

## RESEARCH ARTICLE

# The type I interferon receptor is not required for protection in the *Chlamydia muridarum* and HSV-2 murine super-infection model

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**One sentence summary:** Chlamydia pre-infection protects from HSV-2 fatal neurologic disease in mice in the absence of interferon beta signaling, indicating that alternative host immune responses likely promote the protective effect.

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## ABSTRACT

*Chlamydia trachomatis*/HSV-2 vaginal co-infections are seen clinically, suggesting that these sexually transmitted pathogens may interact. We previously established an intravaginal *Chlamydia muridarum*/HSV-2 super-infection model and observed that chlamydial pre-infection protects mice from a subsequent lethal HSV-2 challenge. However, the mechanism of protection remains unknown. The type I interferon, IFN- $\beta$ , binds to the type I interferon receptor (IFNR), elicits a host cellular antiviral response and inhibits HSV replication *in vitro* and *in vivo*. Previous studies have demonstrated that *C. muridarum* infection stimulates genital tract (GT) IFN- $\beta$  production; therefore, we hypothesized that chlamydial pre-infection protects mice from HSV-2 challenge via the IFN- $\beta$ /IFNR-induced antiviral response. To test this prediction, we quantified IFN- $\beta$  levels in vaginal swab samples. Detection of IFN- $\beta$  in *C. muridarum* singly infected, but not in mock-infected animals, prompted the use of the super-infection model in IFNR knockout (IFNR<sup>-/-</sup>) mice. We observed that *C. muridarum* pre-infection reduces HSV-2-induced mortality by 40% in wild-type mice and by 60% IFNR<sup>-/-</sup> mice. Severity of HSV-2 disease symptoms and viral shedding was also similarly reduced by *C. muridarum* pre-infection. These data indicate that, while chlamydial infection induces GT production of IFN- $\beta$ , type I IFN-induced antiviral responses are likely not required for the observed protective effect.

**Keywords:** chlamydia; HSV-2; interferon- $\beta$ ; innate immune response; co-infection; mouse model

## INTRODUCTION

*Chlamydia trachomatis* and HSV-2 are the leading sexually transmitted pathogens in the world causing 101 million and 23.6 million new cases per year, respectively (Looker, Garnett

and Schmid 2008; World Health Organization 2012). *Chlamydia trachomatis* genital tract infections can present with symptoms including cervicitis in women, and urethritis in men, but more than 70% of women and 50% of men experience asymptomatic

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infections (Mackern-Oberti et al. 2013; Menon et al. 2015). Though effective antibiotic treatment for chlamydial infections exists, asymptomatic infections typically go untreated, which can lead to severe complications, especially in women. Over the course of infection, the chlamydiae ascend the female genital tract, causing chronic inflammation that can lead to scarring and long-term consequences such as pelvic inflammatory disease and tubal factor infertility (Menon et al. 2015; O'Connell and Ferone 2016). Genital infections with HSV-2 can present with flu-like symptoms and painful genital ulcers, but these infections are also often clinically mild. Only 10%–25% of people seropositive for HSV-2 realize they have this infection, since obvious symptoms are not always experienced (Wald et al. 2000). Viral shedding occurs without the presence of obvious lesions and at similar levels compared to symptomatic shedders. Therefore, asymptomatic shedders pose an increased risk for perpetuating HSV-2 transmission, since those with obvious symptoms are more likely to seek suppressive therapy (Mark et al. 2008).

Though much is known regarding single infections of either *C. trachomatis* or HSV-2, little is understood regarding *C. trachomatis* and HSV-2 co-infections. Co-detection of these pathogens has been reported in men and women and in similar tissue types. However, these few epidemiological studies usually do not seek to identify these particular co-infections and often sample small populations (Finan, Musharrafieh and Almawi 2006; Shaw et al. 2011; Fageeh 2013). While the prevalence of *C. trachomatis* and HSV-2 co-infections has been estimated to occur in 0.5%–12.8% of the population (Finan, Musharrafieh and Almawi 2006; Shaw et al. 2011; Fageeh 2013), almost nothing has been characterized with respect to the disease progression or outcomes of these co-infections.

We previously established a murine *in vivo* super-infection model to begin to understand the interactions of *Chlamydia* and HSV-2 within the co-infected host. We determined that mice intravaginally infected with *Chlamydia* followed either 3 or 9 days later with HSV-2 infection exhibit significantly increased survival and significantly reduced viral shedding compared to animals singly infected with HSV-2 (Slade et al. 2016). In other words, chlamydial pre-infection protects from subsequent HSV-2-induced mortality and disease. The underlying mechanism of protection remains unknown, but understanding the host responses activated by prior chlamydial infection could potentially lead to new treatment strategies that reduce host susceptibility to HSV-2 infections. Production of type I interferons, in particular IFN- $\beta$ , is a well-known antiviral response known to inhibit HSV replication both *in vitro* and *in vivo* (Domke-Opitz, Straub and Kirchner 1986; Gill, Davies and Ashkar 2008). In response to viral infection, pattern recognition receptors, including toll-like receptors (TLRs), trigger the production of IFN- $\beta$  (Gill et al. 2006). IFN- $\beta$  then binds to the type I interferon receptor (IFNR) and elicits host cellular antiviral responses (Muller et al. 1994). Importantly, *C. muridarum* genital tract infection stimulates the production of IFN- $\beta$  (Prantner et al. 2011). Thus, we sought to determine whether chlamydial pre-infection was activating IFN- $\beta$  production as the primary mechanism of *Chlamydia*-induced protection from subsequent HSV-2 infection in the super-infection model.

## METHODS

### Ethics statement

All animal experiments in this study were conducted in strict accordance with the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals.' The animal protocol

(110602) was approved by the University Committee on Animal Care at East Tennessee State University under the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, US Department of Agriculture and in compliance with the Public Health Service Policy on Human Care and Use of Laboratory Animals.

### Cells, bacteria and viruses

The cell lines used in this study are HeLa 229 cells (cervical adenocarcinoma epithelial cells; ATCC No. CCL2.1, ATCC, Manassas, VA, USA) and Vero cells (African green monkey kidney epithelial cells; ATCC No. CCL-81, ATCC, Manassas, VA, USA). The wild-type HSV-2 333 strain was used for mouse infections. The *Chlamydia muridarum* Weiss strain was obtained from Kyle Ramsey (Midwestern University).

### Animals and infection

Male and female wild-type C57B/6 and type I interferon receptor knockout mice (IFNR<sup>-/-</sup>; strain name B6.129S2-Irfar1<sup>tm1aqt</sup>/Mmjax) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and used to establish breeding colonies in our animal facility. Female C57B/6 and IFNR<sup>-/-</sup> pups obtained from these breeding colonies were used for super-infection experiments as described below. Female wild-type BALB/c were purchased from Envigo, USA (Madison, WI, USA) and used for infection experiments where indicated. All mice used for infection experiments were from 6 to 8 weeks of age.

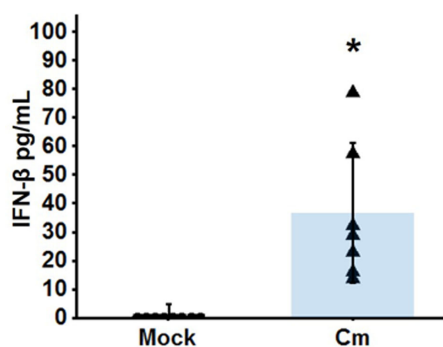
After a 1-week acclimation period, mice were treated with 2.5 mg Depo-Provera (Greenstone LLC, Peapack, NJ, USA) by subcutaneous injection. At 8–10 weeks of age, mice were intravaginally infected with  $1 \times 10^6$  IFU *C. muridarum* on day 0 and/or with  $1 \times 10^5$  PFU HSV-2 on day 3 post chlamydial infection (pci) as previously described (Slade et al. 2016). To monitor pathogen shedding, mice were vaginally swabbed every 3 days until day 21 pci as described elsewhere (Phillips Campbell et al. 2012). Mice were monitored daily for morbidity and mortality induced by HSV-2 infection. Mice exhibiting any degree of hind limb paralysis were sacrificed via cervical dislocation and remaining mice were sacrificed on day 21 pci. All mice were provided food and water *ad libitum* and kept on a standard 12-h light-dark cycle.

### Detection of IFN- $\beta$

Swab samples collected on day 3 pci from BALB/c mice initially infected with  $1 \times 10^6$  IFU *C. muridarum* were used in IFN- $\beta$  ELISA assays (Biolegend Inc, San Diego, CA, USA) according to manufacturer's protocol. Samples were analyzed in triplicate and absorbance was read at 450 nm using a Turner Modulus Multimode Microplate Reader.

### HSV-2 disease scoring and survival determination

Beginning on day 8 pci (day 6 post HSV-2 infection), extrvaginal symptoms of HSV-2-induced disease were scored on a scale of 0–5 as described by Docherty et al. (2005). Briefly, observations were scored as follows: 0, no symptoms; 1, redness of extrvaginal tissue; 2, few lesions, swelling and redness; 3, multiple lesions, swelling and redness; 4, unilateral or bilateral hind limb paralysis and 5, death (Docherty et al. 2005). Mice were considered survivors if alive at the time of observation. Mice that required sacrificing were incorporated into mortality data the following day pci. Days 8, 9, 12, 14, 18 and 21 pci were included



**Figure 1.** IFN- $\beta$  is detected in the genital tracts of *Chlamydia* singly infected animals. Day 3 pci swab samples were subjected to ELISA for detection of IFN- $\beta$ . Bars indicate group average IFN- $\beta$  detection. Symbols indicate individual mouse IFN- $\beta$  detection,  $n = 8$  per group. Data are representative of two independent experiments. Asterisk (\*) indicates statistical difference between mock-infected and *Chlamydia*-infected group means  $\pm$  SEM.

to most accurately represent the range of symptoms observed throughout the course of infection.

### Monitoring of pathogen shedding

Swab samples collected every 3 days pci were processed as previously described (Phillips Campbell et al. 2012; Slade et al. 2016). Processed samples were used to determine pathogen shedding via the chlamydial titer assay and via plaque assay for HSV-2 as described (Slade et al. 2016). Data are expressed as the average inclusion-forming units/mL and plaque-forming units/mL  $\pm$  SEM, respectively.

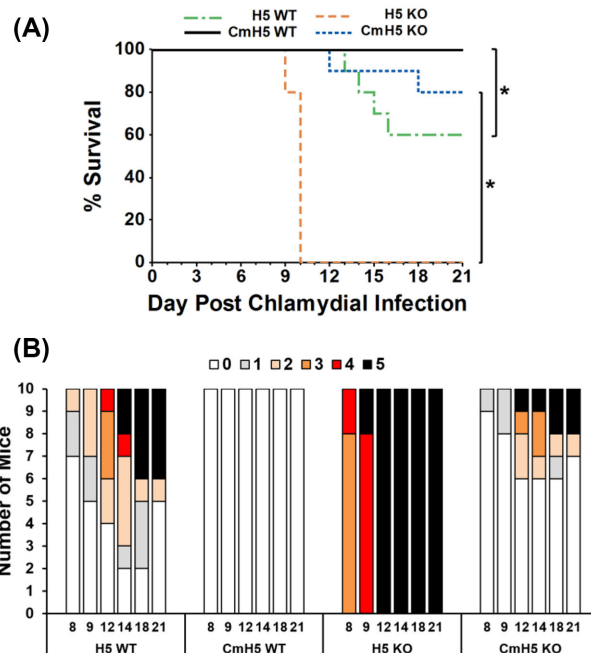
### Statistical analyses

The log-rank test was used to compare group survival trends, and data are displayed using Kaplan–Meier plots generated by Minitab 16 statistical software. Chlamydial and HSV-2 shedding/recovery between groups were analyzed using the unpaired Student's t-test. Values of  $P \leq 0.05$  were considered significant.

## RESULTS

### Interferon beta is detected during chlamydial infection in mice

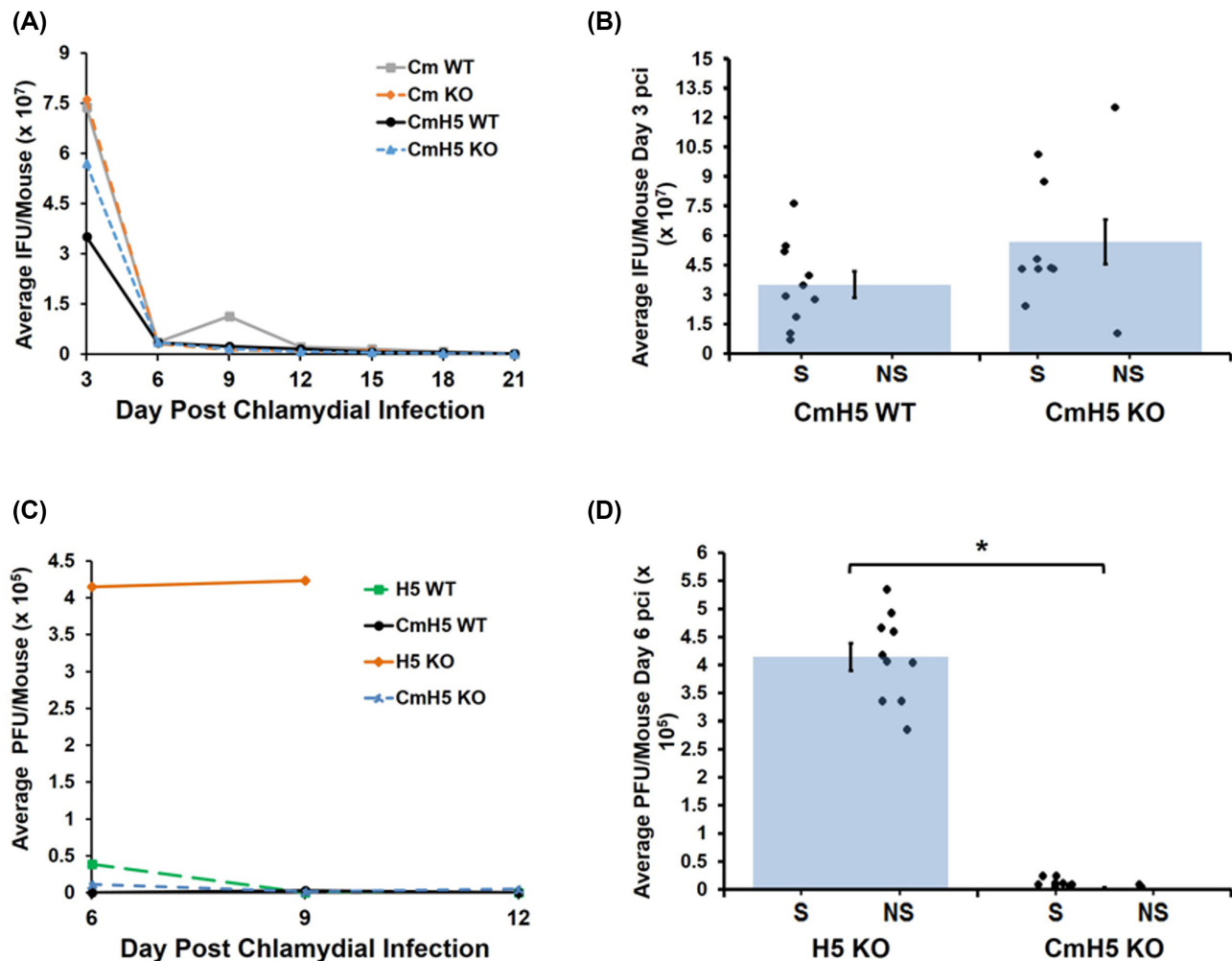
We previously established an *in vivo* super-infection model in which BALB/c mice are intravaginally infected with *C. trachomatis* on day 0, then super-infected with HSV-2 on day 3 pci. We observed that these super-infected animals exhibit significant protection from HSV-2-induced disease and experience significantly reduced viral shedding (Slade et al. 2016). Because IFN- $\beta$  is a prominent and well-known anti-HSV response, we first wanted to determine whether IFN- $\beta$  is produced in *Chlamydia*-infected animals. We used swab samples collected at day 3 pci and performed ELISAs on mock-infected and *Chlamydia* singly infected samples. No IFN- $\beta$  was detected in mock samples; however, significantly more IFN- $\beta$  was detected at day 3 in *Chlamydia* singly infected animals (Fig. 1). These data are consistent with previously published data (Prantner et al. 2011) and suggested that perhaps IFN- $\beta$  was responsible for the protective mechanism we observed in the super-infection model.



**Figure 2.** The type I interferon receptor is not required for protection from HSV-2-induced lethality in the *Chlamydia* and HSV-2 super-infection model. Mice were super-infected with  $1 \times 10^6$  IFU *C. muridarum* on day 0 and with  $10^5$  PFU HSV-2 on day 3 pci in either wild type (CmH5 WT) or type I interferon receptor knockout mice (CmH5 KO). As controls, mice were singly infected with Cm (not shown) or HSV-2 in either WT or KO mice (H5 WT and H5 KO);  $n = 10$  per group. Morbidity and mortality resulting from HSV-2 was monitored daily, and the % survival between experimental groups was compared using the log rank statistic (A). Significant ( $P < 0.05$ ) difference from HSV-2 singly infected control is indicated by an asterisk (\*). (B) Pathology scoring was conducted as described in the methods and representative days between day 8 and day 21 pci are shown.

### The interferon beta receptor is not required for *Chlamydia*-induced-protection from subsequent HSV-2 infection

Because we detected IFN- $\beta$  in *Chlamydia*-infected day 3 murine swab samples, we applied our super-infection model to type I interferon receptor knockout (IFNR<sup>-/-</sup>) mice. IFNR<sup>-/-</sup> mice are able to produce IFN- $\beta$ , but due to lack of functioning receptors, IFN- $\beta$  can no longer exert its antiviral effects (Muller et al. 1994). As controls, we used *Chlamydia* singly infected C57BL/6 wild type and IFNR<sup>-/-</sup> mice (Cm WT and Cm KO). The Cm WT and Cm KO groups both demonstrated 100% survival (data not shown). As additional controls, we included both HSV-2 singly infected and *Chlamydia*/HSV-2 co-infected wild-type groups. We observed significantly more survival in the co-infected wild-type mice compared to HSV-2 singly infected wild-type mice. These data recapitulate our original observations in BALB/c mice (Slade et al. 2016), demonstrating that Cm-mediated protection from HSV disease is not mouse strain specific. However, a higher inoculum of  $1 \times 10^5$  PFU HSV-2 was required to infect C57BL/6 mice, which are more resistant to HSV infection (Brenner, Cohen and Moynihan 1994), compared to the  $5 \times 10^3$  PFU previously used to infect BALB/c mice (Slade et al. 2016). By day 10 pci, the HSV-2 singly infected knockout mice (H5 KO) experienced 100% mortality and demonstrated significantly less survival compared to the 60% survival observed in the co-infected knockout group (CmH5 KO;  $P < 0.001$ ; Fig. 2A). HSV-2 pathology scoring indicated that HSV-2 infection in IFNR<sup>-/-</sup> mice is more severe compared to that in WT C57BL/6 mice. As expected, the onset of symptoms of



**Figure 3.** *Chlamydia* pre-infection significantly decreases HSV-2 shedding in co-infected IFN $\beta^{-/-}$  mice. Mice were infected as described in Fig. 2. (A) Chlamydial shedding was determined by chlamydial titer assay and is reported as average IFU/mouse  $\pm$  SEM. (B) Average chlamydial shedding at day 3 pci (indicated by bars) and individual mouse chlamydial shedding (segregated according to survival status) are shown;  $n = 10$  per group. (C) HSV-2 shedding was determined by plaque assay and is reported as average PFU/mouse  $\pm$  SEM. (D) Average HSV-2 shedding at day 6 pci (indicated by bars) and individual mouse HSV-2 shedding (segregated according to survival status) are shown;  $n = 10$  per group. Survivors and non-survivors are indicated by S and NS, respectively. Differences in pathogen shedding/recovery between groups in panels A–D were determined with the unpaired Student's *t*-test with  $P < 0.05$  considered significant and are representative of three independent experiments.

HSV-2 at days 8 and 9 pci in the H5 KO group correlated with the abrupt decline in survival observed, whereas minimal symptoms of HSV-2 and low mortality were observed in the CmH5 KO group (Fig. 2B). In examining the combined data from three individual repeats, we observe a similar trend in survival and pathology as reported in Fig. 2. The HSV-2 singly infected mice devoid of IFN- $\beta$  signaling (H5 KO) exhibit 100% mortality by day 10 compared to their *Chlamydia* pre-infected counterparts, which exhibit a combined 20% mortality (Fig. S1A, Supporting Information). These data corresponded to pathology scoring data, which again demonstrated increased severity of HSV-2 disease in H5 KO mice compared to the CmH5 KO mice (Fig. S1B, Supporting Information).

With respect to pathogen shedding, chlamydial titer assays demonstrated no significant differences between Cm WT and Cm KO or between CmH5 WT and CmH5 KO groups at any time throughout the course of infection (Fig. 3A). Furthermore, at peak shedding at day 3 pci, similarly elevated chlamydial titers were observed between the CmH5 WT and CmH5 KO, which again exhibited no difference in survival at this time point

(Fig. 3B). As expected, viral plaque assays demonstrated that H5 WT mice exhibited significantly more HSV-2 shedding compared to CmH5 WT animals at peak shedding at day 6 pci ( $P < 0.005$ ; Fig. 3C). Compared to the H5 WT group, the H5 KO group displayed nearly 10-fold more viral shedding. Interestingly, the H5 KO group exhibited similarly elevated viral shedding levels at day 9 pci, when all of the remaining HSV-2-infected groups exhibit a decline in viral shedding. No plaque assay data were available for the H5 KO group past day 9 pci, since these animals all succumbed to HSV-2 infection prior to day 12 (Fig. 2A), the next sampling time. Despite the high levels of HSV-2 shedding detected in the H5 KO mice, the CmH5 KO mice still exhibited significantly more survival and significantly reduced viral shedding (Fig. 3C and D), indicating that a robust IFN- $\beta$ -independent mechanism is promoting the protective effect.

## DISCUSSION

Altogether, our data demonstrate that the absence of IFN- $\beta$  signaling does not abolish the protective effect established

by chlamydial pre-infection in HSV-2 super-infected animals. Compared to HSV-2-infected IFN $\beta$ <sup>-/-</sup> mice, the *Chlamydia* pre-infected animals exhibit (i) significantly higher survival; (ii) less severe HSV-2 disease symptoms and (iii) significantly reduced viral shedding. Not only do these data recapitulate the observations made in our original experiments, but they rule out IFN- $\beta$  as the primary host response involved in the protective mechanism. Therefore, there must be other mechanisms involved that mediate the protective effect.

We previously postulated that TLRs were involved in the protective mechanism, largely due to their ability to induce the production of IFN- $\beta$ . Though we have ruled out IFN- $\beta$ , it is still possible for TLRs to play a role in the protective mechanism through the production of other chemokines and cytokines. For example, during *C. muridarum* infection in mice, activation of TLR2, TLR3 and TLR4 can lead to the production of the pro-inflammatory cytokines IL-6 and MIP-2 (Darville et al. 2003; Derbigny et al. 2012). These particular cytokines are also known to inhibit HSV infection (LeBlanc et al. 1999; Eo et al. 2001), and could therefore aid in downstream effects that prevent subsequent HSV-2-induced disease. Additionally, IL-6 is a chemoattractant for influx of polymorphonuclear leukocytes (Romano et al. 1997). These cells represent the dominant immune cell type during early chlamydial infection, but are also required for HSV clearance *in vivo* (Milligan, Bourne and Dudley 2001; Rank et al. 2008).

Like IFN- $\beta$ , the type III interferon, IFN- $\lambda$  induces antiviral responses through activation of the JAK/STAT signaling pathway (Bierne et al. 2012); however, IFN- $\lambda$  exerts its effects through a unique receptor comprised of IL-10R $\beta$  and IL-28R $\alpha$ . While the type I IFN receptor is ubiquitously expressed, expression of the IFN- $\lambda$  receptor is limited to epithelial cells, hepatocytes and some immune cells (Ank et al. 2008; Bierne et al. 2012). During HSV-2 infection, production of IFN- $\lambda$  occurs via stimulation of TLR3 and TLR9 and reduces HSV-2 replication in keratinocytes and vaginal epithelial cells (Ank et al. 2008). IFN- $\lambda$  has not been as extensively examined in the context of chlamydial infection. One study demonstrated that infection of LoVo colonic carcinoma cells with *Chlamydia trachomatis* serovar L2 induced an 8-fold increase in IFN- $\lambda$  mRNA compared to uninfected cells, but IFN- $\lambda$  protein production was not assessed (Bierne et al. 2012). Since *Chlamydia* infection also stimulates TLR3 and TLR9 (O'Meara, Andrew and Beagley 2014), it is possible that IFN- $\lambda$  produced in response to *C. trachomatis* infection may be sufficient to combat subsequent HSV-2 co-infection in the absence of signaling via the type I IFNR. The redundancy in these two systems suggests that dual inactivation of the type I and type III IFNRs may be required to determine whether antiviral IFN responses are mediating the protective effect exhibited in the *Chlamydia*/HSV-2 super-infection model.

Natural killer (NK) cells are a major source of the type II interferon, IFN- $\gamma$ . Thus, NK cells largely contribute to IFN- $\gamma$ -dependent T cell activation and subsequent clearance of single infections of either *Chlamydia* or HSV-2 (Milligan and Bernstein 1997; Tseng and Rank 1998; Ashkar and Rosenthal 2003). Though IFN- $\beta$  has been reported to be required for NK cell activation during HSV infection in mice, the combination of IL-12 and IL-18 exposure can activate NK cells in the absence of IFN- $\beta$  (Hook, Matyszak and Gaston 2005). Furthermore, *C. muridarum* can stimulate the production of IL-12 and IL-18 in culture (Hook, Matyszak and Gaston 2005). Therefore, it may be possible for *C. muridarum* to induce the production of IL-12 and IL-18 to activate NK cells in an IFN- $\beta$ -independent manner, thereby establishing protection from HSV-2 in the super-infection model.

Although IFN- $\beta$  signaling does not appear to be required for *C. muridarum* to establish protection from subsequent HSV-2 infection in the murine *C. muridarum*/HSV-2 super-infection model, there are still many avenues to explore. Future experiments will aim at determining the role of TLR activation, cytokine production beyond IFN- $\beta$  and IFN- $\beta$ -independent immune cell infiltration and activation, as these additional host innate immune responses could be promoting the protective effect elicited by prior chlamydial infection.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSPD](#) online.

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**Conflict of interest.** None declared.

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