

Cell Intrinsic Factors Modulate the Effects of IFN γ on the Development of *Chlamydia trachomatis*

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Abstract

Chlamydia trachomatis is an obligate intracellular bacterial pathogen that cannot synthesize several amino acids, including tryptophan. Rather, *C. trachomatis* acquires these essential metabolites from its human host cell. Chlamydial dependence on host-provided tryptophan underlies a major host defense mechanism against the bacterium; namely, the induction of the host tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO1) by interferon gamma (IFN γ), which leads to eradication of *C. trachomatis* by tryptophan starvation. For this reason, IFN γ is proposed to be the major host protective cytokine against genital *C. trachomatis* infections. The protective effect of IFN γ against *C. trachomatis* can be recapitulated *in vitro* using epithelial cell-lines such as the cervical carcinoma derived cell-line Hela, the Hela subclone HEP-2, and the cervical carcinoma derived cell-line ME180. Addition of IFN γ to these cells infected with *C. trachomatis* results in a strong bactericidal or bacteriostatic effect dependent on the concentration of IFN γ administered. Unlike Hela, HEP-2, and ME180, there are other human epithelial, or epithelial-like cell-lines where administration of IFN γ does not affect chlamydial replication, although they express the IFN γ receptor (IFNGR). In this report, we have characterized the mechanisms that underlie this dichotomy using the cell-lines C33A and 293. Akin to Hela, C33A is derived from a human cervical carcinoma, while 293 cells were produced by transfection of adenovirus type 5 DNA into embryonic kidney cells. We demonstrate that although IFNGR is expressed at high levels in C33A cells, its ligation by IFN γ does not result in STAT1 phosphorylation, an essential step for activation of the IDO1 promoter. Our results indicate that although the IFN γ -dependent signaling cascade is intact in 293 cells; the IDO1 promoter is not activated in these cells because it is epigenetically silenced, most likely by DNA methylation. Because polymorphisms in IFN γ , IFNGR, and the IDO1 promoter are known to affect other human infections or diseased states, our results indicate that the effect of allelic differences in these genes and the pathways they activate should be evaluated for their effect on *C. trachomatis* pathology.

Introduction

Chlamydia trachomatis is an obligate intracellular bacterium [1] associated with ocular and genital infections of columnar epithelial cells in humans. There are 15 serovars of *C. trachomatis* [2], which serovars A-C are associated with ocular infections, and as such are a major cause of preventable blindness [3,4]. Genital serovars [D-K] are the most prevalent bacterial sexually transmitted infection (STI) in USA and worldwide [5]. Clearance of infection, without treatment, might take several months to years [6,7]. These undetected and untreated infections can result chronic inflammatory responses, whose consequences include pelvic inflammatory disease, salpingitis, ectopic pregnancy, cervicitis, urethritis, infertility, and chronic pelvic pain [8].

C. trachomatis has a biphasic developmental cycle [9], with the two major bacterial states being elementary bodies (EB) and reticulate bodies (RB) [9]. EBs are infectious particles but metabolically inactive. Upon infection, EBs are incorporated into a host-derived lipid vesicle called an inclusion, in which they differentiate into RBs, which are metabolically active but not infectious [9-11]. RBs replicate by binary fission and finally re-differentiate into EBs that are released and initiate secondary infections of neighboring uninfected cells [9]. Due to its obligate intracellular life-cycle, *C. trachomatis* has lost the capacity to synthesize many metabolites including the amino acid tryptophan

[12,13]. Because human cells also cannot synthesize tryptophan, removal of tryptophan from media blocks chlamydial development and as such is considered bactericidal. The inability of *C. trachomatis* and its host epithelial cell to synthesize tryptophan has rendered the bacterium highly susceptible to the host cytokine IFN γ , which induces expression of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase 1 (IDO1) [14]. Upon binding its receptor (IFNGR), which is a heterodimer of two proteins, IFNGR1/IFNGR2, IFN γ activates the Jak1/Jak2 kinase to phosphorylate the transcription activator STAT1 [15]. Upon phosphorylation, STAT1 dimerizes [15], is translocated into the nucleus [15], and binds cognate GAS sites in the IDO1 promoter to activate transcription of this gene [15]. The IDO1 enzyme irreversibly catabolizes tryptophan to kynurenine [16], thereby starving *C. trachomatis* of this essential amino acid. For this reason, IFN γ is considered to be the major protective host cytokine against *C. trachomatis* infections [10,13,17].

Prior studies indicate that the effect of IFN γ on *C. trachomatis* differs between human cell lines, including Hela, A549, ME180, HEP-2, A2EN, and McCoy [10,17-24], suggesting that cell-intrinsic differences can influence the IFN γ -driven host protective response against *C. trachomatis*. Understanding the nature of these differences is critical to design vaccines against *C. trachomatis* that are minimally affected by genetic differences in humans, or to identify therapeutics

that permits such vaccines to be uniformly effective. In this study we have used two cell-lines, C33A and 293, in which *C. trachomatis* development is not affected by administration of IFN γ to understand some of these cell-intrinsic differences.

Materials and Methods

Cell-lines and culture conditions

The cell-lines HeLa [25], 293 [26], C33A [27,28] and A2EN [18] were used for this study. HeLa and C33A cell-lines are derived from cervical carcinomas. 293 and A2EN cell-lines were constructed *in vitro*. 293 cells were made by transfection of sheared Adenovirus Type 5 DNA into human embryonic kidney cells, followed by a screen for clones that were immortalized. A2EN cells were constructed by transduction of primary human endocervical epithelial cells with a retroviral vector expressing the E6 and E7 oncogenes of human papillomavirus type 16. All cell-lines were routinely passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells infected with *C. trachomatis* were grown in RPMI 1640 supplemented with 10% dialyzed fetal bovine serum.

Chlamydia trachomatis infections

Cells plated 24 hours prior to infection in antibiotic-free DMEM +10% FBS were infected with *C. trachomatis* serovar D (D/UW-3/CX) at m.o.i. of 5 in sucrose-phosphate-glutamic-acid (SPG) buffer. HeLa, A2EN and C33A cells were infected as described previously [17]. Infection in 293 cells was done by gently rocking at 4°C for one hour. SPG was replaced with complete media and incubated for 42 hours. Cells infected with *C. trachomatis* were grown in RPMI 1640 media supplemented with 10% dialyzed fetal bovine serum. The complete medium contains 4 mg/L tryptophan.

Immunofluorescence staining for chlamydial inclusions

Cells were stained with fluorescein isothiocyanate (FITC) conjugated anti-chlamydial lipopolysaccharide (LPS) antibody (Merifluor) and counterstained using Hoechst 33342 (1: 3000 dilution) for two minutes. Cells were visualized using a Zeiss Axiovision AX10 microscope with a 63X oil-immersion objective. Z-stacks containing 20 optical sections were deconvolved using the Landweber positively constrained deconvolution algorithm as described previously [29]. Z-projections of maximum intensity were shown. Inclusion forming units (IFU) were quantified by infecting monolayer of HeLa cells as described previously [17].

Immunoblotting for IDO1, STAT1, p-STAT1, and β -actin

Immunoblots were conducted as described previously [17,30]. The following primary antibodies were used: 1) IDO1–Millipore catalog 05-840; 2) STAT1–Santa Cruz catalog sc-464; 3) p-STAT1–Santa Cruz catalog sc-7988; detects STAT1 phosphorylated at tyrosine 701; 4) β -actin–Sigma-Aldrich catalog A1978.

Flow cytometry for IFNGR1

Flow cytometry was conducted as described previously [30]. Cells were grown for 24 hours in presence of IFN γ (600 U/ml). Cells were lifted from tissue culture dishes using Accutase (MP Biomedicals catalog ICN 1000449), pelleted, and resuspended in 100 μ l of fluorescence-activated cell sorting (FACS) buffer (1% BSA in Ca²⁺/

Mg²⁺-free PBS). Anti-IFNGR1 (BD Pharmingen catalog 558937) antibody (5 μ l) was added and incubated on ice for 30 min. Isotype control and untreated cells were used as control.

IFN γ exposure

IFN γ was obtained from Peprotech (catalog 300-02). IFN γ was added at the indicated concentrations to control cells and infected cells, typically 600 U/ml.

IDO1 promoter transactivation assays

A plasmid, p616, was constructed in which the firefly luciferase gene is under the control of the IDO1 promoter amplified from 293 genomic DNA. The sequence of the IDO1 promoter from 293 cells was identical to promoter sequence in reference human genome (GRCh37). p616 also contains genetic elements from Epstein-Barr virus, namely the cis-acting sequence *oriP*, and the trans-acting protein that binds *oriP*, EBNA1 [31,32]. These elements permit the plasmid to be replicated and maintained stably in proliferating cells [31], thereby allowing a stable derivative of 293 cells, termed 293/p616, to be created containing this reporter plasmid. The EBV elements used do not affect activity of the IDO1 promoter. Transactivation assays were initiated by adding IFN γ to 293/p616 cells; cells extracts made 24-48 hours post-addition of IFN γ were used to assess luciferase expression by luminescence assays conducted as described previously [33].

Statistical analyses

All experiments were conducted at least three times. The Wilcoxon rank sum test was used to perform statistical comparisons using the MSTAT software package [34].

Results

The effect of IFN γ treatment on the development of *Chlamydia trachomatis* is cell-line dependent.

Immediately after infection with *C. trachomatis*, HeLa, A2EN, C33A and 293 cells, grown in complete RPMI media (RPMI 1640 + 10% dialyzed fetal bovine serum), were exposed to vehicle alone (PBS), or 300 U/ml of IFN γ . After an additional 42 hours, cells were fixed and stained using a FITC-conjugated anti-chlamydial LPS antibody or harvested to quantify infectious units released (IFU). The results from this experiment are shown in Figure 1. While the number and size of chlamydial inclusions were dramatically reduced in HeLa and A2EN cells grown in the presence of IFN γ , no such reduction was observed C33A and 293 cells (Figure 1A). Similarly, IFU recovery at 42 hours post-infection (h.p.i) was reduced significantly in HeLa and A2EN cells when grown in presence of 300 U/ml of IFN γ , but not in C33A and 293 cells (Figure 1B).

IDO1 is expressed in HeLa and A2EN cells treated with IFN γ

The observations made above can be explained if the expression of IDO1 differs between cell-lines after addition of IFN γ . Therefore, we evaluated the expression of IDO1 by immunoblot in these four cell-lines after addition of IFN γ . Cells were grown in presence or absence of IFN γ for 24 hours, after which IDO1 protein levels were evaluated. The outcome of this experiment is shown in Figure 2. IDO1 was expressed in HeLa and A2EN cells but not C33A and 293 cells (Figure 2). These results explain why reduced chlamydial development observed in HeLa and A2EN cells upon IFN γ exposure but not in C33A and 293 cells.

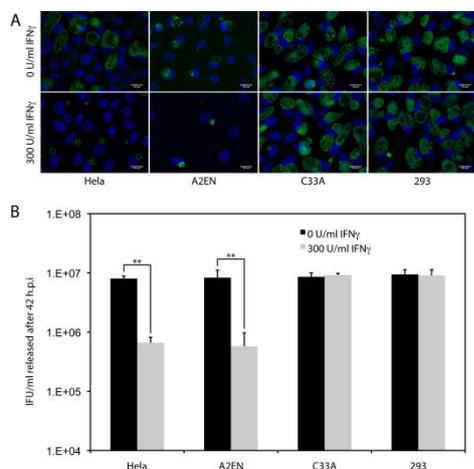


Figure 1: The effect of IFN γ treatment on the development of *Chlamydia trachomatis* is cell-line dependent. HeLa, A2EN, C33A and 293 cells were infected with *C. trachomatis* at an m.o.i. of 5 as described in the methods. After infection, cells were grown in RPMI media in the absence or presence (300 U/ml) of IFN γ . At 42 h.p.i. cells were fixed, permeabilized and stained using a FITC-conjugated antibody against chlamydial, and counter-stained with Hoechst dye. Infected cells were also harvested to measure IFU released by infection of HeLa cells. A) Primary chlamydial inclusions (green) formed in HeLa, A2EN, C33A and 293 cells 42 h.p.i. Hoechst dye (blue) shows cell nuclei and chlamydial DNA. Scale bar indicates 50 μ m. B) IFU/ml released after 42 h.p.i. in HeLa, A2EN, C33A and 293 cells as evaluated by infection of HeLa cells. The mean and standard deviation from three independent experiments were represented as bars (** indicates $p < 0.01$ by Wilcoxon Rank Sum Test).

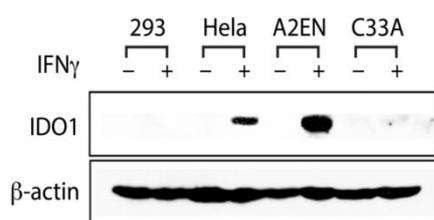


Figure 2: IDO1 is induced in HeLa and A2EN cells but not in C33A and 293 cells treated with IFN γ . HeLa, A2EN, C33A and 293 cells were grown in the absence or presence of IFN γ (600 U/ml) for 24 hours, after which, cells were harvested and lysates were used to evaluate expression of IDO1 by immunoblot using an antibody against IDO1. β -actin expression was evaluated as a loading control.

IFNGR1 is expressed in A2EN, C33A, and 293 cells

Because IDO1 is not expressed in C33A and 293 cells after IFN γ treatment, we tested whether IFNGR is expressed in the surface of these cells. For this, A2EN, C33A, and 293 cells were grown under control conditions or in the presence of 600 U/ml of IFN γ for 24 hours.

At this time, cells were fixed, but not permeabilized and stained using an antibody against IFNGR1. The levels of surface IFNGR1 expression was quantified by flow cytometry. It should be noted that although we only stained for IFNGR1, surface expression of IFNGR1 requires that it form a heterodimer with the IFNGR2 subunit [15]. The results obtained from this experiment are shown in Figure 3. As anticipated, surface expression of IFNGR1 was observed in IFN γ -responsive A2EN cells. We also found surface expression of IFNGR1 in C33A and 293 cells. Therefore, the failure of IFN γ to induce IDO1 expression in these cells does not result from a lack of surface IFNGR. Rather, this failure must result from downstream steps in the signaling pathway that leads to IDO1 expression after administration of IFN γ .

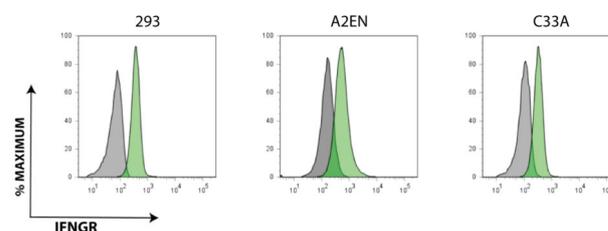


Figure 3: Surface IFNGR1 expression is observed in HeLa, A2EN, C33A and 293 cells. HeLa, A2EN, C33A and 293 cells were grown in the absence or presence of IFN γ (600 U/ml) After 24 hours, cells were lifted, counted, fixed but not permeabilized, and stained with antibody against IFNGR1. Surface expression of IFNGR1 was evaluated by flow cytometry (profile in green). Flow cytometry profiles obtained with the isotype control antibody are shown in grey.

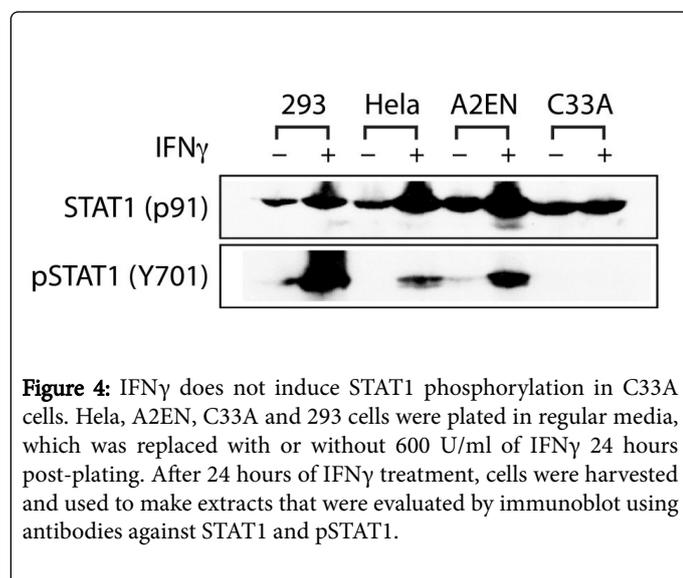
STAT1 is phosphorylated in 293 but not in C33A cells after addition of IFN γ

Activation of STAT1 by phosphorylation after administration of IFN γ is essential for transactivation of the IDO1 promoter [15]. Therefore, we evaluated the capacity of IFN γ to induce STAT1 phosphorylation in all four cell-lines, anticipating that IFN γ addition will induce STAT1 phosphorylation in HeLa and A2EN cells. Cells were grown in the presence or absence of IFN γ (600 U/ml) for 24 hours, after which STAT1 and phospho-STAT1 (pSTAT1) expression was evaluated by immunoblot as described in the Material and Methods section. The outcome of this experiment is shown in Figure 4. IFN γ treatment increased the expression of STAT1 in HeLa, A2EN, and 293 cells but not in C33A cells. As expected, STAT1 was phosphorylated in HeLa and A2EN when cells exposed to IFN γ . STAT1 was not phosphorylated in C33A cells exposed IFN γ , providing an explanation for the inability of IFN γ to induce IDO1 expression in C33A cells. Although IFN γ does not induce IDO1 expression in 293 cells, we were surprised to find that IFN γ induced efficient STAT1 phosphorylation in these cells, indicating that although the IFN γ induced signaling cascade is intact in 293 cells, activated STAT1 cannot or does not induce IDO1 expression.

The WT IDO1 promoter is functional in 293 cells

We considered it possible that that IFN γ administration does not induce IDO1 expression in 293 cells as a consequence of mutations in

the IDO1 promoter within 293 cells. To evaluate this, the IDO1 promoter was cloned and sequenced from 293 cells; this analysis revealed it to perfectly match the IDO1 promoter present in the reference human genome (GRCh37). Because the promoter sequence was unchanged, we wondered whether there were other unknown factors downstream of STAT1 activation that prevented IFN γ from activating IDO1 expression in 293 cells. To test this, we constructed a stable derivative of 293 cells, termed 293/p616, containing an episomal IDO1 promoter-luciferase reporter (p616), which is described in the materials and methods section. Luciferase expression in 293/p616 cells was evaluated 24 hours after addition of IFN γ (600 U/ml); experimental results indicate that IFN γ can efficiently induce the IDO1 promoter in these cells. Importantly, the IDO1 promoter used in p616 was cloned from 293 cells. Therefore, although the entire IFN γ -driven signaling cascade required for IDO1 expression is intact and functional in 293 cells, the native 293 IDO1 promoter in its chromosomal context is not activated by this signaling cascade (Figure 5).



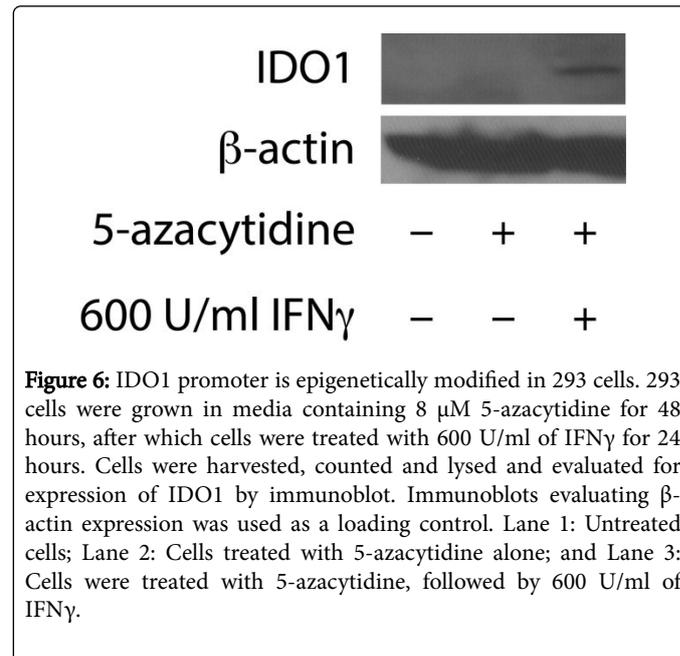
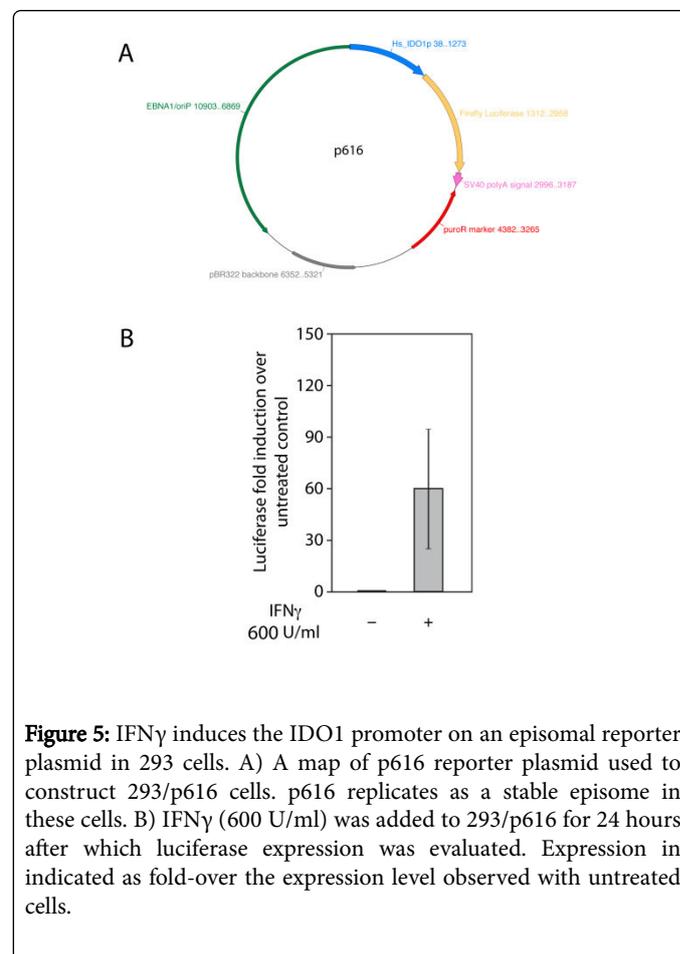
The IDO1 promoter is silenced in 293 cells

Frequently promoters in their chromosomal contexts are epigenetically silenced by methylation at CpG islands [35,36]. Therefore, we tested if CpG methylation underscored the inability of IFN γ to induce IDO1 expression in 293 cells. For this, 293 cells were treated with 5-azacytidine, a well-characterized inhibitor of DNA methyltransferase, for 48 hours [37]. Treated cells were then grown in the absence or presence of IFN γ (600 U/ml) for an additional 24 hours, at which time IDO1 expression was evaluated by immunoblot (Figure 6). Treatment with 5-azacytidine for 48 hours partially rescued induction of IDO1 in 293 cells after addition of IFN γ . Therefore, epigenetic silencing by CpG methylation is one mechanism that underlies the inability of IFN γ to induce IDO1 expression in 293 cells, despite the IFN γ signaling pathway being intact in these cells.

Discussion

We wished to elaborate on cell-intrinsic differences that affect the capacity of IFN γ to induce IDO1 expression because the resultant tryptophan depletion is proposed to be a major determinant of natural immunity against *C. trachomatis* infections [10,11,17]. Our results indicate that cell-intrinsic differences in the capacity of IFN γ to induce

STAT1 activation, and epigenetic silencing of the IDO1 promoter, can both operate as mechanisms that block induction of IDO1 expression after exposure to IFN γ .



Understanding these mechanisms and others is of relevance because IFN γ -dependent pathways protect against multiple infectious agents. We note that IFN γ polymorphisms have been extensively reported in the literature [38-40]. Some of these polymorphisms negatively affect the outcome of disease progression, with a prominent example being the outcome of tuberculosis disease [41]. There is substantial amount of research documenting the effects of IFN γ receptor polymorphisms in the outcome of disease progression. IFN γ receptor polymorphisms have been associated with disease outcome of many intracellular pathogens like *Listeria monocytogenes*, *Salmonella typhi*, *Plasmodium* spp and *Shigella* [42-45].

Allele-specific alterations in the IFN γ -dependent induction of IDO1 are predicted to have a significant effect on the outcome of *C. trachomatis* infection, because *C. trachomatis* is a tryptophan auxotroph, and its human host also cannot synthesize this amino acid. Further, tryptophan is rare amino acid in the human proteome. Tryptophan and its metabolites are essential for the biosynthesis of neurotransmitters such as serotonin [46-49]. In this context, we note that IDO1 polymorphisms are associated with other human conditions including major depressive disorder [47,50], and bipolar disorder [50,51]. We note that IDO1 polymorphisms are also associated with Crohn's disease, a condition that involves interactions between the gut microbiome and the human host [52,53]. The effects of such polymorphisms have not been considered in the context of natural clearance of *C. trachomatis* infections. There are multiple studies indicating that around 20% of *C. trachomatis* infected women can naturally clear this infection within a two-week period without antibiotic therapy [54,55]. While this result is encouraging and indicates that anti-chlamydial vaccines can be made, it is important to note that 80% of infected women do not naturally clear infection. Many factors could underlie this failure; our observations indicate that these factors include allele-specific defects in the IFN γ signaling pathway, or the propensity for the IDO1 promoter to be epigenetically silenced.

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