Herpes simplex virus co-infection-induced *Chlamydia trachomatis* persistence is not mediated by any known persistence inducer or anti-chlamydial pathway

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Several inducers of chlamydial persistence have been described, including interferon- γ (IFN- γ), IFN- α , IFN- β , and tumour necrosis factor- α (TNF- α) exposure, and iron, amino acid or glucose deprivation. A tissue-culture model of *Chlamydia trachomatis*/herpes simplex virus type-2 (HSV-2) co-infection indicates that viral co-infection stimulates the formation of persistent chlamydiae. This study was designed to ascertain whether co-infection-induced persistence is mediated by a previously characterized mechanism. Luminex assays indicate that IFN- γ , IFN- α , and TNF- α are not released from co-infected cells. Semiquantitative RT-PCR studies demonstrate that IFN- β , IFN- γ , indoleamine 2,3-dioxygenase, lymphotoxin- α and inducible nitric oxide synthase are not expressed during co-infection. These data indicate that viral-induced persistence is not stimulated by any persistence-associated cytokine. Supplementation of co-infected cells with excess amino acids, iron-saturated holotransferrin, glucose or a combination of amino acids and iron does not restore chlamydial infectivity, demonstrating that HSV-2-induced persistence is not mediated by depletion of these nutrients. Finally, inclusions within co-infected cells continue to enlarge and incorporate C₆-NBD-ceramide, indicating that HSV-2 co-infection does not inhibit vesicular transport to the developing inclusion. Collectively these data demonstrate that co-infection-induced persistence is not mediated by any currently characterized persistence inducer or anti-chlamydial pathway. Previous studies indicate that HSV-2 attachment and/or entry into the host cell is sufficient for stimulating chlamydial persistence, suggesting that viral attachment and/or entry may trigger a novel host pathway which restricts chlamydial development.

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INTRODUCTION

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are enveloped DNA viruses of the viral family *Herpesviridae*. HSV-2 is the primary cause of genital herpes infection, causing 200 000–500 000 new infections annually. HSV-2 infection usually occurs on the mucous membranes and

skin surrounding the genitals, causing a characteristic lesion. After primary infection, HSV-2 can establish a life-long latent infection in the neurons of the sacral ganglia, which is reactivated, on average, five times each year. Although most genital HSV infections are clinically mild, HSV-2 can cause serious diseases, including keratitis and meningitis (Roizman & Knipe, 2001).

Herpes simplex viruses begin their infection of epithelial cells by binding to the host-cell receptor, heparan sulfate. After this initial binding, viral envelope glycoproteins interact with one of four known co-receptors [herpes viral entry mediator (HVEM), nectin-1 and -2, and 3-O-sulfated heparan sulfate], facilitating viral entry (Spear, 2004). Following fusion of the viral envelope with the host cell, viral tegument proteins are released into the cytoplasm and the capsid is transported to the nucleus, where viral

Abbreviations: EB, elementary body; GM-CSF, granulocyte-macrophage colony-stimulating factor; hTF, holotransferrin (iron-saturated); HSV-1, 2, herpes simplex virus type 1, 2; HVEM, herpes viral entry mediator; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; LT- α , lymphotoxin- α ; MOMP, major outer-membrane protein; NO, nitric oxide; RB, reticulate body; TNF- α , tumour necrosis factor α .

A supplementary table of primers and three supplementary figures are available with the online version of this paper.

genome replication occurs. The viral genome is transcribed and viral proteins are synthesized using host-cell machinery. New virions are then assembled and exit the host cell (Roizman & Knipe, 2001).

Chlamydia trachomatis is a Gram-negative, obligate intracellular bacterium which is responsible for 4 million sexually transmitted infections each year in the USA (Butler, 1997; Weinstock *et al.*, 2004). Genital chlamydial infections are often chronic and asymptomatic, leading to severe complications including pelvic inflammatory disease, ectopic pregnancy and infertility (Darville, 2000). *In vivo* and in culture, chlamydiae carry out a characteristic biphasic developmental cycle. Extracellularly, chlamydiae exist as infectious elementary bodies (EBs). EBs attach to and enter a host cell, where they differentiate within an inclusion into non-infectious reticulate bodies (RBs). The infectious cycle spans 30–72 h (depending upon the chlamydial species/serovar). Near the end of the cycle, RBs develop back into EBs and are released from the infected host cell. When developing chlamydiae are exposed to certain adverse environmental factors [such as interferon- γ (IFN- γ) or penicillin G] or are deprived of nutrients (such as amino acids, iron or glucose), they deviate from the normal developmental cycle into a state termed persistence (Hogan *et al.*, 2004). Once in the persistent state, RBs continue to grow but do not divide, resulting in the formation of enlarged, abnormally shaped RBs. Although they remain viable and continue genomic DNA replication and rRNA synthesis, persistent chlamydiae are no longer infectious. Interestingly, persistent chlamydiae can re-enter the normal developmental cycle and recover infectivity if the environmental stressor is removed. For example, nutrient-deprived, persistent chlamydiae resume development into infectious EBs if the appropriate nutrients are replenished (Harper *et al.*, 2000; Matsushima *et al.*, 1999; Raulston, 1997).

Several host-cell pathways have evolved to limit the spread of chlamydial infections. In human cells, IFN- γ exposure increases host-cell indoleamine 2,3-dioxygenase (IDO) expression, leading to depletion of host-cellular tryptophan. Without this essential amino acid, developing chlamydiae enter into persistence and cease productive replication (Beatty *et al.*, 1994). In murine cells, IFN- γ exposure increases synthesis/activity of the p47 GTPase, Iigp1. Iigp1 inhibits chlamydial development by restricting vesicular trafficking to developing inclusions (Nelson *et al.*, 2005). IFN- α , IFN- β and tumour necrosis factor- α (TNF- α) interfere with chlamydial development by induction of IDO, induction of inducible nitric oxide synthase (iNOS) and/or restriction of intracellular iron stores (Hogan *et al.*, 2004; Matsushima *et al.*, 1999). When iNOS is upregulated, the host cell produces nitric oxide (NO), which kills developing chlamydiae. Although these pathways are intended to eliminate the bacterial infection, chlamydiae are often able to survive the onslaught of the host cell's arsenal by entering into a persistent state until conditions become favourable for their continued development.

Several studies have shown that *C. trachomatis* and HSV-2 co-infections occur *in vivo* (Paavonen *et al.*, 1985; Tait *et al.*, 1985). *In vitro* models of HSV-2/*C. trachomatis* co-infections have indicated that HSV-2 co-infection alters chlamydial development; however, previous studies did not examine the co-infection process in detail (Chiarini *et al.*, 1996; Pontefract *et al.*, 1989; Superti *et al.*, 2001). Data from a tissue culture model of *C. trachomatis* and HSV-2 co-infection established in our laboratory indicate that, during *C. trachomatis* serovar E and HSV-2 co-infection, HSV attachment and/or entry transmits a signal which interrupts the normal chlamydial developmental cycle and induces persistence (Deka *et al.*, 2007). These and other data have led us to hypothesize that HSV attachment to and/or entry activates a novel anti-chlamydial defence pathway in mucosal epithelial cells.

METHODS

Chlamydia, HSV-2 and host cells. A human urogenital isolate of *C. trachomatis* E/UW-5/CX was originally obtained from S. P. Wang and C. C. Kuo (University of Washington, Seattle, WA, USA). The same standardized inoculum of *C. trachomatis* serovar E, propagated in McCoy cells, was used for all experiments (Wyrick *et al.*, 1996). HSV-2 strain 333 and HSV-1 strain KOS stocks were obtained from Mary K. Howett (Drexel University) and Udayasankar Kumaraguru (James H. Quillen College of Medicine), respectively. Viral stocks were prepared in monolayers of Vero cells (African green monkey kidney cells ATCC CCL-81) as described by Duff & Rapp (1971).

Co-infection experimental design and nutrient supplementation. HeLa cells, a human cervical adenocarcinoma epithelial cell line (ATCC CCL2), or HEC-1B cells, a human endometrial epithelial cell line (ATCC HTB-113), were used for all infection experiments. In each experiment, the appropriate host cells were divided into four groups for mock infection, chlamydial infection, HSV infection and *C. trachomatis*/HSV double infection. Host cells were incubated with a dilution of crude EB stock calculated to infect >80% of the cells. After 1 h of adsorption, monolayers were refed with Minimal Essential Medium (MEM; Gibco) and incubated at 35 °C for either 4 or 24 h. Cultures were then infected with either HSV-2 or HSV-1 at a m.o.i. of 10 p.f.u. per cell. Mock-infected HeLa or HEC-1B cells were treated similarly except they were exposed to either 2SPG (0.2 M sucrose, 6 mM NaH₂PO₄, 15 mM Na₂HPO₄, 5 mM L-glutamine, pH 7.2; mock *C. trachomatis* infection) or growth medium (mock viral infection). In most experiments, monolayers were refed with MEM following HSV adsorption. For nutrient supplementation studies, cultures were refed with either MEM or MEM supplemented with excess essential and non-essential amino acids (Gibco), iron-saturated holotransferrin (hTF; Sigma), glucose, or a combination of amino acids and hTF as described in the legend to Supplementary Fig. S3, available with the online version of this paper.

Luminex assay. Aliquots of culture supernatants were examined for IFN- γ , IFN- α , TNF- α , granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) using the BioSource Multiplex Bead Immunoassay (BioSource International) according to the manufacturer's instructions (Deka *et al.*, 2007).

RNA and DNA isolation. Total RNA and DNA were isolated simultaneously from experimental samples using the RNeasy Mini (Qiagen) and QIAmp DNA Blood Mini (Qiagen) kits as described previously (Deka *et al.*, 2006). Total RNA and DNA preparations were

quantified by measuring A_{260} and A_{280} ; all samples had A_{260}/A_{280} ratios >1.9 .

Reverse transcription, PCR and RT-PCR. Reverse transcription of total RNA, PCR and RT-PCR were performed using identical conditions to those previously described (Deka *et al.*, 2006). PCR was performed using purified total cellular DNA or cDNA as a template. Control and experimental template DNAs were diluted from 1/10 to 1/1000 (in double-distilled H_2O) and synthetic control DNA targets diluted from 10 to 0.1 pg ml^{-1} , ensuring that each reaction was quantified in the linear amplification range. Published primer sets included chlamydial 16S rRNA, human glyceraldehyde-3-phosphate dehydrogenase and IDO (Deka *et al.*, 2006; Nelson *et al.*, 2005). We designed specific primer sets and, in some cases, synthetic DNA targets for chlamydial *trpA*, human 18S rRNA, *ifn- β* , *inf- γ* , *lymphotoxin- α* and *inos* (see Supplementary Table S1 for sequences). All synthetic DNA targets and primer sets were designed using Ensembl and Vector NTI Advance V10 (Invitrogen). After PCR, all reactions were electrophoresed and quantified as described previously (Deka *et al.*, 2006).

Griess reaction. Aliquots of select culture supernatants (50 μl) were examined for nitrite using the Griess Reagent System (Promega) according to the manufacturer's instructions.

Fluorescence microscopy and image analysis. Fluorescence analyses were performed as described previously (Deka *et al.*, 2006) except that FITC-conjugated monoclonal antibodies generated against *C. trachomatis* major outer-membrane protein (Pathfinder *Chlamydia trachomatis* monoclonal MOMP antibody 30702, Bio-Rad) were used to stain chlamydial inclusions. Images were captured using a Zeiss Axiovert S100 microscope and Axiocam camera. Measurement of inclusion sizes was performed as described by Deka *et al.* (2006) except that selected photomicrographs were converted to greyscale using Adobe Photoshop V5.0. The relative area of each inclusion was determined using Dymension II Imaging Software V2.06 (SynGene).

Cer₆-NBD-ceramide labelling. HeLa cells were plated in 24-well plates (1.5×10^5 cells per well), and mock, singly- or co-infected as described above. At 16.5 h post-HSV-2 infection, cells were stained with 200 $\mu g\ ml^{-1}$ BSA pre-conjugated C₆-NBD-ceramide stain (Molecular Probes) for 30 min at 4 °C. The stain was then back-extracted for 3 h at 37 °C until 20 h post-HSV-2 infection (Alzhanov *et al.*, 2007; Hackstadt *et al.*, 1995). Cells were mounted with GelMount (Fisher) and examined by fluorescence microscopy using FITC filters. Images were captured as described above with a 4 s exposure and 320 $\times$ magnification.

Chlamydial titrations by subpassage. Chlamydial titrations were performed as previously described (Deka *et al.*, 2006) using Pathfinder anti-chlamydial stain (Bio-Rad) to stain chlamydial inclusions formed from subpassaged EBs. The number of inclusion-forming units (i.f.u.) in the undiluted inoculum was then derived from triplicate counts and expressed as i.f.u. ml^{-1} .

Spectro Ferritin ELISA. Intracellular ferritin in cell lysates collected from mock-, chlamydiae-singly-infected with or without 50 μM Desferal, and *C. trachomatis*/HSV-2 co-infected cells was measured using the Spectro Ferritin ELISA kit (Ramco) as described in the legend to Supplementary Fig. S3. Addition of the iron-chelating agent Desferal (Sigma) to chlamydiae-infected cultures was used as a positive control for iron depletion (Raulston, 1997).

Statistical analyses. Statistical analyses were performed using Microsoft Excel. Comparison of means was done by using a 2-sample *t*-test for independent samples. *P* values ≤ 0.05 were considered

significant. All plotted values are means $\pm$ SEM of either eight or nine biological replicates divided between three separate experiments.

RESULTS AND DISCUSSION

C. trachomatis/HSV co-infected cells do not produce cytokines known to alter chlamydial development

Exposure to cytokines, in particular IFN- γ , is one of the most extensively studied mechanisms of inducing chlamydial persistence. However, other cytokines can also negatively affect *C. trachomatis* development. TNF- α , IL-1 α and IL-1 β can synergize with IFN- γ , lowering the concentration at which IFN- γ exposure affects developing chlamydiae (Carlin & Weller, 1995; Shemer-Avni *et al.*, 1988; Summersgill *et al.*, 1995). Lymphotoxin- α (LT- α), TNF- α and IFN- α/β exposure can also upregulate iNOS expression and cellular NO production, inhibiting chlamydial development (Devitt *et al.*, 1996; Matsushima *et al.*, 1999). Cycloheximide exposure experiments (Deka *et al.*, 2007) suggest that HSV-induced persistence occurs in the absence of *de novo* host protein synthesis. However, the observation that cycloheximide exposure did not completely abrogate host protein synthesis (Deka *et al.*, 2007) made it important to confirm that 'anti-chlamydial' cytokines were not released from co-infected cells. HSV-infected human corneal epithelial cells produce IFN- β , as well as the cytokines TNF- α , interleukin-8 (IL-8) and IL-6 (Li *et al.*, 2006). However, co-infected epithelial cells may produce cytokines that are not elicited by infection with either pathogen alone (or by other commonly utilized experimental stimuli, such as cytokine exposure). Therefore, production of all cytokines known to elicit an anti-chlamydial response was examined in triplicate mock-, singly- or co-infected cultures as described in Methods. At 20 h post-viral infection, supernatants were collected and assayed for IFN- γ , IFN- α , TNF- α , GM-CSF, IL-1 α/β and IL-6. IFN- γ , IFN- α , TNF- α and GM-CSF were not detected in HeLa supernatants, regardless of infection status. IL-1 α (11.1–13.4 pg ml^{-1}), IL-1 β (34.7–46.9 pg ml^{-1}) and IL-8 (1.7–3.6 pg ml^{-1}) were detected, but at similar levels in *C. trachomatis* singly-infected and co-infected HeLa cultures, indicating that they were unlikely to be involved in HSV-2-induced persistence. As expected, IL-6 was strongly induced by HSV-2 infection and was increased in HSV singly- and co-infected HeLa cultures compared to that in mock- and *C. trachomatis* singly-infected cultures (Fig. 1a). However, in duplicate singly- and co-infected HEC-1B cultures, similar quantities of IL-6 were detected in all experimental samples (Fig. 1b). Because HSV-induced persistence also occurs in HEC-1B cells, it is unlikely that IL-6 production stimulates HSV-induced persistence (Deka *et al.*, 2007). Co-infection experiments using HSV-1 yielded similar results (data not shown).

Expression of IFN- γ , IFN- β and LT- α mRNAs during co-infection was measured by semiquantitative RT-PCR. As shown in Fig. 1(c), *ifn- γ* , *ifn- β* , and *lt- α* were not expressed

Fig. 1. *C. trachomatis*/HSV co-infected cells do not produce cytokines known to alter chlamydial development. (a, b) The concentration of IL-6 present in culture supernatants from mock-, singly- and co-infected HeLa (a) and HEC-1B (b) cells was determined by Luminex assays. Cytokine concentrations are expressed as pg ml⁻¹ ± SEM; *n*=3. Asterisks (*) indicate cytokine concentrations that are significantly different (by *t*-test) compared to those from *C. trachomatis* singly-infected cells (*P*<0.05). (c) Total cellular RNA was subjected to semiquantitative RT-PCR using primers specific to human *ifn-β*, *ifn-γ* and *It-α*. A dilution series of synthetic DNA targets was amplified for each gene as a positive control. *n*=8.

in mock-, singly- or co-infected cells. Amplification of diluted synthetic DNA targets specific to the *ifn-γ*, *ifn-β*, and *It-α* genes demonstrated that PCR reactions were sensitive and specific. Human 18S rRNA targets were also amplified to ensure that the cDNAs were not degraded (see Supplementary Fig. S1b). Amplification of a dilution series of control HeLa cell genomic DNA ensured that all 18S rRNA amplification reactions were quantified within the linear range of the PCR (Deka *et al.*, 2006). All template-negative (Fig. 1c) and reverse-transcriptase-negative (data not shown) samples were negative, as expected. Finally, all amplimers were of the expected size and sequence (data not shown). Taken together, these data indicate that HSV/*C. trachomatis* co-infection does not stimulate host epithelial cells to produce any known 'anti-chlamydial' cytokine.

***C. trachomatis*/HSV-2 co-infected cells do not produce iNOS**

The iNOS/NO pathway is another important component of the anti-chlamydial defence (Devitt *et al.*, 1996; Matsushima *et al.*, 1999). Although iNOS activity is not

stimulated by either IFN-γ or chlamydial infection in cultured human epithelial cells (Roshick *et al.*, 2006), the possibility that the additional stimulus provided by viral co-infection might activate iNOS/NO production in human cells is untested and should be evaluated. Therefore, *C. trachomatis*/HSV-2 co-infected cells were examined for iNOS mRNA upregulation by semiquantitative RT-PCR and for nitrite accumulation in supernatants using the Griess reaction. Neither iNOS mRNA nor nitrite was detected in any experimental cell cultures (Supplementary Fig. S1a and data not shown), confirming that HSV-2-induced chlamydial persistence is not mediated by iNOS induction or NO production.

Inclusions within co-infected cells continue to enlarge and incorporate C₆-NBD-ceramide

In murine cells, IFN-γ exposure activates the p47 GTPase, Iigp1, which halts *C. trachomatis* development by interrupting sphingomyelin transport and vesicular trafficking to the inclusion. If *C. trachomatis*-infected murine cells are exposed to IFN-γ early in the developmental cycle,

inclusions remain small (Nelson *et al.*, 2005). Recent studies indicate that Iigp1 is upregulated in HSV-1-infected murine cells at 3 h post-infection (Pasička *et al.*, 2006). Although a human Iigp1 homologue has not been identified, it is possible that HSV/host cell interaction activates human p47 GTPases that function similarly. Therefore, *C. trachomatis*-infected HeLa cells were co-infected with HSV-2 at either 4 or 24 h post-chlamydial infection. Replicate cultures were harvested either immediately after HSV adsorption or 20 h post-HSV-2 infection. Monolayers were fixed, immunostained with anti-MOMP antibodies, photographed, and relative inclusion size was determined (Deka *et al.*, 2006). Mean inclusion size was similarly increased in both singly- and co-infected cells over the course of the experiment when HSV-2 was added 4 h post-chlamydial infection (Fig. 2a). In cells co-infected with HSV-2 at 24 h post-*C. trachomatis* infection, the starting inclusion size was identical in both cultures. However, inclusions in co-infected cells were significantly larger than those in singly-infected controls after the 20 h HSV-2 infection period, as previously reported (Deka *et al.*, 2006). Additional cultures were infected as described above, labelled with BSA-conjugated C₆-NBD-ceramide

stain, and visualized by fluorescence microscopy (Alzhanov *et al.*, 2007; Hackstadt *et al.*, 1995). No obvious difference was observed in the intensity or distribution of ceramide staining between chlamydial inclusions in singly- and co-infected cells at either time interval tested (Fig. 2b and data not shown). Although it is possible that vesicular trafficking to the inclusion is subtly altered by HSV infection, these data demonstrate that neither gross vesicular trafficking nor sphingomyelin transport to the inclusion is disrupted by viral co-infection.

HSV-2 induction of chlamydial persistence is not mediated by IDO activity or global nutrient deficiencies

Degradation of the essential amino acid tryptophan by the host enzyme IDO strongly induces chlamydial persistence (Hogan *et al.*, 2004). In response to tryptophan deprivation, chlamydiae upregulate expression of the tryptophan synthase gene, *trpA* (Belland *et al.*, 2003). To determine whether our HeLa cell clone can express IDO, mock- and *C. trachomatis*-infected HeLa monolayers were exposed to IFN- γ (50 U ml⁻¹) for 48 h followed by RNA isolation.

Fig. 2. Inclusions within co-infected cells continue to enlarge and incorporate C₆-NBD-ceramide. (a) Cultures of mock-, singly- and co-infected HeLa cells were harvested immediately after HSV adsorption or 20 h later, fixed, subjected to IFA, and photographed at 320 $\times$. Relative inclusion size from 25 random fields was determined. Values are means of triplicate determinations. Asterisks (*) indicate values that are significantly different (by *t*-test) compared to those from *C. trachomatis* singly-infected cells immediately post-HSV adsorption or a significant difference (**) between *C. trachomatis* singly-infected and co-infected cells at 20 h post-HSV infection ($P < 0.05$). (b) Cells were *C. trachomatis*-infected, HSV-2 co-infected at 24 h post-chlamydial infection and C₆-NBD-ceramide stained, as described. Cells were photographed at 320 $\times$ (reproduced here at 225 $\times$) at 20 h post-HSV infection. All data shown are representative of three independent experiments.

Expression of *ido* and *trpA* mRNA in IFN- γ -exposed control cultures was then evaluated by semiquantitative RT-PCR. IDO expression was significantly increased in both mock- (data not shown) and *C. trachomatis*-infected HeLa cells in response to IFN- γ , compared to that in unexposed cells (Supplementary Fig. S2a). A significant increase in *trpA* expression was also observed in *C. trachomatis*-infected cells following IFN- γ exposure versus unexposed, chlamydiae-infected cultures (data not shown).

To determine if co-infection similarly elevates *ido* or *trpA* levels, total RNA was isolated from triplicate sets of mock-, singly- or co-infected HeLa cells and subjected to reverse transcription and semiquantitative RT-PCR with primers specific for the human *ido* and chlamydial *trpA* genes. Again, amplification of a dilution series of cDNA from *C. trachomatis*-infected, IFN- γ -exposed HeLa cells indicated that the IDO RT-PCR was specific and sensitive. IDO mRNA was not detected in any of the experimental samples tested, regardless of infection status (Supplementary Fig. S2b). Various dilutions of chlamydial genomic DNA were amplified to ensure that all *trpA* amplification reactions were quantified within the linear range of the PCR (data not shown). Additionally, chlamydial genome copy number in each sample was determined by amplification of the chlamydial 16S rRNA gene (data not shown). All *trpA* RT-PCR values were normalized to the chlamydial genome copy number (Deka *et al.*, 2006). In contrast to *trpA* expression during IFN- γ exposure, no statistical difference in *trpA* expression was observed between *C. trachomatis* singly-infected and co-infected samples (Supplementary Fig. S2c). These data confirm that *C. trachomatis*/HSV-2 co-infected cells are not deprived of tryptophan by the action of IDO.

Global deprivation of amino acids and other nutrients, especially iron and glucose, can also cause developing chlamydiae to enter persistence (Harper *et al.*, 2000; Raulston, 1997). To investigate the possibility that HSV-2 co-infection induces chlamydial persistence by limiting iron availability, we first determined whether co-infected cells were iron-deprived. Intracellular ferritin levels can be used as an indirect measure of host intracellular iron levels (Dill & Raulston, 2007). As a positive control for iron depletion, duplicate *C. trachomatis*-infected HeLa cultures were exposed to the iron-chelating agent Desferal following chlamydial infection. Intracellular ferritin levels were not significantly reduced in chlamydiae singly-infected or co-infected cells, compared to the level in mock-infected controls (Supplementary Fig. S3a), indicating that co-infected cells were not iron-deprived. In contrast, Desferal exposure significantly reduced intracellular ferritin levels, as expected (Supplementary Fig. S3a).

During HSV-2 replication, cellular amino acids are depleted through the synthesis of viral proteins (Roizman & Knipe, 2001). While it is possible that co-infection induces persistence by depriving the chlamydiae of amino

acids, it remains unlikely, given previous data that UV-inactivated, replication-incompetent HSV-2 stimulates chlamydial persistence (Deka *et al.*, 2007). However, several incoming HSV virion proteins, such as the virion host shut-off protein (VHS), produce significant host cellular physiological alterations in the absence of viral replication (Roizman & Knipe, 2001). Thus, the possibility that viral infection induces nutrient deprivation via an indirect pathway cannot be excluded. Notably, supplementation of nutrient-starved chlamydiae with amino acids, iron or glucose reverses the negative effect of the nutrient deficiency (Hogan *et al.*, 2004; Raulston, 1997). Additionally, the anti-chlamydial effect stimulated by IFN- α can only be completely reversed by addition of both excess amino acids and iron (Devitt *et al.*, 1996). Based upon this information, we performed co-infection supplementation studies with excess amino acids, iron, glucose, or a combination of amino acids plus iron. Immediately following HSV-2 infection, mock-, singly- or co-infected cells were refed with MEM or MEM supplemented with a $5 \times$ concentration of both essential and non-essential amino acids, 6 mg ml^{-1} iron-saturated holotransferrin (hTF), $450 \text{ mg glucose ml}^{-1}$, or a combination of $5 \times$ amino acids plus 6 mg hTF ml^{-1} . This concentration of hTF has been previously shown to rescue chlamydial infectivity in iron-deprived, infected epithelial cells (Raulston, 1997). Twenty hours after HSV-2 infection, the monolayers were collected and processed for chlamydial titration. The titre of infectious EBs was significantly reduced in epithelial cells co-infected with HSV-2 compared to that from cultures singly-infected with *C. trachomatis* (Supplementary Fig. S3b). Supplementation of co-infected host cells with hTF did not rescue chlamydial infectivity (Supplementary Fig. S3b), indicating that supplementation with excess iron was not able to restore production of infectious chlamydial progeny during co-infection. Similar results were obtained with excess amino acids, glucose (Supplementary Fig. S3c) and a combination of amino acids plus iron supplementation (data not shown). These data demonstrate that co-infection-induced chlamydial persistence is not mediated by limitation of amino acids, iron, glucose, or the combination of amino acids plus iron within the host cell. Because the anti-chlamydial effect of IFN- α is mediated by restriction of both tryptophan and iron, these results support previous data indicating that co-infection does not induce persistence through an IFN- α -dependent mechanism.

Concluding remarks

Collectively, our data demonstrate that co-infection-induced persistence is not mediated by: (i) any known anti-chlamydial cytokine; (ii) activation of iNOS or IDO; (iii) inhibition of vesicular trafficking or sphingomyelin transport to the inclusion or (iv) amino acid, iron or glucose deprivation. The reduction in chlamydial infectivity stimulated by HSV-2 co-infection is of smaller magnitude than that observed in other models of persistence (Beatty *et al.*, 1994; Pantoja *et al.*, 2001).

Additionally, the negative effects of HSV-2 co-infection on chlamydial development are observed when the stimulus is added at 24 h post-*C. trachomatis* infection, whereas IFN- γ must be added to chlamydia-infected cultures either before infection or very early during the developmental cycle for maximal effect (Nelson *et al.*, 2005). Taken together, these observations support the hypothesis that HSV-2 co-infection induces *C. trachomatis* persistence through an undescribed, and therefore novel mechanism.

Previously, we reported that both cycloheximide exposure during co-infection with replication-competent HSV-2 and co-infection with UV-inactivated HSV-2 can stimulate chlamydial persistence, indicating that early events during HSV-2 infection are sufficient for this effect (Deka *et al.*, 2007). During the host-cell entry process, HSV binds to one of several co-receptors: HVEM, nectin-1, nectin-2 or 3-O-sulfated heparin sulfate (Spear, 2004). Previous studies have demonstrated that each of these co-receptors can alter cellular signalling pathways when bound to their natural ligands (Granger & Rickert, 2003; Hsu *et al.*, 1997; Mauri *et al.*, 1998; Nakanishi & Takai, 2004). Therefore, HSV-2 attachment to the host cell during co-infection could alter cellular signalling and activate a novel host anti-chlamydial response, leading to persistence. Another possibility is that HSV stimulates Toll-like receptors, initiating such an anti-chlamydial pathway (Aravalli *et al.*, 2005; Compton *et al.*, 2003; Kurt-Jones *et al.*, 2005; Pyles *et al.*, 2002; Wang *et al.*, 2005). Conversely, there is the potential that viral tegument proteins, released into the cell during entry, could stimulate chlamydial persistence by altering the host cytoskeleton, degrading host mRNAs or altering host gene transcription (Elliott & O'Hare, 1998; Kwong & Frenkel, 1989; Roizman & Knipe, 2001). Investigations of anti-chlamydial host cell responses have provided valuable information regarding *C. trachomatis* biology, pathogenesis and interactions with the host cell. Illumination of the mechanism behind HSV-2-induced persistence will allow us to evaluate the contribution of this novel anti-chlamydial response to *C. trachomatis* pathogenesis and host defence.

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