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TLR3 Deficiency Leads to a Dysregulation in the Global Gene-Expression Profile in Murine Oviduct Epithelial Cells Infected with *Chlamydia muridarum*

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Article Info

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Abstract

Chlamydia trachomatis replicates primarily in the epithelial cells lining the genital tract and induces the innate immune response by triggering cellular pathogen recognition receptors (PRRs). Our previous studies showed that Toll-like receptor 3 (TLR3) is expressed in murine oviduct epithelial (OE) cells, is the primary PRR triggered by *C. muridarum* (*Cm*) early during infection to induce IFN- β synthesis, and that TLR3 signaling regulates the chlamydial induced synthesis of a plethora of other innate inflammatory modulators including IL-6, CXCL10, CXCL16 and CCL5. We also showed that the expression of these cytokines induced by *Chlamydia* was severely diminished during TLR3 deficiency; however, the replication of *Chlamydia* in TLR3 deficient OE cells was more robust than in WT cells. These data suggested that TLR3 had a biological impact on the inflammatory response to *Chlamydia* infection; however, the global effects of TLR3 signaling in the cellular response to *Chlamydia* infection in murine OE cells has not yet been investigated. To determine the impact of TLR3 signaling on *Chlamydia* infection in OE cell at the transcriptome level, we infected wild-type (OE-WT) and TLR3-deficient (OE-TLR3KO) cells with *Cm*, and performed transcriptome analyses using microarray. Genome-wide expression and ingenuity pathway analysis (IPA) identified enhanced expression of host genes encoding for components found in multiple cellular processes encompassing: (1) pro-inflammatory, (2) cell adhesion, (3) chemoattraction, (4) cellular matrix and small molecule transport, (5) apoptosis, and (6) antigen-processing and presentation. These results support a role for TLR3 in modulating the host cellular responses to *Cm* infection that extend beyond inflammation and fibrosis, and shows that TLR3 could serve a potential therapeutic target for drug and/or vaccine development.

Keywords: TLR3; Chlamydia; RNA; Micro-array; Epithelial; RANTES; Pathogenesis; Transcriptome; Genital tract.

Introduction

Chlamydia trachomatis is a gram-negative, obligate intracellular bacterial pathogen causing the most common sexually transmitted infections (STI) worldwide, particularly among young women [1]. In women, chronic infection with the urogenital serovars (D-K) can cause pelvic inflammatory diseases (PID) and chronic pelvic pain, which can culminate into scarring and fibrosis of the Fallopian tubes leading to infertility or ectopic pregnancy [2-4]. According to the Centers for Disease Control and Prevention's (CDC's) report in 2016, a total of 1,598,354 *Chlamydia* infections were reported in USA alone, which was 4.7% higher than the reported number of cases in 2015 [5]. As per CDC's estimate, nearly 20 million healthy individuals are infected every year, accounting for almost \$16 billion in

health care costs annually (CDC 2016) [5]. The asymptomatic nature of *C. trachomatis* infection means that the pathogen persists for a long time in some individuals, which suggests the effective evasion of host immune systems [6,7].

The innate immune system is known for recognizing a vast variety of pathogens, including viruses, bacteria and fungi via sensing the specific pathogen associated molecular patterns (PAMPs). The innate immune response induced by PAMPs includes the cellular production of a wide range of antimicrobial and inflammatory mediators. The recognition of PAMPs by pattern recognition receptors (PRRs) expressed by innate immune cells is crucial for maintenance of homeostatic immunity as well as an effective induction of an adaptive immune response [8-10]. However, an overly activated innate immune response can cause the overabundant production of inflammatory mediators which can result in tissue damage [11,12]. Among the PRRs, Toll-like receptors (TLRs) play a major role in innate immunity by recognizing structurally conserved microbial components [13]. TLRs are membrane bound PRRs that have been shown to be triggered by PAMPs from various bacterial, viral and fungal pathogens [14]. Engagement of the TLRs by the PAMPs can lead to the activation of phagocytosis and the production of inflammatory cytokines including TNF- α , IL-6, and GM-CSF, as an important step prior to the switch from an innate immunity and the onset of an adaptive immune response [15-18].

Epithelial cells lining the mucosal surfaces of the female genital tract serve as the sentinels to the invading *C. trachomatis* by expressing TLRs that trigger the innate immune response by inducing a multitude of pro-inflammatory cytokines and chemokines during infection [19]. These cytokines and chemokines mediate resolution of *Chlamydia* infection and are responsible for polarizing the innate and adaptive immune responses [18-23]. We previously showed that *Chlamydia muridarum* (*Cm*), an orthologue of human *C. trachomatis*, induces TLR2 dependent secretion of acute-phase inflammatory cytokines including IL-6, GM-CSF, and TNF- α in murine oviduct epithelial (OE) cells [8]. Further, we showed that *Chlamydia* infection mediates IFN- β secretion in a mostly TLR3-dependent manner, and demonstrated a role for TLR3 in regulating the expression of a plethora of other innate-inflammatory modulators including IL-6, CXCL10, CXCL16 and CCL5 [24-26]. The *Chlamydia*-induced expression of these cytokines was severely diminished in TLR3 deficient OE cells whereas; replication of *Cm* in TLR3 deficient OE cells was more robust than in WT OE cells. These findings suggest that triggering the TLR3 pathway in OE cells during *Cm* infection invokes cellular mechanisms that inhibit the chlamydial developmental cycle, and thereby implicate TLR3 in regulating cellular processes that extend beyond the syntheses of chemo tactic and inflammatory mediators.

In order to help identify other cellular pathways that are regulated by TLR3 signaling during *Chlamydia* infection, we conducted transcriptome analyses and comparative gene expression profiling on WT and TLR3-deficient murine OE cells that were infected with *Cm*. In the present study, we aim

to identify target genes that are affected by *Chlamydia* infection of OE cells and to ascertain a role for TLR3 in regulating those pathways by using genome-wide microarray analysis followed by ingenuity pathway analysis (IPA). Our results indicate that TLR3 plays a significant role in modulating the host cellular responses that encompass a diverse subset of biological functions during *Cm* infection.

Materials and Methods

Oviduct epithelial cells and culture conditions

The murine oviduct epithelial cell lines OE-129TLR3^{-/-} (C19) and OE-129WT [25] were grown at 37°C in a 5% CO₂ humidified incubator in epithelial-cell media {Dulbecco's modified Eagle medium and F12K (Sigma-Aldrich) in 1:1 ratio}, supplemented with 10% fetal bovine serum (HyClone), 2mM L-alanyl-L-glutamine (Glutamax I; Gibco/ Invitrogen, Carlsbad, CA), 5 μ g bovine insulin/ml, and 12.5 ng/ml recombinant human KGF (keratinocyte growth factor; Sigma-Aldrich) as previously described [18,24].

Chlamydia stocks

Mycoplasma-free *C. muridarum* Nigg, previously known as *C. trachomatis* strain (MoPn), was grown and titrated in McCoy cells (ATCC) as described [18, 27, 28]. The elementary bodies were harvested from infected McCoy cells, were re-suspended in SPG buffer (250mM sucrose, 10 mM sodium phosphate, and 5mM L-glutamic acid, pH 7.2), and quantified on McCoy cells using methodology described previously [18,26,29].

In vitro infection of oviduct epithelial cells

OE-129WT and OE-129TLR3^{-/-} (C19) cells were seeded in 6-well tissue culture plates and grown until confluence. The cells were infected with 5 inclusion forming-units (IFU) of *C. muridarum*/cell in 900ul of culture medium as described previously [24, 25]. Mock-treated controls were incubated with an inoculum containing equivalent volume of SPG buffer, but were not infected with *Chlamydia*. Each cell type was seeded in duplicate wells of the 6-well plate and were infected when confluent. The experiments were repeated 3 times on different days to provide the appropriate number of biological replicates for proper statistical analyses.

Total RNA purification and Microarray analysis

Total RNA was purified from OE-129WT and OE-129TLR3^{-/-} (C19) cells infected with *C. muridarum* (5 IFU/cell) at 24h post-infection using the Norgen Total RNA Purification Kit (Norgen Biotek; Thorold, Ontario). During purification, all RNA samples were treated with RNase-free DNase I (Qiagen) to remove all traces of genomic-DNA contamination. The RNA quantity and quality were measured using the Nano Drop 2000c spectrophotometer (Thermo Scientific; Pittsburgh, PA). The RNA purified from mock-infected cells was used as control sample. Purified total RNA was submitted to the gene-expression profiling services of Phalanx Biotech Group, Inc. (Belmont, CA) for microarray analysis. Genes that were up or down regulated \geq 2.5-fold with *p*-values <0.05 were considered in the final analysis (Tables 1 and 2; and Supplementary Tables 1 and 2).

Table 1: Differentially expressed genes up-regulated in OE-WT-Cm vs OE-WT-Mock and down-regulated in OE-TLR3KO-Cm vs OE-WT-Cm^(a)

| OE-WT-Cm/OE-WT-Mock | | | | | OE-TLR3KO-Cm/OE-WT-Cm | |
|---------------------|---------------------------|---|-------------|-------------|-----------------------|-------------|
| Entrez Gene ID (mM) | Gene Symbol (Entrez Gene) | Entrez Gene Name | Fold Change | p-value | Fold Change | p-value |
| 100038882 | Isg15 | ISG15 ubiquitin-like modifier | 82.104 | 0 | 2.528 | 0.025 |
| 20304 | Ccl5 | C-C motif chemokine ligand 5 | 80.053 | 0 | -47.826 | 2.07E-30 |
| 16782 | Lamc2 | laminin subunit gamma 2 | 71.848 | 0 | -8.295 | 0.000153 |
| 20310 | Cxcl2 | C-X-C motif chemokine ligand 3 | 70.603 | 0 | -10.952 | 0.000000151 |
| 16819 | Lcn2 | lipocalin 2 | 67.864 | 0 | -36.169 | 7.21E-29 |
| 22169 | Cmpk2 | cytidine/uridine monophosphate kinase 2 | 31.845 | 0 | -46.343 | 0 |
| 12985 | Csf3 | colony stimulating factor 3 | 27.161 | 0 | -3.455 | 0.000632 |
| 80981 | Arl4d | ADP ribosylation factor like GTPase 4D | 21.484 | 5.01E-26 | -19.014 | 2.08E-24 |
| 20311 | Cxcl5 | C-X-C motif chemokine ligand 6 | 15.617 | 5.02E-19 | -4.31 | 0.019 |
| 214854 | Neur13 | neuralized E3 ubiquitin protein ligase 3 | 14.48 | 3.22E-28 | -2.66 | 0.0111 |
| 18788 | Serp1nb2 | serpin family B member 2 | 14.088 | 1.91E-12 | -24.308 | 1.76E-10 |
| 16193 | Il6 | interleukin 6 | 13.337 | 3.58E-37 | -17.969 | 1.18E-09 |
| 229898 | Gbp5 | guanylate binding protein 5 | 11.081 | 0 | -2.56 | 0.000694 |
| 58203 | Zbp1 | Z-DNA binding protein 1 | 10.234 | 0 | 10.005 | 3.49E-27 |
| 20303 | Ccl4 | C-C motif chemokine ligand 4 | 9.458 | 0.00338 | -6.068 | 0.0000371 |
| 240327 | Gm4951 | predicted gene 4951 | 8.929 | 6.93E-17 | 14.813 | 1.86E-15 |
| 11847 | Arg2 | arginase 2 | 8.562 | 3.02E-10 | -4.601 | 0.00000186 |
| 17858 17857 | Mx1 | MX dynamin-like GTPase 1 | 7.365 | 6.83E-38 | 5.369 | 0.0405 |
| 20296 | Ccl2 | chemokine (C-C motif) ligand 2 | 7.36 | 2.2E-20 | -3.483 | 1.01E-33 |
| 19288 | Ptx3 | pentraxin 3 | 7.12 | 3.18E-08 | -33.12 | 7.32E-36 |
| 20656 | Sod2 | superoxide dismutase 2 | 6.045 | 0 | -6.336 | 1.16E-26 |
| 20306 | Ccl7 | chemokine (C-C motif) ligand 7 | 5.505 | 7.13E-24 | -2.946 | 0.00000187 |
| 14102 | Fas | Fas cell surface death receptor | 4.848 | 7.88E-08 | -10.915 | 6.99E-26 |
| 23961 | Oas1b | 2'-5' oligoadenylate synthetase 1B | 4.845 | 9.07E-17 | 8.129 | 1.22E-17 |
| 16913 | Psmb8 | proteasome subunit beta 8 | 4.558 | 3.62E-10 | -4.651 | 2.52E-09 |
| 11988 | Slc7a2 | solute carrier family 7 member 2 | 4.507 | 0.00000086 | -27.433 | 0 |
| 327959 | Xaf1 | XIAP associated factor 1 | 4.474 | 3.04E-35 | -37.868 | 0 |
| 16452 | Jak2 | Janus kinase 2 | 4.419 | 0.000000098 | -3.011 | 1.78E-18 |
| 16912 | Psmb9 | proteasome subunit beta 9 | 4.367 | 0.0067 | -4.443 | 8.21E-11 |
| 65221 | Slc15a3 | solute carrier family 15 member 3 | 4.252 | 3.31E-12 | -2.58 | 0.00000651 |
| 243771 | Parp12 | poly(ADP-ribose) polymerase family member 12 | 4.18 | 9.21E-12 | -15.479 | 9.2E-24 |
| 17123 | Madcam1 | mucosal vascular addressin cell adhesion molecule 1 | 4.075 | 1.79E-08 | -4.034 | 9.95E-10 |

Table 1(a): Differentially expressed genes up-regulated in OE-WT-Cm vs OE-WT-Mock and down-regulated in OE-TLR3KO -Cm vs OE-WT-Cm (Continued)^(a)

| OE-WT-Cm/OE-WT-Mock | | | | | OE-TLR3KO-Cm/OE-WT-Cm | |
|---------------------|---------------------------|--|-------------|-------------|-----------------------|-------------|
| Entrez Gene ID (mM) | Gene Symbol (Entrez Gene) | Entrez Gene Name | Fold Change | p-value | Fold Change | p-value |
| 16666 | Krt16 | keratin 16 | 3.732 | 0.00432 | -3.812 | 0.000724 |
| 17133 | Maff | MAF bZIP transcription factor F | 3.602 | 5.23E-34 | -4.715 | 2.21E-13 |
| 14283 | Fosl1 | FOS like 1, AP-1 transcription factor subunit | 3.586 | 0.000139 | -3.475 | 0.00155 |
| 14313 | Fst | follistatin | 3.525 | 6.82E-10 | -6.758 | 0.00000863 |
| 114229 | Kiss1r | KISS1 receptor | 3.504 | 1.96E-22 | -7.478 | 1.88E-23 |
| 16169 | Il15ra | interleukin 15 receptor subunit alpha | 3.468 | 3.75E-17 | -3.046 | 0.000000158 |
| 50527 | Ero1l | endoplasmic reticulum oxidoreductase 1 alpha | 3.343 | 1.63E-15 | -3.126 | 0.00000163 |
| 17476 | Mpeg1 | macrophage expressed 1 | 3.265 | 0.00431 | 21.94 | 1.41E-23 |
| 63872 | Zfp296 | zinc finger protein 296 | 3.239 | 0.000000657 | 4.454 | 3.47E-10 |
| 17384 | Mmp10 | matrix metalloproteinase 10 | 3.183 | 6.66E-08 | -10.946 | 5.38E-20 |
| 13874 | Ereg | epiregulin | 3.172 | 1.93E-08 | -6.62 | 0.000606 |
| 12642 | Ch25h | cholesterol 25-hydroxylase | 3.156 | 0.00037 | -98.991 | 0 |
| 18606 | Enpp2 | ectonucleotide pyrophosphatase/phosphodiesterase 2 | 3.138 | 0.000161 | -100 | 0 |
| 22029 | Traf1 | TNF receptor associated factor 1 | 3.105 | 0.000000562 | -2.578 | 0.0000198 |
| 18035 | Nfkb1a | NFKB inhibitor alpha | 2.971 | 1.22E-34 | -2.722 | 2.27E-26 |
| 64654 | Fgf23 | fibroblast growth factor 23 | 2.919 | 1.43E-09 | -87.556 | 0 |
| 17082 | Il1rl1 | interleukin 1 receptor like 1 | 2.916 | 0.00469 | -4.115 | 0.00854 |
| 320207 | Pik3r5 | phosphoinositide-3-kinase regulatory subunit 5 | 2.915 | 0.000602 | -26.596 | 1.34E-24 |
| 11770 | Fabp4 | fatty acid binding protein 4 | 2.91 | 0.000000619 | -10.759 | 5E-10 |
| 18787 | Serp1e1 | serpin family E member 1 | 2.902 | 0.000000258 | -4.264 | 3.49E-25 |
| 12475 | Cd14 | CD14 molecule | 2.825 | 8.79E-14 | -2.52 | 3.16E-09 |
| 117167 | Steap4 | STEAP4 metalloproteinase | 2.825 | 0.00389 | -14.747 | 9.46E-08 |
| 19698 | Relb | RELB proto-oncogene, NF-kB subunit | 2.697 | 7.01E-31 | -3.018 | 0 |
| 11639 | Ak4 | adenylate kinase 4 | 2.691 | 3.04E-13 | -6.141 | 8.57E-10 |
| 77113 | Klhl2 | kelch like family member 2 | 2.65 | 3.49E-08 | 4.316 | 0.00000534 |
| 26399 | Map2k6 | mitogen-activated protein kinase kinase 6 | 2.608 | 4.68E-19 | -7.841 | 1.86E-18 |
| 59028 | Rcl1 | RNA terminal phosphate cyclase like 1 | 2.598 | 0.00000325 | -2.984 | 3.29E-09 |
| 15894 | Icam1 | intercellular adhesion molecule 1 | 2.579 | 0 | -14.551 | 0 |
| 225642 | Grp | gastrin releasing peptide | 2.575 | 1.75E-18 | -11.28 | 2.28E-14 |
| 21354 | Tap1 | transporter 1, ATP binding cassette subfamily B member | 2.57 | 2.83E-14 | -4.642 | 0.000298 |

Table 2: Differentially expressed genes downregulated in OE-WT-Cm vs OE-WT-Mock and upregulated in OE-TLR3KO-Cm vs OE-WT-Cm ^(b)

| OE-WT-Cm/OE-WT-Mock | | | | | OE-TLR3KO-Cm/OE-WT-Cm | |
|---------------------|---------------------------|--|-------------|-----------|-----------------------|-----------|
| Entrez Gene ID (mM) | Gene Symbol (Entrez Gene) | Entrez Gene Name | Fold Change | p-value | Fold Change | p-value |
| 76441 | Daam2 | disheveled associated activator of morphogenesis 2 | -3.603 | 0.0000152 | 3.375 | 1.02E-08 |
| 14537 | Gcnt1 | glucosaminyl (N-acetyl) transferase 1, core 2 | -3.313 | 1.86E-12 | 5.13 | 0 |
| 56338 | Txnip | thioredoxin interacting protein | -2.817 | 6.55E-20 | 14.882 | 4.93E-30 |
| 213121 | Ankrd35 | ankyrin repeat domain 35 | -2.798 | 0.00472 | 2.574 | 7.04E-38 |
| 11668 | Aldh1a1 | aldehyde dehydrogenase 1 family member A1 | -2.787 | 0.00756 | 12.645 | 2.32E-12 |
| 320472 | Ppm1e | protein phosphatase, Mg ²⁺ /Mn ²⁺ dependent 1E | -2.778 | 0.000302 | 3.608 | 6.14E-08 |
| 13924 | Ptpv | protein tyrosine phosphatase, receptor type, V | -2.622 | 7.51E-10 | 4.994 | 1.67E-19 |
| 17022 | Lum | lumican | -2.565 | 0.000194 | 5.108 | 0.0000977 |
| 23876 | Fbln5 | fibulin 5 | -2.564 | 0.0000277 | 15.858 | 0 |
| 76454 | Fbxo31 | F-box protein 31 | -2.561 | 0.00114 | 5.979 | 2.22E-10 |
| 103172 | Chchd10 | coiled-coil-helix-coiled-coil-helix domain containing 10 | -2.54 | 0.000863 | 32.532 | 1.68E-11 |

Note: OE-WT-Cm/OE-WT-Mock refers to the gene-expression levels in *Chlamydia muridarum* (Cm) infected OE-WT cells derived versus the basal gene-expression levels in the Mock-infected OE-WT cells at the 24h time-point. OE-TLR3KO-Cm/OE-WT-Cm refers to the gene-expression levels in Cm-infected OE-WT cells versus the gene-expression levels in the Cm-infected OE-WT cells at the 24h time-point. For all genes, only fold changes above 2.5-fold with a p-value of <0.05 for changes in expression between above groups are shown, - (negative) sign with fold-change indicates down-regulated genes whereas; fold-change without - (negative) sign indicates up-regulated genes. **(a) Table 1:** represents differentially expressed genes up-regulated in OE-WT-Cm vs OE-WT-Mock and down-regulated in OE-TLR3KO-Cm vs OE-WT-Cm. **(b) Table 2:** represents differentially expressed genes down-regulated in OE-WT-Cm vs OE-WT-Mock and up regulated in OE-TLR3KO-Cm vs OE-WT-Cm.

Functional and canonical pathway analyses

The microarray gene expression data were analyzed by Qiagen’s ingenuity pathway analysis (IPA; Ingenuity Systems, <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>; Qiagen Inc.) to determine whether genes are associated with particular diseases, exhibit prominent biological function, or if canonical signaling pathways were preferentially up- or down-regulated in murine OE cells [30]. Genes were selected for analyses if they had a p-value <0.05 and absolute fold change ≥2.5. The data are presented as the comparison of: (1) *C. muridarum*-infected wild-type (OE-WT-Cm) vs Mock-infected wild-type (OE-WT-Mock) and (2) *C. muridarum*-infected wild-type (OE-WT-Cm) vs *C. muridarum*-infected TLR3-deficient OE cells (OE-TLR3KO-Cm) (Table 3, Supplementary Table 3, and Table 4).

Table 3: Ingenuity Canonical Pathway Analysis (IPA): Significant pathways predicted to be activated or inhibited based on the calculated z-score. A positive z-score predicts activation; a negative z-score predicts inhibition. Genes listed in Bold and *Italic* show up-and down-regulation, respectively.

| Group | OE-WT-Cm vs OE-WT-Mock | | | OE-TLR3KO-Cm vs OE-WT-Cm | | |
|--|------------------------|---------|--|--------------------------|---------|---|
| | -log (p-value) | z-score | Molecules | -log (p-value) | z-score | Molecules |
| Acute Phase Response Signaling | 4.46 | 3 | MAP2K6, NFKBIA, SOD2, CFB, IL6, LBP, JAK2, SERPINE1, IL1RAP(9) | 5.02 | -1.886 | <i>MAP2K6, MAPK1, SERPINF1, VWF, CEBPB, IL1R1, STAT3, JAK2, IL6, MAPK11, NR3C1, C5, HP, NFKBIA, SOD2, ITIH2, F8, AKT3, PIK3CB, OSMR, SERPINE1, RBP4, AGT, TNFRSF11B(24)</i> |
| Dendritic Cell Maturation | 4.03 | 3 | ICAM1, NFKBIA, RELB, PIK3R5, STAT2, IL6, JAK2, TLR3, CREB5 (9) | 5.54 | -0.577 | <i>ICAM1, MAPK1, IFNB1, PIK3R5, HLA-DQA1, LTB, IL6, JAK2, MAPK11, CD1D, NFKBIA, CREB1, AKT3, PLCB1, TLR3, COL18A1, FCGR3A/FCGR3B, TNFRSF11B, PIK3C2B, FCGR2A, RELB, TLR2, GAB1, PLCG2, PIK3R6, PIK3CB, CSF2(27)</i> |
| Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses | 7.13 | 2.828 | PTX3, IRF7, OAS2, DDX58, Oas1b, PIK3R5, IL6, CCL5, TLR3, EIF2AK2, RIPK2(11) | 6.83 | 0.688 | <i>PTX3, PIK3C2B, MAPK1, IFNB1, Oas1b, PIK3R5, CCL5, IL6, OAS3, EIF2S1, RNASEL, C5, TLR2, GAB1, PLCG2, CREB1, PIK3R6, TLR7, CASP1, PIK3CB, TLR3, CSF2, PRKD3, PRKD1(24)</i> |
| IL-6 Signaling | 5.45 | 2.646 | MAP2K6, NFKBIA, IL1RL1, PIK3R5, CD14, IL6, LBP, JAK2, IL1RAP(9) | 5.02 | -1.606 | <i>MAP2K6, PIK3C2B, MAPK1, TNFAIP6, IL1RL1, PIK3R5, CEBPB, IL1R1, STAT3, JAK2, IL6, MAPK11, IL18RAP, NFKBIA, GAB1, PIK3R6, AKT3, CD14, PIK3CB, TNFRSF11B(20)</i> |
| Interferon Signaling | 7.41 | 2.449 | IFI35, STAT2, IRF9, PSMB8, JAK2, TAP1, ISG15(7) | 1.43 | -1.342 | <i>IFNB1, PSMB8, JAK2, TAP1, ISG15(5)</i> |
| TREM1 Signaling | 4.12 | 2.449 | CXCL3, ICAM1, IL1RL1, IL6, JAK2, TLR3(6) | 5.16 | -1.291 | <i>SIGIRR, ICAM1, MAPK1, IL1RL1, JAK2, IL6, STAT3, TLR2, CXCL3, PLCG2, TLR7, CASP1, AKT3, TLR3, CSF2(15)</i> |
| VDR/RXR Activation | 4.02 | 2.236 | CXCL10, CAMP, IL1RL1, CD14, IGFBP5, CCL5(6) | 4.94 | -1.633 | <i>IGFBP6, WT1, CCNC, IL1RL1, HES1, CCL5, CEBPB, HOXA10, MXD1, IGFBP3, CD14, VDR, CSF2, PRKD3, PRKD1(15)</i> |
| p38 MAPK Signaling | 2.25 | 2.236 | MAP2K6, IL1RL1, CREB5, IL1RAP, FAS(5) | 2.03 | -1.155 | <i>MAP2K6, PLA2G6, MAPKAPK3, IL1RL1, PLA2G5, CREB1, RPS6KA2, IL1R1, EEF2K, MAPK11, FAS, PLA2G12A, IL18RAP (13)</i> |

Table 3(a): Ingenuity Canonical Pathway Analysis (IPA): Significant pathways predicted to be activated or inhibited based on calculated z-score. A positive z-score predicts activation; a negative z-score predicts inhibition. Genes listed in **Bold** and *Italic* show up-and down-regulation, respectively. (Continued)

| Group | OEWT- <i>Cm</i> vs OE-WT- <i>Mock</i> | | | OE-TLR3KO- <i>Cm</i> vs OE-WT- <i>Cm</i> | | |
|--|---------------------------------------|---------|---|--|---------|--|
| | -log (p-value) | z-score | Molecules | -log (p-value) | z-score | Molecules |
| LPS-stimulated MAPK Signaling | 2.86 | 2 | MAP2K6, NFKBIA, PIK3R5, CD14, LBP(5) | 3.28 | -0.277 | <i>MAP2K6, PIK3C2B, NFKBIA, MAPK1, GAB1, CREB1, PIK3R5, PIK3R6, CD14, PIK3CB, MAPK11, PRKD3, PRKD1(13)</i> |
| Th1 Pathway | 2.75 | 2 | MAP2K6, ICAM1, PIK3R5, CD274, IL6, JAK2(6) | 3.17 | -0.535 | <i>MAP2K6, PIK3C2B, NOTCH3, ICAM1, HAVCR2, HLA-DQA1, PIK3R5, STAT3, NFATC4, JAK2, IL6, NFIL3, GAB1, PIK3R6, APH1B, PIK3CB, ICOSLG/LOC102723996(17)</i> |
| LPS/IL-1 Mediated Inhibition of RXR Function | 2.29 | 2 | <i>ALDH1A1, IL1RL1, CD14, FABP4, LBP, ALDH3A1, IL1RAP(7)</i> | 2.77 | 0.277 | <i>APOE, IL1RL1, NR1H4, NR1H3, ABCG1, IL1R1, ABCA1, PAPSS2, IL18RAP, SULT2B1, CHST2, ALDH1A1, NR1H2, NROB2, UST, FABP4, CD14, HS3ST1, FMO1, ABC3, HS3ST5, TNFRSF11B, GSTK1(23)</i> |
| JAK/Stat Signaling | 2.1 | 2 | PIK3R5, STAT2, IL6, JAK2(4) | 2.44 | -0.302 | PIK3C2B, GAB1, MAPK1, PIK3R5, PIK3R6, AKT3, PIK3CB, IL6, CEBPB, JAK2, STAT3(11) |
| HMGB1 Signaling | 2.07 | 2 | MAP2K6, ICAM1, PIK3R5, IL6, SERPINE1(5) | 2.84 | -0.775 | <i>MAP2K6, PIK3C2B, ICAM1, MAPK1, PIK3R5, RHOJ, IL6, IL1R1, MAPK11, GAB1, PIK3R6, AKT3, PIK3CB, SERPINE1, CSF2, TNFRSF11B(16)</i> |
| PI3K/AKT Signaling | 1.51 | 2 | NFKBIA, MAP3K8, PTGS2, JAK2 (4) | 0.442 | -1.134 | <i>NFKBIA, SYNJ1, GAB1, MAPK1, INPP5J, AKT3, PIK3CB, JAK2(8)</i> |
| Tec Kinase Signaling | 1.11 | 2 | PIK3R5, STAT2, JAK2, FAS(4) | 1.45 | -1.069 | PIK3C2B, PIK3R5, TNFSF10, RHOJ, JAK2, STAT3, FAS, LCK, ACTA2, GAB1, PLCG2, PIK3R6, PIK3CB, PRKD3, PRKD1(15) |
| Toll-like Receptor Signaling | 7.39 | 1.89 | MAP2K6, NFKBIA, IL1RL1, CD14, TNFAIP3, LBP, TLR3, EIF2AK2, TRAF1(9) | 3.29 | -1.265 | <i>TLR2, MAP2K6, UBD, SIGIRR, NFKBIA, MAPK1, IL1RL1, TLR7, CD14, TLR3, MAPK11, TRAF1(12)</i> |
| Activation of IRF by Cytosolic Pattern Recognition Receptors | 10.8 | 1.508 | DHX58, TANK, IRF7, NFKBIA, ZBP1, DDX58, STAT2, IRF9, IL6, IFIT2, ISG15(11) | 0.643 | -0.447 | <i>NFKBIA, ZBP1, IFNB1, IL6, ISG15(5)</i> |

Table 3(b): Ingenuity Canonical Pathway Analysis (IPA): Significant pathways predicted to be activated or inhibited by z-score. A positive z-score predicts activation; a negative z-score predicts inhibition. Genes listed in **Bold** and *Italic* show up-and down-regulation, respectively. (Continued)

| Group | OE-WT- <i>Cm</i> vs OE-WT- <i>Mock</i> | | | OE-TLR3KO- <i>Cm</i> vs OE-WT- <i>Cm</i> | | |
|---|--|---------|--|--|---------|---|
| | -log (p-value) | z-score | Molecules | -log (p-value) | z-score | Molecules |
| NF-κB Signaling | 4.25 | 1.414 | MAP2K6, TANK, NFKBIA, RELB, PIK3R5, TNFAIP3, MAP3K8, TLR3, EIF2AK2(9) | 3.32 | 0.218 | <i>MAP2K6, SIGIRR, PIK3C2B, FLT1, RELB, PIK3R5, IL1R1, TLR2, TNIP1, LCK, GHR, NFKBIA, GAB1, PLCG2, PIK3R6, TLR7, PDGFRA, AKT3, PIK3CB, TLR3, TNFRSF11B(21)</i> |
| Production of Nitric Oxide and Reactive Oxygen Species in Macrophages | 1.43 | 1.342 | NFKBIA, PIK3R5, ARG2, MAP3K8, JAK2 (5) | 4.37 | -1 | MAP3K15, APOE, MAPK1, PIK3R5, ARG2, JAK2, MAPK11, NFKBIA, HOXA10, AKT3, NOS2, PRKD3, PRKD1, TNFRSF11B, PIK3C2B, MAP3K9, RHOJ, TLR2, GAB1, PLCG2, PIK3R6, PIK3CB, SIRPA, CLU, RBP4(25) |
| Cardiac Hypertrophy Signaling | 1.14 | 1.342 | MAP2K6, PIK3R5, MAP3K8, IL6, ADRB2(5) | 4.11 | -0.756 | <i>MAP2K6, MAP3K15, MAPK1, PIK3R5, IL6, MAPK11, IGF1, PPP3R1, CREB1, PRKAR1B, PLCB1, PIK3C2B, MAP3K9, MAPKAPK3, ADCY3, CACNA1C, RHOJ, PPP3CC, NFATC4, MYL7, MYL9, PRKAR2B, ADRA2A, GAB1, PLCG2, PIK3R6, PIK3CB, ADCY7(28)</i> |
| TGF-β Signaling | 2.03 | 1 | MAP2K6, IRF7, SERPINE1, INHBA(4) | 0.783 | -1.342 | <i>MAP2K6, MAPK1, VDR, HOXC9, SERPINE1, MAPK11, INHBB(7)</i> |
| LXR/RXR Activation | 4.71 | -2.121 | IL1RL1, CD14, ARG2, IL6, LBP, PTGS2, TLR3, IL1RAP(8) | 6.17 | 1.091 | KNG1, APOE, IL1RL1, NR1H4, SERPINF1, NR1H3, ARG2, ABCG1, IL1R1, IL6, ABCA1, IL18RAP, NR1H2, MYLIP, CD14, TLR3, NOS2, CLU, MMP9, TNFRSF11B, RBP4, AGT(22) |
| PPAR Signaling | 1.9 | -2 | NFKBIA, IL1RL1, PTGS2, IL1RAP(4) | 1.24 | 1 | <i>NFKBIA, NROB2, MAPK1, IL1RL1, PDGFRA, NR1H3, IL1R1, IL18RAP, TNFRSF11B(9)</i> |
| PPARα/RXRα Activation | 2.14 | -1.342 | MAP2K6, NFKBIA, IL1RL1, IL6, JAK2, IL1RAP(6) | 2.45 | -0.5 | <i>MAP2K6, MAPK1, IL1RL1, ADCY3, JAK2, IL6, IL1R1, ABCA1, IL18RAP, GHR, PRKAR2B, NFKBIA, NROB2, PLCG2, PRKAR1B, PRKAA2, PLCB1, ADCY7, ITGB5(19)</i> |

Table 4: Top disease and cell-function pathways predicted to be increased or decreased bases on calculated z-score. A positive z-score predicts an increase and a negative z-score predicts a decrease in disease and other functional pathways.

| Categories | Diseases or Functions | OE-WT- <i>Cm</i> vs OE-WT- <i>Mock</i> | | | | OE-TLR3KO- <i>Cm</i> vs OE-WT- <i>Cm</i> | | | |
|-------------------------------------|-------------------------------|--|----------------------------|---------|-------------|--|----------------------------|---------|-------------|
| | | p-Value | Predicted Activation State | z-score | # Molecules | p-Value | Predicted Activation State | z-score | # Molecules |
| Inflammatory Response | Inflammatory Response | 1.39E-15 | Increased | 4.367 | 44 | 1.28E-16 | Decreased | -1.597 | 136 |
| Organismal Injury and Abnormalities | Inflammation of organ | 3.29E-13 | Increased | 0.061 | 54 | 5.82E-12 | Increased | 2.324 | 178 |
| Tissue Development | Growth of epithelial tissue | 0.000000578 | Increased | 2.862 | 27 | 1.71E-20 | Decreased | -2.302 | 130 |
| Cell Death and Survival | Necrosis of epithelial tissue | 1.1E-11 | Increased | 0.812 | 32 | 3.31E-16 | Increased | 2.133 | 109 |

Results

Identification of differentially-regulated genes in murine oviduct epithelial cells

Our previous studies showed that TLR3 is the primary PRR in OE cells triggered by *Cm* infection in the early synthesis of IFN- β , and in the syntheses of a multitude of other innate inflammatory modulators such as IL-6, CXCL10, CXCL16 and CCL5. Our data show that TLR3 has a biological impact on the innate immune response to *Chlamydia* infection; however, the comprehensive impact of TLR3 deficiency during *Chlamydia* infection of murine OE cells remains unclear. To determine the global significance of TLR3 signaling on *Cm* infection in OE cell lines, we infected wild-type and TLR3-deficient OE cells (here forth referred to as OE-WT and OE-TLR3KO cells, respectively) with 5 IFU/cell *Cm* for 24h post-infection (PI) and performed transcriptome analysis using microarray. We used a minimum significance criterium of $p < 0.05$ and an absolute fold change ≥ 2.5 for comparative gene analyses (See Materials and Methods). Out of the several hundred genes that were differentially affected by *Cm* infection, candidate gene selection based on this criterion yielded 152 genes up-regulated and 56 genes down-regulated when comparing OE-WT-*Cm* vs the OE-WT-*Mock* control (Supplementary Tables 1 and 2). Interestingly, there were 600 genes up-regulated and 616 genes down-regulated when comparing OE-TLR3KO-*Cm* vs OE-WT-*Cm* cells, which shows that TLR3 deficiency results in the differential regulation of a multitude of cellular processes during *Chlamydia* infection of the OE cells.

We selected genes that were up-/or down-regulated > 2.5-fold in both comparative groups: [OE-WT-*Cm* vs OE-WT-*Mock*] and [OE-TLR3KO-*Cm* vs OE-WT-*Cm*] for our analyses (Tables 1 and 2). The most prominent genes up-regulated in OE-WT-*Cm* vs OE-WT-*Mock* are listed in Table 1. As indicated, many of the most highly up-regulated genes in the OE-WT-*Cm* cells such as *Ccl5* (80.050-fold), *Lamc2* (71.848-fold), *Cxcl2* (70.603-fold), *Lcn2* (67.864-fold), *Cmpk2* (31.845 fold) and *Csf3* (27.161-fold) were substantially and significantly down regulated in OE-TLR3KO-*Cm* cells. Not surprisingly, pro-inflammatory cytokines and chemokines showed the highest level of up regulation during *Cm* infection in the OE-WT cells. Amongst the most highly up-regulated pro-inflammatory mediators were *Ccl5* (RANTES), *Ccl4* (MIP-1b), and the monocyte chemo attractant proteins *Ccl2* and *Ccl7* (or MCP 1 and 3, respectively) belonging to C-C chemokine family. The

C-X-C motif chemokines *Cxcl2* (also called macrophage inflammatory protein2-alpha) and *Cxcl5* (or epithelial neutrophil activating protein-78), and the acute inflammatory cytokines *Csf3* and *Il6* were also included in the most highly upregulated inflammatory mediators. These highly up-regulated cytokine and chemokines were severely down-regulated in the OE-TLR3KO-*Cm* cells relative to OE-WT-*Cm* cells (Table1).

The extracellular matrix (ECM) proteins play a critical role in the cell invasion, adhesion, cell patterning and architectural changes, and "outside-in" signal transduction [31]. Our microarray data showed that many host ECM moieties were differentially expressed, suggesting a rapid and dramatic remodeling of the extracellular milieu in response to *Chlamydia* infection (Tables 1 and 2; Supplementary Tables 1 and 2). The genes involved in remodeling included glycol proteins, metalloproteinases, numerous collagens, and several fibrosis-associated moieties that were highly up-regulated in our data set (Table 1). The *Cm* induced ECM genes included *Lamc2* (LAMC2), *Fst* (Follistatin), *Mmp10* (metalloproteinase MMP10), *Ereg* (epiregulin EREG), *Enpp2* (ATX or autotaxin, ENPP2), *Fgf23* (fibroblast growth factor FGF23), *Steap4* (metalloreductase STEAP4), *Madcam-1* (mucosal vascular address in cell adhesion molecule-1, MAdCam-1) and *Icam-1* (intracellular adhesion molecule-1, ICAM-1). However, as we have seen with many of the highly up regulated pro-inflammatory mediators, transcription of these ECM genes in the *Cm*-infected OE-TLR3KO cells resulted in a substantial down regulation or inhibition when compared to the *Cm*-infected OE-WT cells.

Other genes that were highly up regulated in response to *Cm* infection in WT-OE cells but attenuated during TLR3 deficiency included components of various cellular metabolic pathways (LCN2, CMPK2, ARG2, SOD2, ERO1- α [or endoplasmic reticulum oxidoreductase1- α], CH25H, FABP4, and AK4), and proteins involved in membrane tracking and protein processing (ARL4D, NEURL3, PTX3, SERPINB2 [or PAI-2], and SERPINE1 [or PAI-1]). Interferon-sensitive genes (ISGs) are known for their role in cell-intrinsic immunity against diverse pathogens, such as viruses and intracellular bacterial species including *Chlamydia*, *Mycobacteria*, *Listeria*, *Salmonella*, and *Toxoplasma* [32]. We found several ISG including *Gbp5* (a member of IFN-inducible subfamily of GTPases) [33] and *Parp12* [34-37] that were highly induced in the *Cm* infected OE-WT cells, but differentially regulated during TLR3 deficiency.

Some of the more moderately up-regulated genes encoding the pro-apoptotic proteins (FAS and XAF1), proteins related to MHC Class 1 antigen presentation (PSMB-8, PSMB-9 and TAP1), solute carrier (SLC) transporters (SLC7A2 and SLC15A3), and the JAK2 protein [38]. Other genes moderately up regulated included those encoding the TNF- α mediated pro-inflammatory proteins KRT16 (keratin), activator protein-1 (AP-1), family members of MAFF (MafBZIP transcription factor F) and FOSL1 (Fos-like 1 or FRA1) [39], TRAF1, NF- κ B (inhibitor of nuclear factor kappa-B or I κ B- α), NF- κ B/RELB, MKK6 (Mitogen-activated protein kinase 6), and GRP (Gastrin releasing peptide). Finally, our data also showed some moderate but significantly up-regulated expression of genes encoding various receptors and signaling molecules including KISS1R, IL15R- α , IL1RL, CD14, PIK3R5 (phosphatidylinositol 3-kinase, regulatory subunit 5), and the RCL1 (RNA terminal phosphate cyclase like-1) protein that is involved in eukaryotic 18S RNA biogenesis [40]. However, much like the highly up regulated genes, these moderately induced genes in *Cm*-infected OE-WT cells were differentially regulated during TLR3-deficiency. Interestingly, not all genes induced during *Cm* infection of WT-OE cells were attenuated or down regulated during TLR3 deficiency. Results depicted in Table 1 show that transcription levels for *Isg15*, *Mx1*, *Gm4951*, *Oas1b*, *Mpeg1*, *Zfp296*, *Zbp1*, and *Klhl2* were not reduced in the *Cm*-infected OE-TLR3KO cells relative to *Cm*-infected OE-WT cells; instead, their expression levels were significantly increased during TLR3-deficiency. *Isg15*, *Mx1*, *Oas1b* and *Mpeg1* are type I and II interferon responsive genes and have been implicated in the host protection against various pathogenic organisms [32, 41-45].

Table 2 shows 11 genes that were down-regulated more than 2.5-fold during *Cm* infection of the OE-WT cells. *Aldh1a1*, *Ankrd35*, *Chchd10*, *Daam2*, *Fbln5*, *Fbxo31*, *Gcnt1*, *Lum*, *Ppm1e*, *Ptprv* and *Txnip* were all down regulated during *Cm* infection in wild-type OE cells; however, their expression levels in OE-TLR3KO cells was several-fold higher. Interestingly, 8 of the 11 genes (*Aldh1a1*, *Ankrd35*, *Chchd10*, *Daam2*, *Fbln5*, *Fbxo31*, *Gcnt1*, and *Ppm1e*) have been reported to be prognostic markers in a variety of metastatic malignancies and other cancer-related diseases [46-54]. *Ptprv* and *Txnip* exhibit tumor-suppressor function and has involvement in cell proliferation and apoptosis [55, 56]. The *Lum* gene encodes the extracellular matrix protein lumican (LUM) that has a role in the bacterial clearance [57, 58], and its expression was up-regulated 5.108-fold in *Cm*-infected OE-TLR3KO cells when compared to *Cm* infected OE-WT cells.

Functional and canonical pathway analyses

Based on the gene expression data, a set of cellular pathways predicted to be either activated or inhibited during *Cm* infection was identified by IPA software. Table 3 lists what are predicted to be the most significantly changed pathways based on the expression levels of the input genes. As indicated, most of the *Cm* infection-induced pathways in the OE-WT cells were found to be significantly inhibited in the *Cm*-infected OE-TLR3KO cells. Pathways regulating the role

of PRRs in recognition of bacteria and viruses, LPS/IL-1 mediated inhibition of RXR function, and NF- κ B signaling were not inhibited in the *Cm*-infected OE-TLR3KO cells when compared to *Cm*-infected WT-OE cells like the other pathways listed in Table 3; however, activation of these pathways was significantly lower in the OE-TLR3KO cells. The lower portion of Table 3 shows the canonical pathways that were predicted to be inactivated in *Cm*-infected OE-WT cells e.g., LXR/RXR activation, PPAR signaling, and PPAR α /RXR α activation pathways. In contrast, LXR/RXR activation and PPAR signaling pathways are predicted to be activated in the *Cm*-infected OE-TLR3KO cells when compared to the *Cm*-infected OE-WT cells.

Upstream regulator analysis

IPA predicts upstream transcriptional regulators based on experimentally observed relationships between regulators and genes in the dataset. The calculated z-score predicts either activation or inhibition of regulators on the basis of the relationship with dataset genes and direction of expression change of input genes. There are 7 categories: immunological (cytokines and chemokines), chemical, kinase, transcription factor, trans membrane receptor, translation regulator, and transporter, where several *Cm* activated and inhibited upstream regulators are grouped together and listed in Supplementary Table 3 (Excel file). Among the 7 regulator categories, cytokine is the top-most significantly activated upstream regulator during *Cm* infection of OE cells, and the group includes IFN- γ , TNF, IL-1 β , OSM, IL-1 α , IL-17A, TNFSF-11, IL-1, IL-6, CD40LG, TNFSF-12, CSF-2, IL-18, IL-2, EDN1, CCL5, IL-15, MIF, IL-17F, CXCL2, IL-5, CXCL3, C5, CCL2, CXCL12, IL-7, IL-33, CXCL-8, TNFSF-14, TNFSF-13B, and IL-36A. Our data showed that these 31 cytokines were activated ≥ 2.5 -fold in *Cm*-infected OE-WT cells when compared to the Mock-infected controls. In corroboration with our previously published results [25, 26, 59], many of these cytokines were predicted to be either severely reduced or inhibited in *Cm*-infected OE-TLR3KO cells relative to *Cm*-infected OE-WT cells in IPA. It is noteworthy that one particular cytokine (IL-10) was different from the other cytokines listed in Supplementary Table 3 in that it was predicted to be down regulated in the *Cm*-infected OE-WT cells relative to its expression in the Mock control OE-WT cells, but predicted to be activated in *Cm*-OE-TLR3KO cells relative to *Cm*-infected OE-WT cells. We previously reported significant increases in the genital tract secretion of IL-10 of TLR3-deficient mice when compared to wild-type control mice [59]; thus, our findings in the IPA support our *in vivo* data regarding this pleiotropic regulatory cytokine [60]. We hypothesized that IL-10 plays a role in attenuating the *in vivo* synthesis of IFN- β during in TLR3-deficient mice and likely promotes the increased *Cm* replication that we have observed in the TLR3-deficient mice.

Upstream regulator analyses showed 61 chemical regulators that were either activated or inhibited in *Cm*-infected OE-WT cells when compared to Mock-infected OE-WT cells, but were differentially regulated in the *Cm*-infected OE-TLR3KO cells. The chemical categories encompass

endogenous non-mammal, endogenous mammal, other, drug, reagent, toxicant, and kinase inhibitors. Further analyses showed that 17 kinase regulators were activated i.e.; CHUK, IKKBK, IKBK, MAP3K7, RIPK2, MAPKAPK2, JAK2, JAK1, MAP3K14, MAPK8, PRKCD, MAP2K1, PRKCA, SRC, RET, GSK3B, SPHK1, whereas; one kinase (MAPK1) was found to be down-regulated in *Cm*-infected OE-WT cells. Among transcription factors (TFs), 16TFs: NF- κ B [complex], REL-A, JUN, STAT4, FOXL2, CEBPB, EGR1, TP63, ECSIT, HMGB1, IRF6, FOXO1, CEBPA, HIF1A, CEBPE, and JUNB) were activated and one TF (ZFP36) was inhibited. Other upstream regulators activated during *Cm* infection of OE-WT cells included 10 transmembrane receptors (TLR4, TLR3, TLR2, TLR5, CD40, CD14, TNFRSF1A, ICAM1, IL17RA, and TNFRSF1B), one translation regulator (EIF4E), and two transporters (LCN2 and TNNI3). The remaining upstream regulators spanning the 7 categories shown in Supplementary Table 3 were all inhibited in *Cm*-infected OE-WT cells when compared to Mock-infected OE-WT cells. However, they were also differentially regulated when compared to *Cm*-infected OE-TLR3KO cells in that they were either inhibited to a lesser degree, or the pathways were activated instead.

Disease pathogenesis and cellular function

Based on the gene expression input data, IPA predicted the pathways associated with disease pathogenesis and cellular function that are most affected in the cell during *Cm* infection of OE cells (Table 4). The inflammatory response and growth of epithelial tissue were not surprisingly the most affected pathways that are predicted to be affected when *Chlamydia* invades epithelial cells lining the female genital tract. As shown in Table 4, these pathways are the most highly up regulated in the *Cm*-infected OE-WT cells when compared to the Mock-infected OE-WT controls. As indicated, these two pathways are predicted to be significantly diminished or inhibited in the *Cm*-infected OE-TLR3KO cells when compared to the *Cm*-infected OE-WT cells. In support of our previously published reports showing that TLR3-deficiency leads to increased genital tract pathology in mice during *Cm*-infection [59], IPA showed that the pathways associated with organ inflammation and tissue necrosis were predicted to be significantly increased in the *Cm*-infected OE-TLR3KO cells when compared to *Cm*-infected OE-WT cells. Collectively, transcriptome and IPA results show role for TLR3 in modulating host cellular responses to *Cm* infection that extend beyond inflammation and fibrosis, and implicate TLR3 as a major component in the cellular response to *Chlamydia* infection in genital tract epithelial cells.

Discussion

We previously showed that *Cm* infection induces IFN- β secretion in OE cells in a mostly TLR3-dependent manner, and that TLR3 deficiency lead to the dysregulation in syntheses of a plethora of other innate inflammatory modulators including IL-6, CXCL10, CXCL16 and CCL5. Although the expression of these cytokines induced by *Chlamydia* was severely diminished in TLR3 deficient OE cells and mice, replication of

Cm during TLR3-deficiency was more robust than in OE-WT cells and mice. These data suggested that TLR3 had a biological impact on the innate immune response to *Chlamydia* infection; however, the impact of TLR3 signaling on the global cellular response to *Chlamydia* infection in OE cells remained unanswered. In the present study, we performed transcriptomics on wild-type and TLR3-deficient OE cells and conducted IPA to understand the complexity of genes and pathways in murine OE cells that are affected during *Cm* infection. In this regard, we are hoping to identify the spectrum of pathways that are either activated or inhibited during *Cm* infection, in order to ascertain those critical cellular mechanisms that can be exploited for the development of better treatments for chlamydial disease and therapeutic agents.

Microarray results uncovered a multitude of genes that were either up-or down-regulated ≥ 2.5 -fold in *Cm* infected OE-WT cells when compared to the mock-infected controls, and revealed that many of those genes were differentially regulated during TLR3-deficiency when comparing transcriptome data generated in *Cm*-infected OE-WT cells to results generated in the *Cm*-infected OE-TLR3KO cells. Among the most highly up-regulated genes during *Cm* infection of OE cells, the overwhelming majority were pro-inflammatory cytokines and chemokines (Table 1 and Supplementary Table 3, excel file). Our data showed that many of these pro-inflammatory mediators were either severely attenuated in their expression levels during TLR3 deficiency, or that their expression was down regulated below basal expression levels. Because epithelial cells lining the genital tract serve as immunological sentinels to microbial pathogens, and are responsible for initiating the primary phases of the host immune response [19, 21, 23], a comprehensive understanding of the cascade of cellular events that are triggered in OE cell during *Cm* infection would significantly enhance our knowledge and increase our understanding of the cellular mechanisms induced during chlamydial pathogenesis.

Cytokines and chemokines are crucial in regulating a variety of molecular and cellular events specifically inflammation, scarring, and fibrosis. We found that CCL5 (RANTES), CXCL2 (MIP2- α), CSF3, CXCL5 (ENA-78), IL-6, CCL4 (MIP-1b), CCL2 (MCP-2) and CCL7 (MCP-3) were among the highest up-regulated cytokine and chemokines. CCL5 showed the highest up-regulation (80.053-fold) in WT-OE cells during *Cm* infection in comparison to mock-infected WT-OE cells, corroborating results from previous studies [26, 61]. CCL5 is a chemotactic factor that is secreted by a variety of cells including epithelial cells, and functions by recruiting leukocytes to the inflamed sites via binding to its CCR3 receptor. CCL5 also recruits macrophages to the site of inflammation by binding the CCR5 receptor. CCL5 is also a key chemokine in the induction of other Th1 cytokines, and has a functional role in the humoral immune responses against chlamydial pathogens [62, 63]. The C-X-C motif chemokine ligand 2, CXCL2 (MIP2- α) was also shown to be highly induced and was the next highest upregulated chemokine behind CCL5 in the microarray analyses. CXCL2 (along with CCL4) has been demonstrated to be involved in the recruitment of both

lymphocytes and neutrophils to the inflamed sites in mouse model of *Chlamydia* infection [64, 65]. Other significantly up-regulated cytokine and chemokine markers were colony stimulating factor 3 (CSF3), IL-6, CXCL5, CCL2 and CCL7. Induction of CSF3 and CXCL5 has been reported to be involved in attraction of neutrophils and other acute inflammatory cells against *Chlamydia* infection [18, 21, 66-68]. IL-6 is an important mediator of fever and major component of the acute inflammatory response; however, the role IL-6 synthesized during chlamydial genital tract immunopathology in mice is somewhat lesser defined and variable [69, 70]. CCL2 (MCP-1) and CCL7 (MCP-3) were also up-regulated by *Cm* infection in OE-WT cells, and was shown to play a crucial role in recruiting neutrophils, macrophage/monocytes, and dendritic cells to the site of chlamydial infection by other researchers [18, 68]. Because all of these highly up regulated cytokines and chemokines were severely down-regulated in TLR3-deficient OE cells when compared to WT-OE cells during *Cm* infection, the microarray results provides more comprehensive evidence that TLR3 deficiency results in a significantly impaired inflammatory immune response to *Cm* infection in OE cells, and supports our findings that TLR3 alters the pathogenicity of *Chlamydia* infection *in vivo* [59].

In addition to the vast array of inflammatory mediators that were affected by *Cm* infection in OE cells, there were other classes of genes that were significantly up-regulated as part of the cellular response to infection. IPA data showed that *Cm* infection in OE-WT cells induced genes encoding the glycoprotein laminin subunit gamma-2(LAMC2), follistatin (FST), matrix metalloproteinase (MM10), epiregulin (EREG), autotaxin (ATX or ENPP2), fibroblast growth factor23 (FGF23), metalloredutase (STEAP4), MAdCAm, and ICAM1, which are known to function in remodeling of extracellular matrix (ECM) proteins [31]. The ECM provides structural and biochemical support of surrounding cells, and although the actual composition of ECM varies between multicellular structures, properties including cell adhesion, cell-to-cell communication, and cell differentiation are common functions of the ECM [71, 72]. The transcriptome data show that *Cm* infection in OE-WT cells substantially up regulates these ECM genes, and thereby can trigger or disrupt cellular processes that are essential for processes including cellular growth, wound healing, and fibrosis, but are highly suggestive that *Chlamydia* infection can also have impact on processes such as cell migration, gene expression, and cellular differentiation in macrophages and other hematopoietically-derived cells [73,74]. Our findings support the investigations of others who have implicated several members of this subset of ECM proteins in various disorders associated with genital tract *Chlamydia* infections such as oviduct fibrosis and scarring, uterus and oviduct distention, and tubal damage caused by ectopic pregnancy [75-80]. Because the transcriptome data show that TLR3-deficiency causes dysregulation in the gene expression levels of most of the ECM proteins identified by IPA, these data provides insight into a possible mechanism that expounds our recent report demonstrating that TLR3 deficient mice suffer more severe genital tract pathology than wild-type mice during *Cm* infection [59].

Other genetic factors that were significantly up regulated during *Cm* infection of OE-WT cells included a subset of host-cell metabolism related genes encoding LCN2, CMPK2, ARG2, SOD2, ERO1- α , CH25H, FABP4, and AK4. Lipocalin-2 (LCN2) is an iron-sequestering multifunctional protein. Iron is an essential nutrient for many intracellular pathogens including *Chlamydia*. LCN2 binds to bacterial siderophores and thereby limiting the availability of iron for bacteria, inhibiting their growth, and thus protecting host from intracellular pathogens [81-83]. Intracellular pathogens including *Chlamydia* induce a strong Th1 immune response, which exerts pro-inflammatory effects by stimulating production of free radicals such as reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) [84]. L-arginine is a crucial component for both inducible nitric oxide synthase (iNOS) and ARGase 1/2. It has been hypothesized that the enhanced expression of host SOD2 and ARG2 protects the host cells from the damaging ROS and RNOS [85-87], and both genes were found to be up-regulated in our analyses. FABP4 (fatty acid binding protein 4) is primarily expressed in adipocytes and macrophages, and has roles in modulating immune responses and in lipid metabolism [88]. Walenna NF. *et al* demonstrated that *C. pneumonia* exploits host FABP4 for lipid metabolism in order to obtain ATP and lipids from the host cell, in order to facilitate its robust replication in adipocytes [89]. Not surprisingly, our findings show that TLR3 deficiency either severely attenuates or down regulated the expression of all of these metabolic factors, and presents the hypothesis that dysregulation of these metabolic factors will have a negative impact on the ability of the OE cells to control chlamydial replication. We showed in our previous reports that *Cm* replication in OE-TLR3KO cells was significantly more robust, and that the chlamydial inclusions were larger and more aberrantly shaped when compared to infection in OE-WT cells [26]. Collectively, our data show that TLR3 signaling does indeed play a role controlling chlamydial replication within the cell, and the data generated in IPA supports the hypothesis that TLR3 mediates this function by regulating the transcription of many of these host cell metabolism related genes.

Other genes that were highly up-regulated in the *Cm*-infected OE-WT cells but were differentially regulated in the *Cm*-infected OE-TLR3KO cells included genes that encode proteins that are involved in other areas of innate immunity such as ARL4D, NEURL3, PTX3, SERPINE1 and SERPINB2. These proteins include GTPases, ligases, and protease inhibitors that play important roles in actin remodeling, tissue repair, and ECM degradation [90-98]. In addition to the genes mentioned above, we observed significant increased expression levels of the interferon-responsive genes *Gbp5* (a member of IFN-inducible subfamily of GTPases) and *Parp12*. GBP5 protects host against diverse pathogens [33, 99] and PARP12 localizes to the stress granules under stress condition, mediates cell survival/growth, and induces an anti-viral response by inhibiting protein translation at both viral and cellular protein levels [34-37]. We also saw up-regulated expression of the pro-apoptotic genes *Fas* and *Xaf1* (XIAP associated factor 1) in our analysis, and increased transcription

of genes encoding proteins involved in antigen processing including TAP1, LMP7, and LMP2.

We found 8 genes that were induced during *Cm* infection in OE-WT cells but were not reduced or inhibited in the *Cm*-infected OE-TLR3KO cells when compared to the *Cm*-infected WT-OE cells. In contrast to the large majority of genes that were either down regulated or inhibited in TLR3's absence, *Isg15*, *Mx1*, *Gm4951*, *Oas1b*, *Mpeg1*, *Zfp296* and *Klhl2* were all significantly up regulated in *Cm*-infected TLR3-deficient OE cells when compared to *Cm*-infected OE-WT cells. Because the absence of TLR3 results in the *increased* expression of these genes during *Chlamydia* infection, these results suggest a repressor role for TLR3 in the transcription of these particular genes in OE cells. The repressor function of TLR3 was also observed in the expression of *Aldh1a1*, *Ankrd35*, *Chchd10*, *Daam2*, *Fbln5*, *Fbxo31*, *Gcnt1*, *Lum*, *Ppm1e*, *Ptpmv* and *Txnip* in *Cm*-infected OE-WT cells. These genes were either significantly up regulated in the TLR3-deficient OE cells, or their expression levels no longer repressed as they were in OE-WT cells (see Table 2). Although the role of TLR3 as an activator of gene expression has been well described such as in the direct activation of IFN- β in response to dsRNA [100], or in the indirect activation of CCL5 via TLR3-induced IFN- β [101], a mechanism to describe TLR3 signaling as a repressor pathway remains unclear and requires further study.

IPA identified several inflammatory pathways such as acute phase response signaling, dendritic cell maturation, role of PRR in recognition of bacteria and viruses, IL-6 signaling, interferon signaling, TREM1 signaling, VDR/RXR-activation, p38 MAPK signaling, LPS-stimulated MAPK signaling, Th1 signaling, LPS/IL-1 mediated inhibition of RXR function, JAK/STAT signaling, HMGB1 signaling, PI3K/AKT signaling, Tec kinase signaling and TLR signaling pathway to be most crucial in *Chlamydia* pathogenesis. These pathways were predicted to be significantly activated in *Cm*-infected OE-WT cells, but were predicted to be either severely attenuated or inhibited in TLR3-deficient OE cells during *Cm* infection. In contrast, we also found some canonical pathways that were predicted to be inactivated in *Cm*-infected OE-WT cells such as LXR/RXR activation, PPAR signaling and PPAR α /RXR α activation pathway, but were activated in OE cells by *Cm* during TLR3-deficiency (LXR/RXR activation and PPAR signaling). Not surprisingly, there were several genes affected by *Cm* infection that are common amongst the various activated and inhibited pathways suggesting that many of these gene products are pleiotropic in their function; however, their impact in their respective pathways were differentially impacted based on the presence or absence of TLR3. These IPA data corroborates the hypothesis that TLR3 functions as a regulator in cellular responses to *Chlamydia* infection in OE cells, and can function as either an activator or repressor of numerous pathways associated with the inflammatory immune response.

Finally, IPA can be used to identify a cascade of upstream transcriptional regulators from transcriptome data identifying the genes that are activated or repressed during *Cm* infection of OE cells. In our analyses, we identified a multitude of

upstream regulators that were either activated or repressed during *Cm* infection of OE cells including immunological (cytokines and chemokines), chemical, kinase, transcription factor, transmembrane receptor, translation regulator, and transporter regulators. Among the most highly activated upstream regulators, cytokines were at the top of the list, suggesting *Chlamydia* was able to modulate the cellular immune responses by altering the expression patterns of some critical immune system molecules including cytokines. Additionally, the main anti-inflammatory-cytokine, IL-10, was shown to be down regulated in *Cm*-infected OE-WT cells whereas; it was significantly activated in the *Cm*-infected TLR3-deficient OE cells which supports our recently published *in vivo* results [59]. IL-10 has been identified as a key player in the establishment and perpetuation of viral persistence, is known to be negatively regulated by IFN- β production, and promotes a suppressive environment that diminishes the antiviral response [102-104]. Because the *Cm*-induced synthesis of IFN- β is severely diminished in TLR3-deficient OE cells, IPA showing that IL-10 synthesis was significantly up regulated during *Cm* infection of OE-TLR3KO cells fits the paradigm that its synthesis is regulated by TLR3-dependent IFN- β .

IPA results show that other upstream-activated regulators belonging to classes of kinases, transcription factors, transmembrane receptors, translation regulators, and transporters groups were also impacted by *Cm* infection in OE cells. As was demonstrated with the immunological regulators, these additional upstream regulators were also negatively regulated in the TLR3-deficient OE cells during *Cm* infection when compared with *Cm*-infected OE-WT cells. Further, IPA results show that the *Cm* infection induced regulators either activated or inhibited cellular pathways in OE-WT cells that were more likely to restrict chlamydial pathogenesis. The IPA data was suggestive that the upstream regulators affected during *Cm* infection of OE-WT cells were more likely to attenuate chlamydial replication and promote growth of epithelial tissue; whereas, *Cm* infection in TLR3-deficient OE cells showed activation or inhibition in upstream regulators controlling 3-4 times the number of pathways involved in organ inflammation, fibrosis, and necrosis of epithelial tissue than that in the *Cm*-infected OE-WT cells. Collectively, the transcriptome and IPA results further validates our previous findings that TLR3 could elicit and regulate host protective cellular responses that limit bacterial proliferation and genital tract pathologies caused by *Chlamydia* infection [26, 59], and implicate TLR3 as a potential therapeutic target for drug and/ or vaccine development.

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Competing interests

The authors have declared that no competing interests exist.

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