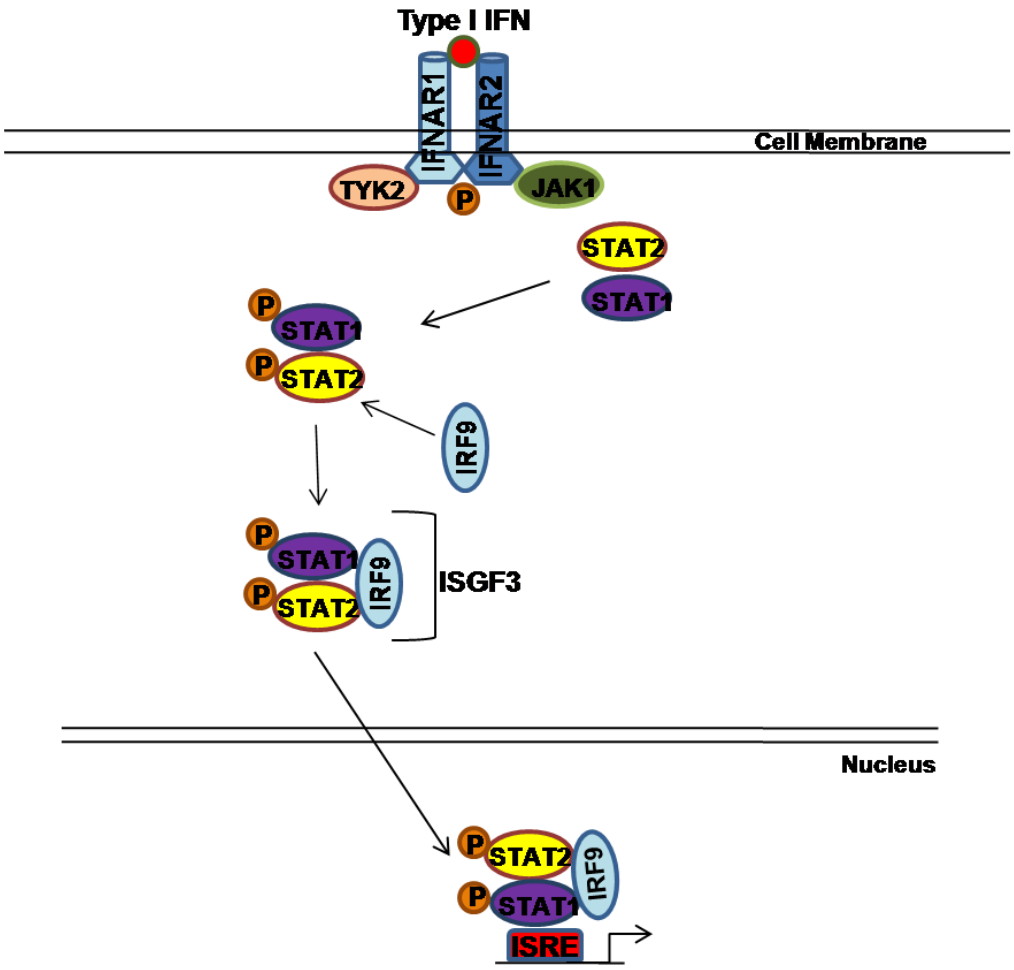
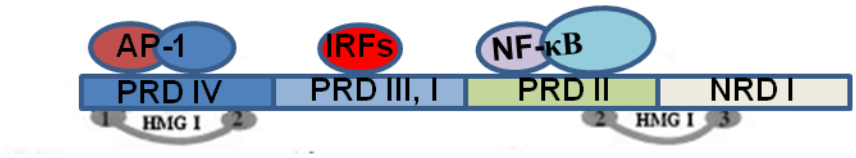


Figure 3. Type I IFN signaling

Type I IFNs (i.e. IFN-β, IFN-α) bind to the heterodimeric IFNAR1-IFNAR2 complex stimulating the activation of permanently bound JAKs, TYK2 and JAK1. The activated JAKs transphosphorylate tyrosine residues on the cytoplasmic tail of the IFNAR1-IFNAR2 receptor complex; and serve as recruitment sites for cytoplasmic STAT1 and STAT2 proteins. Upon tyrosine phosphorylation via the JAKs, STAT1 and STAT2 form heterodimers which are further complexed with cytoplasmic IRF9. This STAT1-STAT2-IRF9 complex forms the transcription factor ISGF3.

Activated ISGF3 translocates to the nucleus where it associates with ISRE promoter sequences to initiate the transcription of ISGs. Adapted from [178].



IFN-β Gene Promoter Region

Figure 4. Ifnb gene enhanceosome

The induction of Ifnb gene transcription requires the assembly, activation and binding of several transcription factors to specific DNA-sequences in the gene promoter region; as well as the assembly of mammalian architectural proteins called high mobility group I (HMG I) [200, 201]. Upon pathogen stimulation, the transcription factors AP-1, NF-κB, and the IRFs (IRF3 and/or IRF7) become active, translocate to the nucleus, and bind to specific positive regulation domains (PRDs): PRD IV, PRD II, and PRD –III –I; respectively, where along with coactivators, they initiate the transcription of the Ifnb gene [202]. The HMG I proteins are required for recruitment of the transcription factors to their respective PRDs [200]. Negative regulation domains (NRDs) are also found in the promoter region. Figure adapted from [201].

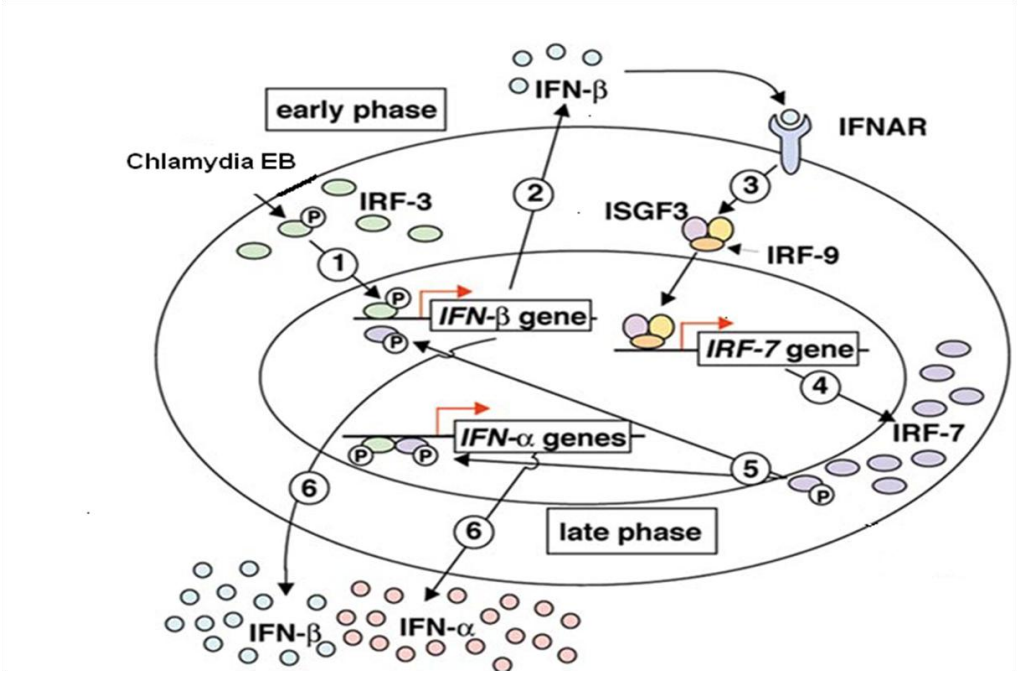


Figure 5. Type I IFN positive-feedback amplification loop

To amplify the production of type I IFNs, IFN-β (and some IFN-α subtypes) employs the use of a positive feed-back mechanism in which (1) pathogen-induced IRF3 activation leads to the initial expression of small quantities of IFN-β. (2) The secreted type I IFNs bind to the IFNAR heterodimeric complex of the same cell (or neighboring cells) ultimately activating the ISGF3 transcription complex that translocates to the nucleus and (4) binds to the ISRE promoter sequence of the IRF7 gene; initiating transcription and translation of IRF7. (5) IRF7 is phosphorylated/activated in the cytoplasm via kinases associated with PRR signaling pathways [203, 204], and upon activation translocates to the nucleus to initiate optimal transcription of IFN-β and some IFN-α subytypes [195]. Figure adapted from [178, 198].

The antiviral properties of type I IFNs are well documented and provided the initial evidence of the biological functions of these interferons upon pathogen stimulation. The antibacterial effects of type I IFNs are becoming of increased interest, and have proved to be more complicated. The first antibacterial effect of type I IFNs was documented in *Chlamydia* species where de la Maza et al, showed a significant decrease in *Chlamydia* replication in non-hematopoeitic cells treated with IFN-α [205]. Furthermore, type I IFNs were also shown to inhibit the *Chlamydia* growth cycle by inhibiting the primary differentiation of the EB to RB [206]. In addition, Devitt et al, showed the significant production of type I IFNs as well as inducible nitric oxide synthase, an enzyme required to produce the free radical nitric oxide, upon *C. trachomatis* infection [207]. Additional intracellular bacteria such as *Legionella pneumophila* possess the limited ability to replicate in macrophages dependent on autocrine type I IFN signaling [170].

V. STAT1

As suggested by their nomenclature, STATs possess dual roles as both signal transducers and transcription factors. STAT1 was the first STAT identified as a latent cytoplasmic factors activated by IFNs upon extracellular stimulation [208-211]. Since then six additional mammalian STAT proteins (STATs 1-4, 5a, 5b, and 6) have been identified and shown to be activated upon the ligation of specific cytokines to their appropriate receptors [182, 212-214]. Both type I and type II interferons are known to induce transcriptional responses mediated predominately by the JAK/STAT signaling pathway, with STAT1 playing a crucial role in both instances [169, 182]. Alternatively, STAT1 is among the few STATs that have been shown to become directly or indirectly

activated via the stimulation of other receptors that possess intrinsic receptor tyrosine kinase activity (i.e. growth factor receptors); independent of IFN signaling [182, 215].

The basic model of activation for STAT1 and the other STAT proteins involves a series of three tyrosine phosphorylations via the JAKs, that ultimately result in the phorphorylation of a single tyrosine (Y) residue (residue Y701) [169, 182, 208, 216-218]. STAT1 and STAT3 have also displayed a requirement for phosphorylation of a serine residue 727 to achieve maximal transcriptional activity, and can occur independently of tyrosine phosphorylation [216, 219, 220]. STAT1 inactivation has been linked to several biological events including nuclear import/nuclear export signals, the upregulation and expression of genes encoding specific phosphatases, suppressors of cytokine signaling (SOCS) and more recently, acetylation via the histone acetyltransferase (HATs) CREB-binding protein (CBP) [221-224]. In addition, studies have revealed mechanisms by which cytoplasmic or "latent" STAT1 proteins remain inactive and sequestered in the cytoplasm. Lackmann et al, showed that unphosphorylated STAT1 and STAT3 proteins can form dimers in the cytoplasm in association with additional factors. Furthermore, these unphosphorylated STAT1 dimers conform to an anti-parallel state inhibiting required protein-protein interactions and exposing tyrosine residues for dephosphorylation if necessary [182, 225-227].

Upon activation and subsequent dimerization, STATs are recruited to the nucleus where they associate with specific DNA sequences (i.e. ISREs), and initiate the transcription of target genes [182, 228]. Transcriptional regulation by STAT1 is achieved via several mechanisms that modulate protein-protein interactions, post-translational modification (i.e. serine 727 phosphorylation), and the recruitment of specific co-

activators (i.e. CBP) [217, 220, 229-231]. Interestingly, studies in human epithelial cell models have shown an accumulation of unphosphorylated STAT1 in the nucleus that is involved in upregulating, specifically, immunoregulatory genes persisting for days following a low-concentration of IFN-β stimulation [232]. The modulation of STAT1 function is critical for its biological impact and is directly related to the structural domains of the protein (Figure 6); which is conserved among the STAT protein family.

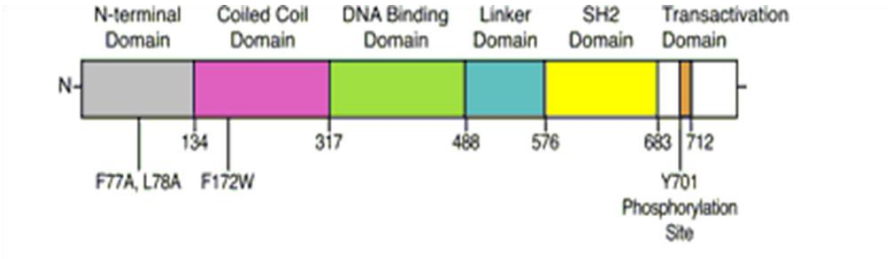


Figure 6. STAT1 protein structure

The functional activities of STAT1 and all STATs (i.e. signal transduction, nuclear import/export, and transcriptional activity) are directly correlated to the structural domains of the proteins. STAT1 protein structure (amino acids 130-712) consists of a transactivation domain (TAD) encompassing the Y701 phosphorylation site, and serves as the site for the recruitment of key coactivators that aid in efficient transcriptional activity. The Src-homology 2 (SH2) domain is required for the initial recruitment of STAT1 to the receptor-associated kinases or JAKs for initial phosphorylation, and for the subsequent dimerization of the STAT proteins. The DNA-binding domain (DBD) is a key requirement for STAT1 transcriptional activity. The amino (N)-terminal domain, linker domain and coiled-coil domain have been implicated in critical protein-protein interactions among STATs, between STATs and other co-factors, and in nuclear import/export; regulating activation-deactivation cycles and transcriptional activity [213]. Adapted from [227].

VI. STAT1 and Pathogen-induced Immune Responses

STATs are implicated in a wide variety of biological occurrences including embryonic development in mice, the regulation of cellular growth, and the modulation of pathogen-induced immune responses in mice and humans [233-236]. Though STAT1 and STAT2 are activated upon IFN stimulation, STAT1 can be phosphorylated upon the initial recognition of pathogens and independent of IFN-stimulation, whereas STAT2 is activated predominantly by the "early" type I IFNs [216, 237]. STAT1 activation has been shown to mediate IFN-induced gene expression in virally stimulated macrophages [112]. STAT1 mutations in human and mouse models have been associated with severe susceptibility to *Mycobacterial* and viral infections which coincides with severely diminished innate immune responses [238, 239] and a complete lack of responsiveness to IFN-α and IFN-γ [239], respectively. In a *Vesicular Stomatitis Virus* (VSV) model, the STAT1-mediated induction of nuclear pore proteins has been shown to overcome the inhibition of proteins translocating to the nucleus as a possible antiviral strategy [240]. Other studies utilizing viral models such as VSV, have shown increased susceptibility and mortality to infection in both IFNAR -/- and STAT2 -/- [237]. However, influenza virus models have demonstrated a requirement for STAT1 in tissue tropism and the ultimate clearance of the pathogen [241, 242]. These findings highlight the overall importance of STAT-mediated IFN signaling in viral infections.

During bacterial infections the precise actions of IFNs and the STATs that mediate them are more complicated and tend to vary between microorganisms. Unlike some viral infections, STAT2 -/- and IFNAR -/- mice revealed similar mortality rates compared to wild-type controls upon *Listeria* infection [237, 243]. Though STAT1 has

been implicated in bacterial clearance, some studies have shown STAT1-dependent cell death of macrophages infected with *Listeria* [244]. Other groups have shown decreased bacterial pathology upon infection with *Mycobacteria* and *Chlamydia* species in epithelial cells utilizing IFNAR -/- mouse models [163, 245]. In contrast, many intracellular pathogens including *Mycobacterium*, *Salmonella*, and *Chlamydia* have been shown to disrupt STAT1 activity; as the host relies heavily on STAT1-mediated defenses to achieve optimal clearance and protective immunity [239, 246-249].

VII. Present Studies

Using *C.muridarum*, formerly known as mouse pneumonitis strain (MoPn), and a murine oviduct epithelial (OE) cell infection model as a surrogate for *C. trachomatis* infection in humans, we previously showed that *Chlamydia* infection of OE cells results in the TLR-dependent synthesis of proinflammatory cytokines and chemokines including: CCL5, IL-6, GM-CSF, and IFN-β [155]. Subsequent studies identified TLR3 as the primary PRR stimulated in oviduct epithelial cells resulting in the *Chlamydia*-induced synthesis of IFN-β [155, 250, 251]. However, our data revealed that the OE cells were still capable of producing residual amounts of IFN-β during *Chlamydia* infection, despite the absence of a 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. Others have demonstrated the existence of multiple pathways that *Chlamydia* uses to induce type I IFN synthesis in various cell types including the MYD88-dependent pathways observed in peritoneal macrophages [160], pathways that include signaling through the "rat sarcoma" (RAS) signaling adaptor molecule cytosolic phospholipase A2 (cPLA2) in mouse embryo fibroblasts [252], and TLR-independent pathways that signal via NOD 1 and stimulator of IFN gene (STING) protein [253]. The multitude of pathways induced by *Chlamydia* to synthesize type I IFN underscores the redundancy in the mechanisms of immune response to infection, and highlights the importance of type I IFNs in *Chlamydia* pathogenesis.

The investigation into the role of STAT1 in response to *Chlamydia*-induced type I 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 appeared to be essential for restricting pathogen replication [254]. In this study, we investigate 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 observed in the HeLa 229 cells. Based on our previous observations that *C. muridarum*-induced IFN-β synthesis is largely TLR3-dependent in OE cells, we hypothesize that STAT1 is upregulated and activated subsequent to the TLR3-dependent response in OE cells, in order to sustain and amplify the type I interferon response throughout the course of infection. We also hypothesize that JAK/STAT signaling possibly serves as a compensatory mechanism for *Chlamydia*-induced IFN-β production in the absence of TLR3.

Herein, we show that STAT1 expression and activation is indeed upregulated in response to *C. muridarum* infection in OE cells, and we show that STAT1 is critical for IFN-β production at late stages of infection. We demonstrate that TLR3-deficeincy diminishes the expression and activation of STAT1 in infected OE cells during late stage infection, suggesting a dependence on the initial TLR3-dependent production of IFN-β. We also demonstrate that STAT1 may be involved in differentially modulating *Chlamydia*-induced mRNA expression of key components of the type I interferon signaling pathway, as well as, type I interferons (IFNβ/α) during early and/or late stages of infection. Our studies show that STAT1 plays a critical role in mediating the modulation of the innate inflammatory immune response in oviduct epithelial cells during late stage *Chlamydia* infections; specifically in amplifying type I interferon signaling; and regulating type I interferon production.

MATERIALS AND METHODS

I. Mice

C57BL/6 (control) and C57BL/6 (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 were immediately sacrificed upon receipt and primary oviduct epithelial cells harvested from these mice were used for experiments in this study. The Indiana University Institutional Animal Care and Utilization Committee approved all experimental protocols.

II. Reagents

Recombinant murine IFN-γ was purchased from PeproTech (Rocky Hill, NJ). The lyophilized IFN-γ was suspended in PBS supplemented with 0.1% bovine serum albumin (BSA) and frozen at -20°C until use. Immediately before use, recombinant murine IFN-γ was thawed and diluted to10 ng/ml in fresh epithelial cell media. Recombinant murine IFN-β was purchased from R&D Systems (Minneapolis, MN). The recombinant murine IFN-β was reconstituted, stored, and used as previously described [255]. Purified anti-mouse type I interferon-alpha receptor 1 (IFNAR1) neutralizing antibody against subunit 1 of the heterodimeric type I interferon receptor, and the mouse IgG1 isotype control were purchased from Biolegend (San Diego, CA). The neutralizing antibody and isotype control were stored according to manufacturers' recommendations. IFNAR neutralizing antibody and mouse isotype control were diluted to two times a working concentration of 4.61 ng/ml in fresh epithelial cell media for neutralization experiments. Rat monoclonal neutralizing antibody against murine IFN-β was purchased from Abcam, Inc. (Cambridge, MA). The neutralizing antibody was aliquoted and frozen at -80°C until use.

Rat monoclonal neutralizing antibody against murine IFN-β was thawed and diluted to two times a working concentration of 3.6 ng/ml in fresh epithelial cell media immediately before use. The isotype control antibody, as described above, was used at a similar concentration as the IFN-β neutralization antibody as a negative control. Fludarabine, a nucleoside analog and potent inhibitor of STAT1 [256] was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1 µg/ml for inhibitor experiments.

III. BMDM Isolation

The derivation and maintenance of BMDMs from C57BL/6 wild-type (WT) mice and C57BL/6 STAT1 (-/-) was achieved by carefully removing the femurs, tibias, and fibulas from the sacrificed mice followed by sterilization with 70% ethanol and repeated washes with complete RPMI media obtained from Sigma (St. Louis, MO) supplemented with 10% characterized fetal bovine serum (FBS) purchased from HyClone (Logan, UT), 2mM L-alanyl-L-glutamine (Glutamax I) purchased from Invitrogen (Grand Island, NY), 1 ml mercaptoethanol (Invitrogen) and 50 µg/ml of gentamicin (Sigma). Next, the epiphyses and diaphysis were cut from the femurs and tibias, exposing the marrow cavity. Using a 27 gauge needle, 3 ml of ice cold complete RPMI media was injected into the cavity to recover bone marrow into sterile Petri plates. The removed epiphyses and diaphysis were crushed in .5 ml of ice cold complete RPMI media to obtain residual marrow. Bone marrow red blood cells were lysed by adding 3.5 ml of ammonia/chloride/potassium (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.001 mM EDTA buffer) lysing buffer (Invitrogen) for 3 minutes at room temperature. The lysate was strained through a 40 µm screen column and centrifuged at 1200 rpm for 5 minutes. The pellet was then washed with and cells resuspended in RPMI media supplemented with

10% characterized fetal clone serum (FC3) purchased from HyClone (Logan, UT), Glutamax I (Invitrogen), 1 ml mercaptoethanol (Invitrogen) per 500 ml of RPMI media and 50 µg/ml of gentamicin (Sigma). The cells were plated at a concentration of 2 x 10^6 cells/ml of the above RPMI media. The cells were further supplemented with an equal volume of RPMI media containing 40 ng/ml GM-CSF, and plated in a 24-well culture plate until 70-80% confluency, at which time cells were passaged until needed.

IV. Cells and bacteria

The derivation and maintenance of cloned oviduct epithelial cell lines OE129 TLR3 (-/-) and OE129 WT has previously been described [44, 155, 250], and a new cloned epithelial cell line described in this report, designated OEB6 STAT1 (-/-) and OEB6 WT, were derived using a similar method. Reproductive tract tissues from C57BL/6 (control) and C57BL/6 (STAT1-deficient) female mice, encompassing a small cuff of the ovary, were harvested. Luminal epithelial cells were released with a pancreatin-hyaluronidase-collegenase mixture. The resulting epithelial cells were expanded in vitro and cloned by limiting dilution. Resulting clones were screened and epithelial cell lineage confirmed by a previously described method [155]. Selected clones were expanded and designated OEB6 STAT1 (-/-) and OEB6 WT from STAT1-deficient and WT, respectively. The upper reproductive tract epithelial cells were grown at 37°C in a 5% CO_2 humidified incubator and maintained in epithelial media: 1:1 DMEM:F12K (Sigma), supplemented with 10% characterized FBS (HyClone), 2mM L-alanyl-L-glutamine (Glutamax I; Invitrogen), 5 µg of bovine insulin/ml, and 12.5 ng/ml of recombinant human fibroblast growth factor-7 (keratinocyte growth factor; Sigma) as previously described [44, 155, 251]. WT and STAT1 (-/-) BMDMs were grown at 37°C

in a 5% CO_2 humidified incubator and maintained in RPMI media supplemented with 10% FC3 (HyClone), Glutamax I (Invitrogen), 1 ml mercaptoethanol (Invitrogen) per 500 ml of RPMI media and 50 mg/ml of gentamicin (Sigma).

Mycoplasma-free *C. muridarum*, previously known as *C. trachomatis* strain mouse pneumonitis (*MoPn),* were given to us as stocks that came from the Johnson lab originally purchased from American Type Culture Collection (Manassas, VA); and grown and titered in McCoy cells as previously described [44, 257].

V. RNA interference: generation of STAT1-knockdown OEs

Small interfering RNA (siRNA) control plasmid (psiLucGL3) and mouse STAT1 plasmid (psimSTAT1) were purchased from Invivogen (San Diego, CA). Both plasmids were used at a concentration of 1 µg and transformed into competent *E. coli* GT116 cells (Invitrogen) using the standard transformation conditions and according to the manufacturer's protocol. The transformants were grown on agar plates supplemented with 25 µg/ml of Zeocin (Invivogen); to which both plasmids were designed to confer resistance (Figure 7), and subsequent colonies selected and amplified per manufacturer's protocol using the Quick Plasmid Midi Prep Kit purchased from Qiagen (Valencia, CA). Post-amplification, the plasmid DNA concentrations were quantified via spectrophotometric analysis using the Nanodrop 2000 (Thermo Scientific; Wilmington, DE), and 10 µg of each plasmid was transfected per manufacturer's protocol into OE129 WT cells using the Amaxa Nucleofector Kit R purchased from Lonza (Basel, Switzerland). Cells were designated OE129 WT (psimSTAT1) and OE129 WT (psiLucGL3), and maintained in epithelial media at 37°C in a 5% CO_2 humidified

incubator until 70% confluent. Transfection efficiencies were determined by flow cytometric analysis for green fluorescent protein (GFP)-labeled cells.

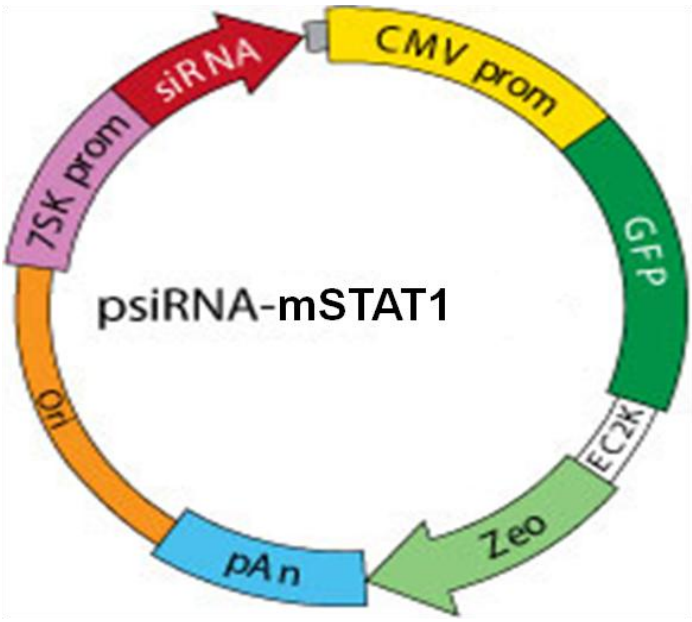


Figure 7. Basic plasmid map of psimSTAT1

The basic backbone of the ready-made plasmid used for small interfering Stat1 mRNA in OE cells is composed of Zeocin (Zeo)-resistant DNA sequences, as well as GFP; and expression is driven by the 7SK and Cytomegalovirus (CMV) promoters. The psiLucGL3 control plasmid was composed of similar DNA sequences and expression driven under similar promoters. Figure adapted from Invivogen; at www.invivogen.com.

VI. *Chlamydia muridarum* infections

All cells were plated in 24-well tissue culture plates and used when 70-80% confluent. For all experiments, the cells were infected with 10 IFUs of *C. muridarum*/cell in 750 µl of epithelial cell culture medium. The plates were centrifuged at 1200 rpm (200 × g) in a table-top centrifuge for 1 hour then incubated at 37°C in a 5% CO_2 humidified incubator without subsequent change of medium for 24 hours. Mock-infected and cytokine and/or neutralizing antibody-treated only wells received an equivalent volume of epithelial cell culture medium lacking *C. muridarum*.

VII. Enzyme-linked immunosorbent assay (ELISA) determination of cytokine production

OE129 WT, OE129 TLR3 (-/-), OEB6 WT, OEB6 STAT1 (-/-), WT BMDMs, STAT1 (-/-) BMDMs, or OE129 WT and TLR3 (-/-) transfected with either (psimSTAT1) and/or the control plasmid; were plated in 24-well tissue culture plates to 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 24 hour or the indicated timepoints, and analyzed for cytokine content using ELISAs for IFN-β and/or IL-6, as previously described [155]. All standards and experimental samples were analyzed in triplicate. The lower range of assay sensitivity for individual cytokines was 50 pg/ml and 10 pg/ml for IL-6 and IFN-β; respectively. Optical densities taken at 450 nm for quantification were measured using a microplate reader (Bio-Rad; Hercules, CA).

VIII. RT-PCR

Total RNA was isolated from mock-infected, IFN-γ-treated and *C. muridarum*-infected OE129 WT, OE129 TLR3 (-/-), OEB6 WT, and OEB6 STAT1 (-/-) cells using the RNeasy kit (Qiagen). During purification, all RNA samples were treated with RNase-free DNase I (Qiagen) to remove genomic-DNA contamination and were quantified by the Nanodrop 2000 (Thermo Scientific). Total RNA was then reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturer's protocol, using standard cycling conditions. Specific primer pairs for Stat1 and β-actin were used to amplify 1-2 μg of cDNA. Optimized primer pairs were designed by using the Primer 3 design tool [258]. The specific primer pairs (Invitrogen) used for Stat1 and β-actin are as follows: sense 5′ TCA GAA ATC CGC CTG TCT CT 3′, antisense 5′ TCA TTT CAT TTT GGC ATG GA 3′ (Stat1) and sense 5′ TAC GTA GCC ATC CAG GCT GT 3′, antisense 5′ AAG GAA GGC TGG AAA AGA GC 3′ (β-actin), respectively. Using 1-2 μg of cDNA as the template for each reaction, RT-PCR was accomplished by using iScript RT-PCR kit (Bio-Rad) as described in the manufacturers' protocol. The cycling conditions were as follows: 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 RT-PCR. Reactions were also amplified in the absence of reverse transcriptase as negative controls.

IX. Western blotting

OE129 WT, OE129 TLR3 (-/-), OEB6 WT, and OEB6 STAT1 (-/-) cells were plated in 24-well tissue culture plates to 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 µl/ml (Sigma); incubated on ice for 30 minutes. Cell lysates were clarified by centrifugation at 14,000 rpm (2400 × g) and proteins were quantified and separated by SDS-PAGE as previously described [251]. After proteins were transferred to nitrocellulose transfer membranes (Bio-Rad), the transfer membranes were blocked according to the manufacturer's protocol with a protein-free Tris-buffered saline (pH 7.4) containing 0.05% Tween-20 blocking buffer purchased from Pierce (Rockland, IL). The proteins were stained by immunoblotting with either a 1:2000 dilution of a murine antibody against total STAT1, a 1:2500 dilution of phosphorylation-specific (pY701) STAT1 antibody (both obtained from BD Bioscience (San Jose, CA)), or a 1:3000 dilution of phosphorylation-specific (Serine residue 396) IRF3 antibody purchased from Cell Signaling (Boston, MA). Protein-antibody complexes were detected by secondary blotting with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (Pierce). Proteins were visualized using the ECL western blotting substrate (Pierce) as described in the manufacturer's protocol.

X. Recombinant cytokine treatment

To serve as a positive control for the induction of STAT1 protein activation and STAT1 protein/Stat1 gene expression, recombinant murine IFN-γ was added directly to the media of OE129 WT, OE129 TLR3(-/-), OEB6 WT, and OEB6 STAT1(-/-) cells at 10 ng/ml for 4-6 hours (18-20 h post mock-infection). Recombinant IFN-β treatment was

achieved by adding recombinant IFN-β directly to the medium of the cells at 10 U/ml for 4 hours (20 h post mock-infection) for most experiments.

XI. Neutralization Experiments

For IFNAR neutralization experiments, the OE cells (OE129 WT, TLR3 -/-, STAT1 -/-) were either infected with *C. muridarum* or treated with IFN-β, and the IFNAR activity blocked with a neutralizing antibody against murine IFNAR1 at both early-stage infection (6hr) and late-stage infection (16hr). For 6 hour infections, cells were infected with *C. muridarum* and treated with IFNAR neutralizing antibody added to the cell supernatants at time 0 (T_0) at the indicated concentration for the duration of the infection. The cells treated with recombinant IFN-β only received equal volumes of epithelial media, incubated at 37°C for 1 hour; after which, recombinant IFN-β was added to the cell supernatants at the indicated concentration and cell lysates and supernatants were harvested 5 hours later (6 hour infection). The cells treated with recombinant IFN-β and IFNAR neutralizing antibody received each treatment at the indicated concentrations, simultaneously, at T_0 and cell lysates and supernatants harvested at 6 hours. Normal mouse IgG1 isotype control antibody was used in a similar fashion in conjunction with recombinant IFN-β to serve as a negative control. For 16 hour infections, cells were infected with *C. muridarum* and treated with IFNAR neutralizing antibody added to the cell supernatants 11 hours post-infection time 11 (T_{11}) at the indicated concentration; and cell lysates and supernatants were harvested 5 hours later (16 hour infection). The cells treated with recombinant IFN-β only received equal volumes of epithelial media, incubated at 37°C for 1 hour; after which, recombinant IFN-β was added to the cell supernatants at the indicated concentration for 9 hours. After the 9

hour incubation, the epithelial media was removed and replaced with fresh media. The cells incubated at 37°C for 1 hour; after which, recombinant IFN-β was added to the cell supernatants at the indicated concentration and cell lysates and supernatants were harvested 5 hours later (16 hour infection). The cells treated with recombinant IFN-β and IFNAR neutralizing antibody received equal volumes of epithelial media, incubated at 37°C for 1 hour; after which, recombinant IFN-β was added to the cell supernatants at the indicated concentration for 9 hours. After the 9 hour incubation, the epithelial media was refreshed and the cells incubated at 37°C for 1 hour; after which, IFNAR neutralizing antibody was added to the cell supernatants at the indicated concentrations and incubated at 37°C for 1 additional hour. Following the 1 hour incubation of the IFNAR neutralizing antibody, recombinant IFN-β was added to the cell supernatants at the indicated concentration and cell lysates and supernatants were harvested 4 hours later (16 hour infection). Normal mouse IgG1 isotype control antibody was used in a similar fashion in conjunction with recombinant IFN-β to serve as a negative control.

IFNAR neutralization experiments were also performed for OE129 WT cells infected with *C. muridarum* for 24 hours and treated with IFNAR neutralizing antibody at the indicated concentrations; at early (T_0) versus late (T_{16}) points in infection. The cells were mock-infected or infected with *C. muridarum* and supernatants harvested at 24. Cells were also *Chlamydia*-infected and treated with IFNAR neutralizing antibody or the normal mouse isotype control at T_0 and supernatants collected at 24 hours. Additionally, the WT cells were *Chlamydia*-infected and treated with IFNAR neutralizing antibody or the normal mouse isotype control at T_0 and supernatants collected at 16 hours; followed by the addition of fresh epithelial cell media and treatment with IFNAR neutralizing

antibody or the normal mouse isotype control at T_{16} and cell supernatants collected 8 hours later (24 hour infection). All supernatants collected were analyzed for IFN-β production via ELISA.

For IFN-β neutralization experiments OE129 WT cells were either infected with *C. muridarum* or treated with IFN-β and the IFN-β activity blocked with a monoclonal neutralizing antibody against murine IFN-β at both early-stage infection (6hr) and late-stage infection (16hr). For 6 hour infections, OE129 WT cells were infected with *C. muridarum* and treated with IFN-β neutralizing antibody added to the cell supernatants 1 hour post-infection at time 1 (T_1) at the indicated concentration for the duration of the infection. Next, recombinant IFN-β and the IFN-β neutralizing antibody were allowed to incubate together at the indicated concentrations for 1 hour at room temperature in order to form specific antigen-antibody (Ag-Ab) complexes before being added to the culture media. Cell lysates and supernatants were harvested 5 hours later (6 hour infection). Normal mouse IgG1 isotype control antibody was used in a similar fashion in conjunction with recombinant IFN-β to serve as a negative control. For 16 hour infections, OE129 WT cells were infected with *C. muridarum* and treated with IFN-β neutralizing antibody added to the cell supernatants 1 hour post-infection (T_1) at the indicated concentration and allowed to incubate for 6 hours; after which, the epithelial media was removed and replaced with fresh media. The cells incubated at 37°C for 1 hour. At 8 hours post-infection, IFN-β neutralizing antibody was added to the supernatant and cell lysates and supernatants were harvested 8 hours later (16 hour infection). Recombinant IFN-β and the IFN-β neutralizing antibody were allowed to form Ag-Ab complexes as previously described and added to the cells supernatants at 8 hours post-

incubation at 37°C; cell lysates and supernatants were harvested 8 hours later (16 hour infection). Normal mouse IgG1 isotype control antibody was used in a similar fashion in conjunction with recombinant IFN-β to serve as a negative control. The cells treated with recombinant IFN-β only were treated in a similar manner as in the IFNAR neutralization experiments for both 6 and 16 hour time-points.

XII. Quantitative real-time PCR

Total RNA was purified from OE129 WT, OE129 TLR3 (-/-), and OEB6 STAT1 (-/-) cells using the RNeasy Plus kit (Qiagen). Specialized spin columns provided in the kit were used for all samples during purification to ensure the removal of genomic-DNA contamination; however, each sample was subjected to an addition on-column DNase I treatment to ensure complete removal of cellular and bacterial DNA. The purified RNA was quantified by spectrophotometric analysis. Total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol and using standard cycling conditions. Optimized primer pairs were designed from published data and reputable databases [259, 260]. The cDNA product was diluted 1:20, and the Ifnb-specific, Irf3-specific, Irf7-specific, Stat1-specific, Stat2-specific, Ifna4-specific, Ifna2-specific, and β-actin-specific control primers (Table I) were adjusted to 2 pmol/µl working stock. Quantitative real time PCR 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), and as described [251]. Dissociation curves were recorded after each run to ensure primer specificity.

Primer Name	Sense Primer	Antisense Primer	Product Size (bp)
Ifnb	*AAGAGTTACACTGCCTTTGCCATC*	*CACTGTCTGCTGGTGGAGTTCATC*	110
Ifna2	*AGCAGATCCAGAAGGCTCAA*	*CATTCCAAGCAGCAGATGAA*	99
Ifna4	*TTCTGCAATGACCTCCATCA*	*TATGTCCTCACAGCCAGCAG*	101
Stat1	*CGGAGTCGGAGGCCCTAAT*	*ACAGCAGGTGCTTCTTAATGAG*	140
Stat2	*TTTGGCTACCTGGATTGAAGAC*	*GGCTGAATTTTCGCAAGTTATGC*	170
Irf7	*CAATTCAGGGGATCCAGTTG*	*AGCATTGCTGAGGCTCACTT*	112
Irf3	*GATGGCTGACTTTGGCATCT*	*ACCGGAAATTCCTCTTCCAG*	104
β-actin	*GGCTGTATTCCCCTCCATCG*	*CCAGTTGGTAACAATGCCATGT*	154

Table 1. Primers used in quantitative real-time PCR experiments. All primers are listed in the 5′ to 3′ orientation. Control reactions were set up with the β-actin primers as the loading control.

XIII. 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 hours post infection, the infected cells were then fixed with 200 µl of methanol and incubate at room temperature for 10 minutes. The fixed cells were stained with a murine anti-*Chlamydia* LPS antibody, provided by Dr. Wilbert J. Newhall [261], diluted 1:500 in PBS and incubated for 1 hour 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) diluted 1:50 in PBS.

XIV. STAT1 Inhibitor Experiment

In 24-well tissue culture plates, OE129 WT cells were mock-infected, infected with *C. muridarum*, or *C.muridarum*-infected with fludarabine-treatment at 4-, 6-, or 16-hours post-infection at a concentration of 1 μg/ml and infections carried out to the 24-hour time-point. The cell supernatants were harvested and analyzed for IFN-β production.

XV. Statistical Analysis

Data are expressed as means ± SD. All experiments were repeated at least three times, and statistical significance determined using Student's two-tailed *t* test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

CHAPTER I. ESTABLISH A ROLE FOR STAT1 IN REGULATING *CHLAMYDIA*-INDUCED TYPE I IFN RESPONSES IN OE CELLS

A. *C. muridarum* induces increased STAT1 protein activation/expression and Stat1 gene expression

Reports have shown that *C. trachomatis* induced significantly increased STAT1 protein activation and expression in HeLa 229 cells, albeit in an IFN-β-dependent manner [254]. In an effort to identify a role for STAT1, in the context of type I interferon synthesis and signaling in *C. muridarum*-induced OE cells, we first examined Stat1 gene expression and STAT1 protein activation in wild-type OE cells via RT-PCR and western blot analysis, respectively. We utilized two wild-type OE cell lines designated OE129 WT and OEB6 WT that were either mock-infected, treated with IFN-γ (10 ng/ml), or infected with 10 IFU/cell of *C. muridarum*. Representative data was taken from the 24 hour time point whereby we assessed Stat1 expression and STAT1 expression/activation by RT-PCR and by western blot. *C. muridarum*-infection significantly increased Stat1 mRNA levels (as compared to mock-infected controls) in both wild-type OE cell lines (Figure 8). Interferon-gamma is a well-known inducer of Stat1 expression and STAT1 activation [182, 216] and *C. muridarum*-induced Stat1 mRNA expression at a level comparable to that of the IFN-γ-treated controls (Figure 8). Increased gene expression does not necessarily correspond to increased protein expression or activity. We performed western blot analysis to examine total STAT1 protein expression and activation (phosphorylation) upon *C. muridarum* infection. *C. muridarum*-infected wild-type OE cells expressed significantly greater total STAT1 and increased STAT1

phosphorylation as compared to mock-infected controls (Figure 9). These increases in total STAT1 protein expression and STAT1 activation were also comparable to those of the IFN-γ-treated controls (Figure 9). Collectively, the data indicate that *C. muridarum* induces Stat1/STAT1 expression and STAT1 activation in OE cells in a manner similar to that which was observed in studies describing STAT1 activation in HeLa 229 cells infected with *C. trachomatis* [254].

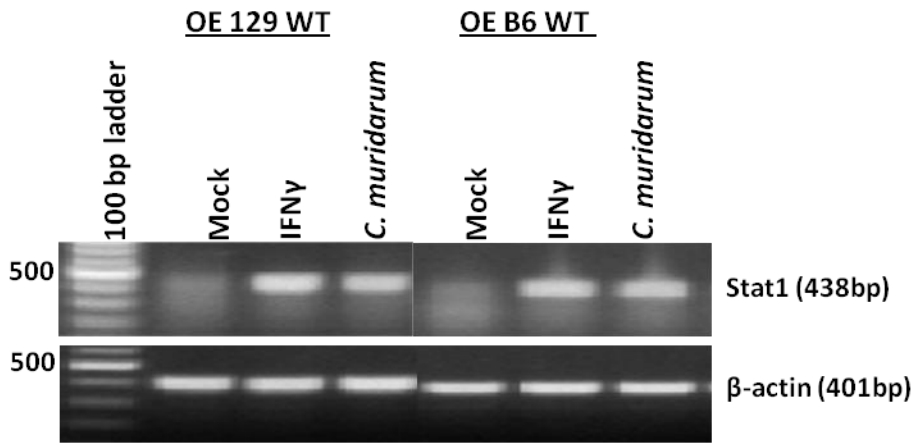


Figure 8. *C. muridarum* infection increases Stat1 mRNA message relative to mock-infected controls in WT oviduct epithelial cells

Semi-quantitative RT-PCR was performed using 1 µg of cDNA reverse transcribed from RNA isolated from WT OEs that were mock-infected, infected with *C. muridarum* at 10 multiplicity of infection (MOI) for 24 hours, or treated with 10 ng/ml of IFN-γ for 4-5 hours. Stat1 primers were used to amplify Stat1 mRNA message; and β-actin primes were used to amplify β-actin mRNA message as a loading control.

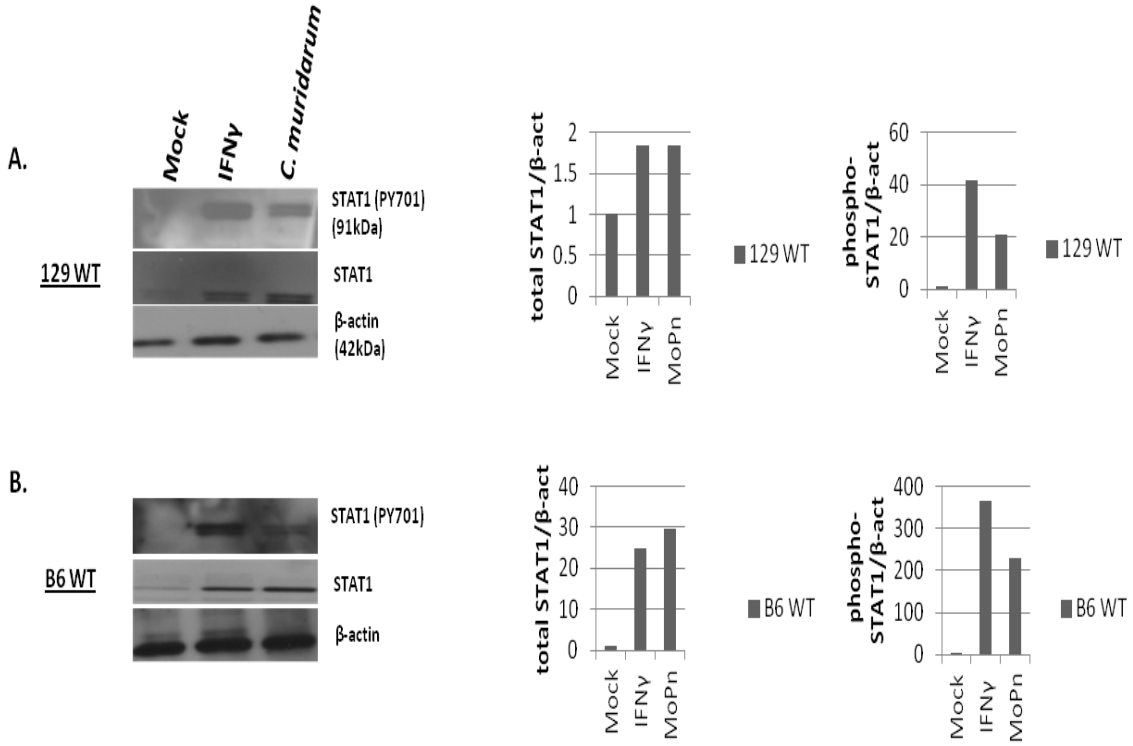


Figure 9. *C. muridarum* infection increases STAT1 protein expression and activation relative to mock-infected controls in WT OEs

Twenty microgram of protein in whole cell lysates from: (A.) OE129 WT or (B.) B6 WT OEs that were mock-infected, infected with *C. muridarum* at 10 MOI for 24 hours, or treated with 10 ng/ml of IFN-γ for 4-5 hours; were separated on a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with antibody to phosphorylated STAT1 (tyrosine residue 701), total STAT1 and β-actin. β-actin served as a loading control. The results shown are representative of three independent experiments. Densitometric analysis of total STAT1 expression and STAT1 phosphorylation are shown for each cell line relative to the β-actin control (indicated as β-act in the graphs), and *C. muridarum*-infection indicated by MoPn.

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

We described an upregulation in Stat1/STAT1 expression and STAT1 activation in response to *C. muridarum* infection of wild-type OE cells that were harvested at 24hr post-infection (PI) in the previous data set. Therefore, we sought to determine if STAT1 played a functional role in *Chlamydia*-induced IFN-β synthesis. To ascertain whether the increased Stat1 gene expression and STAT1 protein activation contributed to the *C. muridarum*-induced IFN-β response in OE cells, OEB6 WT and OE STAT1 (-/-) cells derived from C57BL/6 control and STAT1-deficient mice respectively, were infected with *C. muridarum*, and the amount of IFN-β secreted in supernatants at 24 hr PI was measured (Figure 10). As shown, IFN-β production was significantly increased upon *Chlamydia* infection in B6 WT cells (compared to mock-infected cells); however, the amount of *C. muridarum* induced IFN-β synthesis at 24 hr PI in the OE STAT1 (-/-) cells was substantially reduced and was not significantly different than that of mock-infected controls. We observed similar results using BMDMs that were isolated from wild-type and STAT1-deficient mice and were infected with 10 IFU/cell *C. muridarum* (data not shown).

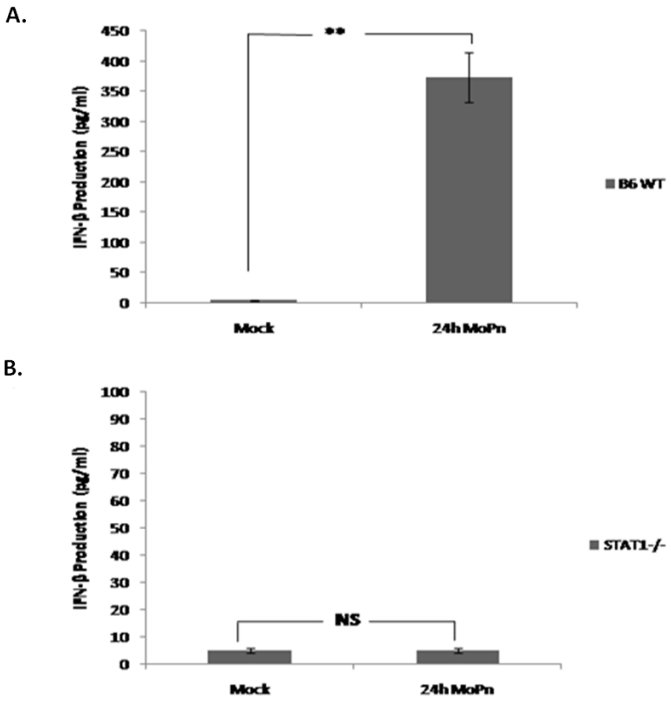


Figure 10. *C. muridarum*-induced IFN-β production is diminished in STAT1-deficient OEs

OEB6 WT cells and STAT1-deficient (STAT1-/-) OE cells were mock-infected and/or infected with 10 MOI of *C. muridarum* (denoted as MoPn), for 24 hours. ELISA was used to measure infection-induced IFN-β secreted into the supernatants of mock-infected and *C. muridarum*-infected: (A.) B6 WT cells, and (B.) STAT1 (-/-) cells. The data represent mean ± SD and are representative of three different experiments conducted in triplicate. Significance was determined using Student's T test; **=p value <0.005 for mock-infected vs MoPn-infected; NS=not significant.

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

We have provided data that highlights STAT1 as a critical mediator in the synthesis of type I interferons (IFN-β) in *C. muridarum*-infected oviduct epithelial cells; particularly, in the secretion of IFN-β at late stages of infection and in accordance with previous studies implicating a TLR3-dependent IFN-β response in these cells. In addition, we have provided both *in vitro* and *in vivo* evidence implicating the TLR3-dependent IFN-β response in restricting *C. muridarum* growth and/or replication [250, 255]. Our data in those studies demonstrate substantially increased *Chlamydia* replication in TLR3-deficient mice and OE cells that likely reflects the significant decrease in the amount of IFN-β produced when compared to wild-type mice and OE cells. Therefore, we wondered if STAT1 would have a functional role in restricting *C. muridarum* replication in OE cells. To indirectly assess the requirement for STAT1 in *Chlamydia*-induced IFN-β production we observed the *in vitro* effects of STAT1-deficiency on *C. muridarum* growth and replication in OE cells. Wild-type and STAT1-deficient OE cells were mock-infected or infected with 10 MOI *C. muridarum* for 24 hours followed by methanol fixation, and the detection of viable *Chlamydia* IFUs with a *Chlamydia*-specific antibody against LPS. As shown, *Chlamydia* IBs were detected in many of the *C. muridarum*-infected WT OEs as small round inclusions within the cell compared to mock-infected WT controls (Figure 11). However, the STAT1-deficient OE cells displayed aberrantly shaped *Chlamydia* IBs that appeared to span the entire area of the cells and were detectable in most of the cells compared to WT OEs (Figure 11); suggesting that STAT1 plays a critical role in restricting *C. muridarum* growth and

further supporting our observations that STAT1 is a key mediator in *C. muridarum*-induced type I IFN production.

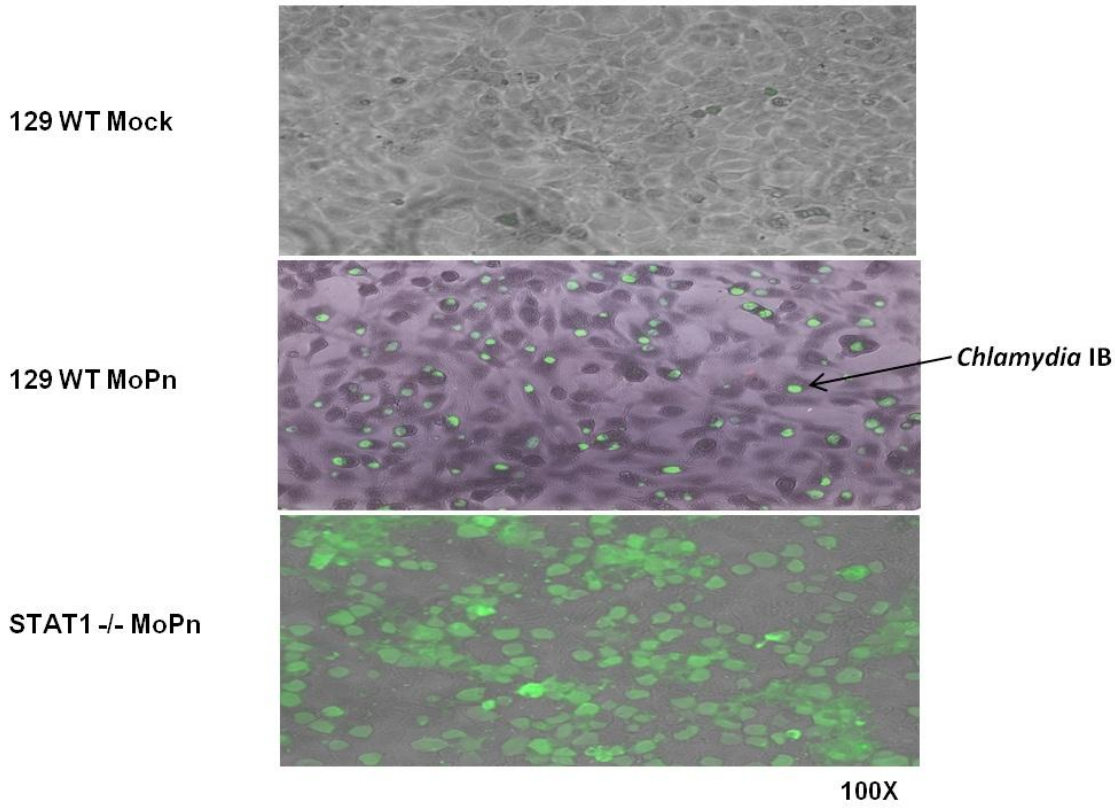


Figure 11. *C. muridarum*-infected STAT1-deficient OE cells display aberrantly shaped *Chlamydia* inclusion-forming units; implicating uncontrolled *C. muridarm* replication

OE129 WT and OE STAT1 (-/-) cells were mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for 24 hours. 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* inclusion bodies (IBs) via fluorescent microscopy. Note that OE 129 WT cells were used as the control instead of OE B6 WT cells due to delayed cellular growth rates and faster *Chlamydia* clearance rates in the OE B6 WT compared to the OE 129 WT cells. Original magnification was at 100X.

D. STAT1 is critical for up-regulating Ifnb transcription during late-stage *C. muridarum* infection

Our previous data reveals that STAT1 plays a role in *Chlamydia*-induced IFN-β production in OE cells. These data were analyzed at 24 hours post-infection; representative of late-stage *Chlamydia*-infection. We sought to determine if STAT1 would also play a role in early-stage *Chlamydia*-induced IFN-β production in OE cells. To address the role of STAT1 in sustaining and amplifying *Chlamydia*-induced type I IFN production in OE cells and to delineate its role as either an early-stage or late-stage factor in the synthesis of type I IFNs, we infected wild-type, STAT1-deficient, and TLR3-deficient OE cells with *C. muridarum*, and isolated total cell RNA at either 6 hr (early-stage) or 16 hr (late-stage) time points. Quantitative real-time PCR was used to analyze and compare the subsequent effects on gene transcription of Ifnb and several signaling components that comprise the type I IFN signaling pathway including Stat1, Stat2, and Irf7 [262]. *C. muridarum* induced Ifnb gene expression in both the OE129 WT and TLR3 (-/-) cells at 6 hr PI (Figure 12), and this induction was significantly increased in both cell lines at the 16 hr time-point (Figure 13). The 10-fold higher induction of the Ifnb gene at 16 hr PI in the OE129 WT cells was substantially greater than that of the TLR3-deficient cells (2-fold) (Figure 13), which corroborates our previous studies describing the importance of TLR3 in the *Chlamydia* induced synthesis of IFN-β in OE cells [250]. However, although STAT1-deficient OE cells exhibited a >2-fold induction in the expression of the Ifnb gene in response to *C. muridarum* infection at 6 hr PI,

these cells exhibited no further induction at the 16 hr time-point (Figure 14); corroborating the IFN-β protein data (Figure 10), and suggesting a critical role for STAT1 in upregulating IFN-β synthesis at late times during infection.

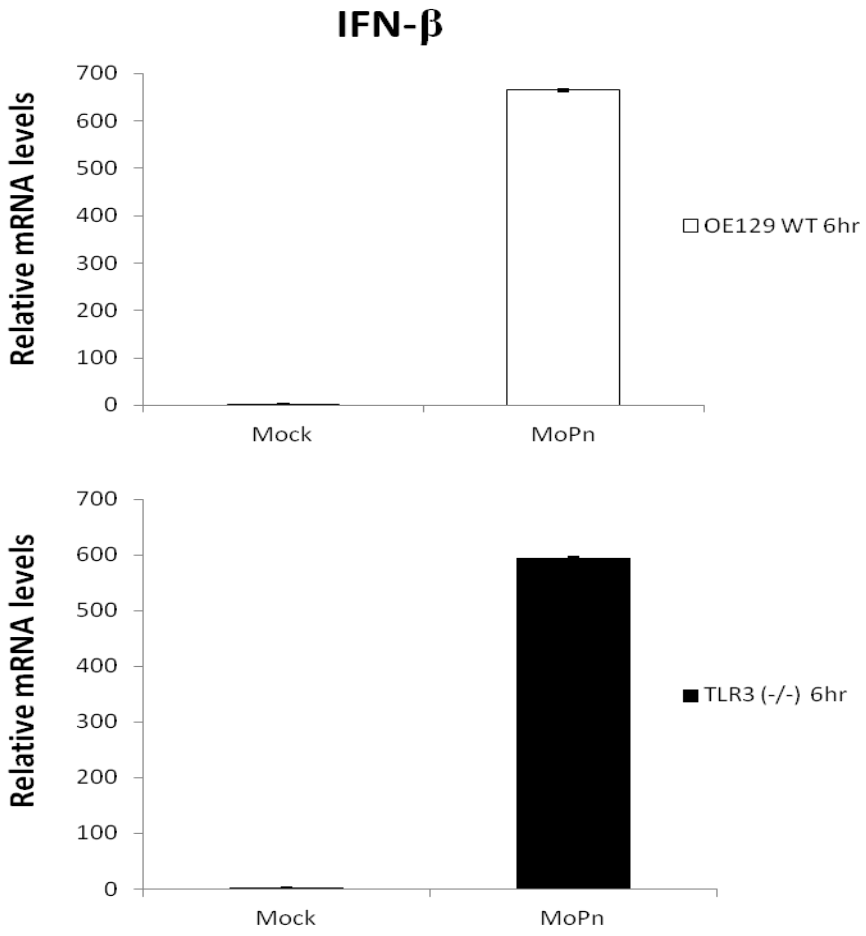


Figure 12. *C. muridarum*-induced Ifnb gene expression is induced to similar levels early in infection in both WT and TLR3-deficient OEs

Quantitative real-time PCR was performed using 1 µg of cDNA reverse transcribed from RNA isolated from OE129 WT and OE TLR3 (-/-) cells that were either mock-infected or infected with 10 MOI of *C. muridarum* (denoted as MoPn) for 6 hours. IFN-β primers were used to amplify Ifnb message mRNA. Control reactions were set up with β-actin primers to ensure equal loading of mRNA. The results are presented as mean fold change ± SD; and are representative data from one of three individual experiments conducted in duplicate.

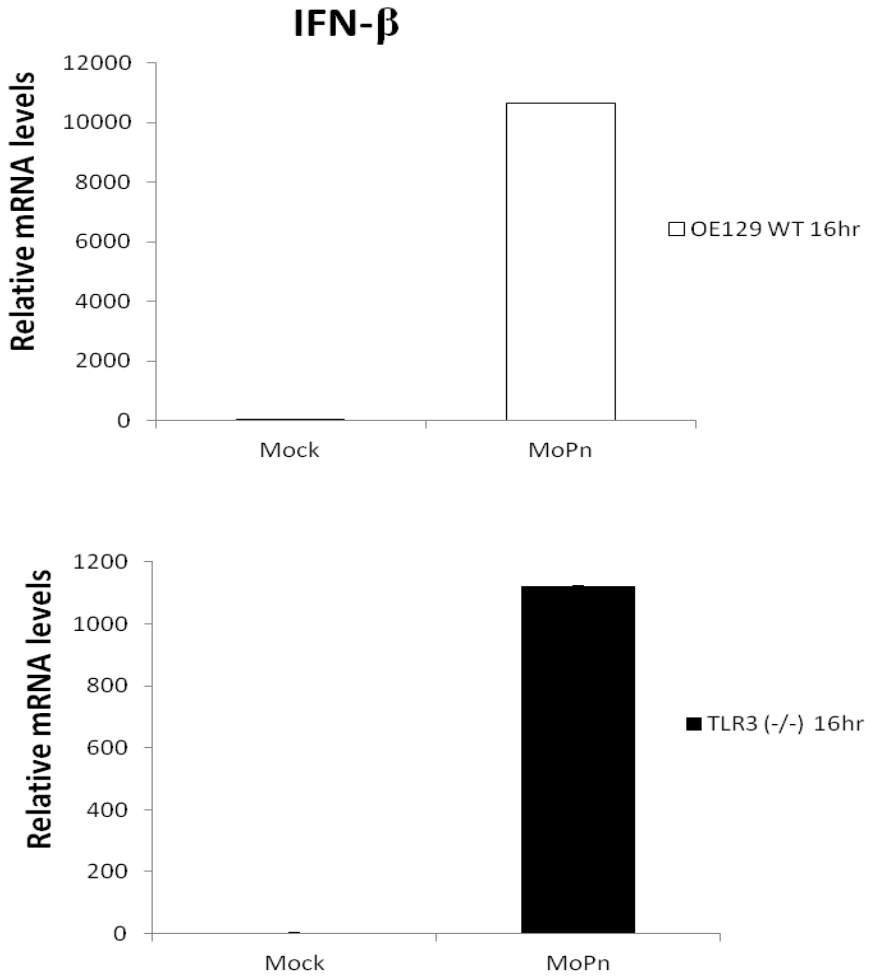


Figure 13. *C. muridarum*-induced Ifnb gene expression substantially reduced in TLR3-deficient OEs at 16 hr post infection

Quantitative real-time PCR was performed using 1 µg of cDNA reverse transcribed from RNA isolated from OE129 WT and OE TLR3 (-/-) cells that were either mock-infected, or infected with 10 MOI of *C. muridarum* (denoted as MoPn) for 16 hours. IFN-β primers were used to amplify Ifnb message mRNA. Control reactions were set up with β-actin primers to ensure equal loading of RNA. The results are presented as mean fold change ± SD; and are representative data from one of three individual experiments conducted in duplicate.

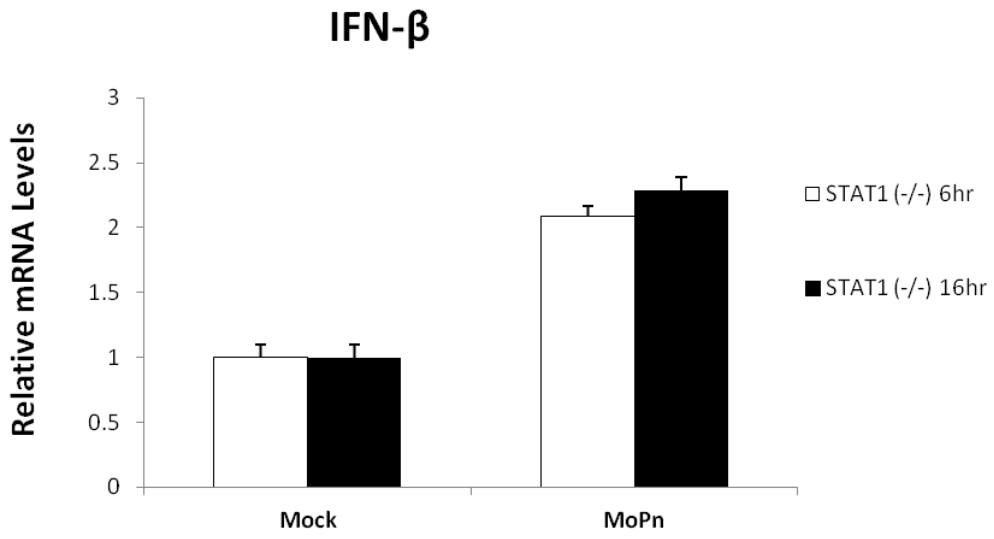


Figure 14. STAT1 is indispensable for effective *Chlamydia*-induced Ifnb gene induction in OE cells

Quantitative real-time PCR was performed using 1 µg of cDNA reverse transcribed from RNA isolated from STAT1-deficient (-/-) OE cells that were either mock-infected, or infected with 10 MOI of *C. muridarum* (denoted as MoPn) for 6 hours (white bars) or 16 hours (black bars). IFN-β primers were used to amplify Ifnb message mRNA. Control reactions were set up with β-actin primers to ensure equal loading of RNA. The results are presented as mean fold change ± SD; and are representative data from one of three individual experiments conducted in duplicate. Because the represented cell line in this figure and cell lines from the previous figures (Figure 12 and Figure 13) were analyzed on the same 96-well plate for each time point, we will refer to Figures 12 and 13 to compare Ifnb mRNA levels of the WT control cell line. Again, OE 129 WT cells were used as the WT control instead of OE B6 WT cells due to delayed cellular

growth rates and faster *Chlamydia* clearance rates in the OE B6 WT compared to the OE 129 WT cells.

We next examined the *Chlamydia*-mediated gene induction of signaling mediators found within the type I IFN signaling pathway. As shown, both Stat2 and Irf7 genes were induced at similar levels, for both time-points, in the wild-type and TLR3-deficient OE cells (Figure 15 B and Figure 15 C). This data suggests that the type I IFN signaling pathway is intact and induced at similar levels, despite the substantial difference in IFN-β synthesis (Figure 13). OE cells deficient in STAT1 showed minimal upregualtion in either Stat2 or Irf7 (Figure 15 B and Figure 15 C); implicating a requirement for STAT1 in the inductions of genes found in the type I IFN signaling pathway. Interestingly, *C. muridarum* mediated Stat1 gene induction at 16 hr PI was substantially lower in the TLR3-deficient cells than the wild type OE cells (Figure 15 A), despite almost equal levels in the gene induction of Stat2 and Irf7 (Figure 15 B and Figure 15 C). Note that all genes, Stat1, Stat2, and Irf7 were substantially induced at late-stage infections (16 hours). The almost 2-fold more induction of Stat1 gene expression in the wild-type versus TLR3-deficient OE cells (Figure 15 A), combined with the equivalent levels of induction of other genes found in the type I IFN signaling pathway, further proposes a role for STAT1 in the *Chlamydia*-mediated synthesis of IFN-β presumably via the type I IFN signaling pathway.

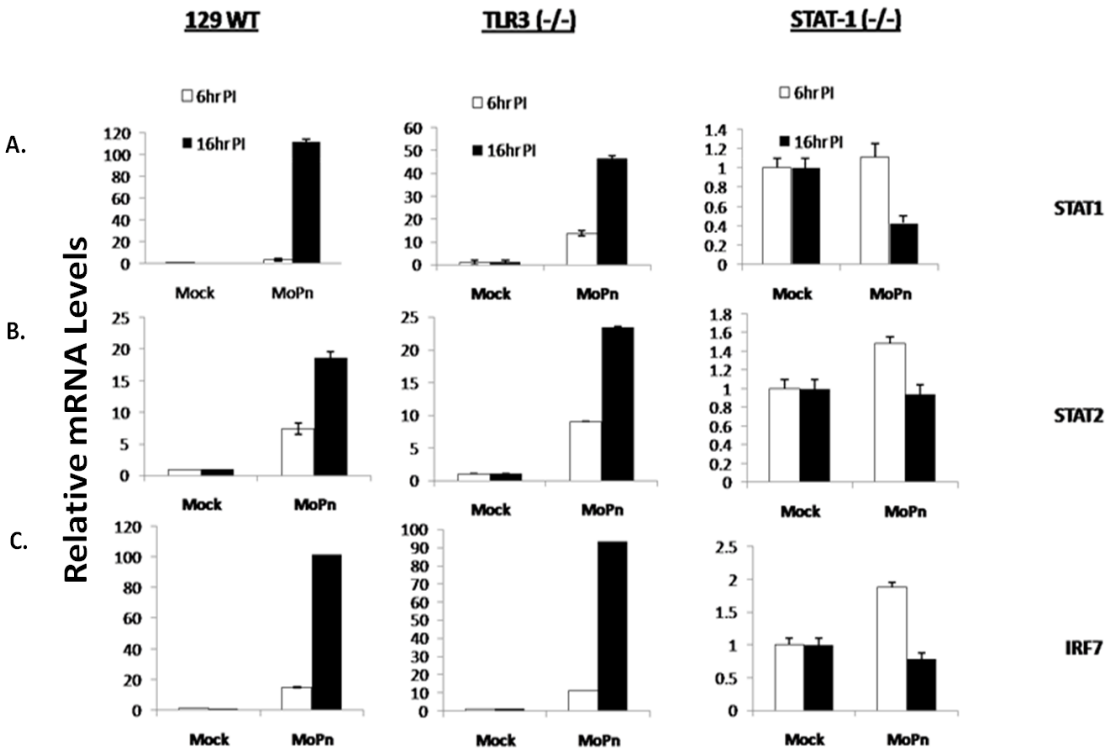


Figure 15. *C. muridarum*-induced upregulation of components of the type I IFN signaling pathway substantially occur during the late stage of infection and is STAT1-dependent

OE129 WT, OE TLR3 (-/-) and OE STAT1 (-/-) cells were mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 (white bars) or 16 (black bars) hours. Quantitative real-time PCR to measure: (A.) Stat1 mRNA, (B.) Stat2 mRNA, or (C.) Irf7 mRNA; was performed using 1 µg of cDNA from total-cell RNA isolated from each cell type for each condition with the respective primers (see 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 ± SD; and are representative data from one of three individual experiments conducted in duplicate.

E. *C. muridarum*-induced type I IFN production is highly dependent on STAT1, but is differentially regulated by type I signaling via the IFNAR in OE cells

Our data proposes a critical role for STAT1 in the optimal synthesis of IFN-β and, presumably, additional type I IFNs during *Chlamydia* infection of OE cells. We also showed that components of the type I IFN signaling pathway were substantially upregulated during late-stage *Chlamydia* infection, interestingly, in a STAT1-dependent manner. We hypothesized that, via a positive-feedback loop initiated by early-stage TLR3-dependent IFN-β and the IFNAR, STAT1's role as a mediator of type I IFN signaling is crucial for the optimal production or amplification of IFN-β during late-stage *Chlamydia*-infections in OE cells. To examine whether the type I IFN pathway is involved in the *Chlamydia*-induced IFN-β production and in the synthesis of other type I IFNs in OE cells, we measured levels in the gene expression of candidate type I IFN genes induced by *C. muridarum* at 6 hr and 16 hr PI, in the presence or absence of neutralizing antibody specific for the interferon-α/β receptor (IFNAR). OE129 WT, OE TLR3 (-/-) and OE STAT1 (-/-) cells were either mock-infected, or *C. muridarum*-infected with or without neutralizing antibody specific for IFNAR. *C. muridarum* infection induced expression of the Ifna2 gene in wild type and TLR3-deficient OE cells at early and late times during infection, though the OE cells made substantially more Ifna2 early in the infection (6 hr) (Figure 16). As shown, the synthesis of Ifna2 at early times during infection appeared to be mostly dependent on STAT1 in the OE cells, since OE cells lacking STAT1 were not able to induce Ifna2 gene expression at 6 hr PI (Figure 18 A). Unlike as seen in the induction of the Ifnb gene during *C. muridarum* infection (Figure 13), the induction of Ifna2 appeared to be attenuated by the presence of TLR3 in

OE cells (Figure 16 B); suggesting a possible negative regulation of Ifna2 gene expression by stimulation of TLR3 signaling during *Chlamydia* infection. Blocking the type I IFN pathway with IFNAR neutralizing antibody led to significant reductions in the *C. muridarum*-induced synthesis of Ifna2 in both cell types at early and late infection (Figure 16), implicating an important role for the type I IFN pathway in the *C. muridarum*-induced synthesis of Ifna2. Interestingly, *C. muridarum* was still able to induce expression of Ifna2 at 16 hr PI in the STAT1-deficient OE cells (Figure 18 A); however, its induction appeared to be only minimally dependent on the type I IFN pathway in the absence of STAT1.

We next examined the role of the type I IFN signaling pathway on the expression of Ifna4. *C. muridarum* infection induced Ifna4 gene expression at 6 and 16 hours PI in both wild type and TLR3 deficient OE cells (Figure 17). However, in contrast to what was observed with Ifna2 gene expression, most of the Ifna4 gene expression occurred late (16 hr) in infection (Figure 17). The expression of Ifna4 was also highly dependent on STAT1 at both time points, given that OE cells lacking STAT1 were unable to induce Ifna4 gene expression in response to *C. muridarum* (Figure 18 B). Interestingly, blocking the type I IFN pathway with neutralizing antibody specific for IFNAR led to significant reductions in wild-type OE cells at the 16 hr time point, but not at the 6 hr time point (Figure 17 A). These results suggest that the *Chlamydia*-induced Ifna4 production in wild-type OE cells is highly dependent on STAT1, but occurs through pathways that are distinct from the type I IFN signaling pathway via the IFNAR early during infection. Also, stimulation of the TLR3 signaling pathway during *Chlamydia* infection appears to

attenuate the expression of Ifna4 in the OE cells, which is demonstrated by the >15 fold more induction in the TLR3-deficient OE cells (Figure 17 B).

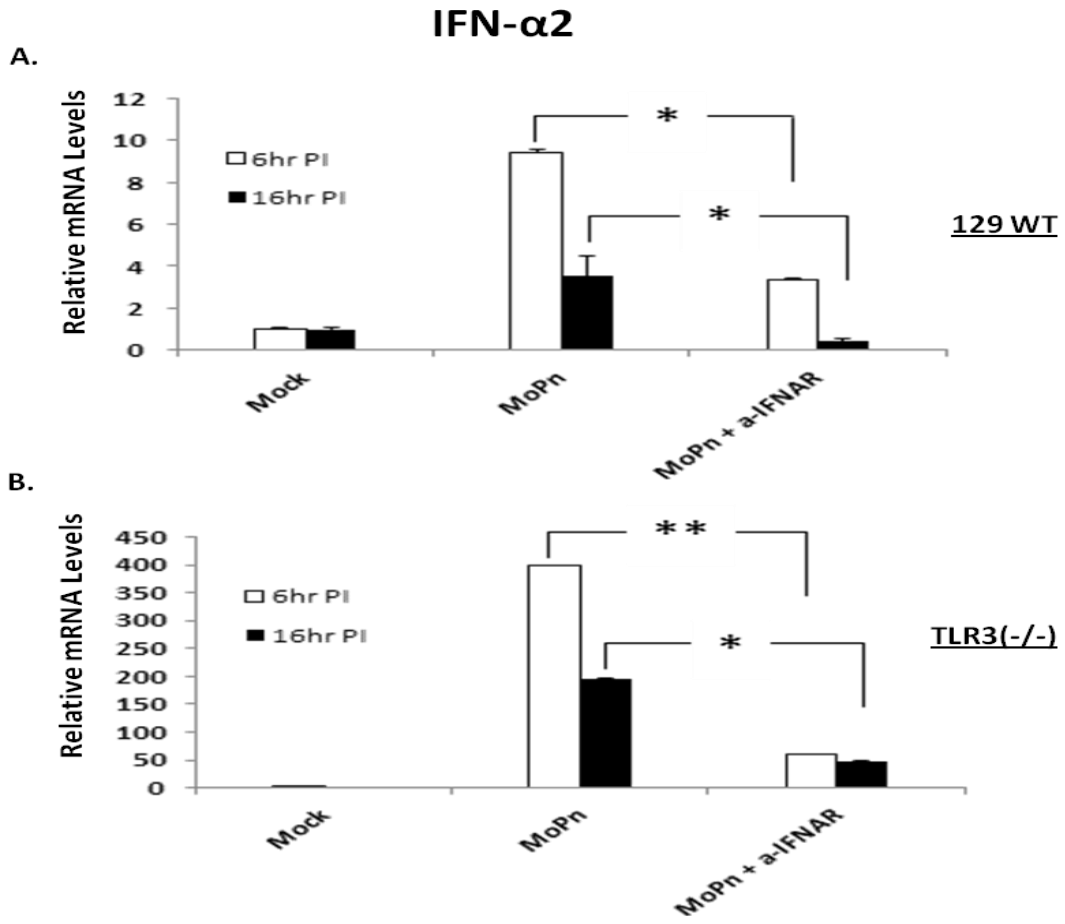


Figure 16. IFNα-2 gene induction occurs preferentially during early-stage *Chlamydia* infection, is IFNAR1-dependent, and is attenuated by TLR3 in OE cells

OE129 WT and OE TLR3 (-/-) cells were mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 (white bars) or 16 (black bars) hours with or without IFNAR neutralizing antibody (denoted as a-IFNAR), as described in Materials and Methods. Quantitative real-time PCR to measure Ifna2 mRNA was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from each cell type for each condition with the respective primers (see 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 ± SD; and are representative data from one of three individual experiments

conducted in duplicate. Significance was determined using Student's T test; *=p value <0.05; **=p value <0.005 for the indicated comparisons (MoPn-infected vs MoPn-infected with IFNAR neutralizing antibody); NS=not significant.

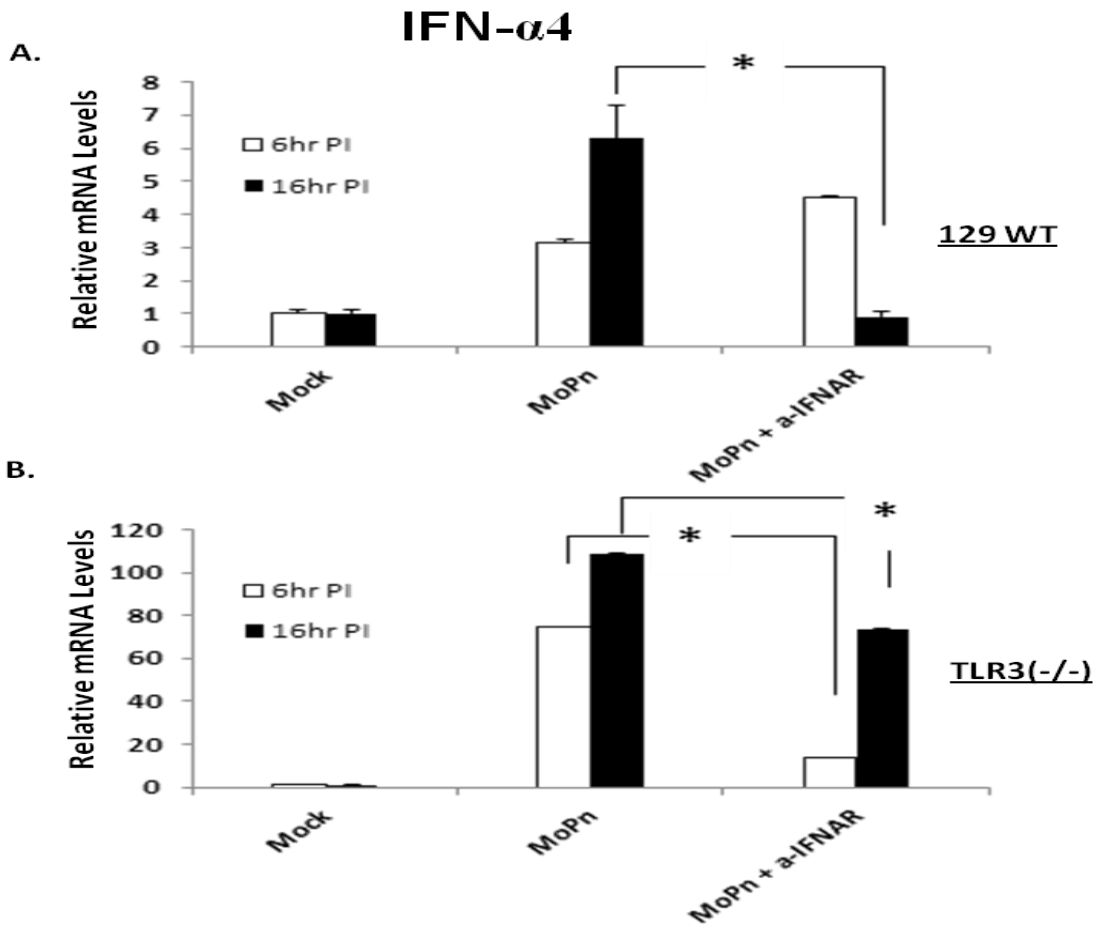


Figure 17. IFNα-4 gene induction occurs preferentially during late-stage *Chlamydia* infection, is IFNAR1-dependent, and is attenuated by TLR3 in OE cells

OE129 WT and OE TLR3 (-/-) cells were mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 (white bars) or 16 (black bars) hours with or without IFNAR neutralizing antibody (denoted as a-IFNAR), as described in Materials and Methods. Quantitative real-time PCR to measure Ifna4 mRNA was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from each cell type for each condition with the respective primers (see 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 ± SD; and are representative data from one of three individual experiments

conducted in duplicate. Significance was determined using Student's T test; *=p value <0.05 for the indicated comparisons (MoPn-infected vs MoPn-infected with IFNAR neutralizing antibody); NS=not significant.

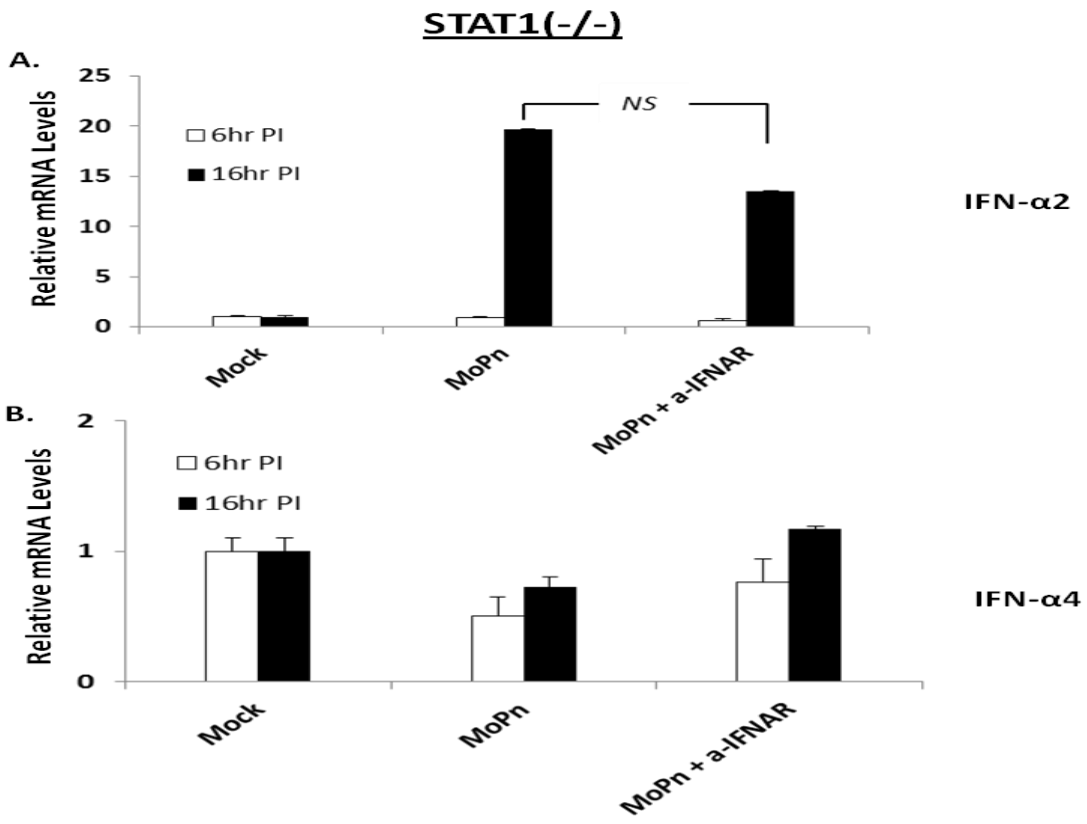


Figure 18. STAT1 is required for Ifna2 and Ifna4 gene induction during *Chlamydia* infection in OE cells

OE STAT1 (-/-) cells were mock-infected, or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 (white bars) or 16 (black bars) hours with or without IFNAR neutralizing antibody (denoted as a-IFNAR), as described in Materials and Methods. Quantitative real-time PCR to measure: (A.) Ifna2 mRNA, and (B.) Ifna4 mRNA was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from the cells for each condition with the respective primers (see 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 ± SD; and are representative data from one of three individual experiments conducted in duplicate. Significance was determined using

Student's T test for the indicated comparisons (MoPn-infected vs MoPn-infected with IFNAR neutralizing antibody); NS=not significant. The cell line represented in this figure and cell lines from the previous figures (Figure 16 and Figure 17) were analyzed on the same 96-well plate for each time point and indicated primer (see Table 1), and can be compared with Ifna2 and Ifna4 levels of the OE WT control cell lines (Figure 16 and Figure 17), respectively.

Contrary to what was observed in the *C. muridarum*-induced synthesis of Ifna gene in OE cells, blocking the type I IFN signaling pathway with IFNAR-specific antibody had no effect on the induction of the Ifnb gene. As shown in Figure 19, the wild-type and TLR3-deficient OE cells both had significant upregulation in the expression of the Ifnb gene in response to *Chlamydia* infection; however, there was no reduction in the level of induction of Ifnb when IFNAR was blocked with specific antibody (Figure 19). Also, the induction of Ifnb was enhanced by the TLR3 signaling pathway, since TLR3-deficient OE cells showed levels of induction that were 10-fold lower than wild-type (Figure 19 B). This finding corroborates our earlier studies describing a dependence on TLR3 for optimal IFN-β synthesis during *C. muridarum* infection of OE cells [250], and presents the hypothesis that the *Chlamydia*-induced expression and the autocrine/ paracrine regulation of IFN-β involves pathways that are distinct from the type-I IFN pathway that signals through IFNAR.

We tested this hypothesis by ascertaining the ability of OE cells to induce type I IFN genes in response to exogenous recombinant IFN-β. As indicated, wild-type OE cells treated with recombinant IFN-β showed substantial inductions in the expression of Ifna2 (Figure 20 A) and Ifnb (Figure 22 a), but only modest induction of Ifna4 (Figure 21 A); however, blocking the type I IFN pathway with antibody specific for IFNAR led to significant reductions in the synthesis of early-stageIfna2 and Ifna4 (Figure 20), but not Ifnb (Figure 22). Interestingly, TLR3-deficient OEs showed diminished expression of Ifnb in response to recombinant IFN-β compared to the wild-type counterparts (Figure 22). Finally, OE cells lacking STAT1 expressed substantially diminished levels of Ifna2 (Figure 23 A), and Ifna4 (Figure 23 B) compared to the levels in both WT and TLR3-

deficient OEs (Figure 20 and Figure 21). There was, though, a modest induction of the Ifna4 gene in the STAT1-deficient OE cells after stimulating the cells with recombinant IFN-β for 16 hrs; however, the Ifna4 induced appeared to be independent of INFAR signaling (Figure 23 B). This data corroborates our findings implicating STAT1 as an important mediator in the syntheses of these cytokines, and supports our theory that type I IFN production may occur independently of type I IFN signaling via the IFNAR. These findings suggest that the IFNAR-dependent type I IFN signaling pathway plays only a minor role in the autocrine/ paracrine induction of Ifnb, and supports our hypothesis that there are other mechanisms involved in the auto-regulation of IFN-β.

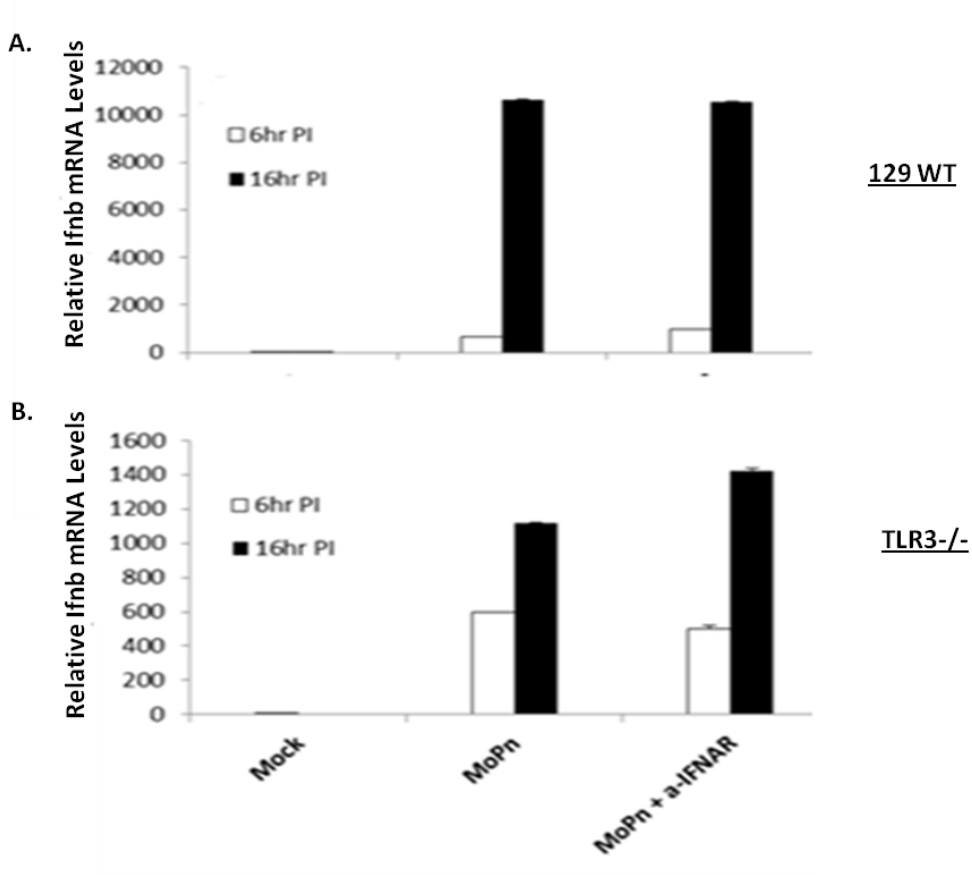


Figure 19. Late-stage *C. muridarum*-induced Ifnb gene induction occurs independently of type I IFN signaling via the IFNAR in OE cells

(A.) OE129 WT, and (B.) OE TLR3 (-/-) cells were mock-infected or infected with 10 MOI of *C. muridarum* (MoPn) for either 6 (white bars) or 16 (black bars) hours with or without IFNAR neutralizing antibody (denoted as a-IFNAR), as described in Materials and Methods. Quantitative real-time PCR to measure Ifnb mRNA levels was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from each cell type for each condition with IFN-β primers (see 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 ± SD; and are representative data from one of three individual experiments conducted in duplicate.

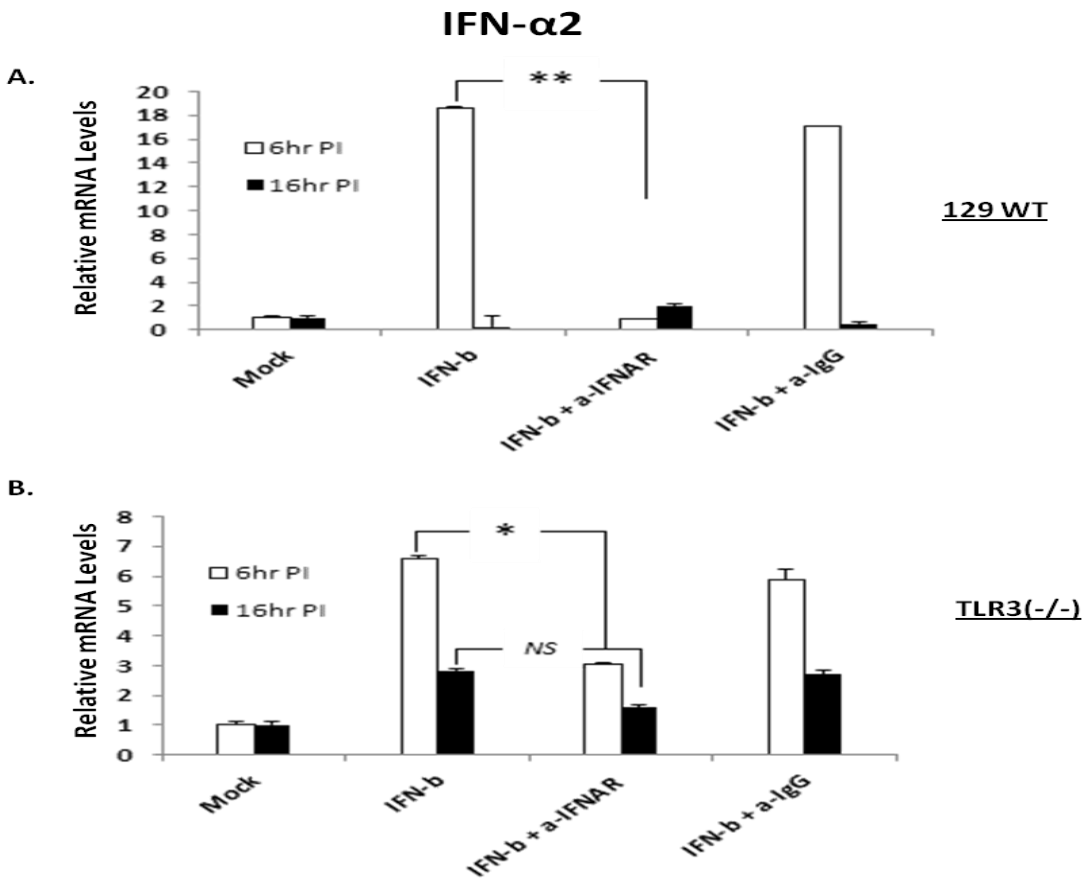


Figure 20. Exogenous IFN-β induces early-stage Ifna2 gene expression through the type I IFN signaling pathway via the IFNAR in OE cells

OE129 WT and OE TLR3 (-/-) cells were mock-infected, or treated with exogenous IFN-β with or without IFNAR neutralizing antibody (denoted as a-IFNAR) or the isotype control (denoted as a-IgG), for either 6 (white bars) or 16 (black bars) hours as described in Materials and Methods. Quantitative real-time PCR to measure Ifna2 mRNA was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from each cell type for each condition with the respective primers (see 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 ± SD; and are representative data from one of three

individual experiments conducted in duplicate. Significance was determined using Student's T test; *=p value <0.05; **=p value <0.005 for the indicated comparisons (IFN-β-treated vs IFN-β-treated with IFNAR neutralizing antibody); NS=not significant.

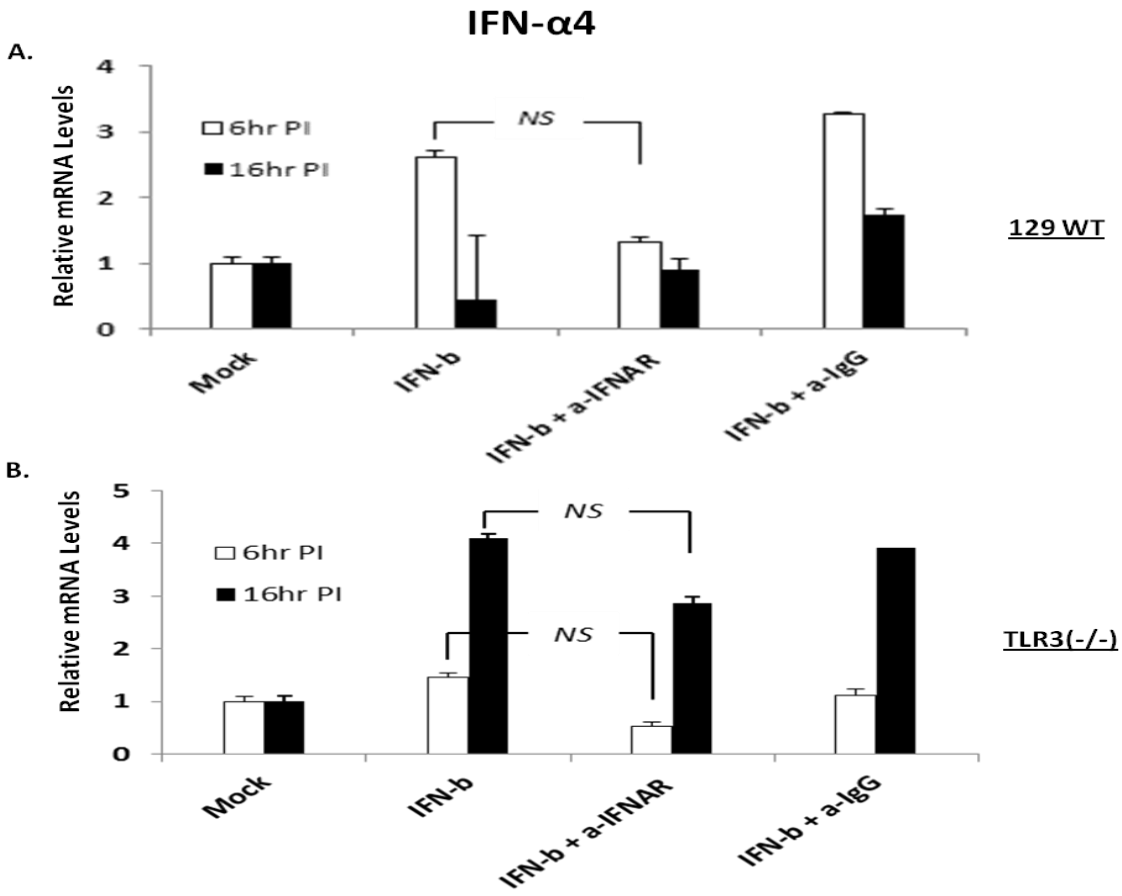


Figure 21. Exogenous IFN-β induces minimal Ifna4 gene expression independent of the type I IFN signaling pathway via the IFNAR in OE cells

OE129 WT and OE TLR3 (-/-) cells were mock-infected, or treated with exogenous IFN-β with or without IFNAR neutralizing antibody (denoted as a-IFNAR) or the isotype control (denoted as a-IgG), for either 6 (white bars) or 16 (black bars) hours as described in Materials and Methods. Quantitative real-time PCR to measure Ifna4 mRNA was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from each cell type for each condition with the respective primers (see 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 ± SD; and are representative data from one of three

individual experiments conducted in duplicate. Significance was determined using Student's T test for the indicated comparisons (IFN-β-treated vs IFN-β-treated with IFNAR neutralizing antibody); NS=not significant.

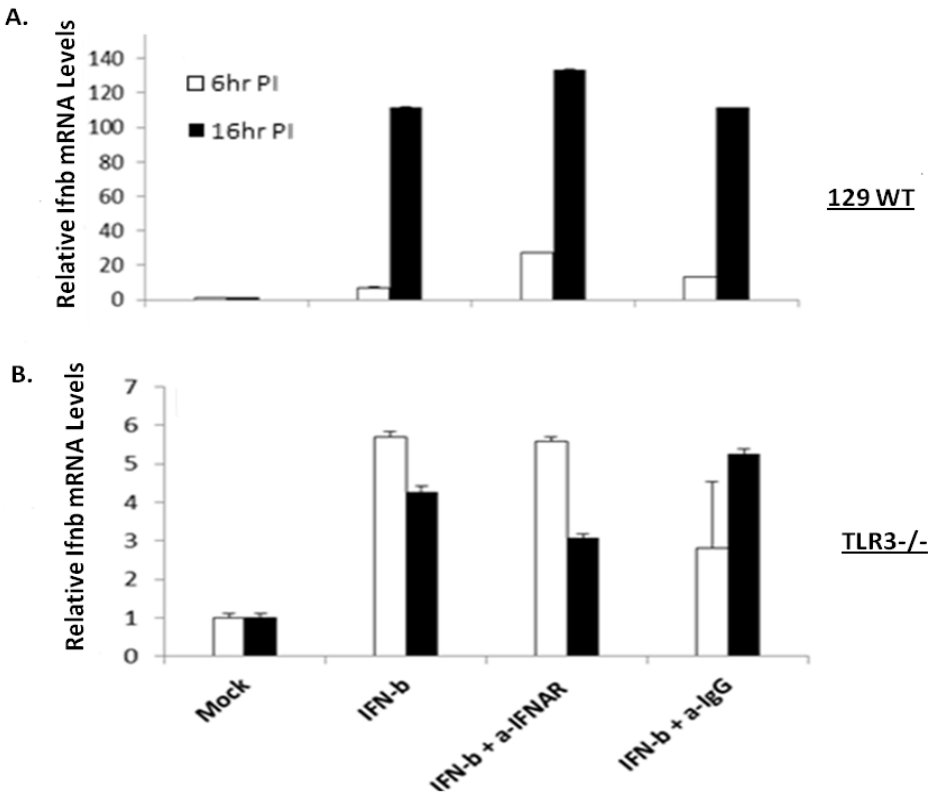


Figure 22. Exogeneous IFN-β upregulates Ifnb gene induction independently of type I IFN signaling via the IFNAR in OE cells

OE129 WT and OE TLR3 (-/-) cells were mock-infected, or treated with exogenous IFN-β with or without IFNAR neutralizing antibody (denoted as a-IFNAR) or the isotype control (denoted as a-IgG), for either 6 (white bars) or 16 (black bars) hours, as described in Materials and Methods. Quantitative real-time PCR to measure Ifnb mRNA levels was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from each cell type for each condition with IFN-β primers (see 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 ± SD; and are representative data from one of three individual experiments conducted in duplicate.

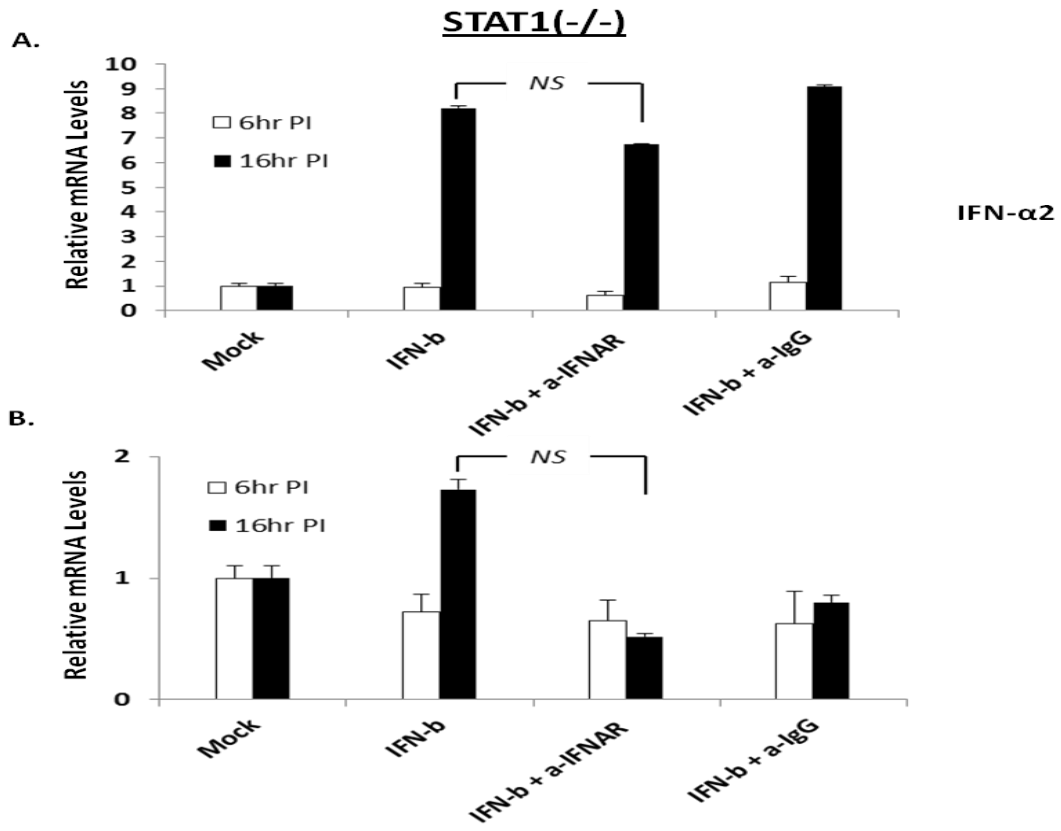


Figure 23. IFN-α subtypes induced with exogenous IFN-β are differentially regulated by STAT1 and induction is independent of type I IFN signaling via the IFNAR in STAT1-deficient OE cells

OE STAT1 (-/-) cells were mock-infected, or treated with exogenous IFN-β with or without IFNAR neutralizing antibody (denoted as a-IFNAR) or the isotype control (denoted as a-IgG), for either 6 (white bars) or 16 (black bars) hours, as described in Materials and Methods. Quantitative real-time PCR to measure: (A.) Ifna2 mRNA, and (B.) Ifna4 mRNA was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from the cells for each condition with the respective primers (see 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 ± SD; and are representative data from one of

three individual experiments conducted in duplicate. Significance was determined using Student's T test for the indicated comparisons (IFN-β-treated vs IFN-β-treated with IFNAR neutralizing antibody); NS=not significant. The cell line represented in this figure and cell lines from the previous figures (Figure 20 and Figure 21) were analyzed on the same 96-well plate for each time point and indicated primer (see Table 1), and can be compared with Ifna2 and Ifna4 levels of the OE WT control cell lines (Figure 20 and Figure 17), respectively.

F. Neutralization of IFN-β activity has no effect on Ifnb gene induction during *C. muridarum* infection in OE cells

We have established a critical role for STAT1-mediated type I IFN signaling in the temporal induction of IFN-α subtypes during *C. muridarum* infection in OE cells; however, our data suggest that type I IFN signaling via the IFNAR plays a minimal role in the positive amplification of the IFN-β response in these cells since blocking the IFNAR (Figure 19) or upon treatment with exogenous recombinant IFN-β (Figure 22), there is relatively no effect on Ifnb gene induction upon *Chlamydia* infection. Furthermore observations taken from the OE cells treated with exogenous recombinant IFN-β; in which the Ifnb gene was induced to similar levels in cells treated with or without the IFNAR neutralizing antibody compared to mock-infected controls (Figure 22), implicate the possibility of additional factors that may recognize and that can be stimulated by IFN-β during *Chlamydia* infection.

To examine whether IFN-β is directly involved in the *Chlamydia*-induced IFN-β production in OE cells, we measured the levels in the gene expression of Ifnb induced by *C. muridarum* at 6 hr and 16 hr PI, in the presence or absence of neutralizing antibody specific for IFN-β. OE129 WT cells were either mock-infected, or *C. muridarum*-infected with or without neutralizing antibody specific for IFN-β. *C. muridarum* infection induced expression of the Ifnb gene in WT OE cells at early and late times during infection compared to mock-infected controls, though the OE cells made substantially more Ifnb transcripts later in the infection (Figure 24); corroborating previous findings. As shown, the expression of Ifnb at early (6 hr) and late (16 hr) times during infection appeared to be independent of IFN-β activity in the OE cells, since neutralization of IFN-β had no

effect on the levels of *C. muridarum*-induced Ifnb gene transcripts (Figure 24); suggesting a minimal role for IFN-β in the positive autocrine regulation of IFN-β during *Chlamydia* infection in oviduct epithelial cells.

We previously demonstrated, in OE129 WT cells that we were able to induce Ifnb gene expression, that when the cells were treated with exogenous recombinant IFN-β blocking the IFNAR with neutralizing antibody prior to the addition of recombinant IFN-β had no effect on cellular Ifnb gene induction (Figure 22). However, contrary to what we observed upon blocking the IFNAR in wild-type OE cells, the blocking of IFN-β functional activity by IFN-β specific neutralizing antibody led to a substantial decreases in Ifnb gene induction at both early- and late-stage treatment (Figure 25). These observations further support the notion that additional factors other than the type I IFN receptor (IFNAR) may recognize and be stimulated by IFN-β. These data further supports our hypothesis that there are other mechanisms involved in the auto-regulation of IFN-β during *Chlamydia* infection in oviduct epithelial cells.

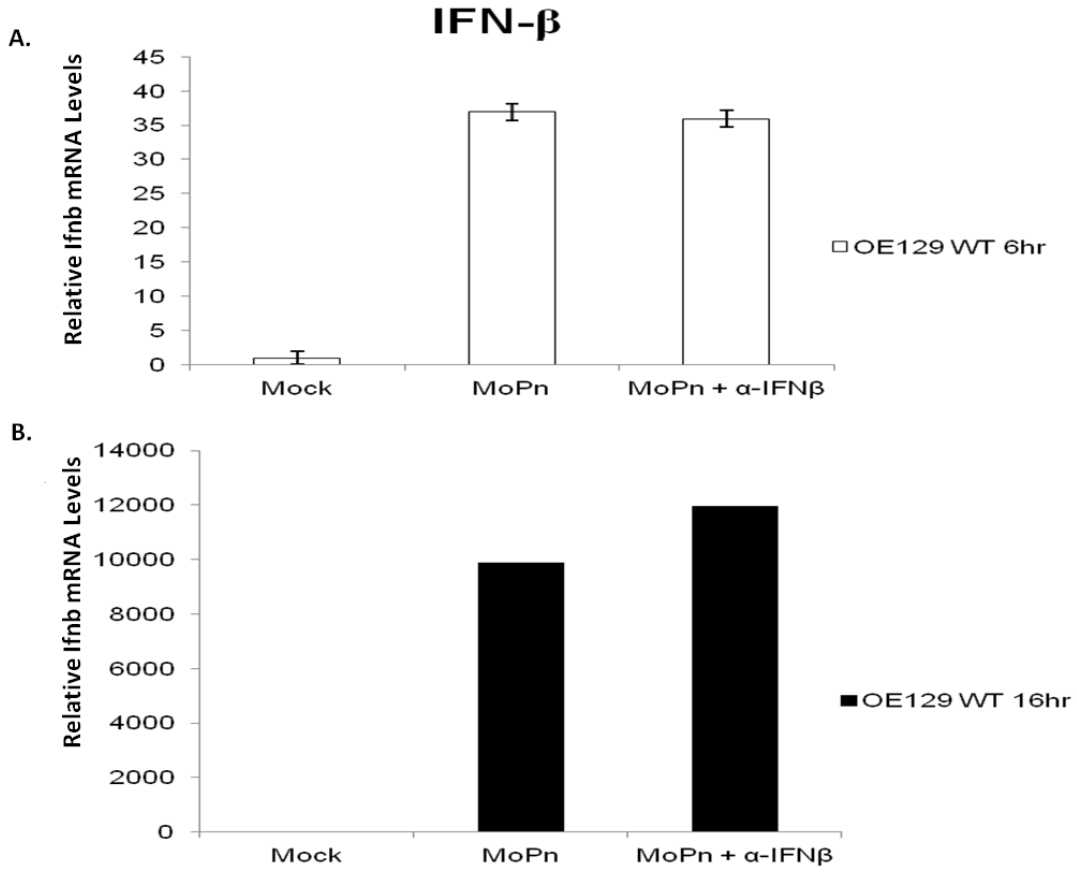


Figure 24. Neutralization of functional IFN-β does not affect *C. muridarum*-induced Ifnb gene induction in OE cells

OE129 WT cells were mock-infected or infected with 10 MOI of *C. muridarum* (MoPn) for: (A.) 6 hours, or (B.) 16 hours with or without IFN-β neutralizing antibody (denoted as α-IFNβ), as described in Materials and Methods. Quantitative real-time PCR to measure Ifnb mRNA levels was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from each conditioned cell with IFN-β primers (see 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 ± SD; and are representative data from one of three individual experiments conducted in duplicate.

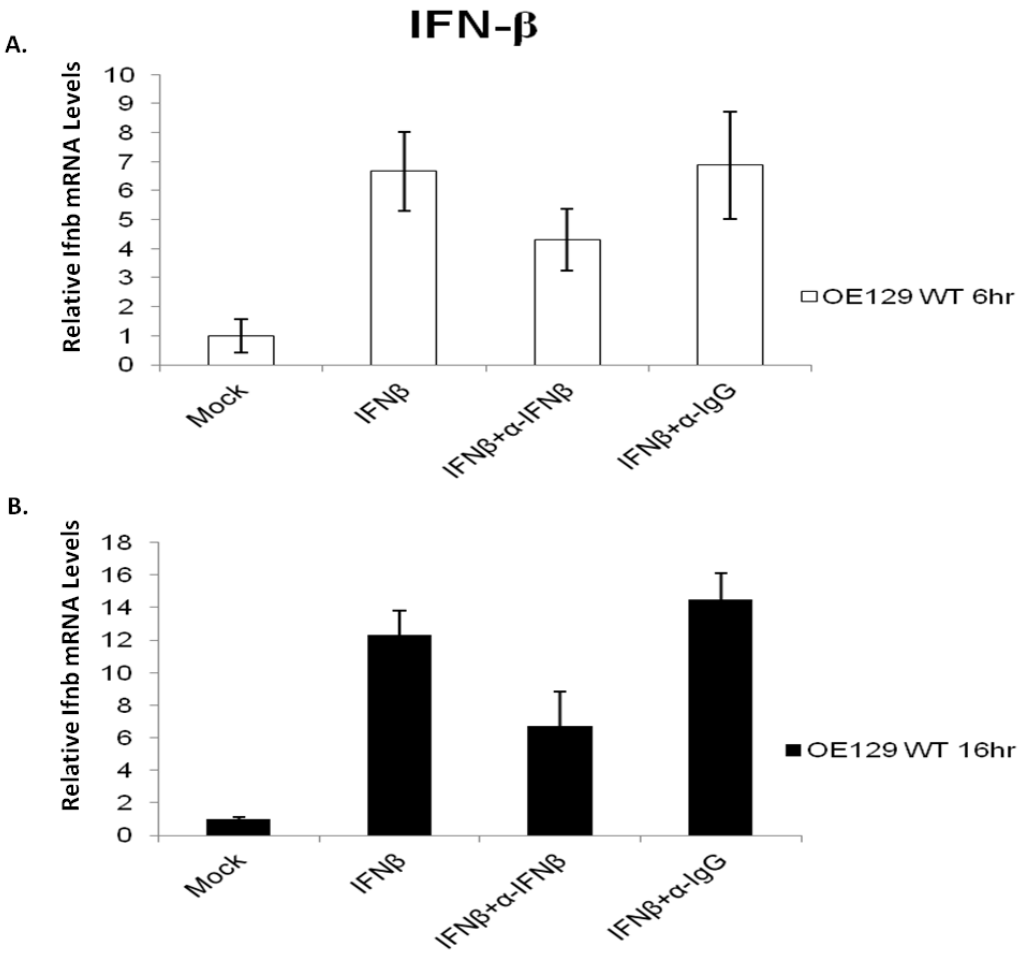


Figure 25. Neutralization of IFN-β substantially reduces exogeneous IFN-β-induced Ifnb gene expression in OE cells

OE129 WT cells were mock-infected or infected with 10 MOI of *C. muridarum* (MoPn) for: (A.) 6 hours, or (B.) 16 hours with or without IFN-β neutralizing antibody (denoted as α-IFNβ), as described in Materials and Methods. Quantitative real-time PCR to measure Ifnb mRNA levels was performed on 1 µg of cDNA reverse transcribed from total-cell RNA isolated from each conditioned cell with IFN-β primers (see 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 ± SD; and are representative data from one of three individual experiments conducted in duplicate.

CHAPTER II. ESTABLISHING THE TEMPORAL ACTIVATION OF STAT1 AND DETERMINING A ROLE FOR STAT1 IN REGULATING PRO-INFLAMMATORY CYTOKINE RESPONSES IN *C. MURIDARUM*-INFECTED OE CELLS

A. IRF3 protein activation is substantially increased during early-intermediate stages of *C. muridarum* infection in OE cells

Our previous work established a major role for TLR3 signaling in the production of *C. muridarum*-induced IFN-β in OE cells. It is well known that TLR3 stimulation ultimately leads to the activation of key transcription factors involved in efficiently initiating Ifnb gene transcription; including IRF3. We hypothesized that the early production of IFN-β occurred predominantly through TLR3 stimulation in *C. muridarum*-infected OE cells. To test this hypothesis we first examined Irf3 gene expression in wild-type OE cells via quantitative real-time PCR. The OE129 WT cells were either mock-infected or infected with 10 MOI of *C. muridarum* for either 6 hr or 16 hr. As shown, *C. muridarum*-infection displayed no significant induction in Irf3 mRNA levels compared to mock-infected controls in wild-type OE cells (Figure 26). We next examined IRF3 protein activation profiles during *C. muridarum*-infection in OE cells. OE129 WT cells were either mock-infected or infected with 10 MOI of *C. muridarum* for 2, 4, 6, 8, 12, 16, 18, or 24 hours. Cell lysates were harvested at the indicated time-points and IRF3 activation assessed by western blot analysis to detect phosphorylated IRF3 (Figure 27). Though mock-infected wild-type OEs expressed basal IRF3 phosphorylation, *C. muridarum*-infection substantially increased IRF3 activation starting at 6 hr PI, and became more notable at 8 hr PI. After which, there was a gradual decrease in IRF3

activation that was completely diminished by 24 hr PI. These findings suggest that while there is no change in Irf3 gene induction in *C. muridarum*-infected WT OE cells, IRF3 protein activation is substantially increased; mostly during the early-intermediate stages of infection. These observations are similar to a recent study in which IRF3 protein was shown to be constitutively expressed and activated at basal levels in unstimulated hematopoetic cells [263].

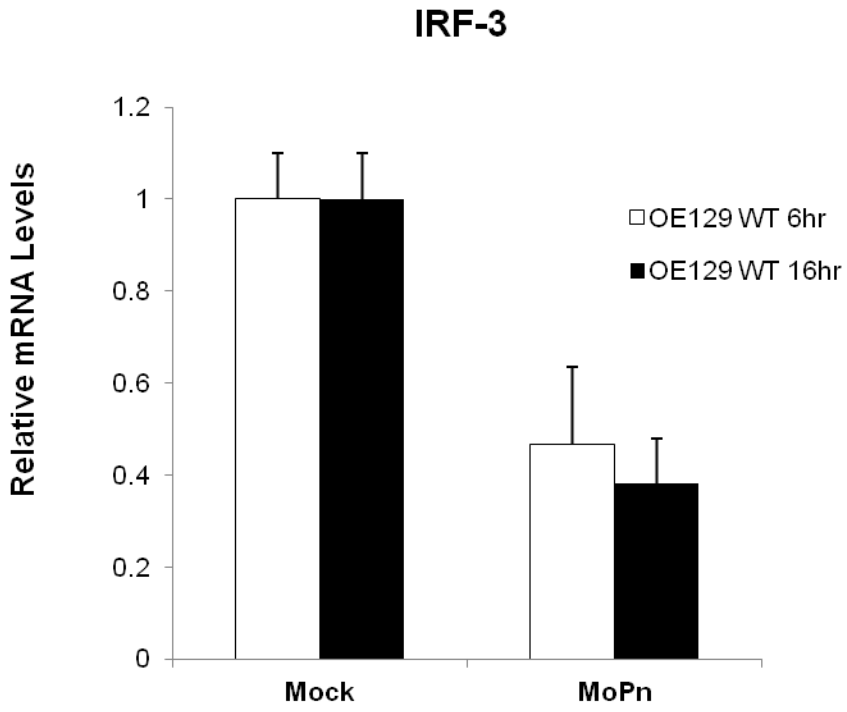


Figure 26. *C. muridarum* infection does not induce Irf3 gene transcription in OE cells

Quantitative real-time PCR was performed using 1 µg of RNA isolated from OE129 WT cells that were either mock-infected, or infected with 10 MOI of *C. muridarum* (denoted as MoPn), for 6 hours (white bars) or 16 hours (black bars). IRF3 primers were used to amplify Irf3 message RNA. Control reactions were set up with β-actin primers to ensure equal loading of RNA. The results are presented as mean fold change ± SD; and are representative data from one of three individual experiments conducted in duplicate.

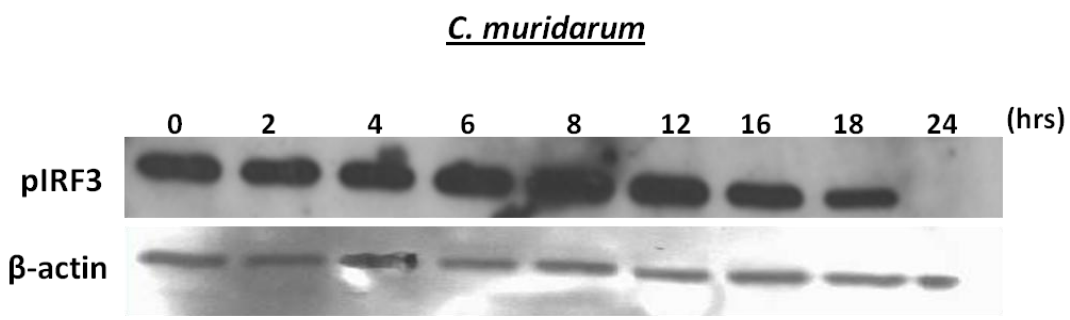


Figure 27. IRF3 activation is substantially increased at early-intermediate stages of *C. muridarum* infection in OE cells

Twenty micrograms of protein from whole cell lysates isolated from OE129 WT cells that were either mock-infected (indicated as 0), or infected with *C. muridarum* at a MOI of 10 IFU/ cell for 2, 4, 6, 8, 12, 16, 18 and 24 hours. The proteins were separated on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with antibody to phosphorylated IRF3 (serine residue 396) and β-actin. β-actin served as a loading control. The results shown are representative of three independent experiments.

B. STAT1 protein activation/expression and Stat1 gene expression are disrupted in *C. muridarum*-infected TLR3-deficient OE cells

In Chapter I we showed that STAT1 plays a major role in the IFN-β response in *Chlamydia*-infected OE cells. We hypothesized that this was due to its involvement in mediating type I IFN signaling. However we showed that Ifnb induction occurs independently of the type I IFN signaling pathway via the IFNAR (Figure 19); suggesting that STAT1 may play a more prominent role in the IFN-β response during early *Chlamydia* infections. We previously reported that *C. muridarum*-induced IFN-β production in oviduct epithelial cells was dependent on TLR3 signaling [251], and in this study we hypothesize that this response is initiated early in infection. We sought to ascertain the role of TLR3 signaling on STAT1 activation and expression during *Chlamydia* infections in OE cells. OE129 WT and OE TLR3 (-/-) cells were either mock-infected, IFN-γ-treated, or infected with 10 IFU/cell *C. muridarum* prior to analyses by RT-PCR for Stat1 gene expression and western blot analyses for STAT1 protein expression and activation. *C. muridarum* infection significantly increased Stat1 mRNA levels compared to mock-infected controls in OE129 WT cells; however, Stat1 mRNA induction was significantly diminished in TLR3-deficient OEs when compared to the wild-type OE cells (Figure 28). We performed western blot analysis to validate the RT-PCR data (Figure 28). Indeed, the notably diminished induction of Stat1 mRNA in *C. muridarum*-infected OE TLR3 (-/-) cells, correlated to diminished levels of total STAT1 protein and a corresponding loss of STAT1 protein activation upon *C. muridarum* infection (Figure 29). In Figure 29 the OE129 WT cells exhibited significant increases in

both total STAT1 expression and activation (as compared to mock-infected controls) upon *Chlamydia* infection, and these levels were comparable to IFN-γ-treated controls.

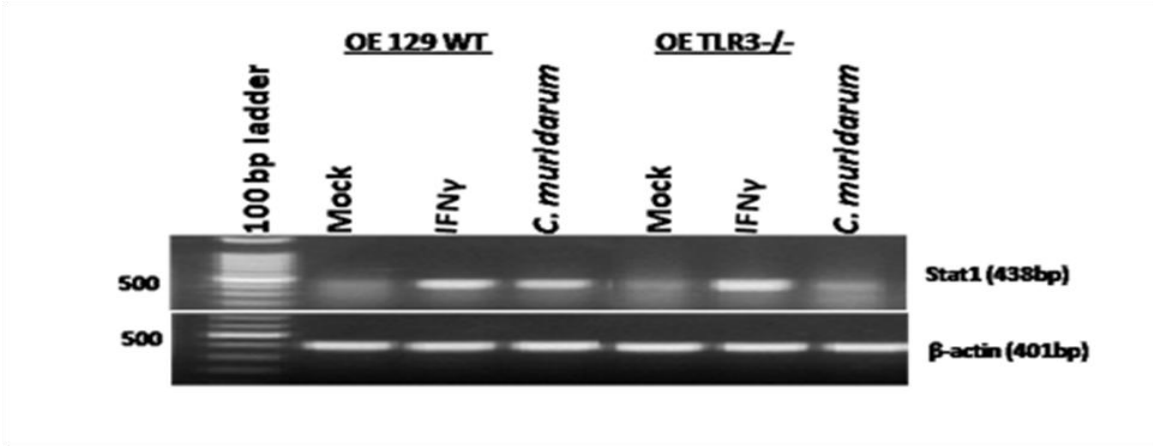


Figure 28. *C. muridarum*-induced Stat1 mRNA message is severely reduced in TLR3-deficient OE cells

Semi-quantitative RT-PCR was performed using 1 µg of cDNA reverse transcribed from RNA isolated from OE129 WTs and OE TLR3-deficient cells that were mock-infected, infected with *C. muridarum* at 10 MOI for 24 hours, or treated with 10 ng/ml of IFN-γ for 4-5 hours. STAT1 primers were used to amplify Stat1 mRNA message; and β-actin primers were used to amplify β-actin mRNA message as a loading control.

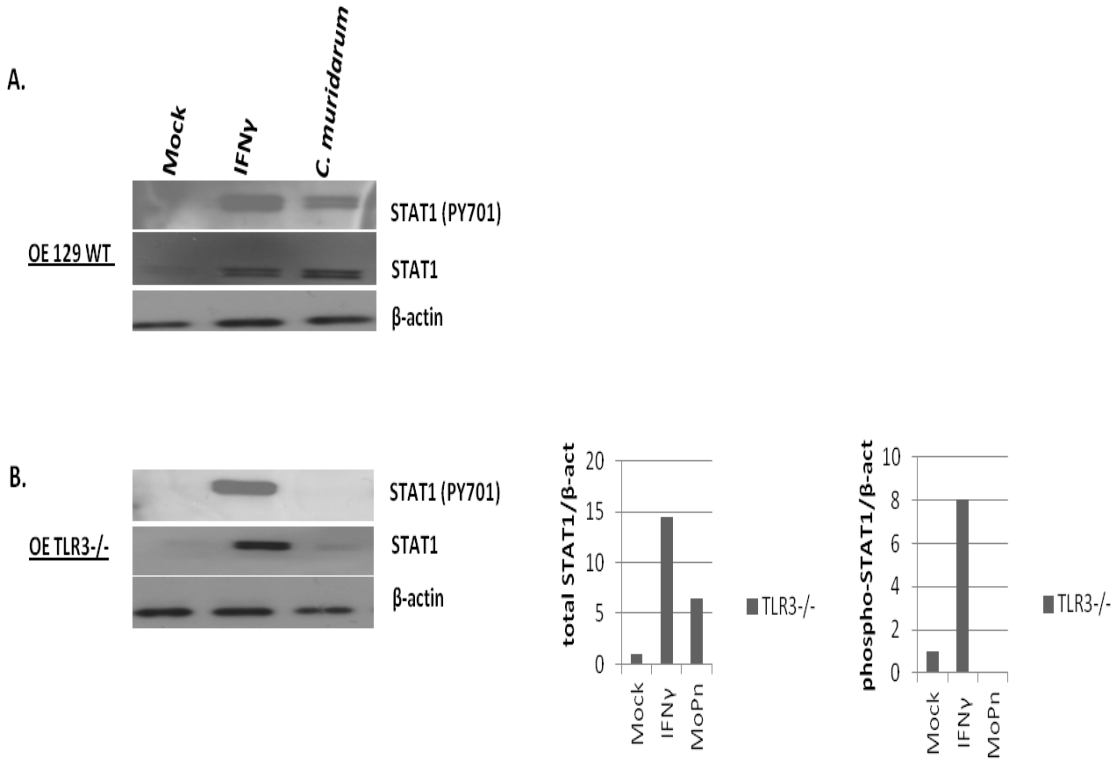


Figure 29. STAT1 protein expression and activation are diminished in *C. muridarum*-induced TLR3-deficient OE cells

Twenty micrograms of protein in whole cell lysates from: (A.) OE129 WT or (B.) TLR3-deficient OEs that were mock-infected, infected with *C. muridarum* at 10 MOI for 24 hours, or treated with 10 ng/ml of IFN-γ for 4-5 hours; were separated on a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and probed with antibody to phosphorylated STAT1 (tyrosine residue 701), total STAT1 and β-actin. β-actin served as a loading control. The results shown are representative of three independent experiments. Densitometric analysis of total STAT1 expression and STAT1 phosphorylation are shown for each cell line relative to the β-actin control (indicated as β-act in the graphs), and *C. muridarum*-infection indicated by MoPn.

C. Early-stage STAT1 activation may contribute to optimal IFN-β production in *C. muridarum*-infected OE cells

The previous data shows that STAT1 activation and total expression are TLR3-dependent (Figure 29) in *Chlamydia*-infected OE cells. We hypothesized that the production of TLR3-dependent IFN-β early during *C. muridarum* infection was required for optimal late-stage production of IFN-β in OE cells, and that the late-stage synthesis was predominantly mediated by STAT1 via the IFNAR signaling. However we showed that Ifnb induction occurs independently of the type I IFN signaling pathway via the IFNAR (Figure 19). This compiled evidence further suggests that STAT1 may play a more predominant role in the type I IFN response during early *Chlamydia* infection in OE cells. To further test this hypothesis, we explored the kinetics of STAT1 activation in *C. muridarum*-infected OE cells at various time-points throughout the infection. OE129 WT cells were either mock-infected (time-point 0) or infected with 10 MOI of *C. muridarum* for 2, 4, 6, 8, 12, 16, 18, or 24 hours. Cell lysates were harvested at the indicated time-points and STAT1 activation assessed by western blot analysis to detect phosphorylated STAT1. Interestingly, mock-infected wild-type OEs showed pronounced STAT1 phosphorylation, while *C. muridarum*-infected wild-type OEs induced notable changes in STAT1 phosphorylation at 2 hours and 16 hours post-infection, when compared to the other post-infection time-points (Figure 30). Additionally, there were notable changes in STAT1 phosphorylation at 6 hours and 24 hours compared to the other post-infection time-points (Figure 30).

Next, we wanted to determine the effects of inhibiting STAT1 activation at early versus late stages of *Chlamydia* infection on IFN-β production in oviduct epithelial cells.

OE129 WT cells were infected with *C. muridarum* alone, or infected with *C. muridarum* and treated with the inhibitor of STAT1 phosphorylation (fludarabine) at 4, 6, or 16 hours; the amount of IFN-β secreted in supernatants at 24 hr PI was measured by ELISA (Figure 31). As shown, IFN-β production was significantly increased upon *Chlamydia* infection in OE129 WT cells (compared to mock-infected cells); however, *C. muridarum*-induced IFN-β was substantially reduced when treated with fludarabine at 4 hours PI compared to *C. muridarum*-infection alone (Figure 31). Fludarabine treatment at 6 hours PI infection slightly reduced IFN-β levels compared to the OE cells infected with *C. muridarum* alone; however, IFN-β levels of OE cells treated with fludarabine at 16 hours PI were comparable to *C. muridarum* infection alone (Figure 31). This observation suggest that while STAT1 activity may be temporally regulated throughout *Chlamydia* infection in OE cells, it likely has the most dramatic effect on IFN-β synthesis during early-stage *Chlamydia* infection in OE cells. Our data show that inhibition of STAT1 phosphorylation early in infection has a more dramatic impact on *C. muridarum*-induced IFN-β synthesis in OE cells than its inhibition at late infection.

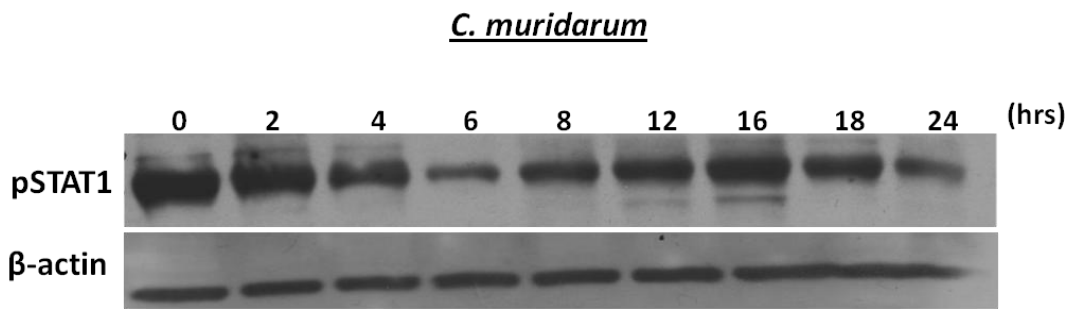


Figure 30. Changes in STAT1 activation during *C. muridarum* infection in OE cells

Twenty micrograms of protein from whole cell lysates isolated from OE129 WT cells that were either mock-infected (indicated as 0), or infected with *C. muridarum* at a MOI of 10 IFU/ cell for 2, 4, 6, 8, 12, 16, 18 and 24 hours. The proteins were separated on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with antibody to phosphorylated STAT1 (tyrosine residue 901) and β-actin. β-actin served as a loading control. The results shown are representative of three independent experiments.

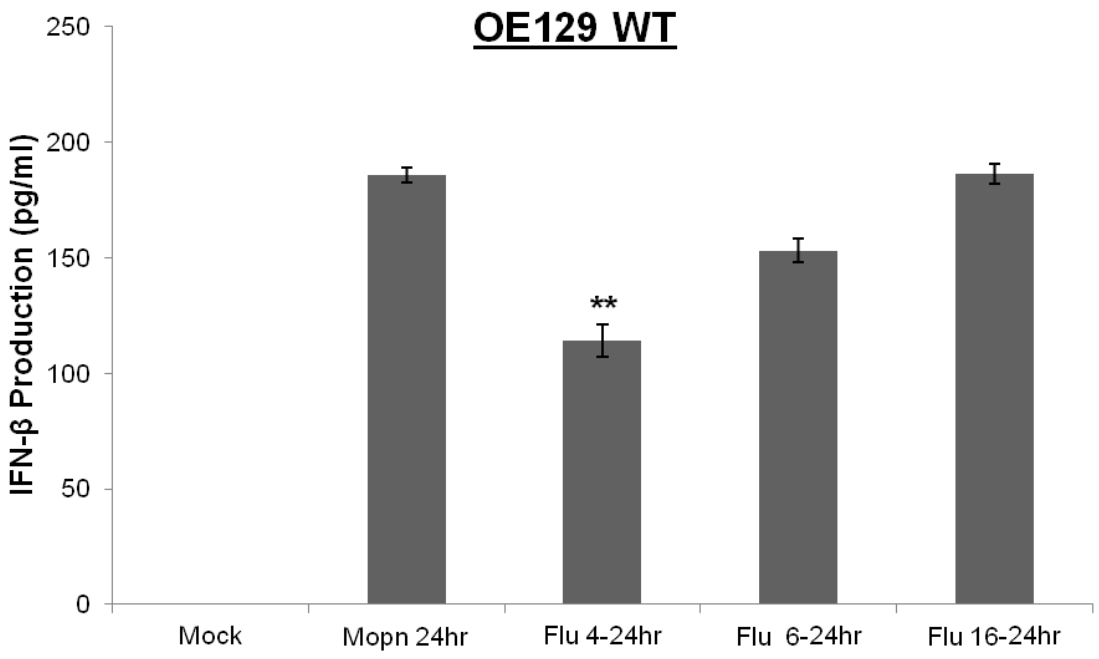


Figure 31. Early inhibition of STAT1 activation substantially reduces *C. muridarum*-induced IFN-β production in OE cells

OE129 WT cells were mock-infected, infected at a MOI of 10 IFU/ cell with either *C. muridarum* alone (denoted as MoPn), or infected with *C. muridarum* and treated with fludarabine (denoted as Flu) at 4, 6, or 16 hours and all supernatants collected at 24 hours. ELISA was used to measure infection-induced IFN-β secreted into the supernatants of the cells. The data represent mean ± SD and are representative of three different experiments conducted in triplicate. Significance was determined using Student's T test; **=p value <0.005 for Mopn 24 hr. versus Flu 4-24hr.

D. STAT1 plays a central role in IL-6 production in *C.muridarum*-infected OE cells

We have previously shown that *C. muridarum*-induced OEs produce robust amounts of acute-phase proinflammatory cytokines; including IL-6, in a TLR2/MyD88-dependent manner [155]. Moreover, recent studies from our lab have shown that *C. muridarum*-infected TLR3-deficienct OE cells secrete significantly reduced amounts of IL-6 compared to wild-type OEs, but that its synthesis was substantially restored by pretreatment with exogenous IFN-β [255]. Based on those studies and our observations in this study showing that STAT1 activation is TLR3-dependent (Figure 29), and that early STAT1 activation may be required for the IFN-β response (Figure 31), we explored the role of STAT1 in the *C. muridarum*-induced IL-6 response *in vitro*. We first utilized BMDMs derived from wild-type C57BL/6 mice and aged matched STAT1-deficient mice to examine the effect of STAT1 deficiency on IL-6 production during *C. muridarum*-induction. The WT and STAT1 (-/-) BMDMs were infected with *C. muridarum*, and the amount of IL-6 secreted in supernatants at 24 hr PI was measured (Figure 32). As shown, IL-6 production was significantly increased upon *Chlamydia* infection in WT BMDMs compared to mock-infected cells; however, the amount of *C. muridarum* induced IL-6 produced at 24hr PI in the STAT1 (-/-) BMDMs was significantly reduced compared to the *C. muridarum*-infected WT BMDMs (Figure 32). Recall, we observed similar secretion patterns in the *Chlamydia*-induced IFN-β response in OE cells isolated from wild-type and STAT1-deficient mice.

Next, OE129 WT and STAT1 (-/-) OEs were infected with 10 MOI *C.muridarum* and the amount of IL-6 secreted in the supernatants was measured at 24 hr PI. IL-6 production was significantly decreased in *Chlamydia*-induced STAT1 (-/-) OEs

compared to WT *Chlamydia*-induced OE cells (Figure 33). To further ascertain the role of STAT1 in the *Chlamydia*-induced IL-6 response in OEs, we knocked-down the expression of STAT1 in OE129 WT cells utilizing a ready-made gene silencing plasmid specific to the STAT1 gene (control plasmid was used to verify specificity). OE129 WT, psiSTAT1 OEs (STAT1 knock-down), and psiLUCGl3 (control plasmid) OEs were mock-infected or infected at a MOI of 10 IFU/ cell with *C. muridarum,* and IL-6 was measured in the supernatants at 24 hr (Figure 34). As shown, *C. muridarum*-induced IL-6 production was significantly decreased in psiSTAT1 OEs compared to OE129 WT cells and OE129 WT OE cells transfected with the psiLUCGl3 control siRNA plasmid (Figure 34). These findings highlight STAT1 as a major player for the induction of IL-6 in *Chlamydia*-induced OEs; suggesting that STAT1 may play a critical role in regulating other acute-phase proinflammatory cytokines. These additional findings corroborates our theory that STAT1 may play a key role in cytokine responses during early-stage *Chlamydia* infection, independent of type I IFN signaling via the IFNAR; that contributes to modulating the overall immune response in oviduct epithelial cells.

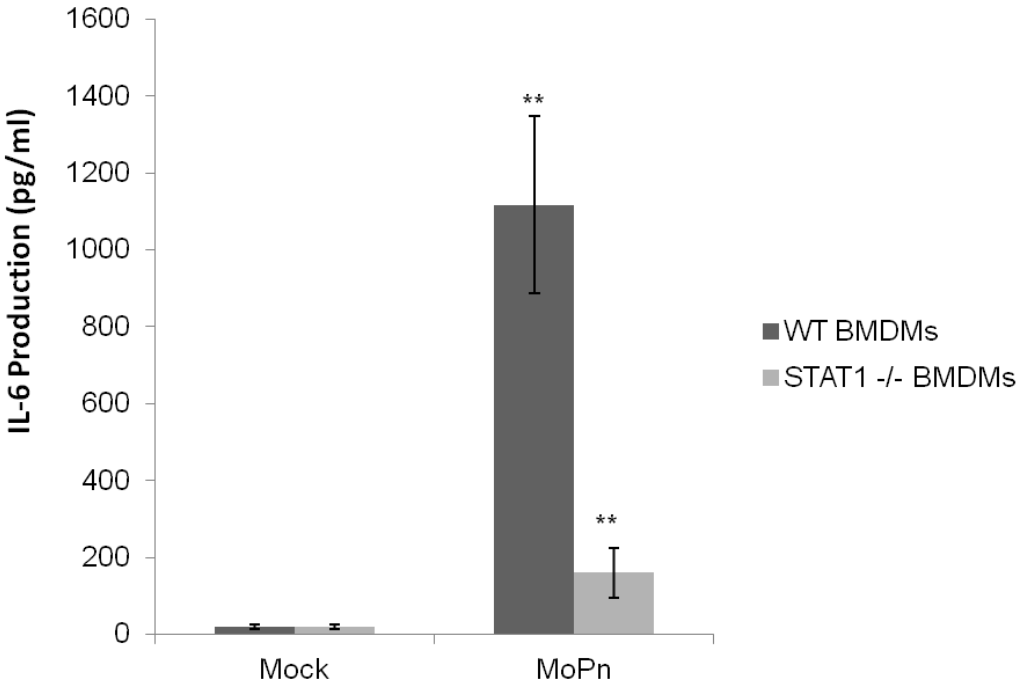


Figure 32. *C. muridarum*-induced IL-6 production is significantly reduced in STAT1-deficient BMDMs

WT BMDMs and STAT1-deficient (STAT1-/-) BMDMs were mock-infected and/or infected at a MOI of 10 IFU/ cell with *C. muridarum* (denoted as MoPn) for 24 hours. ELISA was used to measure infection-induced IL-6 secreted into the supernatants of mock-infected and *C. muridarum*-infected WT BMDMs and STAT1 (-/-) BMDMs. The data represent mean ± SD and are representative of three different experiments conducted in triplicate. Significance was determined using Student's T test; **=p value <0.005 for mock-infected WT BMDMs vs MoPn-infected WT BMDMs; and MoPn-infected WT BMDMs vs MoPn-infected STAT1 (-/-) BMDMs.

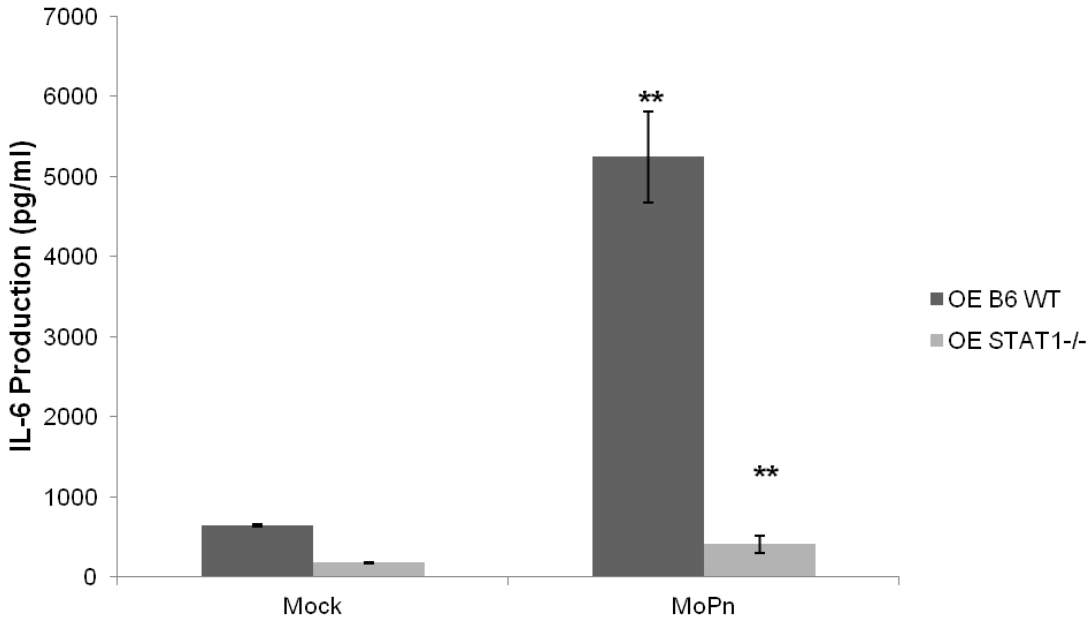


Figure 33. *C.muridarum*-induced IL-6 production is significantly decreased in STAT1-deficient oviduct epithelial cells

OEB6 WT cells and STAT1-deficient (STAT1-/-) OE cells were mock-infected and/or infected at a MOI of 10 IFU/ cell with *C. muridarum* (denoted as MoPn) for 24 hours. ELISA was used to measure infection-induced IL-6 secreted into the supernatants of mock-infected and *C. muridarum*-infected B6 WT cells, and STAT1 (-/-) OE cells. The data represent mean ± SD and are representative of three different experiments conducted in triplicate. Significance was determined using Student's T test; **=p value <0.005 for mock-infected OE B6 WT vs MoPn-infected OE B6 WT; and MoPn-infected OE B6 WT vs MoPn-infected OE STAT1 (-/-) cells.

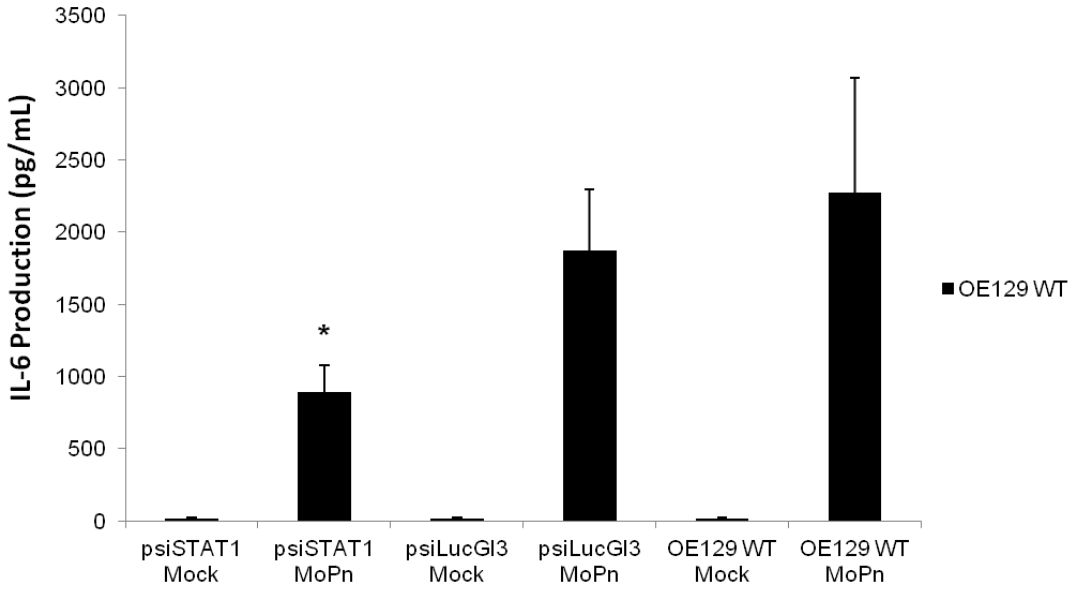


Figure 34. STAT1-knockdown oviduct epithelial cells display significantly reduced *C.muridarum*-induced IL-6 production at 24 hours

OE129 WT cells were either mock-infected, infected at a MOI of 10 IFU/ cell with *C. muridarum* (denoted as MoPn), or transfected with a STAT1-knockdown plasmid (denoted as psiSTAT1) or a control plasmid (denoted as psiLucGl3); respectively, then mock-infected or infected with 10 MOI of *C. muridarum* and all supernatants collected at 24 hours for ELISA analysis to measure infection-induced IL-6 secretion. The data represent mean ± SD and are representative of three different experiments conducted in triplicate. Significance was determined using Student's T test; *=p value <0.05 for MoPn-infected OE129 WT vs MoPn-infected psiSTAT1 OEs.

Collectively, the results presented in Chapters I and II show that STAT1 plays a critical role in the type I IFN and proinflammatory cytokine responses in oviduct epithelial cells during *Chlamydia muridarum* infections. We show that *C. muridarum*-induced STAT1 activation and expression are critical for the synthesis of IFN-β at late-stages of infection, and that TLR3 also contributes to optimal IFN-β synthesis during late-stage *Chlamydia* infections (Figure 35). In addition we show that *C. muridarum* induced the IFN-α subtypes 2 and 4 in a manner that was STAT1-dependent and that required type I IFN signaling through the IFNAR, though at different times during infection and attenuated by TLR3 (Figure 36). Interestingly, we show that the *C. muridarum* induced expression of late-stage Ifnb occurred independently of type I IFN signaling via the IFNAR (Figure 37). Based on these results we speculated as to what role STAT1 would play in the cytokine responses during early stages of infection. We show that the proposed "acute" inducer of the type I IFN response, TLR3, was required for STAT1 activation and expression during *C.muridarum* in oviduct epithelial cells, and interestingly early inhibition of STAT1 activation showed decreased levels of IFN-β secreted from oviduct epithelial cell (Figure 38 A and Figure 38 B). Finally we show that *C. muridarum* infection induced IL-6 secretion in a STAT1-dependent manner (Figure 39).

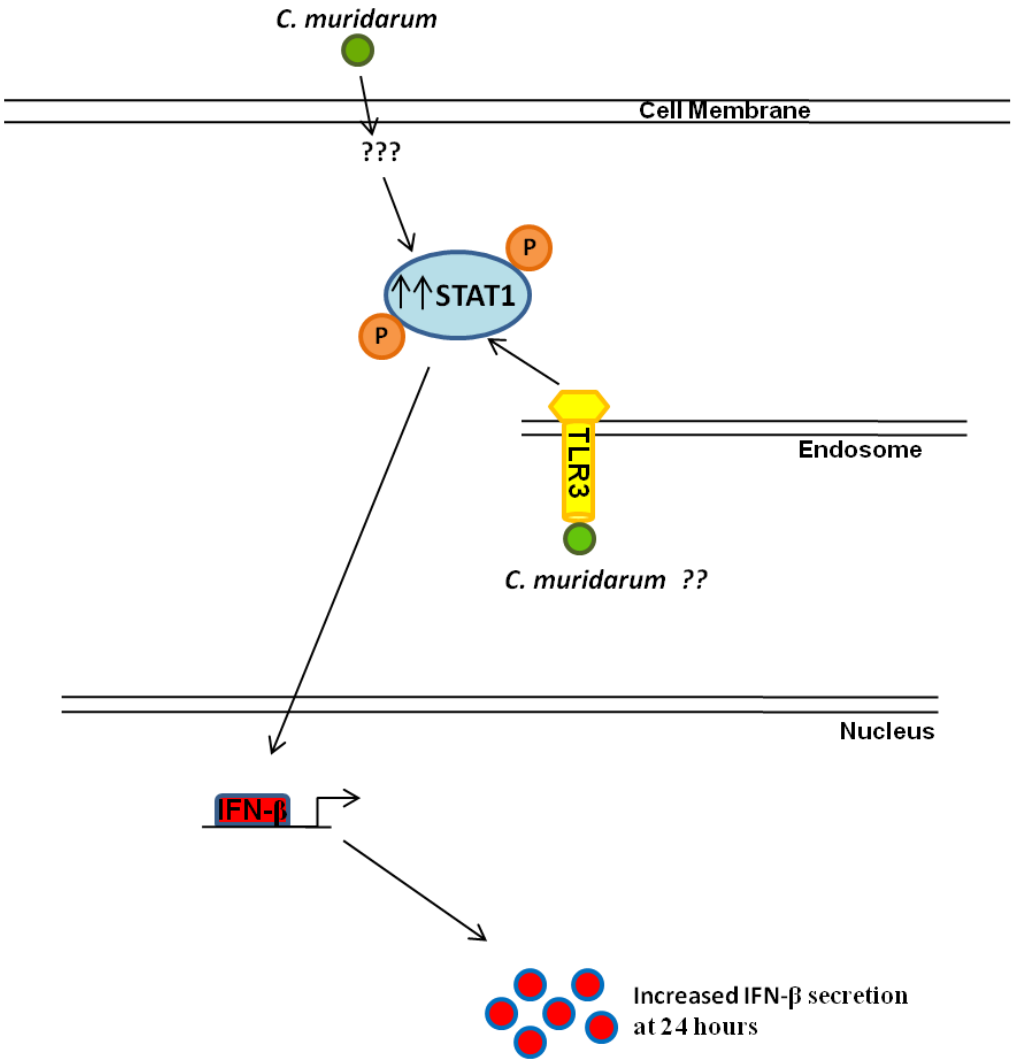


Figure 35. *C. muridarum*-induced TLR3 stimulation and STAT1activation are required for late-stage IFN-β secretion in OE cells

OE cells infected with *C. muridarum* for 24 hours showed increased STAT1 activation and expression resulting in increased IFN-β secretion, similarly to the TLR3-dependent IFN-β response, which may also use STAT1 as a mediator for this response.

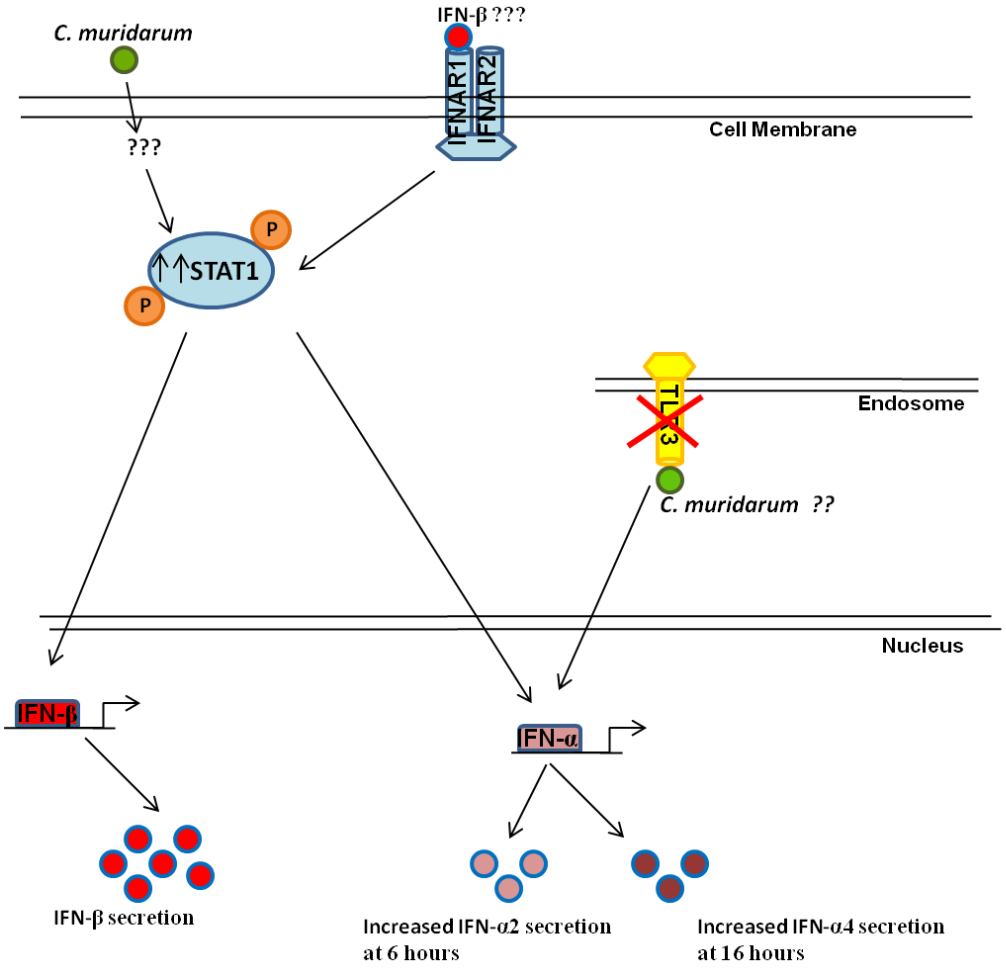


Figure 36. IFN-α subtypes require STAT1 and the IFNAR for induction, and are attenuated by TLR3 during *C. muridarum* infection in OE cells

OE cells infected with *C. muridarum* showed increased Ifna2 induction early during infection and Ifna4 induction late during infection which required STAT1 (presumably activated by type I IFN signaling via the IFNAR or by an unidentified pathway induced by *C. muridrum*) and required the IFNAR of the type I IFN signaling pathway. Upon inhibiting TLR3 signaling by using TLR3-deficient OE cells there was an even more robust induction of the IFN-α subtypes at the indicated times.

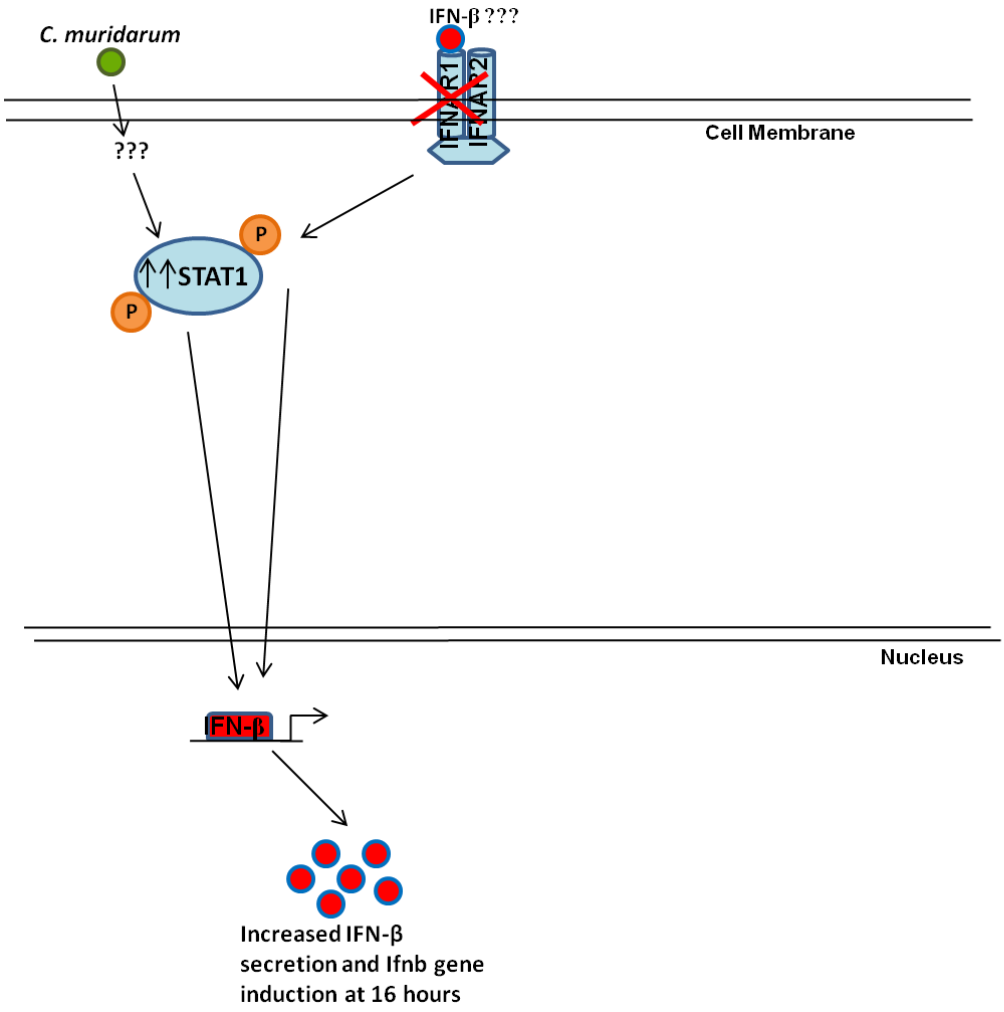


Figure 37. IFN-β requires STAT1, but not the IFNAR1 for induction during late-stage *C. muridarum* infection in OE cells

OE cells infected with *C. muridarum* showed increased Ifnb induction late during infection which required STAT1, and in theory, the initial induction and secretion of IFN-β to serve as a positive-feedback mediator stimulating the type I IFN receptor. However, our results show that increased Ifnb induction late during infection occurred independently of the IFNAR of the type I IFN signaling pathway since blocking IFNAR1 had no effect on the levels of Ifnb gene induction.

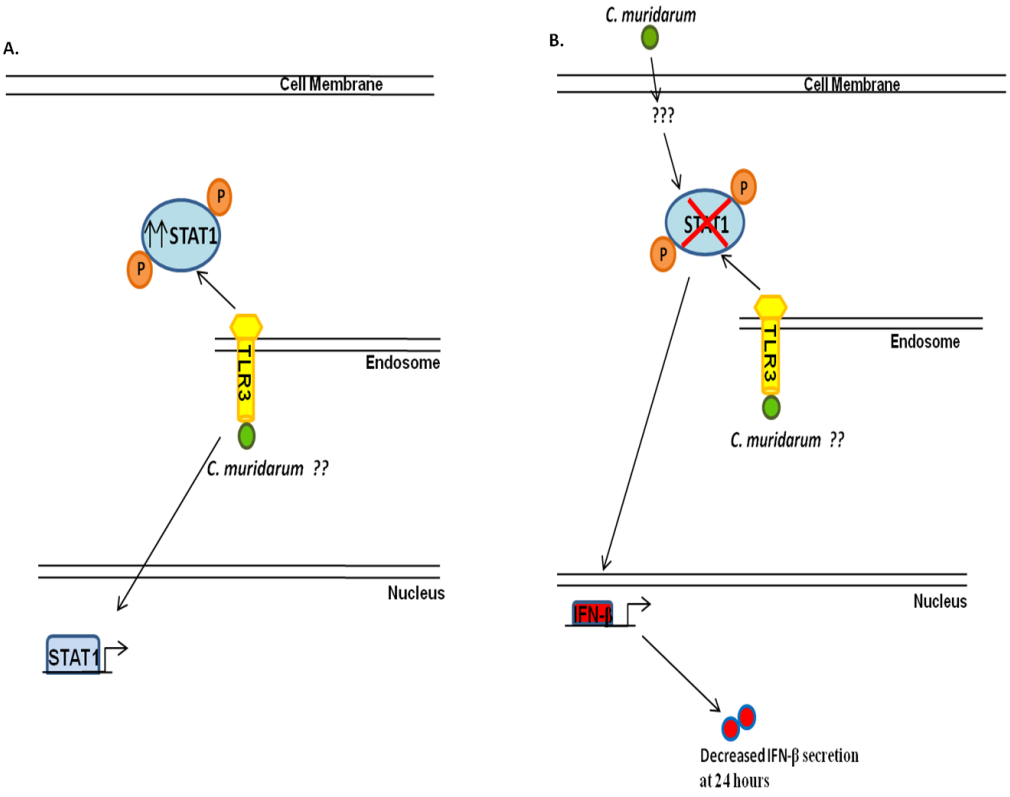


Figure 38. Early-stage STAT1 activation may contribute to optimal "late-stage" IFN-β production during *C. muridarum* infection in OE cells

OE cells infected with *C. muridarum* showed STAT1activation to be TLR3-dependent (A.). We proposed that TLR3 signaling was an early pathway stimulated by an unknown *Chlamydia* PAMP and was responsible for the "early" production of IFN-β. Additionally, when STAT1 activation was inhibited early (4 hours post infection) during *C. muridarum* infection OE cells showed a decrease in late-stage IFN-β secretion (B.).

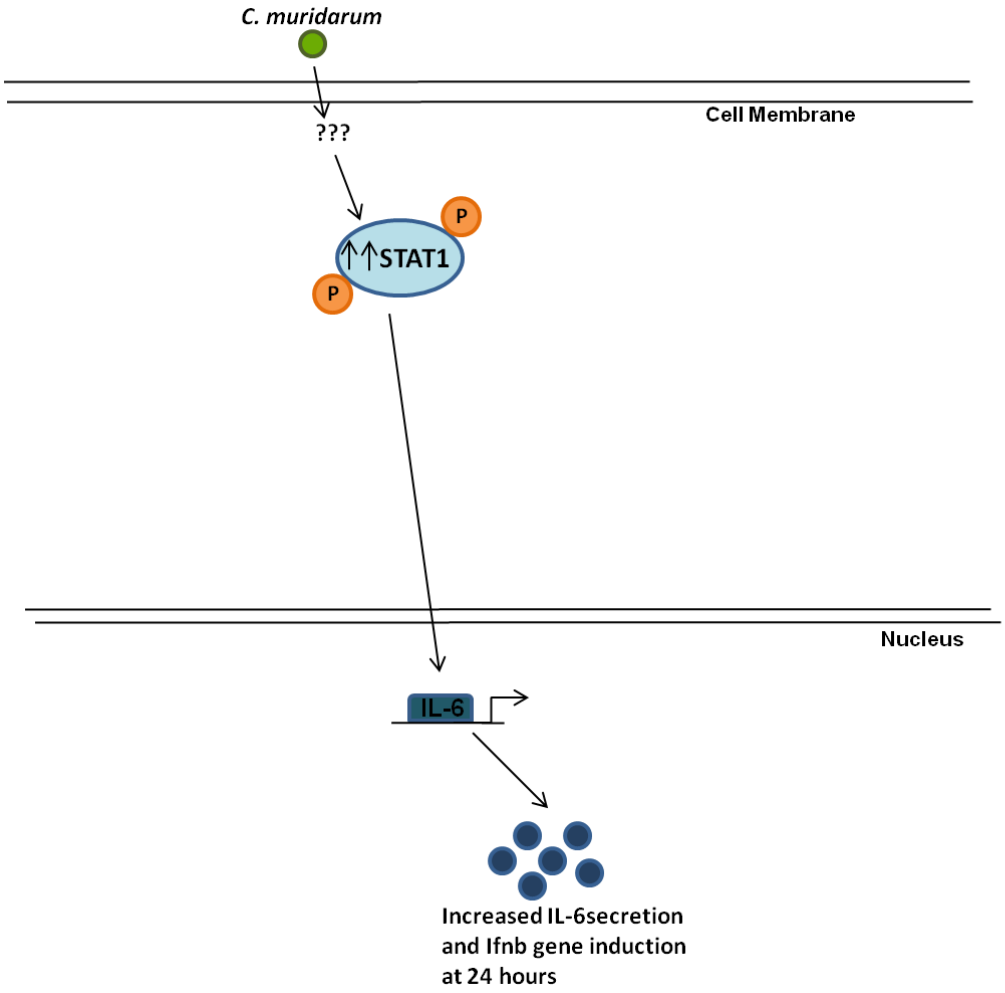


Figure 39. STAT1 is required for IL-6 secretion during *C. muridarum* infection

OE cells infected with *C. muridarum* showed a requirement for STAT1 in the secretion of IL-6, an acute-phase proinflammatory cytokine, presumably via an unidentified pathway stimulated by *C. muridarum*.

DISCUSSION

The epithelial cells' innate immune responses play an important role in the onset of pathogenesis in response to *Chlamydia* infection, and are critical for effective clearance of the pathogen [17, 165]. We have previously shown that *C. muridarum* infected OE cells secrete robust levels of TLR-dependent cytokines and chemokines involved in the innate immune response, including acute phase inflammatory cytokines such as IL-6 and GM-CSF, and the immunomodulatory cytokines such as IFN-β [155]. In subsequent reports, we identified TLR3 as the PRR required for *Chlamydia*-induced IFN-β production in OE cells by showing that OE cells that were either deficient or disrupted in TLR3 signaling, also had severely diminished levels in IFN-β synthesis [250, 251]. Here we show that STAT1, a key component of type I IFN signaling pathway, is also critical for optimal *Chlamydia*-induced IFN-β and IL-6 production in OE cells. We also show that STAT1 plays a major role in the *Chlamydia*-induced synthesis of other type I IFNs in OE cells during early- and late-stage infection. However, our data suggest that STAT1 may have function in type I IFN production, particularly IFN-β production, through pathways that are independent of the type I IFN signaling pathway via the IFNAR. Evidence in this study also suggests a critical role in the early activation of STAT1 that contributes to optimal IFN-β production, and that may contribute to the optimal production of IL-6. Furthermore, we report on a unique expression pattern of type I IFNs during *C. muridarum* infection by demonstrating a differential regulation and dependence on TLR3 between IFN-β and other type I IFNs.

We showed that Stat1 gene expression, STAT1 protein production, and protein activation were significantly increased in wild-type OE cells during *Chlamydia* infection.

Furthermore, we provide evidence in this study that suggests STAT1 is critical for regulating IFN-β secretion, and restricting *C. muridarum* replication in induced OE cells (Figure 10 and Figure 11, respectively). STAT1 is well established as a mediator involved in type I interferon signaling, and in type I interferon-dependent antiviral and antimicrobial responses [169, 238]. Additionally, STAT1 has been implicated as a critical regulator of type I and type II interferon-dependent control of *Chlamydia* infections in nonhemopoeitic cells [254, 264]. Lad et al, demonstrated that HeLa 229 epithelial cells infected with *C. trachomatis* showed an increased STAT1 activation with a resultant reduction in *Chlamydia* replication compared to 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; suggesting that STAT1 activity was IFN-β-dependent in cervical epithelial cells, and proposing that STAT1 has a role for the IFN-β autocrine/paracrine regulatory pathway.

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

This current study's investigation into the role of STAT1 seeks to further define the role of STAT1 in the pathogenesis of *Chlamydia* infection by ascertaining whether STAT1 contributed to the residual amount of *Chlamydia*-induced IFN-β in OE cells lacking TLR3. We hypothesized that STAT1 is an important mediator in the type I IFN response during *Chlamydia muridarum* infection in OE cells, and that it occurs following the TLR3-dependent IFN-β response in order to amplify and sustain the optimal response required to presumably combat infection. The results shown in Figure 13 detail the significant reduction in Ifnb gene expression during *C. muridarum* infection of OE cells

lacking TLR3 compared to wild type OE cells. However; as shown in Figure 14, the lack of STAT1 appeared to have a more dramatic effect in reducing Ifnb synthesis, particularly late during infection. These findings were corroborated in ELISA analysis of supernatants from STAT1-deficient OEs in which we observed significant reductions in *C. muridarum*-induced IFN-β synthesis compared to wild-type OE cells (Figure 10). STAT1 is a critical mediator of type 1 IFN signaling. This data implicates an important role for STAT1 in the synthesis of IFN-β during *Chlamydia muridarum* infections in OE cells; and proposes a possible role for STAT1-mediated type I IFN signaling in the synthesis of type I IFNs during *Chlamydia* infection of OE cells.

B. Temporal and differential upregulation of *C. muridarum*-induced Ifna gene subtypes in OEs requires STAT1, type I IFN-mediated signaling via IFNAR, and is attenuated by TLR3

We show that STAT1 is critical for IFN-β secretion at 24 hours post-*C. muridarum* infection which represents "late-stage" infection in OE cells for this particular laboratory strain. Our hypothesis proposes that this STAT1-dependent IFN-β response occurs, presumably late during infection in OE cells, following the acute response mediated by TLR3. We also propose that STAT1 mediates a similar response for other type I IFNs in OE cells, and that both responses would require type I IFN signaling via the IFNAR. The role of IFN-α has been extensively studied and well documented in viral infections [112, 168, 265-268]; however, the regulatory mechanisms governing IFNα production in *Chlamydia* infections and other similar intracellular bacterial pathogens are poorly understood. In this study we investigated the temporal production of two well characterized IFN-α subtypes; IFN-α2 and IFN-α4 [269-272], and the possible roles that

both IFNAR-dependent type I IFN signaling (presumably initiated by IFN-β) and STAT1 play in regulating their expression in OE cells. Our goal was to ascertain whether *Chlamydia*-induced type I IFN signaling via the IFNAR was critical for the induction of Ifna gene subtypes, and to determine if STAT1 was germane to this process. Our data revealed that there were notable increases in Ifna2 and Ifna4 gene expression in wild-type OE cells upon *C. muridarum* infection and that both sub-types were induced at similar levels in a STAT1 dependent manner. Interestingly, Ifna2 was induced at the early stage of infection while Ifna4 was significantly induced at the late-stage of infection. Both IFN-α sub-types' gene induction were substantially reduced upon inhibition of type I IFN signaling by IFNAR neutralizing antibody; implicating the importance of this pathway in their production during *Chlamydia*-infection in OE cells. Our results reveal a suspected differential induction of Ifna subtypes in OE cells during *C. muridarum* infection; however, our results differ from the investigations of others which typically identify IFNα-4 as the "early subtype" and refer to IFNα-2 as the "late subtype" [195, 269, 273]. Nevertheless, other studies show that the temporal induction of these subtypes can vary dependent on cell type and mode of induction [274, 275].

We showed that both Ifna2 and Ifnb4 were induced in response to exogenous IFN-β, and so we speculate that *Chlamydia*-induced IFN-β regulates the synthesis of other type I IFNs such as the IFN-α subtypes during infection. Other investigations have shown that IFN-β-deficient macrophages produce significantly reduced levels of IFN-α in response to *C. pnuemoniae* infection [159]. Interestingly, we showed that OE cells lacking TLR3 induced both Ifna2 and Ifna4 at considerably higher levels (40-fold and 25-fold, respectively) when compared to wild-type OE cells (Figure 16 and Figure 17).

These results suggest that TLR3 plays a role in regulating the expression of Ifna subtypes, particularly by attenuating its expression during *Chlamydia* infection. Recent studies have identified a plausible mechanism for attenuation of several chemokines and cytokines (including type I IFN), that is dependent on TLR signaling in murine macrophages. One such mechanism involves a TLR-dependent induction of the scaffold/adaptor protein, p62 [276]. Although it is unclear exactly if TLR3 stimulation would lead to p62 activation during *Chlamydia* infection of OE cells, the ability of TLR3 to attenuate expression of *C. muridarum*-induced 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 syntheses of certain type I IFNs are indeed detrimental to the host during *Chlamydia* infection [163], this would represent an important role for TLR3 in *Chlamydia* pathogenesis because of its potential to limit the production of certain type I IFNs; thus limiting 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 [255]. In those studies, we described the ability of TLR3 to modulate the immune response during *C. muridarum* infection in OE cells using mechanisms that were dependent and independent of the synthesis of IFN-β. 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.

C. *C. muridarum*-induced type I IFN and proinflammatory cytokine production is highly dependent on STAT1, but is differentially regulated by the type I signaling pathway in OE cells

Though temporally regulated, the IFN-α subtypes required both STAT1 and the IFNAR for optimal induction during *C. muridarum* infection in OE cells. IFN-β was shown to be STAT1-dependent and induced to optimal levels during late-stage *C. muridarum* infection. We tested the hypothesis that the type I IFN signaling pathway, via the IFNAR, had a critical role in the synthesis of IFN-β during *Chlamydia* infection in OE cells. Our previous data highlights the importance of the type I IFN signaling pathway in the *Chlamydia*-induced synthesis of IFNα-2 and IFNα-4, and could support the conclusions of others describing a critical role for type I IFN signaling pathways in the pathogenesis of *Chlamydia* infection [163, 277]. In those studies, the investigators showed reductions in the levels of *Chlamydia*-induced pathology in both the lung and genital-tract infection models in the IFNAR-deficient mice, and attributed those reductions in pathogenesis to an increase in IFN-γ production and rapid clearance of *Chlamydia*. The results from those earlier studies implicate synthesis of type I IFNs in response to *Chlamydia* infection as being detrimental to the host.

However, we showed that IFNAR-dependent type I IFN signaling pathways had virtually no effect in the synthesis of Ifnb during *C. muridarum* infection in OE cells in experiments in which the IFNAR was blocked by neutralization antibody (Figure 19). It is possible that the concentration of IFNAR neutralizing antibody used may not have been sufficient to block type I IFN signaling; however, we used two times the working concentration of the antibody (4.6 ng/ml) and have observed on average that *C.*

muridarum-infected OE WT cells secrete from 450-700 pg/ml of IFN-β at 24 hours post infection (Reference Figure 10). Also, the autocrine/paracrine gene induction of Ifnb in response to exogenous IFN-β appeared to be completely unaffected by neutralization antibody to the IFNAR (Figure 22), whereas both Ifna2 and Ifn4 showed significant but differential reductions in their expression when the IFNAR1 was blocked with neutralization antibody (Figure 20 and Figure 21). Although all three type I IFNs are highly dependent on STAT1 for optimal induction 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 I IFN signaling pathway that signals through the IFNAR. There are theories of possible intracellular signaling that may contribute to initiating interferon-stimulated genes independently of the IFNAR receptor. One such possibility involves a known transcription factor required for type I IFN production, IRF7. A study has shown that during West Nile viral infections, Irf7 gene was induced in IFNAR-deficient mouse embryonic fibroblasts were it accumulated in the cytoplasm and, additionally, required both IRF3 and IRF9 for induction [278]. It is possible that the coordinated functions of intracellular transcription factors induced by *Chlamydia* infection could initiate type I IFN-stimulated gene induction (including type I IFNs themselves); independently of the IFNAR in OE cells (Figure 40) as this has be shown in myeloid-derived cells [279].

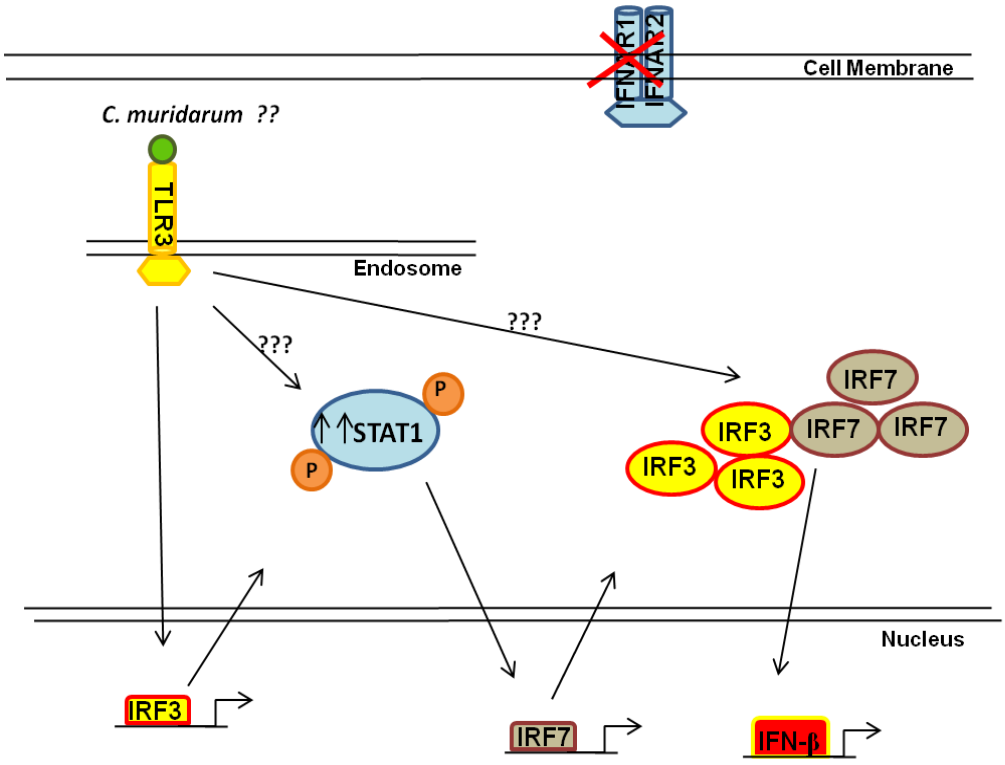


Figure 40. Possible mechanism of STAT1-mediated IFNAR1-indpendent IFN-β production by intracellular transcription factors in OE cells during *C. muridarum* infection

It is possible in OE cells that *C. muridarum* infection can stimulate TLR3 signaling via an unknown PAMP which initiates Irf3 transcription as well as Ifr7 transcription possibly mediated by STAT1. These transcription factors accumulate, and are activated in the cell cytoplasm, possibly via unidentified kinases, and then translocate to the nucleus where they initiate type I IFN gene transcription such as Ifnb independently of the IFNAR.

Furthermore, IFN-β neutralization experiments from this study showed that while neutralization of IFN-β had virtually no effect on *C. muridarum*-induced Ifnb in OE cells (Figure 24), neutralizing exogenous IFN-β using an antibody showed a substantial reduction in autocrine/ paracrine Ifnb expression when compared to treating cells with exogenous recombinant IFN-β alone (Figure 25). This finding was contrary to observations obtained from the experiments whereby the OE cell's IFNAR was blocked with neutralizing antibody before treating with exogenous IFN-β. Our data showed that the levels of Ifnb induced were unaffected when IFNAR was blocked with neutralizing antibody (Figure 22). The differences in IFN-β production between *C. muridarum*-infected OEs and exogenous IFN-β treated OEs treated with IFN-β neutralization antibody could possibly be explained by the ability of *C. muridarum* to stimulate redundant pathways that contribute to IFN-β production such as the recently identified cytosolic nucleic acid sensors, helicase DDX41 and LRRFIP1 [280, 281]. The IFNAR is composed of two heterodimeric receptors, IFNAR1 and IFNAR2. In these experiments we neutralized the IFNAR1, therefore it is possible that there was incomplete blockage of the IFNAR and IFNAR2 could have more affinity for IFN-β thus neutralizing IFNAR1 would have minimal effect on Ifnb induction during *C. muridarum* infection in OE cells. It is also possible that in OE cells IFN-α subtypes particularly IFN-α2 since its gene was induced early compared to IFN-α4 in this study, could initiate STAT1-mediated type I IFN signaling leading to amplified IFN-β production. However, IFN-β has been implicated as a "prerequisite" for viral-induced interferon synthesis, including IFN-α, in mouse embryonic fibroblasts [282]. Nevertheless, our data here suggests that IFN-β may

play a role in its autocrine/paracrine regulation possibly through interactions with novel receptors and/or components that are independent of signaling through the IFNAR.

Our data proposes that STAT1 is critical for the *Chlamydia*-induced synthesis of IFN-β in OE cells, and that the STAT1 activation required for IFN-β synthesis occurs via mechanisms that do not involve JAK/STAT activation through type I IFN signaling via the IFNAR as required for IFN-α synthesis. Recall, we initially proposed that STAT1's contribution to the type I IFN response occurred predominantly during late-stage *Chlamydia* infection. However, we showed an early-stage induction of Ifna2 during *C. muridarum* infection that was STAT1-dependent; suggesting that STAT1 may play a more prominent role early during *Chlamydia* infection in the synthesis of type I IFN and/or other acute-phase cytokines. Our data shows that STAT1 activation and expression are TLR3-dependent during *C. muridarum* infection in OE cells. We have provided additional evidence in this study that suggests noticeable changes in STAT1 activation in OE cells during *Chlamydia* infection (Figure 30). We also show significant reductions in IFN-β secreted from these cells upon early-phase inhibition of STAT1 activation in *Chlamydia*-infected OE cells. It is possible in OE cells that *Chlamydia* instead activates STAT1 through direct stimulation of TLR-induced activation of kinases such as the p38 MAPK known to phosphorylate STAT1 at serine 727 in macrophages upon *Listeria* stimulation [283]; though tyrosine phosphorylation of STAT1 has also been shown to be required for its full transcriptional activity in other cell types [219], or through stimulation of TLRs that synthesize immune factors that are known to activate JAK/STAT signaling pathways which 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, its levels are significantly induced and is detectable in the supernatants as early as 3 hours [44, 155]. 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 interferon regulatory factor 1 (IRF1) gene transcription and potentially type I IFN production [284]. It is possible that upon initially encountering *Chlamydia*, the induction of TLR2-dependent IL-6, or possibly TLR3-dependent IL-6 production, leads to the activation of STAT1-dependent Irf1 induction, that then serves as a primer or co-activator for more robust TLR3-dependent IFN-β synthesis in OE cells (Figure 41). Our results show that *C. muridarum* can induce minimal expression of Ifnb in the absence of STAT1 in the OE cells when compared to either wild-type or TLR3 deficient OE cells (Figure 13 and Figure 14). 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.

However, we do describe observations in this report in which STAT1-deficient BMDMs and OEs show significantly reduced IL-6 production at 24 hours PI compared to wild-type OEs (Figure 32 and Figure 33, respectively). These data were further corroborated by siRNA experiments in which *C. muridarum*-induced STAT1-knockdown OE cells also showed a significant decrease in IL-6 production compared to infected wild-type OEs and OEs transfected with the control plasmid (Figure 34). Published data from our lab has shown a similar decrease in *C. muridarum*-induced IL-6 production in

TLR3-deficient OEs, when compared to *C. muridarum* infected wild-type OE cells [255]. In that earlier investigation, it was suggested that the diminished amount of *Chlamydia*-induced IL-6 synthesis in the TLR3-deficient OE cells were the result of a decreased level of Tlr2 gene upregulation during *Chlamydia* infection, when compared to the level of Tlr2 gene upregulation in the wild-type OE cells. We hypothesized that the Tlr2 gene upregulation during *Chlamydia* infection occurred through mechanisms that involved TLR3-dependent IFN-β synthesis. In additional data from that study we showed that Tlr2 gene expression was upregulated when the TLR3-deficient OE cells were pre-treated with exogenous IFN-β. The current study shows that STAT1 also has a critical role in the synthesis of IFN-β and taken together with the importance of TLR3 in the *Chlamydia*-induced synthesis of IFN-β in OE cells, these observations highlight a role for STAT1 in the positive amplification of other proinflammatory cytokines such as IL-6 during *C. muridarum* infection of OE cells.

.

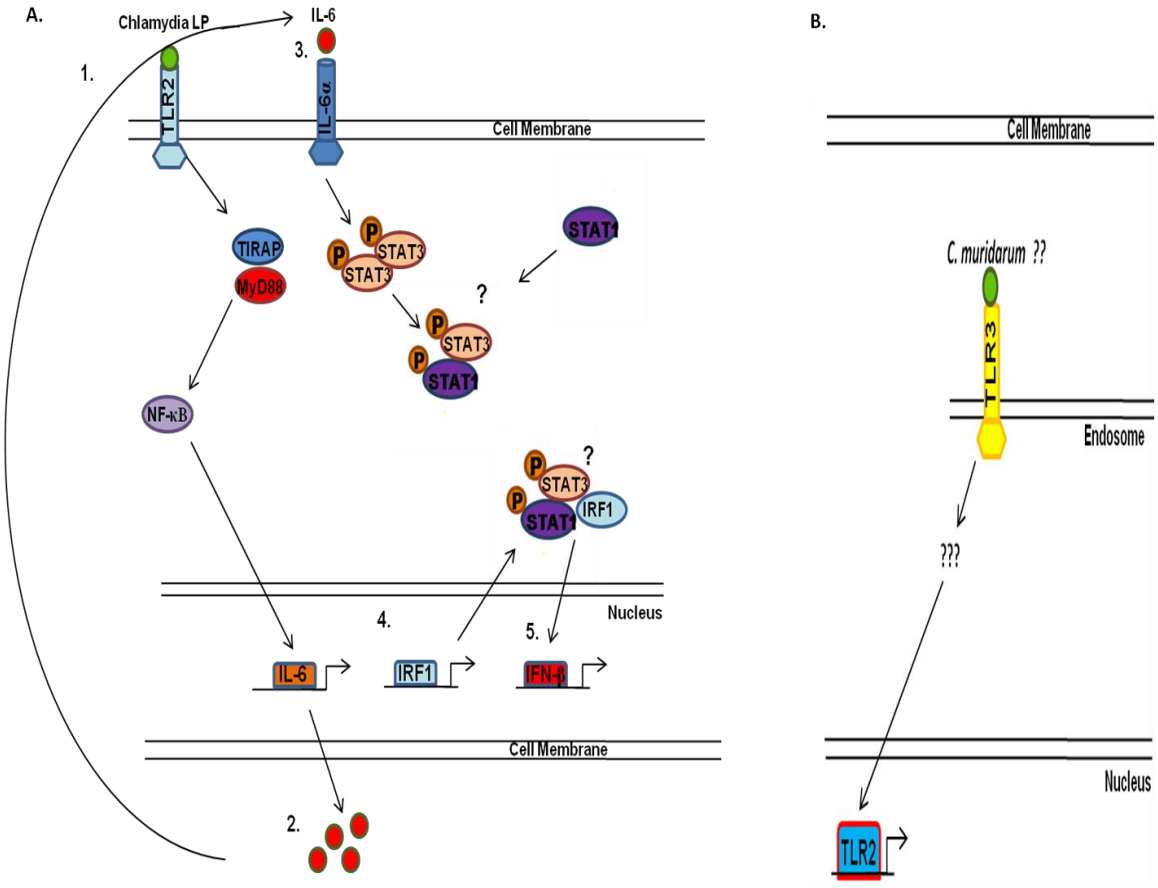


Figure 41. Possible mechanism of early STAT1 activation and optimal IFN-β production through *C. muridarum*-induced IL-6 production in OE cells

(A.) Early STAT1 activation, and ultimately, the production of IFN-β could be induced in OE cells by 1.) stimulation of TLR2 via a *Chlamydia* LP or unidentified PAMP leading to 2.) the expression and secretion of IL-6 which 3.) binds to and stimulates the IL-6α receptor of the same cell or neighboring cells. It is known that IL-6 signaling propagation requires STAT3; therefore, it is possible that phosphorylated STAT3 could recruit, phosphorylated, and form heterodimeric complexes with STAT1, and 4.) translocate to the nucleus initiating the transcription of IRF1. IRF1 is known to bind STAT1 and could possibly complex with the STAT1-STAT3 homodimer and 5.) translocate to the nucleus initiating the transcription of type I IFNs such as IFN-β. (B.) In

addition, TLR3 could indirectly contribute to this response by its role in upregulating Tlr2 gene expression; presumably leading to increased TLR2 expression on the cell surface and ultimately increased IL-6 production.

CONCLUSION

The present study reveals that STAT1 is a critical component of the *Chlamydia*-induced type I interferon and proinflammatory response in oviduct epithelial cells. While IFNAR-dependent type I IFN signaling was shown to play a minimal role in IFN-β production, STAT1 was shown to be critical for optimal induction of IFN-β during the late stages of *C. muridarum* infection in OE cells; presumably dependent on early STAT1 activation. In addition, we show that STAT1 plays a critical role in restricting *C. muridarum* replication in OE cells. We show that STAT1 is required for the *Chlamydia*-induced induction of several genes involved in the type I IFN signaling pathway such as Stat2 and Irf7 during late stages of infection, which is indicative of the type I IFN signaling pathway being most active at the latter stages of *Chlamydia* infection. We showed that STAT1 was also critical for the induction of the Ifna subtypes 2 and 4; which were revealed to be differentially expressed and negatively regulated by TLR3 signaling during *C. muridarum* infection of OE cells. Our data show that IFN-β appears to be regulated in a different manner from other type I IFNs such as IFN-α during *C. muridarum* infection of OE cells, and that Ifna gene subtypes' induction is dependent on type I IFN signaling via the IFNAR signaling pathway. STAT1 was also shown to be required for optimal IL-6 production in *C. muridarum*-induced OE cells. These studies implicate STAT1 as a major modulator of the *Chlamydia*-induced type I interferon and IL-6 response in OE cells, and highlights the possible involvement of multiple and biphasic STAT1-dependent signaling pathways, TLR signaling pathways, and the autocrine/ paracrine pathways involved in regulating their expression.

FUTURE DIRECTIONS

The studies in this thesis have begun to address the contributions of STAT1 in *C. muridarum*-induced IFN-β production in OE cells, and the mechanisms that govern these responses. Elucidation of these pathways of induction and discerning the mechanism(s) involved in the regulation of STAT1 induction during infection will be critical for increased understanding of the overall pathogenesis of *Chlamydia* urogential infections, and could be advantageous in developing future efficacious therapeutics and/or vaccines. However, there are still a number of questions to be answered and observations to be investigated based on results presented in Chapters I-II and discussed above.

For future investigations it is important to identify additional PRRs in OEs that may be induced by *C. muridarum* and contribute to the IFN-β response. Recently, novel cytosolic nucleic acid sensors have been identified in classical immune cells and were shown upon stimulation, induce robust amounts of type I IFN. These PRRs were identified as a DNA helicase, DDX41 [281], and a cytosolic nucleic acid-binding protein, LRRFIP1 [280]. Primers specific for these newly identified cytosolic nucleic acid sensors have been described in the literature, and so it would be advantageous for us to first check for expression of these PRRs in OE cells via RT-PCR. If these PRRs are expressed in OEs we could then observe any changes in their expression upon *C. muridarum* infection. If obvious changes occur, particularly increased expression, we could then knockdown the expressions of either DDX41 or LRRFIP1 through small interfering RNA experiments, and analyze IFN-β secretion via ELISA. Additionally, in an OE cell line named Bm1.11 we previously showed the expression of NOD1 in uninduced conditions and *C. muridarum*-induced expression of NOD2 [155]. We could

further investigate the contributions of these cytosolic nucleotide sensors to *Chlamydia*-induced IFN-β synthesis in OE cells by performing similar analysis.

We have shown and verified a critical role for STAT1 in regulating the production of IFN-β *during C. muridarum* infection in OE cells. Our original hypothesis proposed that STAT1 mediated IFN-β production via type I IFN signaling occurred later in infection following the TLR3-dependent IFN-β response. We showed IFN-β synthesis to be optimal during late-stage infection, but in an IFNAR1-independent manner. To further investigate this phenomenon we could block the IFNAR2 component of the IFNAR and observe the effects on IFN-β production in OE cells during *C. muridarum* infection. To further ascertain STAT1's involvement in this response we could block both IFNAR1 and/or IFNAR2 during *Chlamydia* infection and observe the effects on STAT1 activation and expression in OE cells. Additionally, STAT2 activity could be analyzed in a similar manner to determine its possible contributions to this response since it is a known to form a heterodimeric transcriptional complex with STAT1 that initiates the transcription of type I interferons and other interferon-stimulated genes. While our observations show dramatic changes in Ifnb expression at both early and late stages of *Chlamydia* infection in STAT1-deficient OEs, we provide additional evidence that suggests early activation of STAT1 may play an even more critical role in optimizing the IFN-β response in OE cells. We could further investigate the biphasic activation of STAT1, and the role it may play in *C. muridarum*-induced IFN-β production in OE cells. As mentioned in the Discussion, it is possible that STAT1 may become activated initially by direct *C. muridarum* stimulation of TLRs, leading to the activation of factors that are capable of phosphorylating/activating STAT1. One possibility could be through TLR-

mediated activation of p38 MAPK, which has been shown as required to phosphorylate STAT1 on serine 727 for its full transcriptional activity [208]. We could observe changes in p38 activation upon *C. muridarum* infection in OEs via Western analysis, and additionally, observe STAT1 phosphorylation at serine 727 and IFN-β production upon inhibiting p38 activation at early times during *Chlamydia* infection. Moreover, we could also neutralize TLR2, the likely TLR candidate in OE cells that may lead to p38 activation, and observe the effects of blocking TLR2 signaling on STAT1 activation. Our studies also highlighted a type I IFN signaling-independent mechanism for *C. muridarum*-induced IFN-β production. To further delineated the activation kinetics of STAT1 and address a possible role for STAT1 in that mechanism we could neutralized IFNAR and IFN-β, similarly to experiments performed in this study, and observe STAT1 activation via Western analysis. As discussed above, it has been shown that pathogen-induced IL-6 signaling leads to the transcription of Irf1, a transcription factor implicated in type I IFN gene transcription and known to interact with STAT1 [182, 225]. We could explore the relationship between STAT1 deficiency IL-6-dependent Irf11 gene transcription, which we hypothesis will be directly correlated since in this study we observed decreased levels of IL-6 in STAT1-deficent OEs. Next, we could neutralize the IL-6 receptor similar to experiments in this study, and look at changes in STAT1 activation kinetics. In addition we could neutralize secreted IL-6 using an IL-6 neutralizing antibody to observe changes in STAT1 activity and type I IFN production. It would also be advantageous for us to observe the expression and activation profiles of additional STAT proteins such as STAT3 due to its direct involvement in IL-6 signaling and its ability to form heterodimers with STAT1 [182, 213].

We identified a temporal regulation and production of two distinct IFN-α sub-types, IFN-α2 and IFN-α4 upon *C. muridarum* infection in OEs. Moreover, we showed that production of these cytokines were type I IFN-dependent and attenuated by TLR3, in a manner that contrasts the regulation in the IFN-β response to *C. muridarum* infection. To further investigate this phenomena and the direct involvement of IFN-β, we could initially neutralize IFN-β and observe the changes in Ifna gene transcription compared to observations taken from IFNAR neutralization experiments undertaken in this study. In the Discussion we mentioned a TLR3-induced scaffold protein p62 that could possibly play a role in targeting IFN-α subtypes for degradation via ubiquitin. We could investigate this TLR3-p62-IFN-α axis in OE cells by observing p62 activation at early and late times upon *C. muridarum*-infection in TLR3-deficient cells via Western analysis. Additionally, we could investigate the expression and activity of SOCS via RT-PCR and Western analyses; particularly SOCS1 and SOCS3 in TLR3-deficient cells to determine if they may play a role in TLR3-attenuation of IFN-α2 and IFN-α4.

Upon validation of all in vitro studies and experiments it will be critical to utilized in vivo murine models to observe *C. muridarum*-induced phenotypic outcomes. Importantly, the use of IFN-β and IFN-α knockout mice would be of significance and could identify each cytokine's specific function in the overall pathogenesis of *C. muridarum.* In addition, STAT1 and TLR2 knockout mice would be of equal importance to observe their effects on *C. muridarum*-induced clearance (i.e. IFN-β production, bacterial recovery, leukocyte/lymphocyte recruitment, infection duration, and survival rates), and pathology (i.e. oviduct pathology, and uterine pathology). These future experiments would provide additional validation and potential mechanisms as to how

IFN-β is regulated during *C. muridarum* infections in OE cells, and how these factors contribute to the overall pathogenesis of *Chlamydia* during urogential infections.

REFERENCES

1. Pettersson B, Andersson A, Leitner T, Olsvik O, Uhlen M, Storey C, Black CM: **Evolutionary relationships among members of the genus Chlamydia based on 16S ribosomal DNA analysis.** *J Bacteriol* 1997, **179:**4195-4205.

2. Moulder JW: **Comparative biology of intracellular parasitism.** *Microbiol Rev* 1985, **49:**298-337.

3. Ward ME: **Chlamydial classification, Development and structure.** *British Medical Bulletin* 1983, **39:**109-115.

4. Storz J, Page LA: **Taxonomy of the Chlamydiae: reasons for classifying organisms of the genus Chlamydia, family Chlamydiaceae, in a separate order, Chlamydiales ord. nov.** *International Journal of Systematic Bacteriology* 1971, **21:**332-334.

5. Griffiths E, Ventresca MS, Gupta RS: **BLAST screening of chlamydial genomes to identify signature proteins that are unique for the Chlamydiales, Chlamydiaceae, Chlamydophila and Chlamydia groups of species.** *BMC Genomics* 2006, **7:**14.

6. Everett KD, Hornung LJ, Andersen AA: **Rapid detection of the Chlamydiaceae and other families in the order Chlamydiales: three PCR tests.** *J Clin Microbiol* 1999, **37:**575-580.

7. Everett KD, Andersen AA: **Identification of nine species of the Chlamydiaceae using PCR-RFLP.** *Int J Syst Bacteriol* 1999, **49 Pt 2:**803-813.

8. Everett KD, Bush RM, Andersen AA: **Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms.** *Int J Syst Bacteriol* 1999, **49 Pt 2:**415-440.

9. Peeling RW, Brunham RC: **Chlamydiae as pathogens: new species and new issues.** *Emerg Infect Dis* 1996, **2:**307-319.

10. Stephens RS, Kalman S, Lammel C, Fan J, Marathe R, Aravind L, Mitchell W, Olinger L, Tatusov RL, Zhao Q, et al,: **Genome sequence of an obligate intracellular pathogen of humans: Chlamydia trachomatis.** *Science* 1998, **282:**754-759.

11. Ojcius DM, Bravo de Alba Y, Kanellopoulos JM, Hawkins RA, Kelly KA, Rank RG, Dautry-Varsat A: **Internalization of Chlamydia by dendritic cells and stimulation of Chlamydia-specific T Cells.** *The Journal of Immunology* 1998, **160:**1297-1303.

12. Gervassi A, Alderson MR, Suchland R, Maisonneuve JF, Grabstein KH, Probst P: **Differential regulation of inflammatory cytokine secretion by human dendritic cells upon Chlamydia trachomatis infection.** *Infection and Immunity* 2004, **72:**7231-7239.

13. Kuo CC, Puolakkainen M, Lin TM, Witte M, Campbell LA: **Mannose-receptor positive and negative mouse macrophages differ in their susceptibility to infection by Chlamydia species.** *Microb Pathog* 2002, **32:**43-48.

14. Lee CK: **Interaction between a trachoma strain of Chlamydia trachomatis and mouse fibroblasts (McCoy cells) in the absence of centrifugation.** *Infection and Immunity* 1981, **31:**584-591.

15. Lee CK, Moulder JW: **Persistent infection of mouse fibroblasts (McCoy cells) with a trachoma strain of Chlamydia trachomatis.** *Infection and Immunity* 1981, **32:**822-829.

16. Wyrick PB, Choong J, Davis CH, Knight ST, Royal MO, Maslow AS, Bagnell CR: **Entry of genital Chlamydia trachomatis into polarized human epithelial cells.** *Infection and Immunity* 1989, **57:**2378-2389.

17. Rasmussen SJ, Eckmann L, Quayle AJ, Shen L, Zhang YX, Anderson DJ, Fierer J, Stephens RS, Kagnoff MF: **Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis.** *J Clin Invest* 1997, **99:**77-87.

18. Byrne GI: **Chlamydia trachomatis strains and virulence: rethinking links to infection prevalence and disease severity.** *J Infect Dis* 2010, **201 Suppl 2:**S126-133.

19. Wang SP, Grayston JT: **Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test.** *Am J Ophthalmol* 1970, **70:**367-374.

20. Barnes RC, Suchland RJ, Wang SP, Kuo CC, Stamm WE: **Detection of multiple serovars of Chlamydia trachomatis in genital infections.** *J Infect Dis* 1985, **152:**985-989.

21. Moulder JW: **Interaction of chlamydiae and host cells in vitro.** *Microbiol Rev* 1991, **55:**143-190.

22. Abdelrahman YM, Belland RJ: **The chlamydial developmental cycle.** *FEMS Microbiol Rev* 2005, **29:**949-959.

23. Taraktchoglou M, Pacey AA, Turnbull JE, Eley A: **Infectivity of Chlamydia trachomatis serovar LGV but not E is dependent on host cell heparan sulfate.** *Infection and Immunity* 2001, **69:**968-976.

24. Su H, Raymond L, Rockey DD, Fischer E, Hackstadt T, Caldwell HD: **A recombinant Chlamydia trachomatis major outer membrane protein binds to heparan sulfate receptors on epithelial cells.** *Proc Natl Acad Sci U S A* 1996, **93:**11143-11148.

25. Davis CH, Raulston JE, Wyrick PB: **Protein disulfide isomerase, a component of the estrogen receptor complex, is associated with Chlamydia trachomatis serovar E attached to human endometrial epithelial cells.** *Infection and Immunity* 2002, **70:**3413-3418.

26. Maslow AS, Davis CH, Choong J, Wyrick PB: **Estrogen enhances attachment of Chlamydia trachomatis to human endometrial epithelial cells in vitro.** *Am J Obstet Gynecol* 1988, **159:**1006-1014.

27. Clifton DR, Fields KA, Grieshaber SS, Dooley CA, Fischer ER, Mead DJ, Carabeo RA, Hackstadt T: **A chlamydial type III translocated protein is tyrosine-phosphorylated at the site of entry and associated with recruitment of actin.** *Proc Natl Acad Sci U S A* 2004, **101:**10166-10171.

28. Grieshaber SS, Grieshaber NA, Hackstadt T: **Chlamydia trachomatis uses host cell dynein to traffic to the microtubule-organizing center in a p50 dynamitin-independent process.** *J Cell Sci* 2003, **116:**3793-3802.

29. Grieshaber NA, Fischer ER, Mead DJ, Dooley CA, Hackstadt T: **Chlamydial histone-DNA interactions are disrupted by a metabolite in the methylerythritol phosphate pathway of isoprenoid biosynthesis.** *Proc Natl Acad Sci U S A* 2004, **101:**7451-7456.

30. Hackstadt T, Scidmore-Carlson MA, Shaw EI, Fischer ER: **The Chlamydia trachomatis IncA protein is required for homotypic vesicle fusion.** *Cell Microbiol* 1999, **1:**119-130.

31. Scidmore-Carlson MA, Shaw EI, Dooley CA, Fischer ER, Hackstadt T: **Identification and characterization of a Chlamydia trachomatis early operon encoding four novel inclusion membrane proteins.** *Mol Microbiol* 1999, **33:**753-765.

32. Hackstadt T, Rockey DD, Heinzen RA, Scidmore MA: **Chlamydia trachomatis interrupts an exocytic pathway to acquire endogenously synthesized sphingomyelin in transit from the Golgi apparatus to the plasma membrane.** *EMBO J* 1996, **15:**964-977.

33. Carabeo RA, Mead DJ, Hackstadt T: **Golgi-dependent transport of cholesterol to the Chlamydia trachomatis inclusion.** *Proc Natl Acad Sci U S A* 2003, **100:**6771-6776.

34. Friis RR: **Interaction of L cells and Chlamydia psittaci: entry of the parasite and host responses to its development.** *J Bacteriol* 1972, **110:**706-721.

35. Belland RJ, Zhong G, Crane DD, Hogan D, Sturdevant D, Sharma J, Beatty WL, Caldwell HD: **Genomic transcriptional profiling of the developmental cycle of Chlamydia trachomatis.** *Proc Natl Acad Sci U S A* 2003, **100:**8478-8483.

36. Bavoil PM, Hsia R, Ojcius DM: **Closing in on Chlamydia and its intracellular bag of tricks.** *Microbiology* 2000, **146 (Pt 11):**2723-2731.

37. Miller WC, Ford CA, Morris M, Handcock MS, Schmitz JL, Hobbs MM, Cohen MS, Harris KM, Udry JR: **Prevalence of chlamydial and gonococcal infections among young adults in the United States.** *JAMA* 2004, **291:**2229-2236.

38. Gerbase AC, Rowley JT, Heymann DH, Berkley SF, Piot P: **Global prevalence and incidence estimates of selected curable STDs.** *Sex Transm Infect* 1998, **74 Suppl 1:**S12-16.

39. Centers fDCaP: **Sexually Transmitted Disease Surveillance, 2008.** Atlanta, GA: U.S. Department of Health and Human Services; November 2009.

40. Kaushic C, Grant K, Crane M, Wira CR: **Infection of polarized primary epithelial cells from rat uterus with Chlamydia trachomatis: cell-cell interaction and cytokine secretion.** *Am J Reprod Immunol* 2000, **44:**73-79.

41. Cooper MD, Rapp J, Jeffery-Wiseman C, Barnes RC, Stephens DS: **Chlamydia trachomatis infection of human fallopian tube organ cultures.** *J Gen Microbiol* 1990, **136:**1109-1115.

42. Moller BR, Westrom L, Ahrons S, Ripa KT, Svensson L, von Mecklenburg C, Henrikson H, Mardh PA: **Chlamydia trachomatis infection of the Fallopian tubes. Histological findings in two patients.** *Br J Vener Dis* 1979, **55:**422-428.

43. Agrawal T, Vats V, Salhan S, Mittal A: **The mucosal immune response to Chlamydia trachomatis infection of the reproductive tract in women.** *J Reprod Immunol* 2009, **83:**173-178.

44. Johnson RM: **Murine oviduct epithelial cell cytokine responses to Chlamydia muridarum infection include interleukin-12-p70 secretion.** *Infect Immun* 2004, **72:**3951-3960.

45. Bowie WR, Jones H: **Acute pelvic inflammatory disease in outpatients: association with Chlamydia trachomatis and Neisseria gonorrhoeae.** *Ann Intern Med* 1981, **95:**685-688.

46. Simms I, Stephenson JM: **Pelvic inflammatory disease epidemiology: what do we know and what do we need to know?** *Sex Transm Infect* 2000, **76:**80-87.

47. van Valkengoed IG, Morre SA, van den Brule AJ, Meijer CJ, Bouter LM, Boeke AJ: **Overestimation of complication rates in evaluations of Chlamydia trachomatis screening programmes--implications for cost-effectiveness analyses.** *Int J Epidemiol* 2004, **33:**416-425.

48. Chesson HW, Pinkerton SD: **Sexually transmitted diseases and the increased risk for HIV transmission: implications for cost-effectiveness analyses of sexually transmitted disease prevention interventions.** *J Acquir Immune Defic Syndr* 2000, **24:**48-56.

49. Washington AE, Johnson RE, Sanders LL, Jr.: **Chlamydia trachomatis infections in the United States. What are they costing us?** *JAMA* 1987, **257:**2070-2072.

50. Lau CY, Qureshi AK: **Azithromycin versus doxycycline for genital chlamydial infections: a meta-analysis of randomized clinical trials.** *Sex Transm Dis* 2002, **29:**497-502.

51. Peters DH, Friedel HA, McTavish D: **Azithromycin. A review of its antimicrobial activity, pharmacokinetic properties and clinical efficacy.** *Drugs* 1992, **44:**750-799.

52. Del Rosso JQ: **A status report on the use of subantimicrobial-dose doxycycline: a review of the biologic and antimicrobial effects of the tetracyclines.** *Cutis* 2004, **74:**118-122.

53. Magid D, Douglas JM, Jr., Schwartz JS: **Doxycycline compared with azithromycin for treating women with genital Chlamydia trachomatis infections: an incremental cost-effectiveness analysis.** *Ann Intern Med* 1996, **124:**389-399.

54. Lea AP, Lamb HM: **Azithromycin. A pharmacoeconomic review of its use as a single-dose regimen in the treatment of uncomplicated urogenital Chlamydia trachomatis infections in women.** *Pharmacoeconomics* 1997, **12:**596-611.

55. Tilson EC, Sanchez V, Ford CL, Smurzynski M, Leone PA, Fox KK, Irwin K, Miller WC: **Barriers to asymptomatic screening and other STD services for adolescents and young adults: focus group discussions.** *BMC Public Health* 2004, **4:**21.

56. Bebear C, de Barbeyrac B: **Genital Chlamydia trachomatis infections.** *Clin Microbiol Infect* 2009, **15:**4-10.

57. van de Laar MJ, Morre SA: **Chlamydia: a major challenge for public health.** *Euro Surveill* 2007, **12:**E1-2.

58. Meyers DS, Halvorson H, Luckhaupt S: **Screening for chlamydial infection: an evidence update for the U.S. Preventive Services Task Force.** *Ann Intern Med* 2007, **147:**135-142.

59. Hwang L, Shafer MA: **Chlamydia trachomatis infection in adolescents.** *Adv Pediatr* 2004, **51:**379-407.

60. Brunham RC, Zhang DJ, Yang X, McClarty GM: **The potential for vaccine development against chlamydial infection and disease.** *J Infect Dis* 2000, **181 Suppl 3:**S538-543.

61. Longbottom D, Livingstone M: **Vaccination against chlamydial infections of man and animals.** *Vet J* 2006, **171:**263-275.

62. Olivares-Zavaleta N, Whitmire W, Gardner D, Caldwell HD: **Immunization with the attenuated plasmidless Chlamydia trachomatis L2(25667R) strain provides partial protection in a murine model of female genitourinary tract infection.** *Vaccine* 2010, **28:**1454-1462.

63. Schautteet K, De Clercq E, Vanrompay D: **Chlamydia trachomatis vaccine research through the years.** *Infect Dis Obstet Gynecol* 2011, **2011:**963513.

64. Yu H, Karunakaran KP, Kelly I, Shen C, Jiang X, Foster LJ, Brunham RC: **Immunization with live and dead Chlamydia muridarum induces different levels of protective immunity in a murine genital tract model: correlation with MHC class II peptide presentation and multifunctional Th1 cells.** *Journal of Immunology* 2011, **186:**3615-3621.

65. Hess J, Schaible U, Raupach B, Kaufmann SH: **Exploiting the immune system: toward new vaccines against intracellular bacteria.** *Adv Immunol* 2000, **75:**1-88.

66. Hickey DK, Aldwell FE, Beagley KW: **Transcutaneous immunization with a novel lipid-based adjuvant protects against Chlamydia genital and respiratory infections.** *Vaccine* 2009, **27:**6217-6225.

67. Olive C, Toth I, Jackson D: **Technological advances in antigen delivery and synthetic peptide vaccine developmental strategies.** *Mini Rev Med Chem* 2001, **1:**429-438.

68. Pal S, Theodor I, Peterson EM, de la Maza LM: **Immunization with an acellular vaccine consisting of the outer membrane complex of Chlamydia trachomatis induces protection against a genital challenge.** *Infection and Immunity* 1997, **65:**3361-3369.

69. Su H, Parnell M, Caldwell HD: **Protective efficacy of a parenterally administered MOMP-derived synthetic oligopeptide vaccine in a murine model of Chlamydia trachomatis genital tract infection: serum neutralizing IgG antibodies do not protect against chlamydial genital tract infection.** *Vaccine* 1995, **13:**1023-1032.

70. Sun G, Pal S, Weiland J, Peterson EM, de la Maza LM: **Protection against an intranasal challenge by vaccines formulated with native and recombinant preparations of the Chlamydia trachomatis major outer membrane protein.** *Vaccine* 2009, **27:**5020-5025.

71. Tuffrey M, Alexander F, Conlan W, Woods C, Ward M: **Heterotypic protection of mice against chlamydial salpingitis and colonization of the lower genital tract with a human serovar F isolate of Chlamydia trachomatis by prior immunization with recombinant serovar L1 major outer-membrane protein.** *J Gen Microbiol* 1992, **138 Pt 8:**1707-1715.

72. van Drunen Littel-van den Hurk S, Gerdts V, Loehr BI, Pontarollo R, Rankin R, Uwiera R, Babiuk LA: **Recent advances in the use of DNA vaccines for the treatment of diseases of farmed animals.** *Adv Drug Deliv Rev* 2000, **43:**13-28.

73. Schautteet K, Stuyven E, Beeckman DS, Van Acker S, Carlon M, Chiers K, Cox E, Vanrompay D: **Protection of pigs against Chlamydia trachomatis challenge by administration of a MOMP-based DNA vaccine in the vaginal mucosa.** *Vaccine* 2011, **29:**1399-1407.

74. Donati M, Sambri V, Comanducci M, Di Leo K, Storni E, Giacani L, Ratti G, Cevenini R: **DNA immunization with pgp3 gene of Chlamydia trachomatis inhibits the spread of chlamydial infection from the lower to the upper genital tract in C3H/HeN mice.** *Vaccine* 2003, **21:**1089-1093.

75. Ifere GO, He Q, Igietseme JU, Ananaba GA, Lyn D, Lubitz W, Kellar KL, Black CM, Eko FO: **Immunogenicity and protection against genital Chlamydia infection and its complications by a multisubunit candidate vaccine.** *J Microbiol Immunol Infect* 2007, **40:**188-200.

76. Ulmer JB, Fu TM, Deck RR, Friedman A, Guan L, DeWitt C, Liu X, Wang S, Liu MA, Donnelly JJ, Caulfield MJ: **Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA.** *Journal of Virology* 1998, **72:**5648-5653.

77. Vanrompay D, Hoang TQ, De Vos L, Verminnen K, Harkinezhad T, Chiers K, Morre SA, Cox E: **Specific-pathogen-free pigs as an animal model for studying Chlamydia trachomatis genital infection.** *Infection and Immunity* 2005, **73:**8317-8321.

78. Moss TR (Ed). **International Handbook of Chlamydia**. Doncaster: Euromed Communications Ltd; 2003.

79. el-Asrar AM, Van den Oord JJ, Geboes K, Missotten L, Emarah MH, Desmet V: **Immunopathology of trachomatous conjunctivitis.** *Br J Ophthalmol* 1989, **73:**276-282.

80. Watkins NG, Hadlow WJ, Moos AB, Caldwell HD: **Ocular delayed hypersensitivity: a pathogenetic mechanism of chlamydial-conjunctivitis in guinea pigs.** *Proc Natl Acad Sci U S A* 1986, **83:**7480-7484.

81. Starnbach MN, Bevan MJ, Lampe MF: **Murine cytotoxic T lymphocytes induced following Chlamydia trachomatis intraperitoneal or genital tract infection respond to cells infected with multiple serovars.** *Infection and Immunity* 1995, **63:**3527-3530.

82. van der Snoek EM, Ossewaarde JM, van der Meijden WI, Mulder PG, Thio HB: **The use of serological titres of IgA and IgG in (early) discrimination between rectal infection with non-lymphogranuloma venereum and lymphogranuloma venereum serovars of Chlamydia trachomatis.** *Sex Transm Infect* 2007, **83:**330-334.

83. Dessus-Babus S, Darville TL, Cuozzo FP, Ferguson K, Wyrick PB: **Differences in innate immune responses (in vitro) to HeLa cells infected with nondisseminating serovar E and disseminating serovar L2 of Chlamydia trachomatis.** *Infection and Immunity* 2002, **70:**3234-3248.

84. Brunham RC, Rey-Ladino J: **Immunology of Chlamydia infection: implications for a Chlamydia trachomatis vaccine.** *Nat Rev Immunol* 2005, **5:**149-161.

85. Morrison RP, Caldwell HD: **Immunity to murine chlamydial genital infection.** *Infection and Immunity* 2002, **70:**2741-2751.

86. Read TD, Brunham RC, Shen C, Gill SR, Heidelberg JF, White O, Hickey EK, Peterson J, Utterback T, Berry K, et al,: **Genome sequences of Chlamydia trachomatis MoPn and Chlamydia pneumoniae AR39.** *Nucleic Acids Res* 2000, **28:**1397-1406.

87. Brunham RC (Ed). **Chlamydia: Intracellular Biology, Pathogenesis, and Immunity**. Washington DC: American Society for Microbiology Press; 1999.

88. Caldwell HD, Wood H, Crane D, Bailey R, Jones RB, Mabey D, Maclean I, Mohammed Z, Peeling R, Roshick C, et al,: **Polymorphisms in Chlamydia trachomatis tryptophan synthase genes differentiate between genital and ocular isolates.** *J Clin Invest* 2003, **111:**1757-1769.

89. Joyner JL, Douglas JM, Jr., Foster M, Judson FN: **Persistence of Chlamydia trachomatis infection detected by polymerase chain reaction in untreated patients.** *Sex Transm Dis* 2002, **29:**196-200.

90. Belland RJ, Scidmore MA, Crane DD, Hogan DM, Whitmire W, McClarty G, Caldwell HD: **Chlamydia trachomatis cytotoxicity associated with complete and partial cytotoxin genes.** *Proc Natl Acad Sci U S A* 2001, **98:**13984-13989.

91. Nelson DE, Virok DP, Wood H, Roshick C, Johnson RM, Whitmire WM, Crane DD, Steele-Mortimer O, Kari L, McClarty G, Caldwell HD: **Chlamydial IFN-gamma immune evasion is linked to host infection tropism.** *Proc Natl Acad Sci U S A* 2005, **102:**10658-10663.

92. Barron AL, Rank RG, Moses EB: **Immune response in mice infected in the genital tract with mouse pneumonitis agent (Chlamydia trachomatis biovar).** *Infection and Immunity* 1984, **44:**82-85.

93. Cotter TW, Byrne GI: **Immunity to Chlamydia: comparison of human infections and murine models.** *Res Immunol* 1996, **147:**587-595.

94. Kagnoff MF, Eckmann L: **Epithelial cells as sensors for microbial infection.** *J Clin Invest* 1997, **100:**6-10.

95. Roan NR, Starnbach MN: **Immune-mediated control of Chlamydia infection.** *Cell Microbiol* 2008, **10:**9-19.

96. Porter E, Yang H, Yavagal S, Preza GC, Murillo O, Lima H, Greene S, Mahoozi L, Klein-Patel M, Diamond G, et al,: **Distinct defensin profiles in Neisseria gonorrhoeae and Chlamydia trachomatis urethritis reveal novel epithelial cell-neutrophil interactions.** *Infection and Immunity* 2005, **73:**4823-4833.

97. Ramsey KH, Cotter TW, Salyer RD, Miranpuri GS, Yanez MA, Poulsen CE, DeWolfe JL, Byrne GI: **Prior genital tract infection with a murine or human biovar of Chlamydia trachomatis protects mice against heterotypic challenge infection.** *Infection and Immunity* 1999, **67:**3019-3025.

98. Quayle AJ: **The innate and early immune response to pathogen challenge in the female genital tract and the pivotal role of epithelial cells.** *J Reprod Immunol* 2002, **57:**61-79.

99. Buchholz KR, Stephens RS: **Activation of the host cell proinflammatory interleukin-8 response by Chlamydia trachomatis.** *Cell Microbiol* 2006, **8:**1768-1779.

100. Parr MB, Parr EL: **Antigen recognition in the female reproductive tract: I. Uptake of intraluminal protein tracers in the mouse vagina.** *J Reprod Immunol* 1990, **17:**101-114.

101. Reddy BS, Rastogi S, Das B, Salhan S, Verma S, Mittal A: **Cytokine expression pattern in the genital tract of Chlamydia trachomatis positive infertile women - implication for T-cell responses.** *Clin Exp Immunol* 2004, **137:**552-558.

102. Lu H, Shen C, Brunham RC: **Chlamydia trachomatis infection of epithelial cells induces the activation of caspase-1 and release of mature IL-18.** *Journal of Immunology* 2000, **165:**1463-1469.

103. Zhong GM, de la Maza LM: **Activation of mouse peritoneal macrophages in vitro or in vivo by recombinant murine gamma interferon inhibits the growth of Chlamydia trachomatis serovar L1.** *Infection and Immunity* 1988, **56:**3322-3325.

104. Igietseme JU, Uriri IM, Chow M, Abe E, Rank RG: **Inhibition of intracellular multiplication of human strains of Chlamydia trachomatis by nitric oxide.** *Biochem Biophys Res Commun* 1997, **232:**595-601.

105. Ito K: **The role of gamma-aminobutyric acid (GABA)-benzodiazepine neurotransmission in an animal model of methamphetamine-induced psychosis.** *Hokkaido Igaku Zasshi* 1999, **74:**135-144.

106. Maxion HK, Kelly KA: **Chemokine expression patterns differ within anatomically distinct regions of the genital tract during Chlamydia trachomatis infection.** *Infection and Immunity* 2002, **70:**1538-1546.

107. Nguyen KB, Watford WT, Salomon R, Hofmann SR, Pien GC, Morinobu A, Gadina M, O'Shea JJ, Biron CA: **Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection.** *Science* 2002, **297:**2063-2066.

108. Morrison RP, Feilzer K, Tumas DB: **Gene knockout mice establish a primary protective role for major histocompatibility complex class II-restricted responses in Chlamydia trachomatis genital tract infection.** *Infection and Immunity* 1995, **63:**4661-4668.

109. Hawkins RA, Rank RG, Kelly KA: **A Chlamydia trachomatis-specific Th2 clone does not provide protection against a genital infection and displays reduced trafficking to the infected genital mucosa.** *Infection and Immunity* 2002, **70:**5132-5139.

110. Beatty PR, Stephens RS: **CD8+ T lymphocyte-mediated lysis of Chlamydia-infected L cells using an endogenous antigen pathway.** *Journal of Immunology* 1994, **153:**4588-4595.

111. Starnbach MN, Loomis WP, Ovendale P, Regan D, Hess B, Alderson MR, Fling SP: **An inclusion membrane protein from Chlamydia trachomatis enters the MHC class I pathway and stimulates a CD8+ T cell response.** *Journal of Immunology* 2003, **171:**4742-4749.

112. Takeda K, Kaisho T, Akira S: **Toll-like receptors.** *Annu Rev Immunol* 2003, **21:**335-376.

113. Kawai T, Akira S: **The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.** *Nat Immunol* 2010, **11:**373-384.

114. Netea MG, van der Graaf C, Van der Meer JW, Kullberg BJ: **Toll-like receptors and the host defense against microbial pathogens: bringing specificity to the innate-immune system.** *J Leukoc Biol* 2004, **75:**749-755.

115. Rank RG, Lacy HM, Goodwin A, Sikes J, Whittimore J, Wyrick PB, Nagarajan UM: **Host chemokine and cytokine response in the endocervix within the first developmental cycle of Chlamydia muridarum.** *Infection and Immunity* 2010, **78:**536-544.

116. Akira S, Uematsu S, Takeuchi O: **Pathogen recognition and innate immunity.** *Cell* 2006, **124:**783-801.

117. Jin MS, Lee JO: **Structures of the toll-like receptor family and its ligand complexes.** *Immunity* 2008, **29:**182-191.

118. Barton GM, Medzhitov R: **Toll-like receptors and their ligands.** *Curr Top Microbiol Immunol* 2002, **270:**81-92.

119. Akashi-Takamura S, Miyake K: **TLR accessory molecules.** *Curr Opin Immunol* 2008, **20:**420-425.

120. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee H, Lee JO: **Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide.** *Cell* 2007, **130:**1071-1082.

121. Kang JY, Nan X, Jin MS, Youn SJ, Ryu YH, Mah S, Han SH, Lee H, Paik SG, Lee JO: **Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer.** *Immunity* 2009, **31:**873-884.

122. Kawai T, Akira S: **Toll-like receptor and RIG-I-like receptor signaling.** *Ann N Y Acad Sci* 2008, **1143:**1-20.

123. Kawai T, Akira S: **Innate immune recognition of viral infection.** *Nat Immunol* 2006, **7:**131-137.

124. Kim YM, Brinkmann MM, Paquet ME, Ploegh HL: **UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes.** *Nature* 2008, **452:**234-238.

125. Uematsu S, Fujimoto K, Jang MH, Yang BG, Jung YJ, Nishiyama M, Sato S, Tsujimura T, Yamamoto M, Yokota Y, et al,: **Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5.** *Nat Immunol* 2008, **9:**769-776.

126. Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S: **A toll-like receptor that prevents infection by uropathogenic bacteria.** *Science* 2004, **303:**1522-1526.

127. Bhoj VG, Chen ZJ: **Ubiquitylation in innate and adaptive immunity.** *Nature* 2009, **458:**430-437.

128. Hacker H, Karin M: **Regulation and function of IKK and IKK-related kinases.** *Sci STKE* 2006, **2006:**re13.

129. Hacker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Hacker G, et al,: **Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6.** *Nature* 2006, **439:**204-207.

130. Kawagoe T, Sato S, Matsushita K, Kato H, Matsui K, Kumagai Y, Saitoh T, Kawai T, Takeuchi O, Akira S: **Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2.** *Nat Immunol* 2008, **9:**684-691.

131. Yamamoto M, Okamoto T, Takeda K, Sato S, Sanjo H, Uematsu S, Saitoh T, Yamamoto N, Sakurai H, Ishii KJ, et al,: **Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling.** *Nat Immunol* 2006, **7:**962-970.

132. Yamamoto M, Yamazaki S, Uematsu S, Sato S, Hemmi H, Hoshino K, Kaisho T, Kuwata H, Takeuchi O, Takeshige K, et al,: **Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta.** *Nature* 2004, **430:**218-222.

133. Fernandes-Alnemri T, Yu JW, Juliana C, Solorzano L, Kang S, Wu J, Datta P, McCormick M, Huang L, McDermott E, et al, **The AIM2 inflammasome is critical for innate immunity to Francisella tularensis.** *Nat Immunol* 2010, **11:**385-393.

134. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G: **The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis.** *Nat Immunol* 2009, **10:**241-247.

135. Hornung V, Latz E: **Intracellular DNA recognition.** *Nat Rev Immunol* 2010, **10:**123-130.

136. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K, et al,: **DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response.** *Nature* 2007, **448:**501-505.

137. Yoneyama M, Fujita T: **RNA recognition and signal transduction by RIG-I-like receptors.** *Immunol Rev* 2009, **227:**54-65.

138. Iwasaki A, Medzhitov R: **Toll-like receptor control of the adaptive immune responses.** *Nat Immunol* 2004, **5:**987-995.

139. Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ, Knaus UG: **Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway.** *Nat Immunol* 2000, **1:**533-540.

140. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S: **Unresponsiveness of MyD88-deficient mice to endotoxin.** *Immunity* 1999, **11:**115-122.

141. Takeda K, Akira S: **Regulation of innate immune responses by Toll-like receptors.** *Jpn J Infect Dis* 2001, **54:**209-219.

142. Takeda K, Akira S: **Roles of Toll-like receptors in innate immune responses.** *Genes Cells* 2001, **6:**733-742.

143. Applequist SE, Wallin RP, Ljunggren HG: **Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines.** *Int Immunol* 2002, **14:**1065-1074.

144. Schaefer TM, Desouza K, Fahey JV, Beagley KW, Wira CR: **Toll-like receptor (TLR) expression and TLR-mediated cytokine/chemokine production by human uterine epithelial cells.** *Immunology* 2004, **112:**428-436.

145. Schaefer TM, Fahey JV, Wright JA, Wira CR: **Innate immunity in the human female reproductive tract: antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C).** *Journal of Immunology* 2005, **174:**992-1002.

146. Fichorova RN, Cronin AO, Lien E, Anderson DJ, Ingalls RR: **Response to Neisseria gonorrhoeae by cervicovaginal epithelial cells occurs in the absence of toll-like receptor 4-mediated signaling.** *Journal of Immunology* 2002, **168:**2424-2432.

147. Pioli PA, Amiel E, Schaefer TM, Connolly JE, Wira CR, Guyre PM: **Differential expression of Toll-like receptors 2 and 4 in tissues of the human female reproductive tract.** *Infection and Immunity* 2004, **72:**5799-5806.

148. Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M: **Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide.** *Journal of Immunology* 2001, **167:**1609-1616.

149. Matsuguchi T, Musikacharoen T, Ogawa T, Yoshikai Y: **Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages.** *Journal of Immunology* 2000, **165:**5767-5772.

150. Shuto T, Xu H, Wang B, Han J, Kai H, Gu XX, Murphy TF, Lim DJ, Li JD: **Activation of NF-kappa B by nontypeable Hemophilus influenzae is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK alpha /beta-I kappa B alpha and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells.** *Proc Natl Acad Sci U S A* 2001, **98:**8774-8779.

151. Sweet MJ, Campbell CC, Sester DP, Xu D, McDonald RC, Stacey KJ, Hume DA, Liew FY: **Colony-stimulating factor-1 suppresses responses to CpG DNA and expression of toll-like receptor 9 but enhances responses to lipopolysaccharide in murine macrophages.** *Journal of Immunology* 2002, **168:**392-399.

152. Wang T, Lafuse WP, Zwilling BS: **Regulation of toll-like receptor 2 expression by macrophages following Mycobacterium avium infection.** *Journal of Immunology* 2000, **165:**6308-6313.

153. O'Connell CM, Ionova IA, Quayle AJ, Visintin A, Ingalls RR: **Localization of TLR2 and MyD88 to Chlamydia trachomatis Inclusions.** *Journal of Biological Chemistry* 2006, **281:**1652-1659.

154. Prebeck S, Kirschning C, Durr S, da Costa C, Donath B, Brand K, Redecke V, Wagner H, Miethke T: **Predominant role of toll-like receptor 2 versus 4 in Chlamydia pneumoniae-induced activation of dendritic cells.** *Journal of Immunology* 2001, **167:**3316-3323.

155. Derbigny WA, Kerr MS, Johnson RM: **Pattern recognition molecules activated by Chlamydia muridarum infection of cloned murine oviduct epithelial cell lines.** *J Immunol* 2005, **175:**6065-6075.

156. Darville T, O'Neill JM, Andrews CW, Jr., Nagarajan UM, Stahl L, Ojcius DM: **Toll-like receptor-2, but not Toll-like receptor-4, is essential for development of oviduct pathology in chlamydial genital tract infection.** *Journal of Immunology* 2003, **171:**6187-6197.

157. Bulut Y, Faure E, Thomas L, Karahashi H, Michelsen KS, Equils O, Morrison SG, Morrison RP, Arditi M: **Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway.** *Journal of Immunology* 2002, **168:**1435-1440.

158. Sasu S, LaVerda D, Qureshi N, Golenbock DT, Beasley D: **Chlamydia pneumoniae and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation.** *Circ Res* 2001, **89:**244-250.

159. Trumstedt C, Eriksson E, Lundberg AM, Yang TB, Yan ZQ, Wigzell H, Rottenberg ME: **Role of IRAK4 and IRF3 in the control of intracellular infection with Chlamydia pneumoniae.** *J Leukoc Biol* 2007, **81:**1591-1598.

160. Nagarajan UM, Ojcius DM, Stahl L, Rank RG, Darville T: **Chlamydia trachomatis induces expression of IFN-gamma-inducible protein 10 and IFN-beta independent of TLR2 and TLR4, but largely dependent on MyD88.** *Journal of Immunology* 2005, **175:**450-460.

161. Mackern-Oberti JP, Maccioni M, Cuffini C, Gatti G, Rivero VE: **Susceptibility of Prostate Epithelial Cells to Chlamydia muridarum Infection and Their Role in Innate Immunity by Recruitment of Intracellular Toll-Like Receptors 4 and 2 and MyD88 to the Inclusion.** *Infection and Immunity* 2006, **74:**6973-6981.

162. Commins SP, Borish L, Steinke JW: **Immunologic messenger molecules: cytokines, interferons, and chemokines.** *J Allergy Clin Immunol* 2010, **125:**S53-72.

163. Nagarajan UM, Prantner D, Sikes JD, Andrews CW, Jr., Goodwin AM, Nagarajan S, Darville T: **Type I interferon signaling exacerbates Chlamydia muridarum genital infection in a murine model.** *Infection and Immunity* 2008, **76:**4642-4648.

164. Nagarajan UM, Sikes JD, Yeruva L, Prantner D: **Significant Role of IL-1 Signaling, but Limited Role of Inflammasome Activation, in Oviduct Pathology during Chlamydia muridarum Genital Infection.** *Journal of Immunology* 2012.

165. Stephens RS: **The cellular paradigm of chlamydial pathogenesis.** *Trends Microbiol* 2003, **11:**44-51.

166. Williams DM, Grubbs BG, Darville T, Kelly K, Rank RG: **A Role for Interleukin-6 in Host Defense against Murine Chlamydia trachomatis Infection.** *Infection and Immunity* 1998, **66:**4564-4567.

167. Isaacs A, Lindenmann J: **Virus interference. I. The interferon.** *Proc R Soc Lond B Biol Sci* 1957, **147:**258-267.

168. Trinchieri G: **Type I interferon: friend or foe?** *J Exp Med* 2010, **207:**2053-2063.

169. Decker T, Stockinger S, Karaghiosoff M, Muller M, Kovarik P: **IFNs and STATs in innate immunity to microorganisms.** *J Clin Invest* 2002, **109:**1271-1277.

170. Plumlee CR, Lee C, Beg AA, Decker T, Shuman HA, Schindler C: **Interferons direct an effective innate response to Legionella pneumophila infection.** *J Biol Chem* 2009, **284:**30058-30066.

171. Bornstein J, Lahat N, Kinarty A, Revel M, Abramovici H, Shapiro S: **Interferon-beta and -gamma, but not tumor necrosis factor-alpha, demonstrate immunoregulatory effects on carcinoma cell lines infected with human papillomavirus.** *Cancer* 1997, **79:**924-934.

172. Greiner JW, Guadagni F, Noguchi P, Pestka S, Colcher D, Fisher PB, Schlom J: **Recombinant interferon enhances monoclonal antibody-targeting of carcinoma lesions in vivo.** *Science* 1987, **235:**895-898.

173. Ortaldo JR, Mantovani A, Hobbs D, Rubinstein M, Pestka S, Herberman RB: **Effects of several species of human leukocyte interferon on cytotoxic activity of NK cells and monocytes.** *Int J Cancer* 1983, **31:**285-289.

174. Pestka S: **The interferons: 50 years after their discovery, there is much more to learn.** *J Biol Chem* 2007, **282:**20047-20051.

175. Platanias LC: **Mechanisms of type-I- and type-II-interferon-mediated signalling.** *Nat Rev Immunol* 2005, **5:**375-386.

176. Pestka S, Krause CD, Walter MR: **Interferons, interferon-like cytokines, and their receptors.** *Immunol Rev* 2004, **202:**8-32.

177. Bogdan C, Mattner J, Schleicher U: **The role of type I interferons in non-viral infections.** *Immunol Rev* 2004, **202:**33-48.

178. Decker T, Muller M, Stockinger S: **The yin and yang of type I interferon activity in bacterial infection.** *Nat Rev Immunol* 2005, **5:**675-687.

179. Merlin G, Falcoff E, Aguet M: **125I-labelled human interferons alpha, beta and gamma: comparative receptor-binding data.** *J Gen Virol* 1985, **66 (Pt 5):**1149-1152.

180. Jonasch E, Haluska FG: **Interferon in oncological practice: review of interferon biology, clinical applications, and toxicities.** *Oncologist* 2001, **6:**34-55.

181. Pfeffer LM, Dinarello CA, Herberman RB, Williams BR, Borden EC, Bordens R, Walter MR, Nagabhushan TL, Trotta PP, Pestka S: **Biological properties of recombinant alpha-interferons: 40th anniversary of the discovery of interferons.** *Cancer Res* 1998, **58:**2489-2499.

182. Levy DE, Darnell JE, Jr.: **Stats: transcriptional control and biological impact.** *Nat Rev Mol Cell Biol* 2002, **3:**651-662.

183. Li X, Leung S, Qureshi S, Darnell JE, Jr., Stark GR: **Formation of STAT1-STAT2 heterodimers and their role in the activation of IRF-1 gene transcription by interferon-alpha.** *J Biol Chem* 1996, **271:**5790-5794.

184. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N, Taniguchi T: **IRF-7 is the master regulator of type-I interferon-dependent immune responses.** *Nature* 2005, **434:**772-777.

185. Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, Taniguchi T: **Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction.** *Immunity* 2000, **13:**539-548.

186. Taniguchi T, Takaoka A: **The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors.** *Curr Opin Immunol* 2002, **14:**111-116.

187. Thanos D, Maniatis T: **Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome.** *Cell* 1995, **83:**1091-1100.

188. McWhirter SM, Barbalat R, Monroe KM, Fontana MF, Hyodo M, Joncker NT, Ishii KJ, Akira S, Colonna M, Chen ZJ, et al,: **A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP.** *J Exp Med* 2009, **206:**1899-1911.

189. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA: **Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.** *Nature* 2001, **413:**732-738.

190. Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S: **Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components.** *Immunity* 1999, **11:**443-451.

191. Colonna M, Trinchieri G, Liu YJ: **Plasmacytoid dendritic cells in immunity.** *Nat Immunol* 2004, **5:**1219-1226.

192. Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, Randall RE: **The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter.** *Proc Natl Acad Sci U S A* 2004, **101:**17264-17269.

193. Pandey AK, Yang Y, Jiang Z, Fortune SM, Coulombe F, Behr MA, Fitzgerald KA, Sassetti CM, Kelliher MA: **NOD2, RIP2 and IRF5 play a critical role in the type I interferon response to Mycobacterium tuberculosis.** *PLoS Pathog* 2009, **5:**e1000500.

194. Watanabe T, Asano N, Fichtner-Feigl S, Gorelick PL, Tsuji Y, Matsumoto Y, Chiba T, Fuss IJ, Kitani A, Strober W: **NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway.** *J Clin Invest* 2010, **120:**1645-1662.

195. Marie I, Durbin JE, Levy DE: **Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7.** *EMBO J* 1998, **17:**6660-6669.

196. Levy DE, Marie I, Prakash A: **Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity.** *Curr Opin Immunol* 2003, **15:**52-58.

197. Prakash A, Smith E, Lee C-k, Levy DE: **Tissue-specific Positive Feedback Requirements for Production of Type I Interferon following Virus Infection.** *Journal of Biological Chemistry* 2005, **280:**18651-18657.

198. Sato M, Hata N, Asagiri M, Nakaya T, Taniguchi T, Tanaka N: **Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7.** *FEBS Lett* 1998, **441:**106-110.

199. Stockinger S, Reutterer B, Schaljo B, Schellack C, Brunner S, Materna T, Yamamoto M, Akira S, Taniguchi T, Murray PJ, et al,: **IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism.** *Journal of Immunology* 2004, **173:**7416-7425.

200. Yie J, Merika M, Munshi N, Chen G, Thanos D: **The role of HMG I(Y) in the assembly and function of the IFN-beta enhanceosome.** *EMBO J* 1999, **18:**3074-3089.

201. Yie J, Senger K, Thanos D: **Mechanism by which the IFN-beta enhanceosome activates transcription.** *Proc Natl Acad Sci U S A* 1999, **96:**13108-13113.

202. Merika M, Williams AJ, Chen G, Collins T, Thanos D: **Recruitment of CBP/p300 by the IFN beta enhanceosome is required for synergistic activation of transcription.** *Mol Cell* 1998, **1:**277-287.

203. Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT: **LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF.** *J Exp Med* 2003, **198:**1043-1055.

204. Uematsu S, Sato S, Yamamoto M, Hirotani T, Kato H, Takeshita F, Matsuda M, Coban C, Ishii KJ, Kawai T, et al,: **Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction.** *J Exp Med* 2005, **201:**915-923.

205. de la Maza LM, Peterson EM, Goebel JM, Fennie CW, Czarniecki CW: **Interferon-induced inhibition of Chlamydia trachomatis: dissociation from antiviral and antiproliferative effects.** *Infection and Immunity* 1985, **47:**719-722.

206. Ishihara T, Aga M, Hino K, Ushio C, Taniguchi M, Iwaki K, Ikeda M, Kurimoto M: **Inhibition of chlamydia trachomatis growth by human interferon-alpha: mechanisms and synergistic effect with interferon-gamma and tumor necrosis factor-alpha.** *Biomed Res* 2005, **26:**179-185.

207. Devitt A, Lund PA, Morris AG, Pearce JH: **Induction of alpha/beta interferon and dependent nitric oxide synthesis during Chlamydia trachomatis infection of McCoy cells in the absence of exogenous cytokine.** *Infection and Immunity* 1996, **64:**3951-3956.

208. Darnell JE, Jr., Kerr IM, Stark GR: **Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins.** *Science* 1994, **264:**1415-1421.

209. Fu XY, Schindler C, Improta T, Aebersold R, Darnell JE, Jr.: **The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction.** *Proc Natl Acad Sci U S A* 1992, **89:**7840-7843.

210. Schindler C, Shuai K, Prezioso VR, Darnell JE, Jr.: **Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor.** *Science* 1992, **257:**809-813.

211. Schindler C, Fu XY, Improta T, Aebersold R, Darnell JE, Jr.: **Proteins of transcription factor ISGF-3: one gene encodes the 91-and 84-kDa ISGF-3 proteins that are activated by interferon alpha.** *Proc Natl Acad Sci U S A* 1992, **89:**7836-7839.

212. O'Shea JJ: **Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet?** *Immunity* 1997, **7:**1-11.

213. Schindler C, Levy DE, Decker T: **JAK-STAT signaling: from interferons to cytokines.** *J Biol Chem* 2007, **282:**20059-20063.

214. Copeland NG, Gilbert DJ, Schindler C, Zhong Z, Wen Z, Darnell JE, Jr., Mui AL, Miyajima A, Quelle FW, Ihle JN, et al,: **Distribution of the mammalian Stat gene family in mouse chromosomes.** *Genomics* 1995, **29:**225-228.

215. Vignais ML, Sadowski HB, Watling D, Rogers NC, Gilman M: **Platelet-derived growth factor induces phosphorylation of multiple JAK family kinases and STAT proteins.** *Mol Cell Biol* 1996, **16:**1759-1769.

216. Darnell JE, Jr.: **STATs and gene regulation.** *Science* 1997, **277:**1630-1635.

217. Schindler C, Darnell JE, Jr.: **Transcriptional responses to polypeptide ligands: the JAK-STAT pathway.** *Annu Rev Biochem* 1995, **64:**621-651.

218. Ihle JN, Kerr IM: **Jaks and Stats in signaling by the cytokine receptor superfamily.** *Trends Genet* 1995, **11:**69-74.

219. Wen Z, Zhong Z, Darnell JE, Jr.: **Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation.** *Cell* 1995, **82:**241-250.

220. Zhu X, Wen Z, Xu LZ, Darnell JE, Jr.: **Stat1 serine phosphorylation occurs independently of tyrosine phosphorylation and requires an activated Jak2 kinase.** *Mol Cell Biol* 1997, **17:**6618-6623.

221. Ibarra-Sanchez MJ, Simoncic PD, Nestel FR, Duplay P, Lapp WS, Tremblay ML: **The T-cell protein tyrosine phosphatase.** *Semin Immunol* 2000, **12:**379-386.

222. Kramer OH, Knauer SK, Greiner G, Jandt E, Reichardt S, Guhrs KH, Stauber RH, Bohmer FD, Heinzel T: **A phosphorylation-acetylation switch regulates STAT1 signaling.** *Genes Dev* 2009, **23:**223-235.

223. Krebs DL, Hilton DJ: **SOCS proteins: negative regulators of cytokine signaling.** *Stem Cells* 2001, **19:**378-387.

224. You M, Yu DH, Feng GS: **Shp-2 tyrosine phosphatase functions as a negative regulator of the interferon-stimulated Jak/STAT pathway.** *Mol Cell Biol* 1999, **19:**2416-2424.

225. Chatterjee-Kishore M, Wright KL, Ting JP, Stark GR: **How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene.** *EMBO J* 2000, **19:**4111-4122.

226. Lackmann M, Harpur AG, Oates AC, Mann RJ, Gabriel A, Meutermans W, Alewood PF, Kerr IM, Stark GR, Wilks AF: **Biomolecular interaction analysis of IFN gamma-induced signaling events in whole-cell lysates: prevalence of latent STAT1 in high-molecular weight complexes.** *Growth Factors* 1998, **16:**39-51.

227. Zhong M, Henriksen MA, Takeuchi K, Schaefer O, Liu B, ten Hoeve J, Ren Z, Mao X, Chen X, Shuai K, Darnell JE, Jr.: **Implications of an antiparallel dimeric structure of nonphosphorylated STAT1 for the activation-inactivation cycle.** *Proc Natl Acad Sci U S A* 2005, **102:**3966-3971.

228. Horvath CM: **STAT proteins and transcriptional responses to extracellular signals.** *Trends Biochem Sci* 2000, **25:**496-502.

229. Decker T, Kovarik P: **Serine phosphorylation of STATs.** *Oncogene* 2000, **19:**2628-2637.

230. Look DC, Pelletier MR, Tidwell RM, Roswit WT, Holtzman MJ: **Stat1 depends on transcriptional synergy with Sp1.** *J Biol Chem* 1995, **270:**30264-30267.

231. Zhang JJ, Zhao Y, Chait BT, Lathem WW, Ritzi M, Knippers R, Darnell JE, Jr.: **Ser727-dependent recruitment of MCM5 by Stat1alpha in IFN-gamma-induced transcriptional activation.** *EMBO J* 1998, **17:**6963-6971.

232. Cheon H, Stark GR: **Unphosphorylated STAT1 prolongs the expression of interferon-induced immune regulatory genes.** *Proc Natl Acad Sci U S A* 2009, **106:**9373-9378.

233. Bowman T, Jove R: **STAT Proteins and Cancer.** *Cancer Control* 1999, **6:**615-619.

234. Durbin JE, Hackenmiller R, Simon MC, Levy DE: **Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease.** *Cell* 1996, **84:**443-450.

235. Lee CK, Rao DT, Gertner R, Gimeno R, Frey AB, Levy DE: **Distinct requirements for IFNs and STAT1 in NK cell function.** *Journal of Immunology* 2000, **165:**3571-3577.

236. Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S: **Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality.** *Proc Natl Acad Sci U S A* 1997, **94:**3801-3804.

237. Park C, Li S, Cha E, Schindler C: **Immune response in Stat2 knockout mice.** *Immunity* 2000, **13:**795-804.

238. Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S, Yang K, Chapgier A, Eidenschenk C, Eid P, et al,: **Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency.** *Nat Genet* 2003, **33:**388-391.

239. Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, et al,: **Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway.** *Cell* 1996, **84:**431-442.

240. Enninga J, Levy DE, Blobel G, Fontoura BM: **Role of nucleoporin induction in releasing an mRNA nuclear export block.** *Science* 2002, **295:**1523-1525.

241. Durbin JE, Fernandez-Sesma A, Lee CK, Rao TD, Frey AB, Moran TM, Vukmanovic S, Garcia-Sastre A, Levy DE: **Type I IFN modulates innate and specific antiviral immunity.** *Journal of Immunology* 2000, **164:**4220-4228.

242. Garcia-Sastre A, Durbin RK, Zheng H, Palese P, Gertner R, Levy DE, Durbin JE: **The role of interferon in influenza virus tissue tropism.** *Journal of Virology* 1998, **72:**8550-8558.

243. van den Broek MF, Muller U, Huang S, Zinkernagel RM, Aguet M: **Immune defence in mice lacking type I and/or type II interferon receptors.** *Immunol Rev* 1995, **148:**5-18.

244. Stockinger S, Decker T: **Novel functions of type I interferons revealed by infection studies with Listeria monocytogenes.** *Immunobiology* 2008, **213:**889-897.

245. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, Barry CE, 3rd, Freedman VH, Kaplan G: **Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta.** *Proc Natl Acad Sci U S A* 2001, **98:**5752-5757.

246. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM: **Disseminated tuberculosis in interferon gamma gene-disrupted mice.** *J Exp Med* 1993, **178:**2243-2247.

247. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA: **Multiple defects of immune cell function in mice with disrupted interferon-gamma genes.** *Science* 1993, **259:**1739-1742.

248. Nauciel C, Espinasse-Maes F: **Role of gamma interferon and tumor necrosis factor alpha in resistance to Salmonella typhimurium infection.** *Infection and Immunity* 1992, **60:**450-454.

249. Rottenberg ME, Gigliotti Rothfuchs A, Gigliotti D, Ceausu M, Une C, Levitsky V, Wigzell H: **Regulation and role of IFN-gamma in the innate resistance to infection with Chlamydia pneumoniae.** *Journal of Immunology* 2000, **164:**4812-4818.

250. Derbigny WA, Johnson RM, Toomey KS, Ofner S, Jayarapu K: **The Chlamydia muridarum-Induced IFN-beta Response Is TLR3-Dependent in Murine Oviduct Epithelial Cells.** *Journal of Immunology* 2010, **185:**6689-6697.

251. Derbigny WA, Hong SC, Kerr MS, Temkit M, Johnson RM: **Chlamydia muridarum infection elicits a beta interferon response in murine oviduct epithelial cells dependent on interferon regulatory factor 3 and TRIF.** *Infect Immun* 2007, **75:**1280-1290.

252. Vignola MJ, Kashatus DF, Taylor GA, Counter CM, Valdivia RH: **cPLA2 regulates the expression of type I interferons and intracellular immunity to Chlamydia trachomatis.** *J Biol Chem* 2010, **285:**21625-21635.

253. Prantner D, Darville T, Nagarajan UM: **Stimulator of IFN gene is critical for induction of IFN-beta during Chlamydia muridarum infection.** *Journal of Immunology* 2010, **184:**2551-2560.

254. Lad SP, Fukuda EY, Li J, de la Maza LM, Li E: **Up-regulation of the JAK/STAT1 signal pathway during Chlamydia trachomatis infection.** *J Immunol* 2005, **174:**7186-7193.

255. Derbigny WA, Shobe LR, Kamran JC, Toomey KS, Ofner S: **Identifying a Role for Toll-Like Receptor 3 in the Innate Immune Response to Chlamydia muridarum Infection in Murine Oviduct Epithelial Cells.** *Infection and Immunity* 2012, **80:**254-265.

256. Frank DA, Mahajan S, Ritz J: **Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling.** *Nat Med* 1999, **5:**444-447.

257. Schachter J: **Chlamydiae: exotic and ubiquitous.** *West J Med* 1980, **132:**238-240.

258. Rozen SaS, H.J.: **Primer3 on the WWW for general users and for biologist programmers** <http://jura.wi.mit.edu/rozen/papers/rozen-and-skaletsky-2000-primer3.pdf> *Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology Humana Press, Totowa, NJ* 2000**:**pp 365-386.

259. Roth-Cross JK, Martinez-Sobrido L, Scott EP, Garcia-Sastre A, Weiss SR: **Inhibition of the alpha/beta interferon response by mouse hepatitis virus at multiple levels.** *Journal of Virology* 2007, **81:**7189-7199.

260. Cui W, Taub DD, Gardner K: **qPrimerDepot: a primer database for quantitative real time PCR.** *Nucleic Acids Res* 2007, **35:**D805-809.

261. Newhall WJt, Terho P, Wilde CE, 3rd, Batteiger BE, Jones RB: **Serovar determination of Chlamydia trachomatis isolates by using type-specific monoclonal antibodies.** *J Clin Microbiol* 1986, **23:**333-338.

262. Theofilopoulos AN, Baccala R, Beutler B, Kono DH: **Type I interferons (alpha/beta) in immunity and autoimmunity.** *Annu Rev Immunol* 2005, **23:**307-336.

263. Osterlund P, Veckman V, Siren J, Klucher KM, Hiscott J, Matikainen S, Julkunen I: **Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells.** *Journal of Virology* 2005, **79:**9608-9617.

264. Rothfuchs AG, Trumstedt C, Mattei F, Schiavoni G, Hidmark A, Wigzell H, Rottenberg ME: **STAT1 regulates IFN-alpha beta- and IFN-gamma-dependent control of infection with Chlamydia pneumoniae by nonhemopoietic cells.** *Journal of Immunology* 2006, **176:**6982-6990.

265. Bourne N, Scholle F, Silva MC, Rossi SL, Dewsbury N, Judy B, De Aguiar JB, Leon MA, Estes DM, Fayzulin R, Mason PW: **Early production of type I interferon during West Nile virus infection: role for lymphoid tissues in IRF3-independent interferon production.** *J Virol* 2007, **81:**9100-9108.

266. Szabo G, Dolganiuc A: **The role of plasmacytoid dendritic cell-derived IFN alpha in antiviral immunity.** *Crit Rev Immunol* 2008, **28:**61-94.

267. Barbe F, Saelens X, Braeckmans D, Lefevre F, Reeth KV: **Role of IFN-alpha during the acute stage of a swine influenza virus infection.** *Res Vet Sci* 2010, **88:**172-178.

268. Jonas MM, Ott MJ, Nelson SP, Badizadegan K, Perez-Atayde AR: **Interferon-alpha treatment of chronic hepatitis C virus infection in children.** *Pediatr Infect Dis J* 1998, **17:**241-246.

269. Hoss-Homfeld A, Zwarthoff EC, Zawatzky R: **Cell type specific expression and regulation of murine interferon alpha and beta genes.** *Virology* 1989, **173:**539-550.

270. Greenway AL, Overall ML, Sattayasai N, Rowley MJ, Hertzog PJ, McMullen GL, Cheetham BF, Marzuki S: **Selective production of interferon-alpha subtypes by cultured peripheral blood mononuclear cells and lymphoblastoid cell lines.** *Immunology* 1992, **75:**182-188.

271. Nyman TA, Tolo H, Parkkinen J, Kalkkinen N: **Identification of nine interferon-alpha subtypes produced by Sendai virus-induced human peripheral blood leucocytes.** *Biochem J* 1998, **329 (Pt 2):**295-302.

272. van Pesch V, Lanaya H, Renauld JC, Michiels T: **Characterization of the murine alpha interferon gene family.** *Journal of Virology* 2004, **78:**8219-8228.

273. Le VT, Trilling M, Zimmermann A, Hengel H: **Mouse cytomegalovirus inhibits beta interferon (IFN-beta) gene expression and controls activation pathways of the IFN-beta enhanceosome.** *J Gen Virol* 2008, **89:**1131-1141.

274. Hillyer P, Mane VP, Schramm LM, Puig M, Verthelyi D, Chen A, Zhao Z, Navarro MB, Kirschman KD, Bykadi S, et al,: **Expression profiles of human interferon-alpha and interferon-lambda subtypes are ligand- and cell-dependent.** *Immunol Cell Biol* 2012.

275. Raymond J, Benichou C, de Boissieu D, Mensah K, Bergeret M, Lebon P: **Absence of intrathecal synthesis of some interferon-alpha subtypes in bacterial meningitis.** *J Infect Dis* 1992, **166:**657-659.

276. Kim JY, Ozato K: **The sequestosome 1/p62 attenuates cytokine gene expression in activated macrophages by inhibiting IFN regulatory factor 8 and TNF receptor-associated factor 6/NF-kappaB activity.** *Journal of Immunology* 2009, **182:**2131-2140.

277. Qiu H, Fan Y, Joyee AG, Wang S, Han X, Bai H, Jiao L, Van Rooijen N, Yang X: **Type I IFNs enhance susceptibility to Chlamydia muridarum lung infection by enhancing apoptosis of local macrophages.** *Journal of Immunology* 2008, **181:**2092-2102.

278. Pulit-Penaloza JA, Scherbik SV, Brinton MA: **Type 1 IFN-independent activation of a subset of interferon stimulated genes in West Nile virus Eg101-infected mouse cells.** *Virology* 2012, **425:**82-94.

279. Lazear HM, Lancaster A, Wilkins C, Suthar MS, Huang A, Vick SC, Clepper L, Thackray L, Brassil MM, Virgin HW, et al,: **IRF-3, IRF-5, and IRF-7 coordinately regulate the type I IFN response in myeloid dendritic cells downstream of MAVS signaling.** *PLoS Pathog* 2013, **9:**e1003118.

280. Yang P, An H, Liu X, Wen M, Zheng Y, Rui Y, Cao X: **The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway.** *Nat Immunol* 2010, **11:**487-494.

281. Zhang Z, Yuan B, Bao M, Lu N, Kim T, Liu YJ: **The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells.** *Nat Immunol* 2011, **12:**959-965.

282. Erlandsson L, Blumenthal R, Eloranta ML, Engel H, Alm G, Weiss S, Leanderson T: **Interferon-beta is required for interferon-alpha production in mouse fibroblasts.** *Curr Biol* 1998, **8:**223-226.

283. Stoiber D, Stockinger S, Steinlein P, Kovarik J, Decker T: **Listeria monocytogenes modulates macrophage cytokine responses through STAT serine phosphorylation and the induction of suppressor of cytokine signaling 3.** *Journal of Immunology* 2001, **166:**466-472.

284. Guschin D, Rogers N, Briscoe J, Witthuhn B, Watling D, Horn F, Pellegrini S, Yasukawa K, Heinrich P, Stark GR, et al,: **A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6.** *EMBO J* 1995, **14:**1421-1429.

CURRICULUM VITAE

Kristen L. Hosey

Education:

Doctor of Philosophy (Ph.D.), Microbiology and Immunology, Indiana University at Indiana University-Purdue University Indianapolis, Indianapolis, IN, 2007-2013
Concentrations: Microbiology and Immunology
Dissertation: The Role of STAT1 on Chlamydia-induce Type I Interferon Responses in Oviduct Epithelium

Credits Earned Toward Master of Science (M.S.), Jackson State University, Jackson, MS, 2006-2007
Concentration: Biology
Thesis Research: The Effects of Tobramycin on MG-63 Osteoblast Cell Morphology, Viability and Function

Bachelor of Science (B.S.), Jackson State University, Jackson, MS, 2000-2004
Concentration: Biology

Awards and Honors:

2009	Named Southern Regional Educational Board Doctoral Scholar (SREB)
2008	Best First-Time Presenter Award, Indiana University Purdue University Indianapolis, Department of Microbiology and Immunology, Research In Progress (RIP) Student Seminar Series
2007	Named Edwin T. Harper Scholar, IU School of Medicine Department of Microbiology and Immunology
2007	Inductee Tri-Beta Biological Honor Society, Jackson State University
2006	AmeriCorps Education Award Recipient
2006	Named Bridges to the Doctorate Scholar, Jackson State University
2004	Graduated Suma cum Laude from Jackson State University, Jackson, MS
2003	Bennye Simmons Henderson Biology Book Award Recipient, Jackson State University
2001-2002	Office of Naval Research (ONR) Trainee, Jackson State University

2000 Awarded Full Academic Scholarship, Jackson State University

2000 Delta Sigma Theta Sorority, Inc., Academic Scholarship, Laurel Alumnae Chapter, Laurel, MS

Research Experience:

The Role of STAT1 on Chlamydia-induced Type I Interferon Responses in Oviduct Epithelial Cells
Principle Investigator: Wilbert A. Derbigny, Ph.D., Indiana University School of Medicine, 2008-2012

Chlamydia trachomatis (*C. trachomatis*) is one of the most common bacterial sexually transmitted diseases in the U. S. In women, infection leads to fallopian tube scarring, ectopic pregnancies and infertility. We use *Chlamydia muridarum* (*MoPn*) to investigate the contributions of non-transformed murine oviduct epithelial (OE) cells to *Chlamydia*-specific immunity. Early observations from our lab have shown that these cells, when infected with *MoPn*, secrete a substantial amount of interferon-beta (IFN-β) via a TLR3-dependent mechanism. However, TLR3-deficient OE cells do not completely eliminate *MoPn*-induced IFN-β production; implicating a complementary pathway to IFN-β production. Other studies have shown a *Chlamydia*-induced upregulation of STAT 1 in cervical carcinomas associated with IFN-β production. We hypothesize that there is a *MoPn*-induced upregulation of the JAK/STAT 1 pathway, in part, to serve as a compensatory mechanism for *Chlamydia*-induced IFN-β production in the absence of TLR3. To test this hypothesis we used murine OE cells derived from B6 wild-type, STAT1-deficient, and TLR3-deficient mice; as well as siRNA technology and a series of molecular methodologies, including: Western blot, RT-PCR, and real-time PCR, to observe changes in STAT1 protein/gene activation and expression upon *Chlamydia* induction. Subsequently, we utilized ELISA analysis to observe the effects of STAT1 activation and expression on *Chlamydia*-induced IFN-β production in OE cells. In additional studies designed to investigate this hypothesis we also utilized pathway-specific inhibitors to observe the effects on *Chlamydia*-induced IFN-β production during early and late stages of infection. These data will provide insight into the molecular mechanisms that regulate the production of primary host immununomodulatory responses to *Chlamydia*, and may aid in determining/developing effective therapeutic targets for treatment and vaccination of *Chlamydia*, and associated pathologies.

The Effects of Tobramycin on MG-63 Osteoblast Cell Morphology, Viability and Function
Principle Investigators: Michelle Tucci, Ph.D., Hamed Benghuzzi, Ph.D. and Joseph A. Cameron, Ph.D., University of Mississippi Medical Center and Jackson State University, 2006-2007

Osteomyelitis is an acute or chronic inflammatory process of the bone or an infection of the bone. Systemic antibiotic therapy, due to poor penetration into the bone, does not eradicate the bacteria in most bone infections. Therefore, the local application of aminoglycoside antibiotics at high concentrations to the site of infection is required. Studies have shown at high serum concentrations, aminoglycosides exhibit significant toxicities such as nephrotoxicity and ototoxicity. However, during osteomyelitis treatment, the local concentration of these antibiotics can be up to 100µg/mL or more. MG63 osteosarcoma cells were treated with high and low concentrations of the aminoglycoside Tobramycin and evaluated at different time points for changes in cell morphology, cell damage, and cell number. Results indicate that Tobramycin at concentrations greater than the minimum inhibitory concentration (MIC), > 10µM, resulted in a decrease in osteoblast cell number, osteoblast morphological changes, and increased cellular membrane damage as early as 24 hours. This suggests that Tobramycin-induced toxicities may inhibit the ability of osteoblasts to form bone and, thus, delay healing. These results may have implications for using aminoglycosides as an antibacterial prophylaxis in orthopedic surgeries.

The Identification of Rrp2-Controlled Mammalian Infection-Associated Proteins in Borrelia burgdorferi
Principle Investigator: X. Frank Yang, Ph.D., Indiana University School of Medicine, Summer, 2006

Lyme disease is the most commonly reported arthropod-borne illness in the United States and Europe. The infection is caused by the tick-borne spirochete, *Borrelia burgdorferi*, resulting in a multisystem, multistage, inflammatory illness. Despite its medical importance, very little is known about the virulence determinants of *B. burgdorferi*. In this regard, five new *Borrelia* genes (bb0681, bb0844, bba05, bba07, and bbb09) controlled by the Rrp2 regulatory network, the central network essential for *B. burgdorferi* infection and pathogenesis, were identified. Three of the genes, bb0844, bba05, bba07, were chosen to generate recombinant proteins to determine their regulation at the protein level.

Teaching Experience:

Teacher's Assistant/Experiments Coordinator, Summer 2012
Indiana University School of Medicine Division of Diversity Affairs, Indianapolis, IN
Course: 5th, 6th, 7th, and 8th Grade Science Enrichment/Science Experiments
Responsibilities: developed and conducted hands on science experiments with 5th, 6th, 7th, and 8th grade students, assisted in the development and execution of course syllabus for 7th grade science and health science courses; developed/coordinated hands-on science experiments in Chemistry, Biochemistry, Human Anatomy, and Microbiology,

classroom management and individual nourishment of 5th, 6th, 7th, and 8th grade students' scientific/health-care related interests, working closely with Teachers and TAs in each grade

Teacher's Assistant, Summer 2011
Indiana University School of Medicine Division of Diversity Affairs, Indianapolis, IN
Course: 5th and 6th Grade Science and Mathematics Enrichment/Science Experiments
Responsibilities: developed and conducted hands on science experiments with 5th and 6th grade students, assisted in the development and execution of course syllabus for 6th grade science, math, health science and literature enrichment courses; assisted in the development and execution of hands-on classroom learning modules, classroom management and individual nourishment of 5th and 6th grade students' scientific/health-care related interests

Teacher's Assistant, Spring 2010
Indiana University School of Medicine, Indianapolis, IN
Course: Nursing Microbiology Laboratory
Responsibilities: taught microbiology lab to 1st and 2nd year nursing students at Indiana University Purdue University Indianapolis (IUPUI), explained and conducted experiments with students, conducted test reviews, formulated and graded test questions

Sixth, Seventh and Eighth Grade Title I Instructor, August 2004-July 2005
Blackburn Middle School, Jackson, MS
Course: Intermediate Mathematics
Responsibilities: classroom management, developed and executed course syllabus for 6th, 7th and 8th grade critical needs students based on state mathematics curriculum, set up and conducted hands-on mathematical modules with students, formulated and graded test questions, administered state required testing, and participated in professional development activities

Professional Experience:

Underrepresented Professional and Graduate Student Organization, Indiana University-Purdue University Indianapolis, (Member, 2008-2011)

American Society for Microbiology (ASM) (Student Member, 2011)

American Association for Cancer Research (AACR) (Associate Member, 2009-2010)

Compact for Faculty Diversity: The Institute on Teaching and Mentoring. Atlanta, GA.October 2011 (Attendee)

Midwest Crossroads AGEP Professional Development Conference. Chicago, IL. November 2010 (Attendee)

Compact for Faculty Diversity: The Institute on Teaching and Mentoring. Tampa, FL. October 2010 (Attendee)

Compact for Faculty Diversity: The Institute on Teaching and Mentoring. Arlington, VA. October 2009 (Attendee)

American Society of Hematology (ASH) Annual Meeting. Atlanta, GA. December 2007 (Attendee)

Presenter Indiana University School of Medicine Workshop "Getting You Through" (Spring 2012)

- Charles A. Tindley Accelerated School, Indianapolis, IN Science Interests Outreach Initiative (Spring 2011)

Panelist Summer Research Opportunities Program (SROP) "What I wish I had known before starting graduate school" (Summer 2010)

- Deer Run Elementary School, Indianapolis, IN Outreach Initiative "Scientist for a Day" (Spring 2009)

Abstracts/Conferences Attended:

Kristen L. Hosey, Jasmine Kamran, and Wilbert A. Derbigny. "The Role of STAT1 in *Chlamydia*-induced Type I Interferon Responses in Oviduct Epithelial Cells." National Organization for the Professional Advancement of Black Chemists and Chemical Engineers (NOBCChe) Regional Conference November 2011.

Kristen L. Hosey, Jasmine Kamran, and Wilbert A. Derbigny. "The Role of STAT1 in *Chlamydia*-induced Type I Interferon Responses." Annual Biomedical Research Conference for Minority Students (ABRCMS) November 2009.

Kristen Hosey, Joseph A. Cameron, and Frank Yang. "Identification of Rrp2-Controlled Mammalian Infection-Associated Proteins in *Borrelia burgdorferi*." Mississippi Academy of Sciences (MAS). 2007.

Publications:

Kristen L. Hosey, Hu, Sishun and Derbigny, W.A. Role of STAT1 in *Chlamydia*-induced type I interferon production in oviduct epithelial cells. Journal of Inflammation 2013 (submitted)

Sishun Hu, **Hosey, K.L.** and Derbigny, W.A. Early-stage TLR3-dependent IFN-β and differential signaling pathways are involved in late-stage IFN-β production in *Chlamydia*-induced oviduct epithelial cells. (in preparation)