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THE ROLE OF VIABLE BUT NON-INFECTIOUS DEVELOPMENTAL FORMS IN CHLAMYDIAL BIOLOGY

Topic Editors
Nicole Borel, Andreas Pospischil, Alan P. Hudson, Jan Rupp and Robert V. Schoborg

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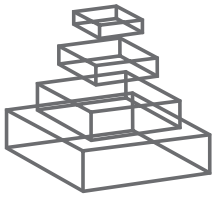
CELLULAR AND INFECTION MICROBIOLOGY



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THE ROLE OF VIABLE BUT NON-INFECTIOUS DEVELOPMENTAL FORMS IN CHLAMYDIAL BIOLOGY

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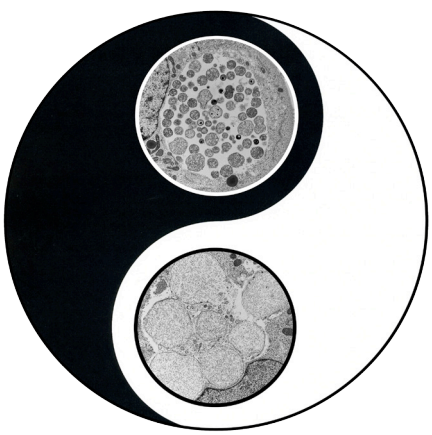

The Yin and Yang of Chlamydial Development.
Five decades of research indicate that environmental changes can induce chlamydiae to transition between "normal", productive, biphasic development and a non-replicative state originally termed persistence and more recently described as the chlamydial stress response. Persistence/stress has been historically defined as a viable but non-infectious developmental state. Persistent/stressed chlamydial organisms can remain in this state for extended periods of time but, when the inducing stressor is removed, can resume replication and ultimately produce infectious progeny (ie. elementary bodies or EB). Top. Transmission electron micrograph (TEM) of a mid-developmental cycle *Chlamydia pecorum* inclusion at 38 hours post-infection, showing a mix of EB, reticulate bodies (RB) and dividing RB (7000 x magnification). Bottom. TEM of a persistent/stressed *C. pecorum* inclusion at 38 hours post-chlamydial infection showing enlarged RB (usually termed aberrant bodies or AB) typical of the viable but non-infectious developmental state (7000 x magnification). Persistence/stress was induced by co-infection of chlamydia-infected Vero cells with porcine epidemic diarrhea virus (PEDV) 14 hours after chlamydial infection.
Prof. Robert V. Schoborg

The chlamydiae are Gram-negative, obligate intracellular bacteria with a complex developmental cycle comprising a metabolically less-active, infectious stage, the elementary body (EB), and a metabolically more active stage, the reticulate body (RB). They are responsible for many acute and chronic diseases in humans and animals. In order to play a causative role in chronic diseases, chlamydiae would need to persist and to re-activate within infected cells/tissues for extended periods of time. Persistence *in vitro* is defined as viable but non-cultivable chlamydiae involving morphologically enlarged, aberrant, and nondividing RBs, termed aberrant bodies (AB). *In vitro*, alterations of the normal developmental cycle of chlamydiae can be induced by the addition of Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and penicillin G exposure as well as amino acid or iron deprivation, monocyte infection and co-infection with viruses.

In vivo, key questions include whether or not ABs occur in infected patients and animals and whether such ABs can contribute to prolonged, chronic inflammation, fibrosis, and scarring through continuing stimulation of the host immune system known from diseases such as trachoma, pelvic inflammatory disease, reactive arthritis and atherosclerosis. To date, the direct causal role in the pathogenesis of chlamydial infection and persistence *in vivo* has been questioned since there was no tractable animal model of chlamydial persistence so far. A very recent study was able to establish an experimental animal model of *in vivo* persistence, when *C. muridarum* vaginally-infected mice were gavaged with amoxicillin. Amoxicillin treatment induced *C. muridarum* to enter the persistent state *in vivo*. Recent *in vivo* data from patients indicate that viable but non-infectious developmental stages are present in the genital tract of chronically-infected women and that the gastrointestinal tract might be a reservoir for persistent chlamydial infections at other sites.

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EDITORIAL
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The role of viable but non-infectious developmental forms in chlamydial biology

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Keywords: the chlamydial stress response, obligate intracellular, viable but non-infectious, aberrant bodies, chronic disease

Chlamydiae are both intriguing obligate intracellular Gram-negative bacteria and highly successful animal and human pathogens. Their unique biphasic developmental cycle is characterized by the infectious but metabolically less-active elementary body (EB), which initiates infection in a susceptible host cell, and the dividing, intracellular reticulate body (RB). The chlamydiae hijack host cells for their own purposes and, after infection and multiplication, emerge from the host cell and spread their hundreds of progeny EB to infect the next cell. Moreover, chlamydiae are able to survive in an alternative form named the aberrant body (AB) under stressful *in vitro* conditions. The AB state has been defined as the hallmark of persistence—which is herein defined as the viable but non-infectious state of the chlamydial developmental cycle. The key questions of whether this AB form occurs *in vivo* and if this alternative chlamydial form contributes to clinically significant chronic diseases processes such as inflammation, scarring, and fibrosis are a matter of debate in the chlamydial research community. These controversies led us to assemble a collection of Opinion, Hypothesis and Theory, Perspective, Review, and Primary Research Articles to shed light on recent research findings. This Research Topic showcases the "chlamydial persistent state" from meaning to mechanism on the cellular and human/animal host level.

The critical dissection of the word "persistence" by Bavoil (2014) points out that much more is hidden behind this term than the definition "to stand still permanently" in a susceptible host. For clinicians, this might represent a bacterial infection that: (i) has a subclinical course, (ii) can escape host immune responses; and (iii) is refractory to antibiotic treatment. The lack of specific diagnostic tests for such "invisible" infections might even complicate the definition and detection of these infections. Ongoing asymptomatic infections may even resemble colonization, as suggested by very recent data on chlamydial infections in the gastrointestinal tract. Notably, asymptomatic gastrointestinal chlamydial infections with recurrent chlamydial fecal shedding have been observed in the Veterinary Medical field for decades. For example, subclinical infections with *Chlamydia (C.) psittaci* in birds are frequent and these "latently" infected birds pose a significant zoonotic risk. Regardless, it is important for investigators in the Chlamydia field to come to agreement regarding the "definition" of what constitutes a persistent chlamydial infection.

The persistence phenotype *in vitro* may be linked to chronic disease processes *in vivo*, such as atherosclerosis. *C. pneumoniae* has been detected in atherosclerotic lesions of human patients by multiple methods and *C. pneumoniae*-induced disease progression has been demonstrated in rodent models—an association that propelled several antibiotic treatment trials. The opinion article by Campbell and Rosenfeld (2014) summarizes the outcome of these clinical trials and critically reviews their limitations. Unfortunately, the mixed results of these trials and the conclusion that anti-chlamydial antibiotics should not be recommended for treatment of patients with coronary heart disease and peripheral artery disease may have led to the erroneous assumption that *C. pneumoniae* did not play a role in the pathogenesis of atherosclerosis. The advanced nature of atherosclerotic lesions questions not only the effectiveness of antibiotic treatment to eradicate *C. pneumoniae* but also implicates how challenging the diagnosis of the agent may be. Puolakkainen (2013) discusses the difficulty in differentiating between acute and chronic infections due to the lack of reliable "persistence" biomarkers and commercially available tests. Whole proteome assays have been developed for research purposes but need careful validation on selected specimens from well-studied patient populations. The most desirable test to detect persistent infections would be serum-based, an optimistic goal that remains frustratingly out of reach without a better understanding of what persistence actually is and the molecular mechanisms that underly induction, maintenance, and recovery from this non-replicative state (Bavoil, 2014).

Azithromycin and tetracycline/doxycycline are considered the first-line antibiotics to treat genital chlamydial infection. *In vitro*, it has been shown that penicillin G exposure induces *C. trachomatis* persistent/aberrant forms. In culture, persistent organisms also resist killing by azithromycin. Because penicillins are commonly utilized to treat other bacterial infections, this may impact patients with concurrent asymptomatic chlamydial infections. Kintner et al. (2014) investigated if commonly prescribed beta-lactam antibiotics are able to induce *C. trachomatis* serovar E persistence/stress in culture. All penicillins tested induced viable but non-infectious chlamydial forms at clinically relevant concentrations, which might represent one mechanism by which chlamydiae resist antimicrobial therapy *in vivo*. In a related study, Ong et al. (2013) determined whether the *C. trachomatis*

serine protease HtrA (CtHtrA) was required for recovery from penicillin-induced persistence/stress response. CtHtrA is essential for the replicative phase and the addition of a chemical inhibitor (JO146) of CtHtrA was lethal when added to the cultures at mid-replicative cycle. The same inhibitor prevented reversion and recovery from penicillin persistence, demonstrating the essential role of CtHtrA during recovery from stressful conditions. Not only is HtrA the first chlamydial gene shown to play a role in recovery from persistence, HtrA inhibitors might prove to be useful for eradicating both productively replicating and persistent chlamydiae.

Persistent infections have been described in a magnitude of experimental cell culture models—all of which involve applying some type of stressor to the chlamydial culture. The most well-described culture model of persistence is the IFN-γ-induced system. In human cells, IFN-γ exposure upregulates indoleamine 2,3-dioxygenase (IDO), decreases intracellular tryptophan (Trp), and induces chlamydial persistence. Chlamydial infectivity can be rescued by addition of exogenous Trp. Four manuscripts in this special issue explore novel aspects of IFN-stimulated persistence. Jerchel et al. (2014) explored *C. trachomatis* persistence and reactivation and host cytokine responses under normoxic and hypoxic conditions. Oxygen concentrations in the female urogenital tract are physiologically low and further diminished by inflammatory processes. This study demonstrated that hypoxia leads to reactivation of INF-γ-induced persistent *C. trachomatis* infections. Moreover, host immune signaling responses were diminished, as reflected by reduced activation of MAP-kinase p44/42 and reduced expression of pro-inflammatory cytokines IL-6 and IL-8. Recent sophisticated comparative evaluation of *Chlamydiales* proteomes for Trp content is reviewed by Bonner et al. (2014). Selection for higher-than-predicted (Up-Trp) Trp content, compared to lower-than-predicted (Down-Trp) content, is key for the persistent state and phylogenetically different for *Chlamydiaceae* compared to other chlamydiae families. Previous studies indicate that there is a significant difference in the capacity of ocular and genital *C. trachomatis* serovars to deal with tryptophan deprivation. Genital serovars encode a functional tryptophan synthase and can use indole to bypass INF-γ-induced tryptophan starvation. The perturbed vaginal microbiome as a source of indole *in vivo* is highlighted in the Hypothesis and Theory article by Aiyar et al. (2014). Indole has been found in vaginal secretions from patients with bacterial vaginosis and indole-producing bacteria have been isolated. Thus, when studying genital chlamydial infections in the future, it will be necessary to consider both the elicited host immune response and the local environmental conditions created by the host microbiome. Transferring such knowledge into the patient's situation is reflected in the article by Lewis et al. (2014). These authors were able to show persistent chlamydial growth forms in the human cervix by novel diagnostic approaches. Local IFN-γ levels corresponded to chlamydial growth pattern and morphology. This study may pave way to the establishment of potential biomarker panel for assessing the disease outcome in *C. trachomatis* infected women.

One ultrastructural feature of persistent chlamydiae observed in many cell culture models is production of abundant membrane vesicles (MVs), which are observed both within the inclusion and in the host cell cytoplasm. The potential role of these vesicles in host-pathogen interactions is explored by Frohlich et al. (2014). The generation of MVs is suggested to be an important mechanism for *C. trachomatis* intracellular survival of stress. MVs are not only present in primary human endocervical epithelial cells infected with *C. trachomatis*, but also in clinical specimens from infected patients (Lewis et al., 2014). The potential function and role of chlamydial MVs in cargo delivery, innate immune response modulation, exchange of genetic material, and intracellular survival under stress conditions calls for more research in this field.

The "chlamydial anomaly" refers to the observation that chlamydial species are sensitive to antibiotics that target the bacterial cell wall, such as penicillin, despite the fact that a functional cell wall has not been detected. This problem is featured in the research article by De Benedetti et al. (2014). The chlamydial genome contains a nearly complete cell wall precursor biosynthesis pathway. *Chlamydiaceae* genomes encode GlyA which in turn can serve as a source of D-alanine, as exemplified in *C. pneumoniae*. In view of this finding, future research might help to clarify the structure of cell wall precursor lipid II and the role of chlamydial penicillin-binding proteins in production of penicillin-induced aberrant chlamydial forms.

Finally, host-pathogen interplay between multiple pathogenic microorganisms, such as the porcine epidemic diarrhea virus (PEDV) co-infection model described in the last paper of this collection (Schoborg and Borel, 2014), might more appropriately reflect the *in vivo* situation than do artificially-induced cell culture models. In particular, this bacterial-viral co-infection has been shown to occur *in vivo*, can be mimicked in experimental porcine animal models and includes the local environmental conditions of the gut microbiome.

In summary, this collection highlights the recent findings in the field of the "persistent" chlamydial state, which might reflect persistence, re-occurrence, re-infection or colonization in an animal or human host. The collection also illuminates the multifaceted nature of chlamydial persistence and the complexity that results from the existence of different models and multiple factors involved. Moreover, many models have been developed from *in vitro* data and applicability to the *in vivo* situation is, as yet, undefined. As outlined by Bavoil (2014), our limited understanding of what persistence is needs further research in the future and will produce greater insight into the host-pathogen interaction.

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PERSPECTIVE ARTICLE
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What's in a word: the use, misuse, and abuse of the word "persistence" in *Chlamydia* biology

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The word persistence was used by *Chlamydia* researchers almost as soon as *Chlamydia* research was born to reflect the propensity of chlamydiae to cause inapparent infection in their hosts, from birds to humans. More recently, the term persistence has been used, misused, and sometimes abused amidst *in vitro* and *in vivo* studies that aim to mimick the ability of chlamydiae to emerge from the presumed inapparent state into clinically detectable infection and disease. Here, I have attempted to provide a global perspective on the state of research on chlamydial persistence, revisiting old observations that may warrant a new look, critically evaluating more recent observations and their shortcomings, and including recent developments that may help redefine chlamydiae as pathogens—or not—of both animals and humans.

Keywords: *Chlamydia*, persistence, recurrence, commensalism, aberrant body

> "Essentially, all models are wrong, but some are useful" (George E. P. Box).

The word persistence derives from the Latin *persistere* where *per* conveys the notion of permanence while the verb *sistere* is most frequently translated as *to stand still*. Thus, the question before us is whether the word persistence is appropriately used to describe chlamydiae that are able to "stand still permanently" in a susceptible host.

Bacterial persistence in plain English has been described often and in many different contexts. A compendium of these descriptions may be summarized as follows: Persistence is an alternative outcome of a bacterial infection whereby a subpopulation of the bacteria becomes "invisible" by variably escaping prolonged antibiotic treatment, warding off innate and adaptive immune responses, causing little or no symptoms in the infected host, and falling below the radar of the diagnostician. Cryptic, latent, covert, dormant, or silent are other terms that have been commonly used to refer to the "persistent" infection state which may consist of an altered form of the bacterium that is inherently physiologically refractory to all the above, or a normal form of the bacterium that is somehow hidden and/or effectively protected from all the above. If one takes away the notion of *infection* from these definitions, then a persistent, asymptomatic infection may closely resemble *colonization*, similar to that by a member of the microbiota, i.e., a commensal organism. Where do *Chlamydia* spp. Belong on the spectrum of commensalism and pathogenesis, and how this impacts the terminology that should be used to refer to persistence are the subject of this commentary.

From a public health point of view, the ability of *Chlamydia trachomatis* to persist is made significant by its ability to occur or recur as an acute infection since persistence on its own would be of little medical interest. In a formal sense, two basic, non-mutually exclusive scenarios should be considered: (1) a clinically detectable infection may recur from a persistent state intervening between two acute episodes; (2) alternatively, a clinically detectable infection may be induced "opportunistically" from a silent state, whereby the host has been colonized or infected at an earlier date without any symptoms or clinical signs of infection. Here, I will respectively refer to these pathways as the *persistence/recurrence* and *colonization* pathways. In either case, the previously "invisible" *C. trachomatis* population becomes "visible" to the host cell, the infected host or to the physician. However, although a distinction between these two scenarios is not usually made, they likely are biologically distinct in the manner chlamydiae become established in the host, how they exit from the "invisible" state, and also in the innate and adaptive responses they elicit, and consequent pathologies. In the absence of evidence for the colonization pathway for *C. trachomatis* to date, far more attention has been given to the persistence/recurrence mechanism owing principally to the emergence in the last two decades of several related *in vitro* models (reviewed in Wyrick, 2010) that recapitulated the basic requirements of the persistence/recurrence phenotype: the induction of infecting chlamydiae into developmental arrest upon exposure to a physiologically relevant stimulus (mimicking *in vivo* persistence), and the subsequent reversal to normal development upon removal of the stimulus (mimicking *in vivo* recurrence) in cultured cells.

However, the concept of persistence did not start with human *C. trachomatis* infections; it started with the avian pathogen, *Chlamydia psittaci*.

The concept of *latent* chlamydial infection was born in the 1930s from observations by Meyer and Eddie who contrasted the frequent occurrence of the psittacosis virus, known today as *C. psittaci*, in companion birds with the relative rarity of overt disease in the birds themselves or the bird handlers (Meyer et al., 1935). Thus, the idea of persistent infection was borne out of the observation that multiple hosts may display completely different outcomes after exposure to the organism, with the majority displaying a latent infection, and a minority displaying overt disease. For infection to recur, it needs to have occurred at least once previously, and it is unclear from these studies whether the latent infected birds had a clinically detectable infection at the onset or were merely colonized. Decades after these early observations, several investigators were able to demonstrate that chlamydial development could be arrested at mid stage by either depriving the chlamydiae of essential metabolites (Bader and Morgan, 1961; Hatch, 1975) or via exposure to aminopterin (Pollard and Sharon, 1963), a folic acid competitive inhibitor and antineoplastic drug once commonly used in chemotherapy. Restitution of the required nutrients, or, in the latter case, supplementation with folinic acid, a vitamer of folate, restored the normal course of development, thereby fulfilling the essential requirements of the persistence/recurrence phenotype. These pioneering studies involved latent infection by *C. psittaci* and a link was not made then with other chlamydial diseases.

Moulder and his colleagues were the first to tackle the question of persistent/recurrent infection in a systematic experimental manner and were, indeed, first to develop *in vitro* models of persistence presumed to represent clinically observed persistence in several seminal publications. When mouse fibroblasts (L cells) were infected with high doses of *C. psittaci* 6BC, most L cells died but a small fraction survived that appeared to be persistently infected with *C. psittaci*, yet were inclusion-free by standard imaging methods of the time (Moulder et al., 1980). The cryptically infected L cells grew poorly, became resistant to super-infection (Moulder et al., 1981, 1982) and the cultures alternated between L cell expansion and chlamydial growth. A subpopulation of L cells with unique properties that enabled persistent infection by cryptic forms of *C. psittaci* was hypothesized. McCoy cell cultures persistently infected with a non-LGV strain of *C. trachomatis* were also obtained upon generating an equilibrium whereby periods of host cell propagation alternated with chlamydial growth and host cell destruction (Lee and Moulder, 1981). While Moulder and colleagues did not have the benefit of PCR, modern omics or imaging techniques, their studies described the ability of chlamydiae to maintain themselves in culture for extended periods of time, representative of a presumed "persistent state" in clinical disease, and to revert upon some unknown stimulus representative of recurrence. Unfortunately, these studies were discontinued around the eighties. Moulder did one last experiment on cryptic bodies that was a precursor of the next era of studies on chlamydial persistence/recurrence. He observed that he could delay the onset of overt chlamydial growth by shifting *C. psittaci*-persistently infected L cells to a nutrient-poor minimal medium or upon exposure to penicillin (Moulder, 1983).

A student of Moulder, Byrne, and his colleagues took the modeling of persistence/recurrence to a new level when they reproduced inducible persistence/recurrence in cultured cells using a physiologically relevant stimulus. Beatty et al. (1993) showed that in the presence of low levels of the cytokine interferon-gamma (IFN-γ), developmental growth of *C. trachomatis* serovar A stopped, producing large atypical reticulate body (RB) forms and that reversion to productive development to infectious elementary bodies (EBs) could be rescued by removal of the cytokine. IFN-γ was shown to exert its activity through tryptophan depletion upon induction of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) (Beatty et al., 1994), fundamentally reducing this mechanism to starvation for an essential nutrient. Similar results, including the observation of abnormally large RBs, consistent with persistence/recurrence were concurrently obtained by Pearce and his colleagues who tested the impact of starvation of several amino acids on chlamydial development (Coles et al., 1993; Pearce et al., 1994) and by Kahane and her colleagues who tested heat shock (Kahane and Friedman, 1992). Abnormally enlarged RBs similar to those observed by these groups had in fact been observed before. Over two decades earlier, Akira Matsumoto had published electron micrographs documenting the dramatic impact of penicillin on *C. psittaci* and *C. trachomatis* development (Matsumoto and Manire, 1970; Matsumoto, 1988). Similar to heat shock and tryptophan depletion, penicillin caused the formation of abnormally enlarged RBs that could be maintained in culture for as long as the host cells were maintained healthy, and that could revert to normal development to infectious EBs upon removal of the drug.

The common denominator of the early reports on chlamydial persistence/recurrence is the observed ability of a chlamydia growing in a cultured cell to withstand a variety of stresses by entering into a non-septating phase for a period of time, from which it can exit and revert to normal development upon removal of the stressor. The formation of abnormally enlarged, multinucleated (Lambden et al., 2006) aberrant RBs[1] (aRBs) may parallel stress-induced non-dividing filamentous forms in other bacterial species that unlike *Chlamydia* are constrained by a rod-shaped peptidoglycan sacculus (e.g., *Escherichia coli*, *Bacillus subtilis*). Other stressors that cause multiple *Chlamydia* spp. growing *in vitro* to produce aRBs have been described since. These include chlamydiaphage superinfection of *Chlamydia caviae* (Hsia et al., 2000), co-infection with viruses (Deka et al., 2006; Borel et al., 2010) or protozoa (Romano et al., 2012, 2013), growth of *C. trachomatis* in monocytes (Koehler et al., 1997), macrophage-like (Nettelnbreker et al., 1998), or fibroblast-like synovial cells (Hanada et al., 2003), iron restriction of *C. trachomatis* (Raulston, 1997), and exposure to a variety of antibiotics (Gieffers et al., 2004). These forms have also been observed *in vivo* in varied

[1] Herein referred to as aberrant RBs or aRBs; although these have been variably named aberrant bodies (ABs), or persistent bodies (PBs), this should be discouraged as it convey that these forms have uniform properties across multiple stressors.

contexts that may or may not relate to a persistent chlamydial infection (Borel et al., 2008; Pospischil et al., 2009; Rank et al., 2011; Phillips Campbell et al., 2012).

In the battleground that is an infection site, it is predictable that both the pathogen and the infected host are experiencing stress. Thus, chlamydiae undergoing a stress response should predictably appear during infection irrespective of whether the infection is acute or persistent. Indeed, aRBs can even be observed, albeit infrequently, in *in vitro* culture in the absence of any apparent stress (Tan et al., 2010). Moreover, the published literature is uneven in reporting the occurrence of typical, highly enlarged aRBs in association with stress-induced *in vitro* persistence/recurrence. In some reports, "aberrant inclusions" are referred to, confusingly implying they contain aRBs or other aberrant forms (e.g., miniature RBs), or that the inclusions themselves are aberrant (e.g., smaller vs. larger than normal). When found, the aRBs may also differ in their general appearance (e.g., Variable vacuolation). An interesting case may be that of *C. trachomatis* serovar L2 strains whereby in some stress-induced persistence systems, typical, highly enlarged aRBs are readily observed (Matsumoto, 1988; Coles et al., 1993; Harper et al., 2000; Lambden et al., 2006; Capmany and Damiani, 2010; McKuen et al., 2013) while they are not readily apparent in others (Rothermel et al., 1983; Shemer and Sarov, 1985; Huston et al., 2008; Skilton et al., 2009). While such differences may reflect experimental discrepancies or systematic differences, they globally reflect that a putative stress response-based persistence phenotype is unlikely to adhere to a single operational mode as highlighted previously by Wyrick (2010). Taken together, the molecular, cellular, and mechanistic diversity of the stress response in different *Chlamydia* strains, serovars and species growing in different cells, and the parallel diversity of conditions, which in different cells and sites of the infected host, can lead to chlamydial stress, signify that the observation of aRBs in an infected site is not sufficient to define a persistent infection. The observation that the requirements of the persistence/recurrence pathway can be fulfilled without the production of aRBs also supports that these forms are not necessary for persistence to occur.

Whether the stress response is involved in clinically observable persistence is an open question for which the model systems have not provided an answer as of yet. What is persistence? Without a clear answer to this question, it is even more difficult to answer the question of how it should be referred to. Without going into the uncertainties on persistent *C. trachomatis* genital infection in humans (i.e., recurrence from a persistent state vs. re-infection from an infected partner), an answer to this question may be provided by expanding our field of vision beyond *C. trachomatis*, to the veterinary *Chlamydia* species. All veterinary *Chlamydia* spp., including the phylogenetically close relatives of *C. trachomatis*, *Chlamydia muridarum*, and *Chlamydia suis* that infect mice and pigs, respectively, are first and foremost residents of the digestive tract of their host. *C. muridarum* is used experimentally to reproduce a genital infection in the mouse that replicates many features of the human *C. trachomatis* infection (Rank, 1994), but is not known to cause disease in wild mice. All veterinary *Chlamydia* spp. are transmitted primarily via the oral-fecal route and are thought to only cause disease in special circumstances, for instance if the innate or immune defenses of a given animal are weakened, or because the nearby animal population is highly infected such that individual animals are constantly re-exposed to infectious chlamydiae (e.g., abortion "storms" caused by *Chlamydia abortus* Pospischil et al., 2002). A possible explanation for the relative inattention to the possibility of an enteric phase for *C. trachomatis* may, therefore, relate to human evolution, which has witnessed a continuous reduction in the role of the fecal-oral route of transmission in the dissemination of infectious diseases in human communities. Recent studies in the mouse: *C. muridarum* model by Yeruva, Rank and colleagues have demonstrated the ability of *C. muridarum* to persist in the gastrointestinal (GI) tract of mice after oral inoculation (Yeruva et al., 2013b). These authors have further shown that the first-line antibiotic, azithromycin, while effective at clearing genital infection, was ineffective at clearing the GI infection (Yeruva et al., 2013a). Jones and colleagues first alluded to the idea that *C. trachomatis* may survive passage through the digestive tract and colonize the lower digestive tract. They observed a correlation between positive pharyngeal and rectal cultures in women who reported no history of rectal intercourse (Jones et al., 1985). These authors also observed a strong association between genital and rectal infection in these women and proposed that "autoinoculation with infected genital secretions may be the primary mechanism by which such [rectal] infections are acquired." In contrast, Yeruva and Rank now propose that, in fact, autoinoculation may be going in the reverse direction, i.e., from the infected GI tract to the female genitalia (Rank and Yeruva, 2014). A sobering thought then is that the practice of oral sex, which is on the rise particularly in adolescent populations where chlamydial infections are also increasing, is providing the inoculum for a reservoir of *C. trachomatis* persisting in the GI tract of humans, thereby compensating for the loss of the fecal-oral route. The model proposed by Yeruva and Rank is remarkable in its simplicity and is consistent with well-known immune down-regulation mechanisms that maintain the gut microbiota and exclude pathogens. Occasional release of infectious EBs from a protected GI site and consequent autoinoculation of the genital tract would also explain the long periods of clinical "invisibility" some individuals experience.

I was asked by the editors of this special issue to attempt to provide new definitions for what *Chlamydia* researchers globally refer to as persistence. Clearly this is not possible without a better understanding of what persistence actually is. The possibility of an enteric phase for *C. trachomatis*, highlighted by the work of Yeruva and Rank, suggests that a *colonization* pathway to persistence I alluded to initially, whereby *C. trachomatis* is primarily a commensal of the GI tract that can occasionally cause disease when in the wrong place, should be seriously evaluated. Whether or not we continue to use, misuse, and abuse the word persistence in chlamydial biology may depend on it.

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Persistent *C. pneumoniae* infection in atherosclerotic lesions: rethinking the clinical trials

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The hypothesis that infectious agents are a risk factor for atherosclerosis has implicated multiple viral and bacterial pathogens in contributing either directly or indirectly to disease progression (Rosenfeld and Campbell, 2011). One of the most vigorously studied organisms has been *Chlamydia pneumoniae*, which has been associated with cardiovascular disease by seroepidemiological studies, detection of the organism by multiple methods in atherosclerotic tissue, and experimental studies demonstrating biological plausibility. Significantly, *C. pneumoniae* accelerates lesion progression in mouse and rabbit models of atherosclerosis (Muhlestein et al., 1998; Hu et al., 1999; Moazed et al., 1999; Fong, 2000; Blessing et al., 2001). Early small clinical trials determined whether treatment with macrolides (Azithromycin, Roxithromycin, and Clarithromycin) would be efficacious in secondary prevention of coronary heart disease. These studies yielded mixed results and had several limitations including the small numbers of patients and short duration of treatment and follow-up period (Grayston, 2003). However, half of them demonstrated some beneficial effects, which provided enthusiasm for the potential of antibiotic intervention in coronary artery disease. There have since been four large clinical trials collectively enrolling over 20,000 patients with stable coronary artery disease (WIZARD, ACES, and CLARICOR) or acute coronary syndrome (PROVE-IT–TIMI) (O'Connor et al., 2003; Cannon et al., 2005; Grayston et al., 2005; Jespersen et al., 2006). As there were short term beneficial effects in the WIZARD trial following a 3 month course of Azithromycin, two subsequent studies addressed whether longer term treatment would be efficacious in reducing coronary events. In the ACES study, patients were treated with Azithromycin for 1 year and followed for 46.8 months (Grayston et al., 2005). The PROVE IT-TIMI trial treated with gatifloxacin for a mean duration of 2 years (Cannon et al., 2005). Overall, none of these well-designed trials demonstrated any long term benefit of antibiotic treatment. Furthermore, two large scale trials subsequently found that treatment with either roxithromycin or rifalizil (PROVIDENCE-1) had no beneficial effects in patients with peripheral artery disease (Joensen et al., 2008; Jaff et al., 2009). Cumulatively, these trials clearly demonstrated that anti-chlamydial antibiotics should not be recommended for treatment of patients with coronary heart disease or peripheral artery disease. Prior to the completion of the PROVE-IT and ACES trials, Grayston commented that if the trials demonstrated a beneficial effect of antibiotics, this would provide additional evidence for a role of *C. pneumoniae* in pathogenesis, but would not prove causality (Grayston, 2003). Alternatively, he predicted that negative results would mostly likely dampen interest in the association of *C. pneumoniae* and atherosclerosis, but noted that failure of the clinical trials would not rule out a pathogenic role (Grayston, 2003). Indeed, the negative outcome led some to conclude that this proved that *C. pneumoniae* did not play a role in the pathogenesis of atherosclerosis (Danesh, 2005) and diminished interest in infectious agents as contributing factors for cardiovascular disease (Epstein et al., 2009).

However, other investigators have underscored several factors that warrant critical evaluation before dismissing *C. pneumoniae* as a contributor to atherosclerotic processes (Anderson, 2005; Taylor-Robinson and Boman, 2005; Deniset and Pierce, 2010; Muhlestein, 2011; Rosenfeld and Campbell, 2011). First, treatment was given to patients with "end stage of disease" that is likely not modifiable. By analogy, antibiotic treatment is not effective in individuals in which inflammation resulting from chronic *C. trachomatis* infection of the upper genital tract or eye has led to the fibrosis and scarring observed in tubal factor infertility and trachoma, respectively. Whether antibiotics would be efficacious in treatment of patients with early atherosclerosis has not been determined as such studies would be difficult to design and execute. Second, it is possible that antibiotic treatment might be ineffective due to pathogen burden as viruses or other bacteria contributing to atherosclerotic processes may not be susceptible to the chosen antibiotics (Epstein et al., 2009; Rosenfeld and Campbell, 2011). Third, the patients in the large scale trials had advanced atherosclerosis and the events being measured were likely due to plaque destabilization and rupture, events that may be independent of plaque progression due to infection. Fourth, a single antibiotic was used in the trials and it is possible that treatment

with a combination of antibiotics might be more effective as shown for patients with chronic *Chlamydia*-induced reactive arthritis (Carter et al., 2010). Last, and the focus of this opinion, is the ability of chlamydiae to establish persistent/chronic infection and the difficulty in treating such infections (Beatty et al., 1994; Grayston, 2003). Chlamydiae undergo a developmental cycle in which the elementary body, an infectious but metabolically inactive form, is not susceptible to antibiotics. The reticulate body, the intracellular replicating form, can establish persistence, a state in which the developmental cycle is arrested rendering the organism refractory to antibiotics. In a continuous cell culture model of *C. pneumoniae* infection thought to more accurately mimic *in vivo* conditions, a mixture of developmental forms, including aberrant forms characteristic of persistent organisms, are observed. In this model, prolonged treatment with antibiotics including azithromycin and clarithromycin failed to eliminate infection (Kutlin et al., 2002a,b). It was also demonstrated in infected monocytes *in vitro* and monocytes isolated from patients undergoing treatment with azithromycin for coronary artery disease that infection was recalcitrant to antibiotic treatment (Gieffers et al., 2001). In addition, various antibiotics can induce chlamydial persistence in cell culture, including azithromycin (Beatty et al., 1994; Gieffers et al., 2004; Wyrick and Knight, 2004). Recently, amoxicillin has been shown to result in the induction of reversible persistent *Chlamydia muridarum* infection in a mouse model of genital tract infection (Phillips Campbell, 2012). The ability of *C. pneumoniae* to establish persistent infection *in vivo* has been experimentally validated in a mouse model of lung infection (Malinverni et al., 1995a; Laitinen et al., 1996). At times post-infection in which the organism can no longer be cultured from the lungs (but pathology persists and the organism can be detected by PCR), treatment with cortisone acetate results in reactivation of infection and the ability to culture organisms. Importantly, in animal models, the organism is frequently detected by PCR or immunohistochemistry following treatment with antibiotics. In acute lung infection, treatment of mice with a single dose of azithromycin or doxycycline resulted in an inability to culture the organism compared to untreated controls. However, 77 and 25% of the culture-negative lungs were positive by PCR, respectively, and no differences in lung histopathology were noted (Malinverni et al., 1995b). This study suggests that infection was not eradicated and raises the question as to whether persistent infection was induced earlier in the course of infection as a result of treatment. In hyperlipidemic apoE-deficient mice, following either a single or three intranasal inoculations starting at 8 weeks of age, *C. pneumoniae* could be cultured from the aorta for 1–2 weeks after the first inoculation, although the aorta remained PCR positive up to 28 weeks of age. These results suggest that the organism can establish persistent infection of the aorta. This was also supported by immunohistochemical detection of the organism in foam cells in 24 week old mice (Moazed et al., 1997, 1999). Significantly, two independent studies were done in this model in which mice were infected and treated with azithromycin at a dose that is comparable to that given to humans for chlamydial respiratory infection (Rothstein et al., 2001; Blessing et al., 2005). In the first study, mice were infected twice, 1 week apart, and treated with azithromycin 2 weeks after each inoculation (Rothstein et al., 2001). In the other study, mice were infected mice three times, 1 week apart and received a 6 week course of azithromycin. In the latter study, mice were treated on days 3, 4, and 5 after the third infection and once a week for 5 subsequent weeks (Blessing et al., 2005). Neither treatment regimen had any beneficial effects on *C. pneumoniae* accelerated atherosclerosis. In the first study, at the endpoint of 26 weeks of age (12 weeks after the second inoculation), *C. pneumoniae* DNA was identified in lung, heart and aorta in 50% of both treated and untreated mice. An earlier study in New Zealand White rabbits treated with azithromycin for 7 weeks immediately following the third infection, demonstrated a decrease in *C. pneumoniae* accelerated intimal thickening. However, *C. pneumoniae* antigen was still detected in 3/10 treated rabbits in comparison to 2/9 untreated animals (Muhlestein et al., 1998). Fong et al. found that the time of treatment with antibiotic was key to mitigating the effect of *C. pneumoniae* infection on atherosclerosis development in rabbits. Early treatment of acute infection with clarithromycin, resulted in reduced effects; however, with delayed treatment there was not a statistically significant reduction in the detection of organism in atherosclerotic tissues in comparison to untreated rabbits. These studies provide further evidence that *C. pneumoniae* establishes persistent infection *in vivo*, which is refractory to antibiotic intervention.

A large number of studies from independent laboratories demonstrated the presence of the organism within human atherosclerotic tissue by detection of *C. pneumoniae* antigen and/or DNA (Campbell and Kuo, 2004; Taylor-Robinson and Boman, 2005). However, isolation of the organism has been rare (Ramirez, 1996; Jackson et al., 1997), suggesting that *C. pneumoniae* establishes persistent infection in the vasculature. Unfortunately, there are no clearly defined markers of persistent infection in humans. To identify such markers, Borel and her colleagues applied tissue microarray (TMA) technology coupled with immunohistochemistry using antibodies prepared against proteins that were differentially expressed *in vitro* in a gamma-interferon induced model of *C. pneumoniae* persistence (Molestina et al., 2002; Mukhopadhyay et al., 2006) and examined archived tissues from patients undergoing heart transplantation. An advantage of this tissue set was that *C. pneumoniae* had been detected in 7 of 12 patients by various methods and the organism was cultured from 1 of these patients (Ramirez, 1996). By TMA analysis, heart tissue from 10 of 12 patients were positively stained with antibodies against proteins upregulated in the persistent state (GroEL and GroES) and all were negative when stained with an antibody against a downregulated protein (Borel et al., 2006). Using a subset of these specimens, "aberrant" forms were visualized by transmission electron microscopy (TEM) and immunogold labeling with antibodies against GroEL and GroES. These forms were confirmed as *C. pneumoniae* by double labeling with other *C. pneumoniae* specific antibodies

providing evidence of persistent infection in atheromas (Borel et al., 2008). A recent prospective study stained coronary heart tissue from patients undergoing heart transplants with a panel of antibodies against *C. pneumoniae* proteins upregulated in "aberrant" forms and detected antigen in 11 of 13 patients, supporting the notion that these antigens may serve as a marker of persistent infection (Borel et al., 2012). However, none of the tissues were PCR positive, nor was any ultrastructural evidence of the organism observed by TEM (Borel et al., 2012). The latter may reflect sampling size and the limited area of the atheroma that can reasonably be analyzed by this method. In our experience with immunohistochemical staining, detection of the organism in atherosclerotic lesions is localized and analysis of sequential sections can yield disparate results. The negative PCR results are more difficult to interpret when compared with the studies of archived tissue, although sampling may again play a role.

In conclusion, the negative outcome of the antibiotic trials should not result in dismissing substantive evidence supporting *C. pneumoniae* infection as a potential contributor to atherosclerotic processes without rigorous investigation of other factors that may alternatively explain the lack of benefits (or not). One of these is the ability of chlamydiae to establish persistent infection, a state that is refractory to antibiotic treatment. The availability of mouse models of persistent chlamydial infection should be exploited to specifically address whether: (1) antibiotics induce persistent *C. pneumoniae* infection in the vasculature; (2) persistent infection of the vasculature can be reactivated by immunosuppression; (3) the absence of an effect of antibiotic intervention on *C. pneumoniae* accelerated atherosclerosis is due to persistent infection and (4) transcriptional profiles that characterize persistence can be demonstrated *in vivo* as recently demonstrated for *C. muridarum* (Carey et al., 2013). More challenging is the identification of diagnostic markers or transcriptional signature patterns of persistent viable *C. pneumoniae* infection in humans, which may differ from those observed in experimental models of persistent infection and vary depending on the environmental factors in the host contributing to persistent infection in different anatomical sites.

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Laboratory diagnosis of persistent human chlamydial infection

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Diagnostic assays for persistent chlamydial infection are much needed to conduct high-quality, large-scale studies investigating the persistent state *in vivo*, its disease associations and the response to therapy. Yet in most studies the distinction between acute and persistent infection is based on the interpretation of the data obtained by the assays developed to diagnose acute infections or on complex assays available for research only and/or difficult to establish for clinical use. Novel biomarkers for detection of persistent chlamydial infection are urgently needed. Chlamydial whole genome proteome arrays are now available and they can identify chlamydial antigens that are differentially expressed between acute infection and persistent infection. Utilizing these data will lead to the development of novel diagnostic assays. Carefully selected specimens from well-studied patient populations are clearly needed in the process of translating the proteomic data into assays useful for clinical practice. Before such antigens are identified and validated assays become available, we face a challenge of deciding whether the persistent infection truly induced appearance of the proposed marker or do we just base our diagnosis of persistent infection on the presence of the suggested markers. Consequently, we must bear this in mind when interpreting the available data.

Keywords: diagnosis, persistent infection, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, proteomics

BACKGROUND

The clinical spectrum of human chlamydial infections includes clinically unapparent infections, acute symptomatic infections as well as persistent infections (defined as the presence of viable but non-cultivable chlamydiae). Persistent *Chlamydia psittaci* infection in cultured cells was described over 30 years ago (Moulder et al., 1980). Similar continuous infection models in cell lines without external induction have later been established for *Chlamydia trachomatis* (Lee and Moulder, 1981) and *Chlamydia pneumoniae* (Kutlin et al., 1999). In cultured cells, persistent infection can also be induced by external factors, including amino acid starvation, interferon-γ-induced tryptophan deprivation, iron chelation, tobacco smoke and viral co-infection as well as by exposure to antimicrobial agents (for review, see Beatty et al., 1994; Hogan et al., 2003). Furthermore, chlamydial infection in monocyte/macrophage cultures has the appearance of a persistent infection (Mannonen et al., 2004, 2011). The presence of large, pleomorphic reticulate bodies, named aberrant bodies, inhibition of binary fission and inability of the aberrant bodies to transform into infectious elementary bodies, characterize *in vitro* persistence. Transcriptomic and proteomic analyses have confirmed that there is continued genome replication and messenger RNA synthesis in the aberrant bodies, but altered cell division (Nicholson et al., 2003; Mäurer et al., 2007).

Although the persistence of *C. trachomatis* and *C. pneumoniae in vitro* is rather well studied, much less is known, if the features typical of *in vitro* persistent infection also characterize persistent experimental or human chlamydial infection. Recently, aberrant bodies were shown to develop in uterine horns of mice infected with *C. muridarum* and treated with amoxicillin (Phillips Campbell et al., 2012). Amoxicillin treatment also reduced vaginal shedding of infectious EB while accumulation of the chlamydial pre—16s rRNA was unaffected (Phillips Campbell et al., 2012). This indicates that chlamydial persistent infection can occur *in vivo*. As the bacteria become dormant and persistent, they are suggested to evade host protective immune responses, although the immune system can be still be stimulated at low-level which may contribute to the development of immune-mediated pathology (Beatty et al., 1994; Phillips Campbell et al., 2012). Follow-up studies on human ocular and recurrent genital *C. trachomatis* infections have shown features common to persistent state, such as the frequent detection of chlamydial nucleic acid while the organisms can infrequently be cultured (Hudson et al., 1992; Dean et al., 2000). Clinical data also suggests that *C. pneumoniae* may persist for months after the initial infection (Grayston, 1992), and persistent lung infections despite of antimicrobial therapy have been reported (Falck et al., 1996; Miyashita et al., 2002). *C. trachomatis* DNA has been demonstrated by *in situ* hybridization in the fallopian tube tissue from infertile women (Campbell et al., 1993; Barlow et al., 2001). Significantly elevated IgA antibody levels considered suggestive of persistent infection were found among the currently or formerly smoking men compared to their non-smoking co-twins (von Hertzen et al., 1998). Moreover, electron micrographs have demonstrated different stages of *C. pneumoniae* in atherosclerotic plaques (Spagnoli et al., 2007; Bobryshev et al., 2008), and

antigens whose expression is up regulated during the persistent state of *C. pneumoniae* have been detected in human atheromas (Borel et al., 2012).

In vitro, chlamydial persistence has also been characterized at molecular level. Although the phenotype of persistent infections is rather similar irrespective of the inducer, the transcriptional studies on persistent chlamydial infection have failed to reveal a profile common for different models (Hogan et al., 2003; Goellner et al., 2006; Klos et al., 2009). This might reflect variation in the experimental systems but it could also indicate variability of the mechanisms acting in the different models of persistent infection. Based on the relative chlamydial rRNA transcript levels in persistently infected monocytes and actively growing epithelial cells, the metabolic rate of *C. trachomatis* in monocytes is lower than in the cells where *C. trachomatis* grows productively (Gérard et al., 2002). When chlamydial transcription and translation were analyzed upon IFN-γ exposure in the epithelial cells, the global chlamydial transcription was shown to be up regulated but the protein synthesis was reduced suggesting uncoupling of transcription and translation (Ouellette et al., 2006). Although this phenomenon can represent a successful survival strategy for *Chlamydiae*, it might also mean less potential chlamydia-derived biomarkers available for detection of the persistent stage. Nevertheless, quantitative proteomic profiling of the persistent and the acute chlamydial infection to identify differentially expressed proteins represents a possibility for providing diagnostic targets for the persistent infection. The proteins associated with persistent infection could be useful as biomarkers for the diagnosis, as therapeutic targets, and ultimately as response-to-therapy markers. However, the initial studies in this area also failed to reveal a common profile between different models and different chlamydial species (Mukhopadhyay et al., 2006). Upon exposure to IFN-γ, the presence of aberrant *C. trachomatis* forms was associated with down-regulation of the outer membrane proteins (OmpA, OmpB) in cell culture, while the expression of chlamydial GroEL was unaltered (Beatty et al., 1993). Attempts to characterize the protein composition of *C. pneumoniae* during persistent stage have shown a marked up-regulation of OmpA and GroEL upon IFN-γ exposure, whereas no significant decrease in bacterial protein expression was observed (Molestina et al., 2002). On the other hand, upon iron limitation unchanged levels of *C. pneumoniae* OmpA and GroEL were observed, while altogether twenty differentially regulated chlamydial proteins were observed (Wehrl et al., 2004). Many of these proteins remained unidentified.

A serum-based assay would be most desirable for the diagnosis of persistent infection. Serum samples are relatively easily obtained whereas tissue samples from the site of persistent infection often require invasive sampling and are thus not readily available. The recent studies on serology during persistent infection suggest that the antibody response could indeed reflect altered protein of chlamydial expression *in vivo* but this needs further studies (see below). Despite of all the recent discoveries, the development of diagnostic tests for persistent infections remains challenging.

In this minireview, diagnostic options of human persistent chlamydial infection will be discussed (see also **Table 1**).

CULTURE

By definition, the detection of persistent chlamydial infection (defined as the presence of viable but non-cultivable chlamydiae) by culture is not possible. Culture has, however, been successfully used in the early studies on persistency. During experimental mouse infection, *Chlamydiae* are able to remain in tissues after cessation of shedding and the infection can be reactivated by cortisone treatment (Yang et al., 1983; Malinverni et al., 1995; Laitinen et al., 1996; Cotter et al., 1997). In these studies, mice were infected and the infection was followed by culture. After the clearance of a culture-positive infection, cortisone was administered. This obviously activated persistent *Chlamydiae*, as infectious bacteria could again be recovered, at least in a proportion of mice.

Although the persistent state is still rather poorly defined *in vivo*, this observation is of potential significance in human persistent infection: Corticosteroids are included e.g., in the treatment of patients with exacerbations of asthma and chronic obstructive bronchitis, conditions associated with persistent *C. pneumoniae* infection (Hahn et al., 1991), and the steroid therapy could reactive infection. If this takes place *in vivo*, it might aggravate infection, and enhance transmission but on the other hand, it might make the bacteria more sensitive to action of antibiotics. There is, however, little, if any clinical data to support this. Partly this could be due to issues related to proper sample collection, as the persistent chlamydial forms can localize in tissues, and not necessarily in the mucosal surface available for swabbing (Cappuccio et al., 1994). Recently, biologic response modifiers have been introduced to the treatment of inflammatory diseases. Whether these new therapies could also reactivate persistent chlamydial infection, remains to be studied.

ANTIGEN DETECTION

To circumvent limitations of the culture, immunological antigen detection methods, such as enzyme immunoassay and direct immunofluorescence staining were developed for diagnosis of chlamydial infections. Theoretically, detection of a chlamydial antigen in clinical specimens could serve as a diagnostic test for persistent infection. However, the general notion is that the detection of chlamydial EB in mucosal smears indicates presence of a current *C. trachomatis* (Havlichek et al., 1990) or *C. pneumoniae* (Grayston et al., 1986) infection. It would obviously require more invasive sampling than swabbing of the mucosa to demonstrate the hallmarks of persistent infection, the aberrant bodies, at the site of infection. Moreover, the amount of aberrant bodies in the specimen is likely to be very small and reagents to visualize them are not readily available.

For research purposes, the detection of chlamydial antigen(s) by immunocytochemistry (ICC) in tissues has proven useful and ICC has assisted studies on clinical features of persistent infections, including post infectious tubal infertility (Patton et al., 1994) and atherosclerosis (Kuo and Campbell, 2000). Instead, detection of a soluble circulating chlamydial antigen could represent a practical tool in diagnosis. The quantification of chlamydial lipopolysaccharide (cLPS) in human sera by enzyme immunoassay represents a potential marker for persistent infection (Tiirola

Table 1 | Potential biomarkers of persistent human chlamydial infection.

Marker	Chlamydial species	Remarks	Condition	References
Chlamydial LPS-containing immune complexes in serum	*C. pneumoniae*	Technically demanding assay	Acute myocardial infarction Chronic coronary heart disease COPD Stroke	Leinonen et al., 1990 Linnanmäki et al., 1993 Von Hertzen et al., 1997 Tarnacka et al., 2002
Chlamydial LPS in tissues/serum	*C. pneumoniae*	Technically demanding assay	Atherosclerosis Acute coronary event Novel cardiovascular event	Kuo et al., 1993; Vikatmaa et al., 2010 Tiirola et al., 2007 Pesonen et al., 2009a,b
Elevated IgG and IgA antibody	*C. pneumonia*		Asthma	Von Hertzen et al., 1997; Hahn et al., 2000
	C. trachomatis		Infertility	Sarov et al., 1986
IgA antibody response to whole bacteria	*C. pneumoniae*	Interlaboratory variation in MIF	Acute coronary syndrome Asthma Predictor of treatment response Increased intima-media thickness in children	Huittinen et al., 2003; Miya et al., 2004 Sävykoski et al., 2004; Von Hertzen et al., 1997; Hahn and Saikku, 1995 Hahn et al., 2006 Volanen et al., 2006
IgE antibody response	*C. pneumoniae*	Immunoblot format	Asthma, severity of asthma	Emre et al., 1995; Hahn et al., 2000, 2012
Chlamydial DNA in mucosal swabs or tissues	*C. pneumoniae*	Not detected in veins or in the vessel wall in the absence of pathology	Atherosclerosis COPD	Kuo et al., 1993; Rosenfeld and Campbell, 2011 Von Hertzen et al., 1997
	C. trachomatis		Trachoma Tubal factor infertility	Mabey and Solomon, 2003 Campbell et al., 1993; Barlow et al., 2001
Chlamydial rRNA in tissues	*C. trachomatis*	Denotes presence of metabolically active bacteria	Reactive arthritis Trachoma	Gérard et al., 1998 Burton et al., 2006
Antibody to GroEL (CPn0134 or CT110)	*C. pneumoniae*	Autoimmunity manifested as antibody to human Hsp60 contributes to pathogeneses	Asthma Coronary heart disease	Huittinen et al., 2001 Huittinen et al., 2002; Mahdi et al., 2002; Biasucci et al., 2003; Pesonen et al., 2009a
	C. trachomatis		Trachoma Subfertility Infertility Tubal factor infertility	Skwor et al., 2010 Karinen et al., 2004 Linhares and Witkin, 2010 Tiitinen et al., 2006; Bunk et al., 2008; Budrys et al., 2012
Antibody to CT858	*C. trachomatis*		Trachomatous trichiasis	Skwor et al., 2010
Seroresponse to a panel consisting of CT110, CT376, CT557, CT443 and absence of response to CT875, CT147	*C. trachomatis*	Some antigens also recognized by sera from acute infection Remains to be confirmed in larger settings	Tubal factor infertility	Budrys et al., 2012
Seroresponse to a panel consisting of antigens CPn0695, CPn0134, CPn0626CPn0702, CPn 449/450, CPn 0854,CPn 0963, CPn1016	*C. pneumoniae*	Remains to be confirmed in larger settings	Presence of *C. pneumoniae* DNA in the coronary artery or in the PBMC	Bunk et al., 2008

et al., 2006). Chlamydial LPS was detected in serum during acute coronary events and its presence correlated with the C-reactive protein levels (Tiirola et al., 2007). Moreover, the circulating cLPS was associated with a new cardiovascular event during the follow-up period (Pesonen et al., 2009b). Also, circulating chlamydial LPS-containing immune complexes have been suggested to be important in the pathogenesis and serve as markers of persistent infection (Leinonen et al., 1990; Linnanmäki et al., 1993), but the assay is technically rather demanding and it has only been used in research.

NUCLEIC ACID AMPLIFICATION

Nucleic acid amplification test kits (NAATs) have revolutionized the diagnosis of acute *C. trachomatis* infections offering high sensitivity and specificity. The NAATs, including polymerase chain reaction (PCR), are also well suitable for the detection of persistent chlamydial infection, when lower quantities of *Chlamydiae* are produced challenging the sensitivity of the detection method. Indeed, the use of amplification technologies and culture has given us clues that persistent, non-cultivable chlamydial infections do occur *in vivo*. Trachoma in children can often be diagnosed by culture, whereas the agent can no longer been cultured in the blinding stage (Grayston and Wang, 1975). Similarly, *C. trachomatis* can be cultured in acute lower genital tract infections, while culture rarely succeeds in upper genital infections, including tubal factor infertility, although *C. trachomatis* DNA can be amplified by PCR (Brunham et al., 1985). The mere demonstration of bacterial DNA does not, however, indicate viability, but could be related to the higher sensitivity of the PCR method *per se*. Identification of chlamydial rRNA primary transcripts indicates that the bacteria are metabolically active (Gérard et al., 1997). Indeed, in the synovial samples from patients with reactive arthritis primary rRNA transcripts and mRNA from chlamydial genes were present (Gérard et al., 1998).

PCR with *C. pneumoniae*-specific primers has played an important role in confirming the presence of DNA from these bacteria in atheromas and circulating blood cells. In combination with serology and ICC, the PCR has been a valuable tool in the studies confirming the link between atherosclerosis and infections, especially with persistent *C. pneumoniae* infection (Campbell and Kuo, 2004; Rosenfeld and Campbell, 2011). Current data supports that this condition represents a persistent infection: *Ex vivo* culture of *C. pneumoniae* from atherosclerosis is rare, while PCR can frequently detect chlamydial DNA in diseased tissues (Campbell and Kuo, 2004). Studies in mice show that the presence of *C. pneumoniae* DNA in tissues is an indicator of prior infection, and when the bacteria are not cultivable it is suggestive of persistence, as DNA in the non-viable bacteria degrades fast *in vivo* (Moazed et al., 1998).

SEROLOGY

Serology has been a valuable tool in epidemiological studies, in studies describing clinical spectrum of chlamydial infections, including persistent infection, and in research. Serology is also commonly used to diagnose acute *C. pneumoniae* infections in clinical practice while its value in the laboratory diagnosis of acute *C. trachomatis* infections is slim. It may, however, prove helpful when studying complications of the acute phase (e.g., reactive arthritis) and manifestations of persistent infections (e.g., tubal factor infertility). Microimmunofluorescence (MIF) test using fixed purified whole bacteria as antigen has been considered a golden standard of chlamydial serology, but automatable enzyme immunoassays (EIA) have taken root in clinical laboratories, partly because the MIF can suffer from subjectivity in interpretation and interlaboratory variation (Peeling et al., 2000). The MIF and most EIAs detect antibody response against proteins on the surface of *Chlamydiae*, and established guidelines on the diagnostic criteria for acute *C. pneumoniae* infections are available: IgM appears in the first infection in 3 weeks, IgG in 6–8 weeks, and if adequately timed paired sera are available, a ≥4-fold IgG titer rise can be seen upon infection and reinfection (Wang and Grayston, 1986).

Despite of considerable efforts, persistent infections are difficult to diagnose, and no widely accepted serological criteria for persistent infection exist at present. IgG antibody can persist long after the acute phase (Puolakkainen et al., 1986; Paldanius et al., 2005), and only reflects (previous) exposure to the organisms. Some markers have yet been brought forward. Elevated IgE levels are almost always associated with asthma, a proposed manifestation of persistent *C. pneumoniae* infection, but the inducing factor for the IgE response has largely remained unknown. In a recent case-control study, *C. pneumoniae*-specific IgE antibody response was strongly associated with asthma and with severity of asthma in adults (Hahn et al., 2012). IgA antibodies are naturally short-lived with a half-life of 5–6 days. Consistently present/elevated *C. pneumoniae* IgA antibody titers (Saikku et al., 1992), and elevated serum *C. pneumoniae* IgA and IgG antibodies together with elevated C-reactive protein (Huittinen et al., 2003; Miya et al., 2004; Sävykoski et al., 2004) have been proposed as serological markers of persistent *C. pneumoniae* infection. IgA response could also be a predictor of treatment response (Hahn et al., 2006). Additionally, an association between *C. pneumoniae* IgA and human Hsp60 antibodies noted in patients with risk of coronary events (Huittinen et al., 2002) suggests that antibodies to human Hsp60 could be induced by bacterial GroEL (*C. pneumoniae* Hsp60 homolog, CPn0134) during persistent infection. Likewise, enhanced antibody response to *C. trachomatis* EB and chlamydial GroEL (*C. trachomatis* Hsp60 homolog, CT110) protein is associated with trachoma (Skwor et al., 2010) subfertility (Karinen et al., 2004), infertility, especially tubal factor infertility, a manifestation of persistent *C. trachomatis* infection (Toye et al., 1993; Witkin et al., 1998; Tiitinen et al., 2006).

Although the performance of the serological assays using whole bacteria as antigen and developed for the detection of acute infection might not be perfect, they have been valuable in epidemiological studies and when patient populations with a given condition have been studied. Moreover, the expanding clinical spectrum of chlamydial diseases, including those associated with persistent infection, was initially based on the findings obtained with the traditional but specific chlamydial serology. The antibodies recognized by the MIF are directed against the surface proteins of the chlamydial EB. OmpA (major outer membrane protein) is a dominant immunogen in *C. trachomatis*, but the (*C. pneumoniae*) proteins contributing to the MIF reactivity have largely remained

unknown (Bunk et al., 2008). Proteomic analyses of chlamydial infection have revealed that a part of the surface proteins are more frequently and a part of them less frequently expressed during persistent than acute infection. Consequently, the MIF test as well as the EIAs using whole bacterial antigen might not be able to adequately discriminate between acute and persistent infections (Bunk et al., 2008). In support of this observation, reactivity of the sera from individuals with persistent infection in the MIF tests is necessarily not reflected by the reactivity of these sera with differentially expressed antigens, including chlamydial GroEL (Huhtinen et al., 2001; Bunk et al., 2008).

Proteomic profiling of chlamydial infection may enable the development of more precise serological assays that could better differentiate responses related to acute infection, cured past infection (serological scar) and persistent infection. The history and clinical status of the patients donating the serum specimens must be characterized appropriately. This is not always possible and straightforward, and may need invasive and extensive examinations. To identify antibody response patterns associated with persistent *C. pneumoniae* infection, Bunk et al. (2008) applied a proteomic approach with 2D-gel electrophoresis combined with immunoblotting. Using serum specimens from patients with probable persistent infection, based on the presence of *C. pneumoniae* DNA in their coronary arteries or in their PBMC, and blood donors without such markers of persistent infection, the researchers could identify a differential response against 12 *C. pneumoniae* antigens, including both increased and decreased reactivity (Bunk et al., 2008). Sera from the PCR-positive individuals reacted more intensely with *C. pneumoniae* CPn0695 (OmpA) and CPn0134 (GroEL) [and also with 6 other antigens, CPn0626 (RpoA), CPn0702 (YscC), CPn449/450 (Pmp10), CPn0854 (PorB), CPn0963 (Pmp21), and CPn1016 (Cpaf)] than sera from controls. The observed antibody response pattern was in accordance with the earlier mentioned *in vitro* proteomic studies showing higher and lower seroreactivity toward the proteins that were shown to be up-regulated and down-regulated, respectively, during IFN-γ-mediated persistence (Molestina et al., 2002; Mukhopadhyay et al., 2006). A recent description of a *C. pneumoniae* ORFeome library covering 99% of the 1052 the ORFs of *C. pneumoniae* provides an additional novel tool for studying the seroresponses during persistent phase in-depth (Maier et al., 2012).

To identify novel *C. trachomatis* proteins or protein combinations associated with different stages of infection, a high-resolution whole genome scale protein array (Wang et al., 2010) has been used to profile serological responses. Sera from patients with tubal factor infertility (TFI) and their controls recognized many *C. trachomatis* proteins (Wang et al., 2010). Comparison of the antibody profiles revealed that the sera from TFI patients preferentially recognized 30 *C. trachomatis* proteins. Those included CT110 (GroEL; 71% of sera reacted with this protein) confirming the earlier findings that response to the Hsp60 homolog is associated with TFI. In this study, no single antigen yielded 100% specificity and >50% sensitivity for detection of TFI, whereas a combination of CT443 (omcB) and CT381 (arginine binding protein) antigens yielded the highest detection sensitivity (68%) for chlamydial TFI while maintaining 100% specificity (Rodgers et al., 2011). However, also *C. trachomatis* antibody-positive individuals with acute *C. trachomatis* infection frequently reacted with GroEL, and diminished the value of this antigen as a marker of persistent infection. Subsequently, panels of *C. trachomatis* antigens that could predict tubal pathology and acute infection have been suggested. Using the same high-resolution approach, reactivity with four proteins [CT110 (GroEL), CT376 (malate dehydrogenase), CT557 (dihydrolipoamide hydrolase), and CT443 (OmcB)] could distinguish women with TFI from fertile women with a sensitivity of 63% and specificity of 100%. Reactivity with a combination of two *C. trachomatis* antigens (CT875 and CT147, both coding for hypothetical proteins) could discriminate between women with acute infection and those with TFI (Budrys et al., 2012) enabling sequential screening to identify patients with these conditions.

CONCLUSION

A serum-based assay (either for antibody detection, antigen detection or nucleic acid amplification) would be desirable for the detection of persistent infection. Serum samples are relatively easily obtained whereas tissue samples from the site of persistent infection are not readily available. The above-mentioned recent studies utilizing proteomics and bacterial genome wide approaches by Bunk et al. (2008), Rodgers et al. (2011) and Budrys et al. (2012) indicate that chlamydial serology can be made more precise and refined. These studies also demonstrate the first evidence of differential serological response as a function of the infection status. Discrimination of antibody response related to persistent infection from that related to acute infection and from a serological scar is important and clinically relevant, and these promising approaches wait to be confirmed in studies with larger populations.

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Commonly prescribed β-lactam antibiotics induce *C. trachomatis* persistence/stress in culture at physiologically relevant concentrations

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Chlamydia trachomatis, the most common bacterial sexually transmitted disease agent worldwide, enters a viable, non-dividing and non-infectious state (historically termed persistence and more recently referred to as the chlamydial stress response) when exposed to penicillin G in culture. Notably, penicillin G-exposed chlamydiae can reenter the normal developmental cycle upon drug removal and are resistant to azithromycin-mediated killing. Because penicillin G is less frequently prescribed than other β-lactams, the clinical relevance of penicillin G-induced chlamydial persistence/stress has been questioned. The goal of this study was to determine whether more commonly used penicillins also induce *C. trachomatis* serovar E persistence/stress. All penicillins tested, as well as clavulanic acid, induced formation of aberrant, enlarged reticulate bodies (RB) (called aberrant bodies or AB) characteristic of persistent/stressed chlamydiae. Exposure to the penicillins and clavulanic acid also reduced chlamydial infectivity by >95%. None of the drugs tested significantly reduced chlamydial unprocessed 16S rRNA or genomic DNA accumulation, indicating that the organisms were viable, though non-infectious. Finally, recovery assays demonstrated that chlamydiae rendered essentially non-infectious by exposure to ampicillin, amoxicillin, carbenicillin, piperacillin, penicillin V, and clavulanic acid recovered infectivity after antibiotic removal. These data definitively demonstrate that several commonly used penicillins induce *C. trachomatis* persistence/stress at clinically relevant concentrations.

Keywords: *Chlamydia trachomatis*, β-lactam, penicillin, antibiotic susceptibility, chlamydial persistence, chlamydial stress response, stressed chlamydiae

INTRODUCTION

The most common bacterial sexually transmitted disease (STD) agent in humans is *Chlamydia trachomatis* (serovars D-K), with 1,412,791 reported cases in the US in 2011 (Centers for Disease Control and Prevention, 2011). *C. trachomatis* genital infection is often chronic in women, with manifestations ranging from mild infection to infertility and ectopic pregnancy (Schachter, 1999). Most women with chlamydia-induced cervicitis are asymptomatic (Schachter et al., 1983) and at higher risk for serious complications. Genital co-infections with *Neisseria gonorrhoeae* and *C. trachomatis* infections are also frequent; in fact, *C. trachomatis* is historically the most common cause of post-gonococcal urethritis and cervicitis (PGU/PGC). Azithromycin and tetracycline/doxycycline are currently the treatments of choice for *C. trachomatis* infections in adults, though amoxicillin remains a recommended treatment for infected, pregnant women (Centers for Disease Control and Prevention, 2010).

C. trachomatis is a Gram-negative, obligate intracellular bacterium with a biphasic developmental cycle. The infectious, extracellular form (the elementary body or EB) enters host genital epithelial cells within an endosome. Following fusion of EB-containing endosomes, EB (0.2 μm diameter) develop into larger (0.8 μm), replicative, non-infectious reticulate bodies (RB). RB use ATP and metabolites from the host cell to grow and subsequently divide within a cytoplasmic inclusion. After 30–70 h, the RB mature into infectious EB and are released from the infected host cell (Wyrick, 2000).

It has become clear that the "biphasic" view of chlamydia development is incomplete. Exposure to certain adverse conditions can divert the developmental cycle into a state referred to as persistence or, more recently, as the chlamydial stress response. In persistence/stress, the chlamydiae are viable, but non-infectious (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). Penicillin G-exposure is one stressor that induces *C. trachomatis* to enter this state in culture. Penicillin G-exposed chlamydiae can remain persistent/stressed for up to 9 months and, upon antibiotic removal, can re-enter the normal developmental cycle (Galasso and Manire, 1961; Matsumoto and Manire, 1970). Persistent/stressed chlamydial inclusions contain enlarged RBs (abberent bodies or AB), and few infectious EBs (Matsumoto and Manire, 1970). Persistent/stressed chlamydiae are also viable, as indicated by: (i) continued synthesis of genomic DNA and unprocessed 16S rRNA; and (ii) their ability to re-enter the developmental cycle. However, they do not divide, are non-infectious, and less metabolically active (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). Published data also indicate that

chlamydiae also enter this altered developmental state *in vivo*. Continued chlamydial infections and repeat infections with the same serovar are common, despite appropriate antibiotic therapy (Patton et al., 1994; Fortenberry et al., 1999; Dean et al., 2000). Chlamydial AB have been observed in samples from patients and infected animals (Nanagara et al., 1995; Skowasch et al., 2003; Pospischil et al., 2009; Rank et al., 2011). Finally, viable but non-infectious organisms are observed in the genital tracts of amoxicillin-treated, *C. muridarum*-infected mice (Phillips-Campbell et al., 2012).

Although the mechanism by which penicillins exert their anti-chlamydial effects has been controversial, *C. trachomatis* does express 3 penicillin-binding proteins (PBPs) (Barbour et al., 1982; Storey and Chopra, 2001). More recent data suggest penicillin G, mecillinam and piperacillin may inhibit chlamydial cell division by binding Pbp2 and Pbp3/FtsI (Ouellette et al., 2012). However, penicillin G and amoxicillin are the only β-lactams demonstrated to render chlamydiae persistent/stressed using all generally accepted critera (Galasso and Manire, 1961; Matsumoto and Manire, 1970; Phillips-Campbell et al., 2012). Ampicillin exposure reduces chlamydial infectivity and alters inclusion morphology (Johnson and Hobson, 1977; Beale et al., 1991; Wolf et al., 2000; Storey and Chopra, 2001), while mecillinam and piperacillin induce AB formation (Storey and Chopra, 2001; Ouellette et al., 2012), suggesting induction of chlamydial persistence/stress. However, the first indication that β-lactams might not be lethal for developing chlamydiae came from clinical observations in the 1960s (Holmes et al., 1967; Richmond et al., 1972).These authors reported frequent cases of *C. trachomatis*-mediated PGU/PGC in patients several months following successful eradication of *N. gonorrhoeae* with penicillin therapy. As a result, in most cases, penicillins are not considered front-line anti-chlamydial drugs.

MATERIALS AND METHODS

CHLAMYDIA AND HOST CELLS

A human urogenital isolate of *C. trachomatis* E/UW-5/CX was obtained from S. P. Wang and C. C. Kuo (University of Washington, Seattle, WA). This strain was propagated in McCoy cells and used for all experiments. HeLa cells, a human cervical adenocarcinoma epithelial cell line (ATCC No. CCL2), were cultivated in growth medium (Minimal Essential Medium (MEM) with Earle's salts containing L-glutamine, 10% fetal calf serum (Atlanta Biologicals) and 1 μg/mL gentamicin) at 37°C and 5% CO_2 on standard tissue culture plates or glass coverslips.

CHLAMYDIAL INFECTION AND ANTIBIOTIC EXPOSURE

Host cell monolayers were either mock-infected (exposed to 2SPG: 0.2 M sucrose, 6 mM NaH_2PO_4, 15 mM Na_2HPO_4, 5 mM L-glutamine, pH 7.2) or *C. trachomatis*-infected at a multiplicity of infection (MOI) of 1, using crude EB stock diluted in 2SPG for 1 h at 35°C. Monolayers used for minimal inhibitory concentration (MIC)/immunofluorescent (IFA) or minimal bactericidal concentration (MBC) analyses were then immediately refed with growth medium plus diluent (ddH_2O) or the antibiotic of interest and incubated for 54 h at 35°C. Monolayers used in all other antibiotic-exposure experiments were first refed with medium and incubated at 35°C for 24 h, after which the supernatant was replaced with medium + diluent or the antibiotic of interest. Cultures were then harvested for analysis at 30 h post-antibiotic addition, a total of 54 h post-infection (hpi). In recovery experiments, replicate antibiotic-exposed cultures were washed at 54 hpi, refed with either antibiotic-replete (non-recovered group) or antibiotic-deficient (recovered group) medium, and incubated at 35°C for 3 additional days (a total of 126 hpi).

FLUORESCENT AND TRANSMISSION ELECTRON MICROSCOPY

IFA and high-contrast transmission electron microscopy (TEM) analyses were performed as described (Deka et al., 2006). Fluorescent photomicrographs were taken using a Zeiss Axiovert S100 microscope/Axiocam camera, and converted to grayscale using Adobe Photoshop V5.0.

CHLAMYDIAL INFECTIVITY ASSAY

Production of infectious *C. trachomatis* EB was assayed using a subpassage titer assay as described (Deka et al., 2006), except that phosphonoformate was omitted from the medium. The number of inclusion forming units (IFU)/mL in the undiluted inoculum was calculated from triplicate determinations.

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 (Deka et al., 2006). Total RNA and DNA preparations were quantified using optical density (OD) at 260 and 280 nm; all samples had OD260/280 ratios >1.9. RNA sample quality was assessed by using a 2100 Bioanalyzer (Agilent) and the RNA 6000 Nano LabChip kit. All samples had RNA Integrity Numbers (RINs) >9.0.

REVERSE TRANSCRIPTION, PCR, AND RT-PCR

Reverse transcription of total RNA, PCR, and RT-PCR were performed as described (Deka et al., 2006). PCR and RT-PCR was performed using purified total cellular DNA or cDNA as templates, respectively. Control and experimental templates were diluted from 1/10 to 1/1000 in ddH_2O to insure that each reaction was quantified in the linear amplification range. Primer sets used to amplify the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and chlamydial 16S rRNA genes, as well as for *C. trachomatis* unprocessed 16S rRNA transcript have been published (Deka et al., 2006). After PCR, all reaction products were electrophoresed, quantified and normalized as described (Deka et al., 2006).

STATISTICAL ANALYSES

Statistics were performed using Microsoft Excel. Means were compared by 2-sample t-test for independent samples. $P = 0.05$ were considered significant. All plotted values are averages of three biological replicates ± standard error of the mean (s.e.m.) and each experiment was performed three times independently.

RESULTS

RATIONALE FOR ANTIBIOTIC-EXPOSURE CONDITIONS

Though penicillin G is a characterized chlamydial persistence inducer, it is less widely used than other β-lactams. Also, as penicillins are no longer recommended for anti-chlamydial therapy in adults, one might question the relevance of penicillin-induced chlamydial persistence *in vivo*. It is important to recognize that penicillins are commonly utilized to treat other bacterial infections—with the result that developing chlamydiae within asymptomatically-infected individuals are exposed to β-lactams during therapy for other concurrent infections. Thus, the penicillins evaluated in this study were chosen primarily according to prescription frequency: amoxicillin (AMX), clavulanic acid (CLA), ampicillin (AMP), and penicillin V (PEN V) were all among the top 200 most prescribed drugs in the US in 2011 (Bartholow, 2011). Carbenicillin (CAR) and piperacillin (PIP) were chosen because of their high efficacy for Gram-negative bacteria. Aztreonam (ATM), a monobactam, was chosen because of its structural dissimilarity to penicillin. The cephalosporin ceftriaxone (CRO) was chosen because it is a recommended treatment for gonococcal infections (Centers for Disease Control and Prevention, 2010), which often occur simultaneously with *C. trachomatis*. Finally, cefotaxime (CTX) was chosen to confirm any effect of CRO on chlamydial development. The standard concentration of each drug used (denoted as 1X in **Table 1**) was equivalent to the serum concentration obtained after administration of a standard dose in clinical trials (McEvoy, 2004), increasing the *in vivo* relevance of any observed effects.

NO PENICILLIN OF INTEREST INHIBITS INCLUSION FORMATION

To determine whether the antibiotics of interest blocked inclusion formation or EB production, chlamydia-infected cells were exposed to 10-fold antibiotic dilutions from 1 to 54 hpi. MIC for the chlamydiae has been variously defined by either inhibition of inclusion formation (Welsh et al., 1992) or by abnormal inclusion morphology (Storey and Chopra, 2001). Because identification of "abnormal inclusions" seemed subjective, we defined the MIC as the minimal drug concentration that prevented inclusion development (Welsh et al., 1992). MBC was defined as the drug concentration required to reduce infectious EB production by >99% (Welsh et al., 1992). As no penicillin tested inhibited inclusion formation, even at 100 times the serum concentration, MICs could not be calculated (**Table 1**). In contrast, the MIC for tetracycline (TET) was 0.043 μg/mL (**Table 1**), similar to published values (Welsh et al., 1992). Exposure to all 6 penicillins tested, as well as to CLA, produced small inclusions

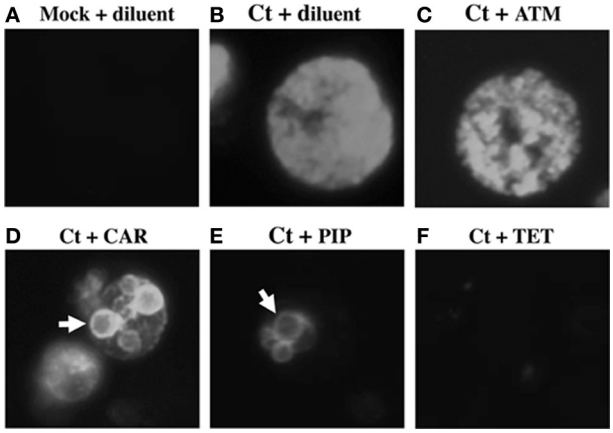

FIGURE 1 | Penicillin-exposure alters chlamydial morphology. HeLa cells were plated on glass coverslips, infected with *C. trachomatis* and refed immediately post-infection with growth medium + various dilutions of each antibiotic of interest. At 54 hpi, infected cells were fixed and stained with FITC-conjugated anti-*C. trachomatis* MOMP as described. **(A)** Mock-infected cells + ddH_2O (diluent). **(B)** *C. trachomatis*-infected (Ct) cells + diluent. **(C)** *C. trachomatis* + ATM. **(D)** *C. trachomatis* cells + CAR. **(E)** *C. trachomatis* + PIP. **(F)** *C. trachomatis* + TET. Panels **(B–E)** are representative inclusions photographed at 1000X magnification. Inclusions in panels **(C–F)** were drug-exposed at the 1X concentration (**Table 1**).

Table 1 | The 1X concentrations, MIC and MBC of all drugs used in this study.

Drug	Class	Serum (1X)* concentration	MIC**	MBC***
Amoxicillin (AMX)	Penicillin	11 μg/mL	>1100 μg/mL	0.011 μg/mL
Ampicillin (AMP)	Penicillin	3.7 μg/mL	>370 μg/mL	0.037 μg/mL
Aztreonam (ATM)	Monobactam	1.8 μg/mL	>180 μg/mL	>180 μg/mL
Carbenicillin (CAR)	Penicillin	1.9 μg/mL	>190 μg/mL	0.019 μg/mL
Cefotaxime (CTX)	Cephalosporin	2.53 μg/mL	>253 μg/mL	>253 μg/mL
Ceftriaxone (CRO)	Cephalosporin	0.93 μg/mL	>93 μg/mL	>93 μg/mL
Clavulanic acid (CLA)	β-lactamase inhibitor	3.5 μg/mL	>350 μg/mL	0.35 μg/mL
Penicillin G (PEN G)	Penicillin	20 U/mL	>2000 U/mL	0.02 U/mL
Penicillin V (PEN V)	Penicillin	0.2 μg/mL	>20 μg/mL	0.002 μg/mL
Piperacillin (PIP)	Penicillin	39 μg/mL	>3900 μg/mL	0.39 μg/mL
Tetracycline (TET)	Tetracycline	4.3 μg/mL	0.043 μg/mL	ND

**The 1X concentration was defined as the peak serum concentration of each drug obtained in clinical trials after administration of a standard dose (McEvoy, 2004).*

***The MIC was defined as the minimal concentration of each drug required to prevent formation of chlamydial inclusions. In most cases, a precise MIC could not be determined because the highest tested drug concentration (100X) did not prevent inclusion formation.*

****The MBC was defined as the minimal concentration of each drug required to reduce infectious EB production in sub-titer assays by >99%. In the case of ATM, CTX, and CRO, MBC could not be determined because the highest tested drug concentration (100X) did not reduce infectious titer by >99%.*

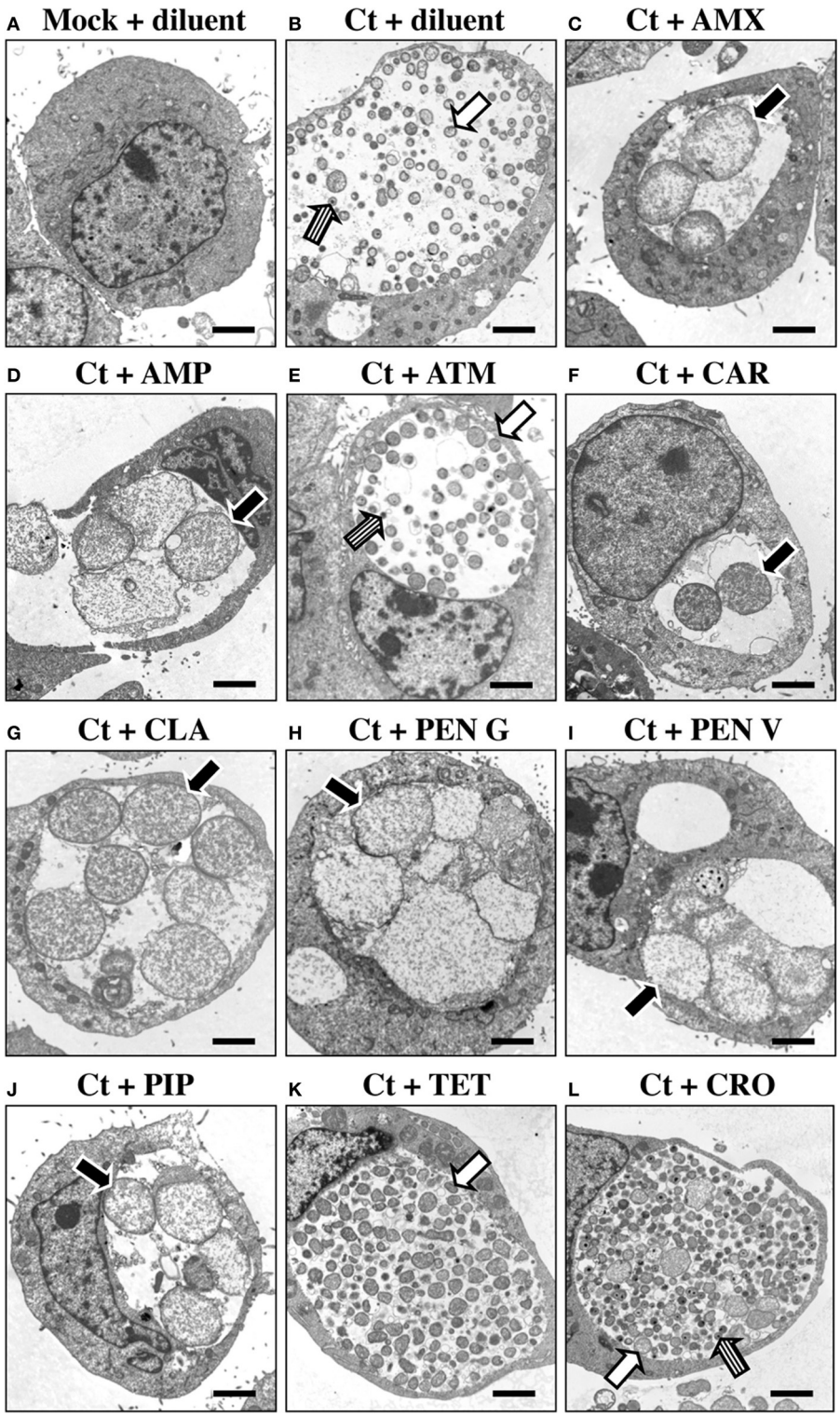

FIGURE 2 | Penicillin-exposure induces chlamydial AB formation. HeLa cells were *C. trachomatis*-infected and incubated in the absence of antibiotic for 24 h. Infected and uninfected cultures were then refed with medium containing each antibiotic of interest at the 1X concentrations (**Table 1**). Cells were incubated for an additional 30 h (a total of 54 hpi), fixed and subjected to TEM. **(A)** Mock-infected cells + ddH_2O (diluent). **(B)** *C. trachomatis*-infected (Ct) cells + diluent. **(C)** *C. trachomatis* + AMX. **(D)** *C. trachomatis* + AMP. **(E)** *C. trachomatis* + ATM. **(F)** *C. trachomatis* + CAR. **(G)** *C. trachomatis* + CLA. **(H)** *C. trachomatis* + PEN G. **(I)** *C. trachomatis* + PEN V. **(J)** *C. trachomatis* + PIP. **(K)** *C. trachomatis* + TET. **(L)** *C. trachomatis* + CRO. Morphologically normal RB and EB are indicated by white and striped arrows respectively. Abberent bodies (AB) are labeled with black arrows. Each photomicrograph is at 7500X magnification; the black bar at the lower right of each panel represent 2 μm.

containing large, spherical structures, similar to AB formed during persistence/stress (**Figures 1D,E**, white arrows). In contrast, diluent- (ddH_2O), ATM- and cephalosporin-exposed inclusions were indistinguishable from each other (**Figures 1B,C** and data not shown). Finally, mock-infected controls contained no inclusions (**Figure 1A**) and TET-exposure prevented inclusion formation (**Figure 1F**), as expected. Thus, all penicillins tested altered inclusion morphology, but did not prevent inclusion development.DNA and unprocessed

PENICILLIN-EXPOSURE INDUCES RB MORPHOLOGIC ALTERATIONS CHARACTERISTIC OF PERSISTENCE/STRESS

PEN G-exposed, persistent/stressed chlamydial RB (termed AB) have a distinctive morphology (Matsumoto and Manire, 1970). To determine whether exposure to other β-lactams induces similar structural alterations, *C. trachomatis*-infected HeLa cells were antibiotic-exposed from 24 to 54 hpi and subjected to TEM. This exposure time was chosen to mimic an *in vivo* situation in which chlamydiae are penicillin-exposed after they have initiated development. Importantly, *C. trachomatis* serovar E under identical culture conditions is primarily in the RB stage, and still susceptible to the effects of penicillin G, up to 44 hpi (Deka et al., 2006, 2007). Inclusions in *C. trachomatis*-infected, diluent-exposed cells contained normal RB (white arrow) as well as intermediate bodies (IB) and EB (striped arrow; **Figure 2B**). ATM-exposed chlamydiae (**Figure 2E**) were indistinguishable from diluent-exposed controls. Though occasional AB were observed in CRO- and CTX-exposed cultures, most RB were normal in appearance and abundant EB were observed (**Figure 2L** and data not shown, white and striped arrows respectively). In contrast, AMX- (**Figure 2C**), AMP- (**Figure 2D**), CAR- (**Figure 2F**), PEN V- (**Figure 2I**), PIP- (**Figure 2J**), and CLA- (**Figure 2G**) exposed chlamydial inclusions contained swollen, irregular AB (black arrows), and few IBs or EBs were observed. PEN G-exposed chlamydiae were similar morphologically to those exposed to the other penicillins (**Figure 2H**), and, as expected, were characteristic of persistent chlamydiae (Matsumoto and Manire, 1970). Finally, though few IB or EB were observed in TET-exposed cultures, AB were not observed (**Figure 2K**, white arrow). These observations indicate that exposure to commonly prescribed β-lactam antibiotics, as well to CLA, induce formation of chlamydial AB.

PENICILLINS REDUCE PRODUCTION OF INFECTIOUS EBs AT PHYSIOLOGICALLY-RELEVANT CONCENTRATIONS

Persistent/stressed chlamydiae, while viable, are non-infectious (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). TEM studies indicated that penicillin-exposed, *C. trachomatis*-infected cells contained few EB (**Figure 2**), suggesting that production of infectious organisms was inhibited by all the penicillins of interest. To confirm these observations, chlamydiae-infected HeLa monolayers were exposed to each antibiotic from 24 to 54 hpi and subjected to sub-passage titration analyses (**Figure 3**). As expected, PEN G-exposure significantly reduced chlamydial infectivity. Likewise, exposure to AMX, AMP, CAR, PEN V, PIP, and CLA at the 1X serum concentration reduced production of infectious *C. trachomatis* EB by >95% compared to the diluent-exposed, chlamydiae-infected controls. In contrast, neither ATM- (**Figure 3**) nor cephalosporin-exposure (data not shown) significantly reduced chlamydial infectivity. As expected, production of infectious EB was essentially abolished by TET-exposure. These data demonstrate that exposure to penicillins or CLA significantly reduces production of infectious EB from infected cells.

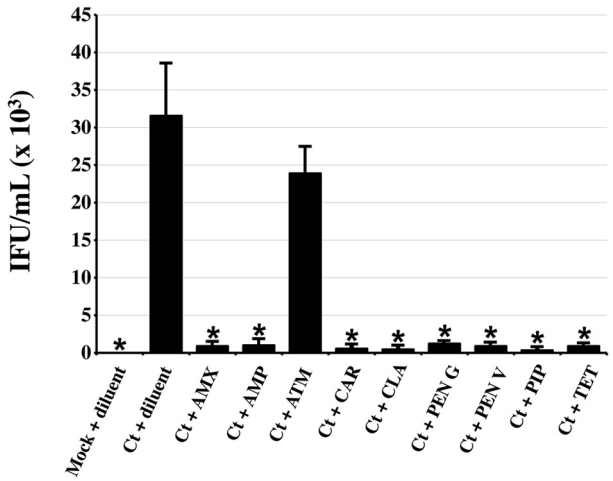

FIGURE 3 | Penicillin-exposure significantly reduces chlamydial infectivity. HeLa cells were *C. trachomatis*-infected and incubated in the absence of antibiotic for 24 h. Infected and uninfected cultures were then refed with medium containing each antibiotic of interest at the 1X concentrations (**Table 1**). Thirty hours post-antibiotic addition (54 hpi total), infected cell lysates were collected and used for infectious titer analyses. Inclusion counts were averaged and used to calculate inclusion forming units (IFU)/mL. The average of three biologic replicates from one of three independent experiments ± s.e.m. is shown. Groups significantly different from the diluent-exposed, Ct-infected control are indicated by asterisks (*), $P \leq 0.05$ was considered significant.

PENICILLIN-EXPOSURE DOES NOT REDUCE ACCUMULATION OF EITHER *C. trachomatis* GENOMIC DNA OR UNPROCESSED 16S rRNA

Though non-infectious, persistent/stressed chlamydiae remain viable and continue to synthesize (and accumulate) both genomic DNA and unprocessed 16s rRNA (Gerard et al., 1998, 2001). To assess chlamydial viability, chlamydiae-infected, HeLa monolayers were exposed to each antibiotic under the conditions described above. Host GAPDH DNA, chlamydial 16S DNA and chlamydial unprocessed 16S rRNA were quantified as described (Deka et al., 2006) and representative gels used for quantification of relative *C. trachomatis* 16S DNA and unprocessed 16S rRNA accumulation are shown in **Figure 4A**. Statistical analysis (**Figure 4B**) indicated that there was no significant reduction in either chlamydial genome accumulation or unprocessed 16S rRNA accumulation in any of the antibiotic-exposed, infected cultures compared to that in diluent-exposed, infected cells. These data are consistent with previous observations that *C. trachomatis* L2 genomic DNA accumulation is unaffected by PEN G-exposure (Lambden et al., 2006). They also suggest that penicillin-exposed chlamydiae continue chromosomal and unprocessed 16S rRNA accumulation and are, therefore, viable.

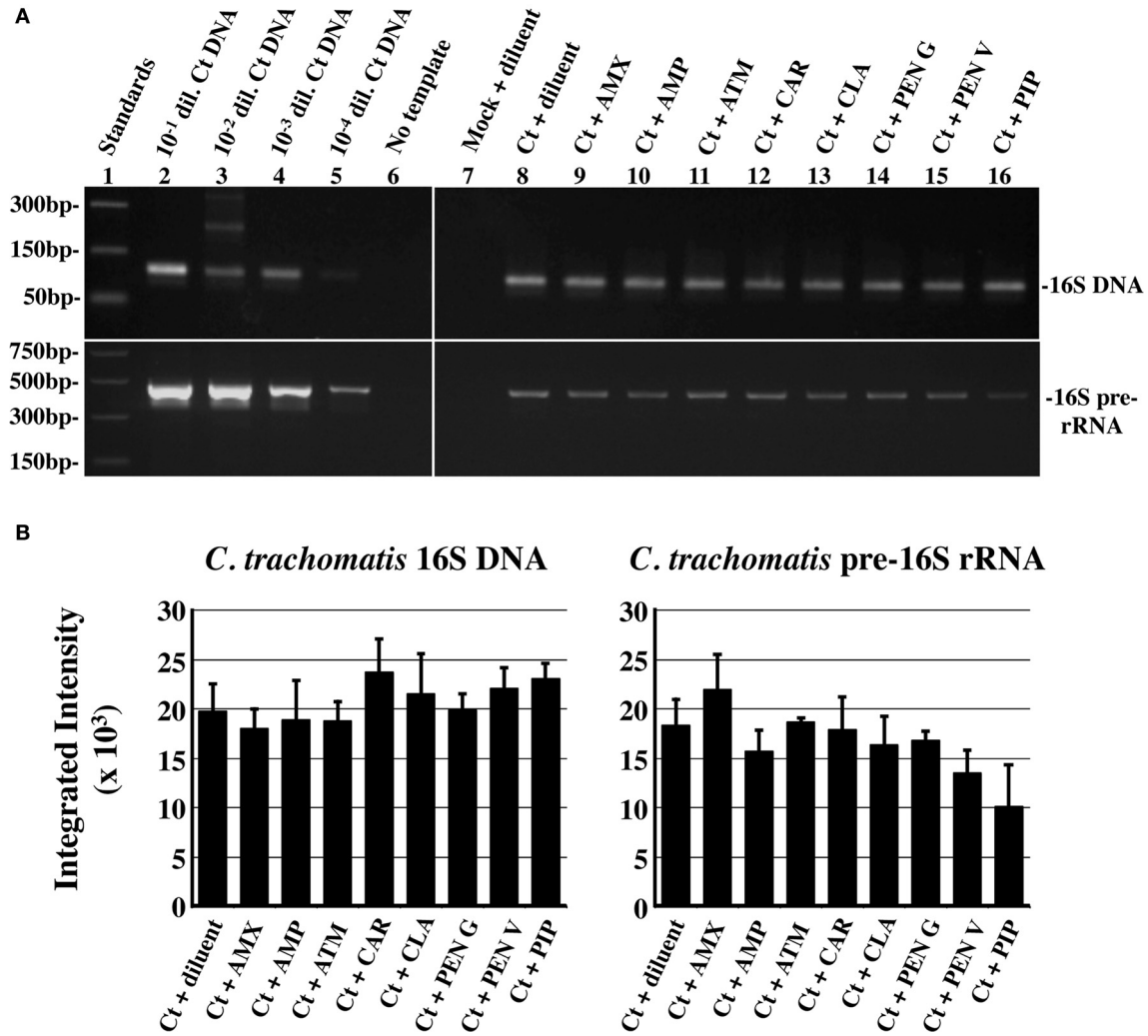

FIGURE 4 | Penicillin-exposure does not reduce genomic DNA or pre-16S rRNA accumulation. Total DNA and RNA from 1X antibiotic-exposed, infected cells was subjected to semi-quantitative PCR (or RT-PCR) using primers specific for human GAPDH (DNA), chlamydial 16S rRNA (DNA), and chlamydial unprocessed 16S rRNA transcripts (RNA). **(A)** Representative PCR gel images. Amplification of + control DNA dilutions are shown to the left. **(B)** Plots of chlamydial genomic DNA amplimer quantity normalized to host genomes (left) and pre-ribosomal RNA-specific amplimer quantity normalized to chlamydial genomes (right). The average of three independent biologic replicates from one of three independent experiments ± s.e.m. is shown. None of the experimental groups were significantly different from the diluent-exposed, infected control at $P \leq 0.05$.

PENICILLIN-EXPOSED CHLAMYDIAE RESUME PRODUCTION OF INFECTIOUS EB AFTER DRUG REMOVAL

One important characteristic of persistent/stressed chlamydiae is that they can re-enter the normal developmental cycle and produce infectious EB when the stressor is removed (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). Therefore, antibiotic recovery experiments were carried out as described in the methods. As expected, the yield of infectious chlamydiae immediately after the initial 30 h antibiotic-exposure (**Figures 5B–E,G–I**) was much lower than that from diluent-exposed, infected controls (**Figures 5A,F**). Chlamydial titers obtained after 3 days of additional antibiotic-exposure ("non-recovered" cultures) remained low in all cases. In contrast, significantly increased chlamydial titers were observed in AMX-, AMP-, CAR-, PEN V-, PIP-, and CLA-exposed cultures after the 3 day recovery period (recovered samples), compared to those obtained either immediately after initial drug-exposure or from parallel "non-recovered" cultures (**Figures 5B–E,G,H**). As expected, PEN G-exposed chlamydiae recovered infectivity after drug removal as well (**Figure 5I**). These data indicate that chlamydiae exposed to commonly prescribed penicillins can recover infectivity when the drugs are removed. Notably, the total amount of infectivity recovered after antibiotic removal was different for each drug tested.

DISCUSSION

AMX-, AMP-, CAR-, PEN V-, PIP-, and CLA-exposed chlamydiae exhibit defining characteristics of persistent/stressed organisms (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). They are viable (as evidenced by continued genome and pre-16S

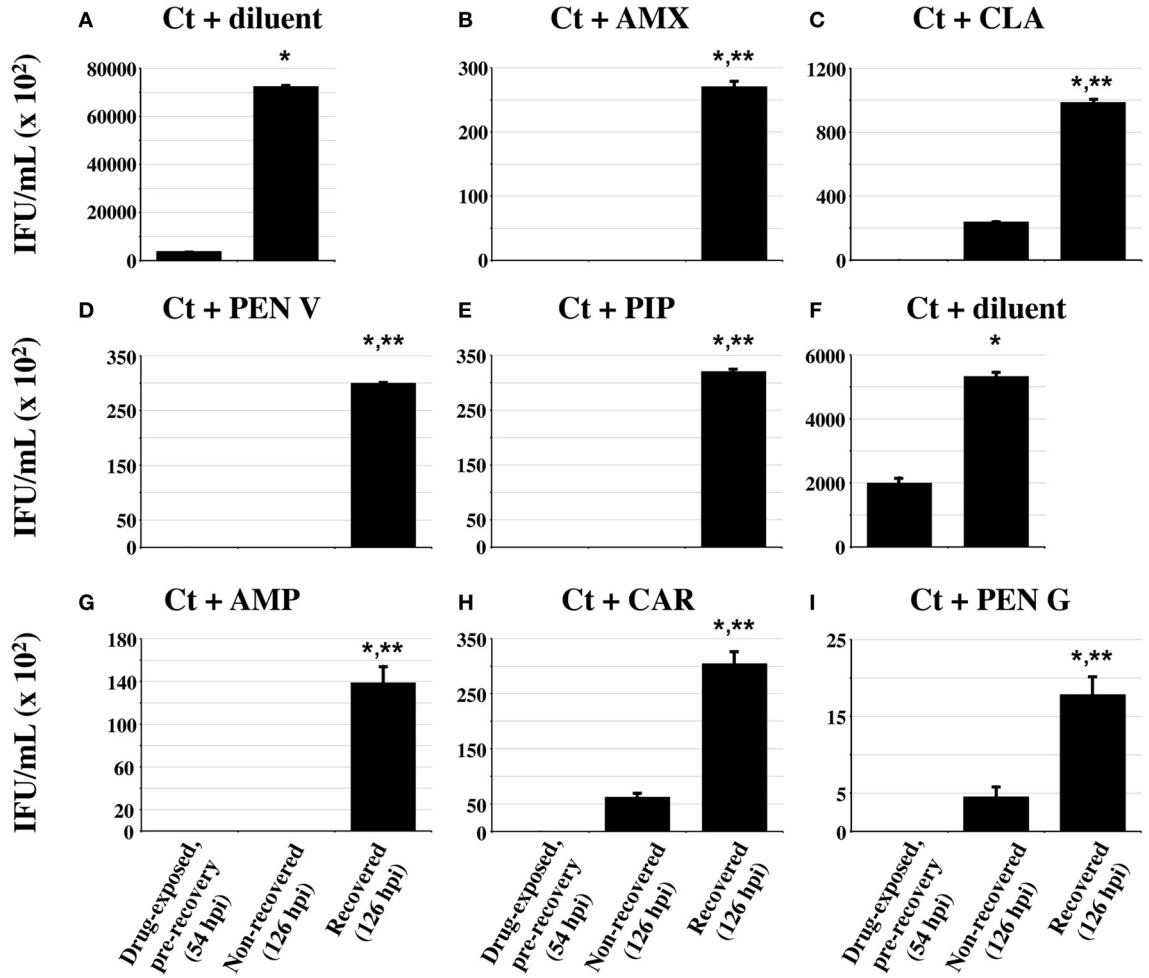

FIGURE 5 | Penicillin-induced stress/persistence is reversible. Replicate cultures of HeLa cells were infected and antibiotic-exposed at concentrations 10-fold higher than the MBC for each drug (**Table 1**) using the timing scheme described in **Figure 3**. At 30 h post-antibiotic addition (54 hpi total), one set of cultures was harvested for "pre-recovery" EB titration as in **Figure 3**. Duplicate antibiotic-exposed and control cultures were washed, refed with either antibiotic-replete (non-recovered samples) or antibiotic-deficient (recovered samples) growth medium and allowed to recover for 3 additional days (a total of 126 hpi). These cultures were then processed for EB titration. Note that each drug-exposure experiment was divided into two separate sets. The diluent-exposed control for panels **(B–E)** is shown in panel **(A)** and the diluent-exposed control for panels **(G–I)** is shown in panel **(F)**. The average of three biologic replicates ± s.e.m. is shown and $p \leq 0.05$ was considered significant. Single asterisks (*) indicate titers that are significantly higher than those observed in the pre-recovery cultures within each drug-exposure group. Double asterisks (**) denote titers that are significantly increased compared to the non-recovered cultures within each group. The experiment shown is one of three independent experiments.

rRNA accumulation and the ability to recover infectivity after drug removal) but non-infectious (as shown by reduced chlamydial titer). These drugs also induce AB formation, which is consistent persistence/stress induction (Matsumoto and Manire, 1970). Thus, these commonly prescribed β-lactams induce chlamydial persistence/stress in culture at physiologically-relevant concentrations. The CDC currently recommends either azithromycin or AMX for treatment of chlamydia-infected, pregnant women (Centers for Disease Control and Prevention, 2010). However, with recent reports linking azithromycin to adverse cardiac outcomes (Ray et al., 2012), more physicians may elect to use AMX in this situation. Whether or not AMX-treated, *C. trachomatis*-infected women have a higher risk of subsequent tubal factor infertility is currently unknown. However, because: (i) chlamydiae can recover from AMX-induced persistence/stress in culture; and (ii) resumption of shedding is observed after AMX-treatment cessation in chlamydia-infected mice (Phillips-Campbell et al., 2012), it seems reasonable to conclude that AMX-treated, infected women may remain at risk for chronic infection and reproductive complications. Thus, if AMX is used, it is important that chlamydial eradication be confirmed post-therapy, as per CDC recommendations (Centers for Disease Control and Prevention, 2010).

CLA, a β-lactam originally isolated from *Streptomyces clavuligerus*, is a strong β-lactamase inhibitor and is used clinically to increase β-lactam activity against penicillinase-producing bacterial strains (Reading and Cole, 1977). CLA also has direct antimicrobial activity, albeit weaker than that of other β-lactams.

Interestingly, the reported CLA MBC for *E. coli* is 25 μg/mL (Neu and Fu, 1978), more than 70 times higher than that for *C. trachomatis* (**Table 1**). Because CLA binds *E. coli* PBP2 (Spratt et al., 1977) and *C. trachomatis* expresses a PBP2 homolog that may function in RB division (Ouellette et al., 2012), it seems likely that CLA induces chlamydial persistence/stress by inhibiting PBP2 function. This would be consistent with published observations that the PBP2-specific drug, mecillinam, also induces AB formation and reduces EB production (Storey and Chopra, 2001; Ouellette et al., 2012). Our data (**Table 1**) also indicate that *C. trachomatis* serovar E is resistant to ATM, CTX and CRO, at least at physiologically achievable concentrations.

Approximately 50% of total antibiotics used in human medicine are β-lactams, most of which are aminopenicillins - like AMX and AMP (Kümmerer and Henninger, 2003; Goossens et al., 2005). Since there are about 1.5 million reported new chlamydial infections per year in the US (Centers for Disease Control and Prevention, 2011), up to 75% of which are asymptomatic (Detels et al., 2011), brief episodes of β-lactam-induced persistence/stress may occur in many patients during treatment for other bacterial infections. There are also other routes by which chlamydia-infected hosts could be chronically exposed to low levels of β-lactam and other antibiotics. For example, there is widespread, low-level β-lactam contamination of milk, meat and other agricultural products. The risks posed by such contamination, most notably penicillin allergy and promotion of antibiotic resistance, have been recognized for decades (Welch, 1956). Antibiotics and their degradation products (DPs) are also found in ground water, the most commonly identified of which are macrolides, sulfonamides, fluoroquinalones and tetracyclines (Heberer, 2002; Kolpin et al., 2002). Notably, exposure to low concentrations of erythromycin (a macrolide), ciprofloxacin and ofloxacin (both fluoroquinalones) also induces persistence/stress in both *C. pneumoniae* and *C. trachomatis* (Dreses-Werringloer et al., 2000, 2001; Gieffers et al., 2004). Though most β-lactams are rapidly converted to DPs by UV light and chlorination, some AMX-DPs, like AMX S-oxide, are environmentally stable and retain an intact β-lactam ring (Gozlan et al., 2010). Therefore, DPs of β-lactams or other antibiotics consumed in treated water could induce chlamydial persistence/stress *in vivo*. However, the effect of antibiotic DPs on chlamydial development, if any, is currently unknown.

Treatment failure is a significant problem in human chlamydial infections (Horner, 2006; Pitt et al., 2013). For example, post-treatment *C. trachomatis* positivity is reported in 10-15% of women on recommended treatment regimens (Wang et al., 2005). Significantly, penicillin G-exposed, persistent/stressed *C. trachomatis* serovar E is resistant to azithromycin killing in culture (Wyrick and Knight, 2004). Likewise, IFN-exposed, persistent/stressed *C. trachomatis* is more resistant to doxycycline (Reveneau et al., 2005). Azithromycin and ofloxacin also do not eradicate persistent/stressed *C. pneumoniae* in culture (Kutlin et al., 1999). Finally, induction of persistence/stress using AMX increases azithromycin treatment failure in *C. muridarum*-infected mice (Phillips-Campbell et al., 2014). These studies suggest that *in vivo* persistence/stress could be one mechanism by which chlamydiae resist antimicrobial therapy *in vivo*. Thus, given the widespread medical use of these antibiotics, as well as their presence in food and water, β-lactam-induced chlamydial persistence/stress may have significantly more *in vivo* relevance than previously assumed.

AUTHOR CONTRIBUTIONS

Jennifer Kintner and Dawn Lajoie performed drug exposure, infectious titer, RT-PCR and recovery experiments; Jennifer Hall assisted with RT-PCR experiments, data analysis and manuscript preparation; Judy Whittimore performed all TEM experiments; and Robert V. Schoborg designed the study, assisted in titer and TEM experiments, analyzed data and prepared the manuscript. All authors have read and approved the manuscript.

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The protease inhibitor JO146 demonstrates a critical role for CtHtrA for *Chlamydia trachomatis* reversion from penicillin persistence

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The *Chlamydia trachomatis* serine protease HtrA (CtHtrA) has recently been demonstrated to be essential during the replicative phase of the chlamydial developmental cycle. A chemical inhibition strategy (serine protease inhibitor JO146) was used to demonstrate this essential role and it was found that the chlamydial inclusions diminish in size and are lost from the cell after CtHtrA inhibition without formation of viable elementary bodies. The inhibitor (JO146) was used in this study to investigate the role of CtHtrA for penicillin persistence and heat stress conditions for *Chlamydia trachomatis*. JO146 addition during penicillin persistence resulted in only minor reductions (∼1 log) in the final viable infectious yield after persistent *Chlamydia* were reverted from persistence. However, JO146 treatment during the reversion and recovery from penicillin persistence was completely lethal for *Chlamydia trachomatis*. JO146 was completely lethal when added either during heat stress conditions, or during the recovery from heat stress conditions. These data together indicate that CtHtrA has essential roles during some stress environments (heat shock), recovery from stress environments (heat shock and penicillin persistence), as well as the previously characterized essential role during the replicative phase of the chlamydial developmental cycle. Thus, CtHtrA is an essential protease with both replicative phase and stress condition functions for *Chlamydia trachomatis*.

Keywords: *Chlamydia*, persistence, HtrA/CtHtrA, protease inhibitor, protease

INTRODUCTION

Chlamydia trachomatis is a unique obligate intracellular bacterial pathogen. The organism is typified by a bi-phasic developmental cycle. This cycle consists of an infectious extracellular form, termed the elementary body (EB), and an intracellular replicative form termed the reticulate body (RB), which divides by binary fission prior to converting back to the infectious progeny (reviewed, Adbelrahman and Belland, 2005). The intracellular form is found inside a unique vacuole inside the host cell that is called the inclusion vacuole. In addition to these two forms, the organism has a "latent" like phase of intracellular growth. This is termed persistence that is defined as viable but non-cultivatable *Chlamydia*. Persistent *Chlamydia* (also called aberrant bodies), are morphologically distinct from the active replicating form with only a few cells visible per inclusion which are much larger size) (Moulder, 1993; Byrne et al., 2001; Hogan et al., 2004; Wyrick, 2010). This ability to become persistent is thought to provide the organism with a survival mechanism to avoid any conditions where they would be unable to survive. Hence, persistence is induced by immune pressure, amino acid deprivation, penicillin, iron limitation, or the presence of other intracellular pathogens (Beatty et al., 1993, 1994a,b; Coles et al., 1993; Byrne et al., 2001; Wyrick and Knight, 2004; CDC, 2005–2009/2010; Deka et al., 2006). Whilst there are numerous means of inducing persistence and the chlamydial cellular morphology appears similar for each of these, it is clear that there are distinct transcriptional and protein profiles associated with the different forms of persistence (reviewed, Wyrick, 2010).

Amino acid deprivation has been shown to induce persistence, which was able to be restored by cysteine and isoleucine (Coles et al., 1993; Harper et al., 2000). The best characterized mechanism of persistence is that induced by IFN- γ (interferon-gamma). IFN- γ (secreted by immune cells) induces a large range of responses in the epithelial cell (Beatty et al., 1993, 1994a). One of the proteins that is highly induced in human epithelial cells in response to IFN- γ is IDO1 (indoleamine 2,3-dioxygenase) (Beatty et al., 1993, 1994a; Ibana et al., 2011). This enzyme catabolizes the host cell tryptophan resulting in reduced tryptophan supply for the auxotrophic pathogen. *C. trachomatis* persistence induced by IFN- γ is able to be reverted by the removal of IFN- γ and addition of tryptophan. IFN- γ aberrant bodies are typified by a loss of expression of genes required for cytokinesis with continuing chromosomal replication (Byrne et al., 2001; Belland et al., 2003). Another commonly used laboratory model of persistence is that which occurs in response to cell wall targeting antibiotics. Models of this form of persistence typically

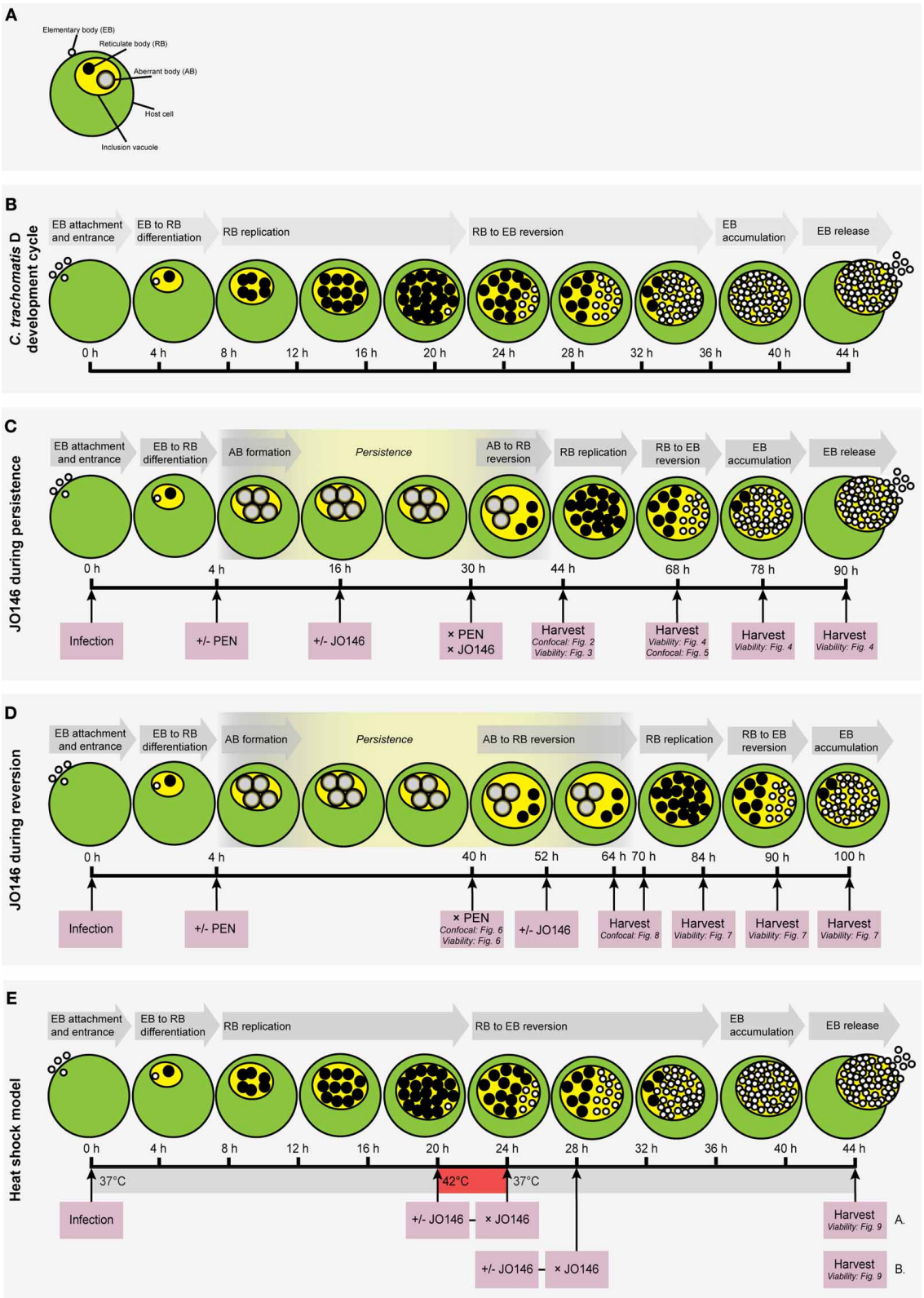

FIGURE 1 | Flow chart diagrams of methodologies used in this manuscript with cartoon representations of the expected growth phases of the *Chlamydia*. (A) Key to the components of a *Chlamydia*-infected host cell. Small, open circles: elementary bodies (EB); large black circles: reticulate bodies (RB); large gray circles: aberrant bodies (AB); green circle: host cell; yellow circle: chlamydial inclusion vacuole. **(B)** The *Chlamydia trachomatis* (serovar D) development cycle is represented by the cartoon. The relative

(Continued)

FIGURE 1 | Continued
time-points are shown below the cells and each stage of the cycle is shown in gray arrows above the cells. **(C)** Experimental conditions used to assess the impact of JO146 addition during penicillin persistence. **(D)** Experimental plan to determine the impact of JO146 on *C. trachomatis* reversion from persistence. **(E)** Experimental plan to use the JO146 inhibitor to determine the role of CtHtrA for viability of *C. trachomatis* during heat stress conditions. The pink boxes represent the experimental actions taken, with arrows extending to the specific time-point for each action. The viability and/or morphological experiments associated with specific time-points are included in small type in the relevant pink boxes. The gray arrows indicate the stage of the development cycle represented by the cartoon. "PEN" represents penicillin. "+/−" indicates that separate experiments were conducted with or without the addition of penicillin ("+/− PEN") or the JO146 inhibitor ("+/− JO146"). "× PEN" indicates that penicillin was removed in the experiments where it was added. The yellow highlighted section of the developmental cycle indicates the expected presence and relative duration of persistence when induced by the addition of penicillin at 4 h PI in the persistence experiments. The cartoon representations of the expected chlamydial developmental cycle phases and the associated time points under these experimental conditions are based on previously published data (Byrne et al., 2001; Miyairi et al., 2006; Skilton et al., 2009).

involve penicillin. Penicillin persistence has been described to result in the *Chlamydia* cells rapidly ceasing cellular division, whilst chromosomal and plasmid replication continue at the same rate regardless of the presence or absence of penicillin (Byrne et al., 2001). The removal of the penicillin then allows reversion, which occurs via an RB budding from the aberrant body, with this only productively occurring in some inclusions (Skilton et al., 2009).

Recently, our team identified a serine protease inhibitor (JO146) against *C. trachomatis* HtrA (CtHtrA) which was lethal when added to cultures during the mid-replicative phase (Gloeckl et al., 2013). The compound is a serine protease inhibitor, which is a tri-peptide with a war-head motif. The compound was firstly identified to be specific to CtHtrA using *in vitro* CtHtrA protease assays (Gloeckl et al., 2013). JO146 was demonstrated to be lethal when added to cultures during the replicative phase of development but not when added early or late during the developmental cycle (Gloeckl et al., 2013). HtrA in many bacteria is a periplasmic protease involved in cell surface protein assembly and extracytoplasmic protein maintenance (reviewed, Clausen et al., 2011). This function is also supported by our data to date for *Chlamydia* HtrA (Huston et al., 2008), and it is likely that this extracytoplasmic protein protection role is particularly critical during the chlamydial penicillin persistence model. Previously, we and others have reported that CtHtrA is highly expressed during penicillin persistence lab models and down regulated during IFN-γ persistence (Belland et al., 2003; Mukhopadhyay et al., 2006; Huston et al., 2008). Therefore, in this project we aimed to test the hypothesis that CtHtrA is essential during penicillin persistence using the CtHtrA inhibitor JO146.

MATERIALS AND METHODS

Chlamydia CULTURE

Chlamydia trachomatis (serovar D UW-E/Cx) was routinely cultured in HEp-2 cells in DMEM (Dulbecco's modified eagle medium), 5% FCS (fetal calf serum), 37°C, 5% CO_2. All cultures were conducted at a 0.3 multiplicity of infection (MOI). A summary of the experimental methods used in this study is shown in **Figure 1**. Penicillin persistence was established by the addition of 100 U/ml of penicillin at 4 h PI (hours post-infection) and JO146 was added at 16 h PI to determine the impact of JO146 treatment during persistence. In order to measure the impact of JO146 on the ultimate viability, the cultures were allowed to revert from persistence by the removal of penicillin. Penicillin was removed by three sequential rounds of media washes and medium was replaced with penicillin-free media at 30 h PI. Viable infectious yield was measured at 68, 78, and 90 h PI. Cultures were also monitored for viability at 44 h PI without the removal of the penicillin to demonstrate lack of viability consistent with persistence (in conjunction with the ability to subsequently rescue these same culture conditions to detectable viability by penicillin removal). Control cultures with no JO146 were included for each experiment and with the solvent DMSO (dimethyl sulfoxide). In order to assess the impact of JO146 on *Chlamydia* during reversion from penicillin persistence, a separate experiment was conducted where persistence was induced in the cultures using penicillin (4 h PI, 100 U ml-1); at 40 h PI the penicillin was removed (washes and media change). At 52 h PI when reversion is likely to be underway in most inclusions, JO146 was added to the cultures (concentrations as indicated on the figures). Reversion is very asynchronous from this form of persistence. Reversion has been reported to take 10–20 h for *C. trachomatis* L2 which has similar (slightly faster) growth kinetics to the strain used in this study. Therefore, 52 h PI is the most logical choice to target reversion based on available data (Skilton et al., 2009). The cultures were harvested and viability was determined at 84, 90, and 100 h PI. Control cultures with no JO146 treatment were included for each experiment. Cultures for immunocytochemistry were conducted on glass coverslips, using the method previously described (Huston et al., 2008).

A heat shock model was used in conjunction with JO146 treatment to evaluate the role of CtHtrA for chlamydial viability during heat stress. Heat shock was conducted at 20 h PI for 4 h (20–24 h PI), as we have previously shown an increase in CtHtrA protein at this time point during heat shock (Huston et al., 2008). JO146 was added at 20 h PI, immediately prior to heat shock at 42°C, 5% CO_2 for 4 h. At the conclusion of the 4 h heat shock, JO146 was removed from the cultures by three washes in 37°C pre-warmed media, prior to returning the culture to 37°C for the remainder of the developmental cycle. In a separate experiment, the role of CtHtrA during recovery from heat shock was also analyzed by the addition of JO146 immediately (at 24 h PI) after the 4 h heat shock treatment and removal of the compound by media washes (three) at 28 h PI. The cultures were harvested at 44 h PI and viable infectious yield was determined (see **Figure 1** summary).

DETERMINATION OF CHLAMYDIAL VIABILITY

Chlamydia viable infectious yield was determined by serial dilution of cultures and infection on HEp-2. At 30 h PI the monolayers were fixed and immunocytochemistry was conducted to

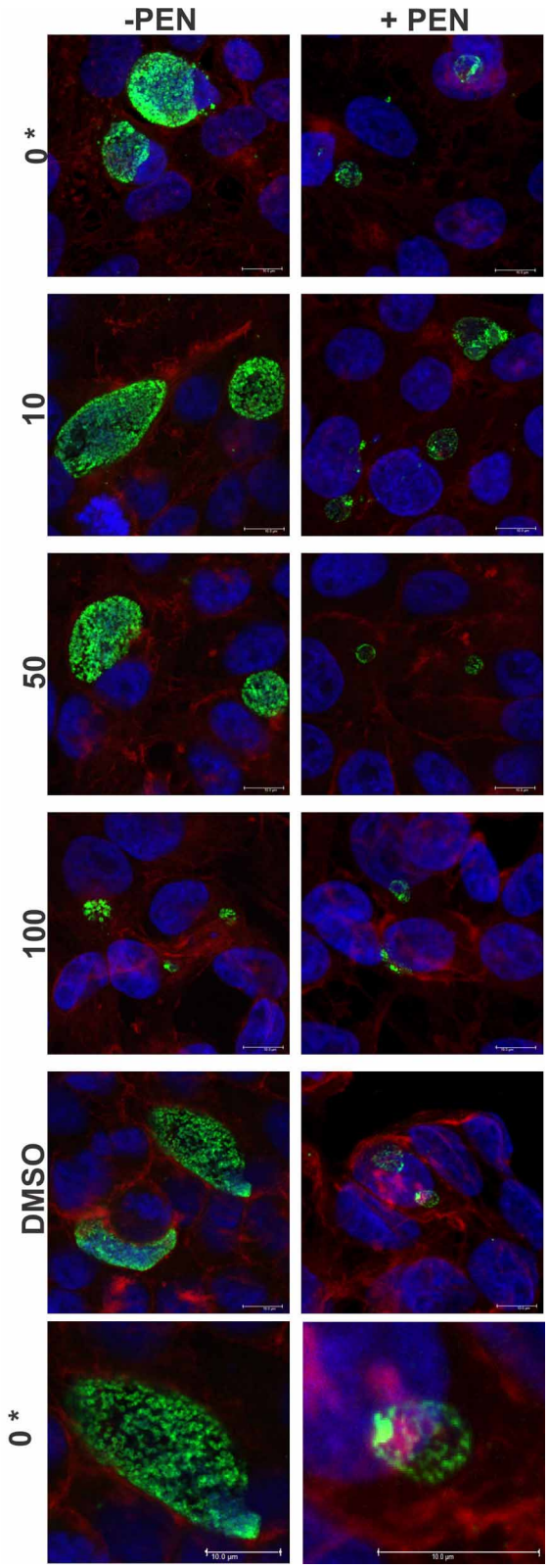

FIGURE 2 | Confocal microscopy images of JO146 treated cultures during penicillin persistence at 44 h PI (+PEN: penicillin added, −PEN: no penicillin). The figure shows representative images from confocal laser *(Continued)*

FIGURE 2 | Continued
scanning microscopy of cultures fixed at 44 h PI, 100 U ml^{-1} penicillin was added at 4 h PI. Representative images from the control culture shown in the left column (−PEN). Penicillin treated conditions are shown in the right column. JO146 treatment conditions are indicated to the right (0, 10, 50, 100 μM, DMSO). The final images at the bottom are enlarged images of the controls which have both had equal contrast adjustments to make the differences more apparent (0 μM JO146 enlarged). The image colors are as follows, green: MOMP (major outer membrane protein) is green, blue: cell nucleus (DAPI), and red: β-actin. Scale bar (bottom right) indicates 10 mm. *Indicates zoomed in images from the 0 μM conditions are shown to allow closer examination of morphology.

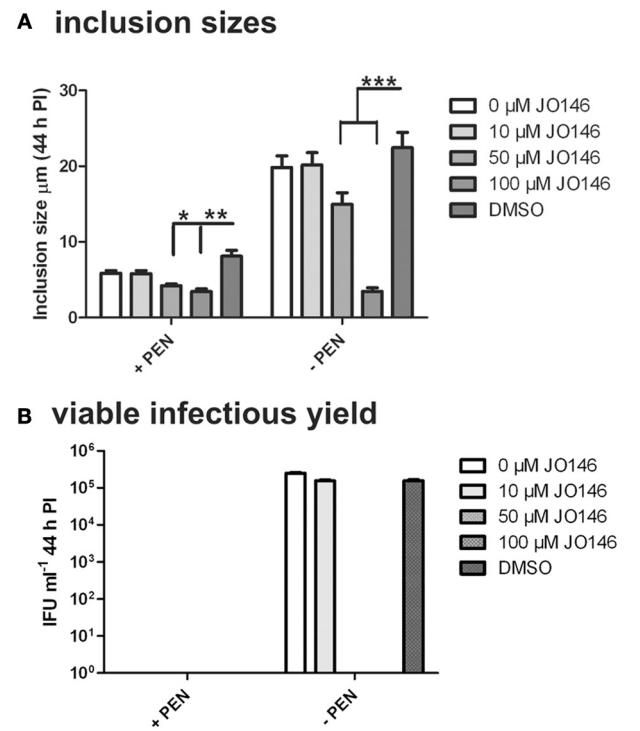

FIGURE 3 | Inclusion sizes and viable infectious yield during penicillin persistence at 44 h PI with and without JO146 treatment. The figure shows morphological analysis of cultures during penicillin persistence and controls by measuring inclusion sizes. The viable infectious yield (44 h PI) in the presence or absence of 100 U ml^{-1} penicillin (4 h PI) is shown on the graph. **(A)** Inclusion sizes are shown from each condition inclusion sizes were measured from independent coverslips, $n = 20$. **(B)** Viable infectious yield with and without penicillin are shown graphically ($n = 27$). Statistics were conducted using Two-Way ANOVA relative to DMSO controls, $p > 0.5$*, $p > 0.01$**, $p > 0.001$**, $P > 0.0001$***.

enable counting of inclusions. The final results are presented as inclusion forming units per ml (IFU ml^{-1}). Statistical analysis was performed using PRISM (GraphPad Software Inc). Results are expressed as mean, with error bars representing standard error of the mean. Two-Way analysis of variance (ANOVA) with a *post-hoc* Bonferroni multiple comparison tests was used to assay the statistical differences relative to the DMSO control.

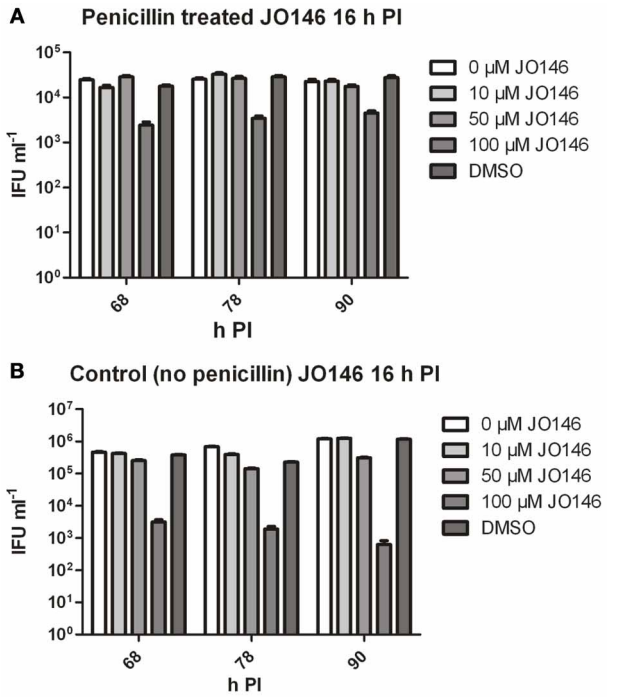

FIGURE 4 | Viable infectious yield of *C. trachomatis* after treatment with JO146 during penicillin persistence. The figure shows viable IFU ml^{-1} from each culture condition at several time points after reversion was commenced (penicillin removal at 30 h PI). Cultures were treated with penicillin 100 U ml^{-1} at 4 h PI, and with JO146 at 16 h PI (concentrations indicated by the gray shading, see key to the right of each graph). Penicillin was removed from the cultures at 30 h PI. **(A)** Viable infectious yield from penicillin treated and restored cultures. **(B)** Viable infectious yield from control cultures which did not have penicillin added. Statistics were conducted using Two-Way ANOVA relative to DMSO controls.

IMMMUNCYTOCHEMISTRY AND MICROSCOPY

Cultures were fixed using 4% paraformaldehyde-PBS (phosphate buffered saline) for 15 min, and then blocked in 1% BSA-PBS overnight at 4°C. The primary antibody (*C. trachomatis* MOMP (major outer membrane proteim), Biodesign International) was added at 1:500 dilutions in 1% BSA (bovine serum albumin) in PBS with 2 μL/coverslip phalloidin 594 (Invitrogen) and 1:40,000 dilution of DAPI (diaminidino phenylindole). Coverslips were incubated for 2 h at room temperature. Three washes with 0.2% Tween 20 in PBS were conducted prior to addition of the secondary antibody (goat anti-mouse IgG H+L-Alexa Flour 488, Invitrogen) at 1:2000 dilutions in 1% BSA in PBS. Coverslips were then washed 4 times with 0.2% Tween 20-PBS and were suspended in PBS prior to mounting with ProLong Gold (Invitrogen). Cultures fixed for immunocytochemistry were examined using the Leica SP5 confocal microscope. Images were prepared using the supplied Leica software suite. Sizes of inclusions at 44 h PI were measured to determine the effect of the inhibitor compound during penicillin-induced persistence using the Leica application suite.

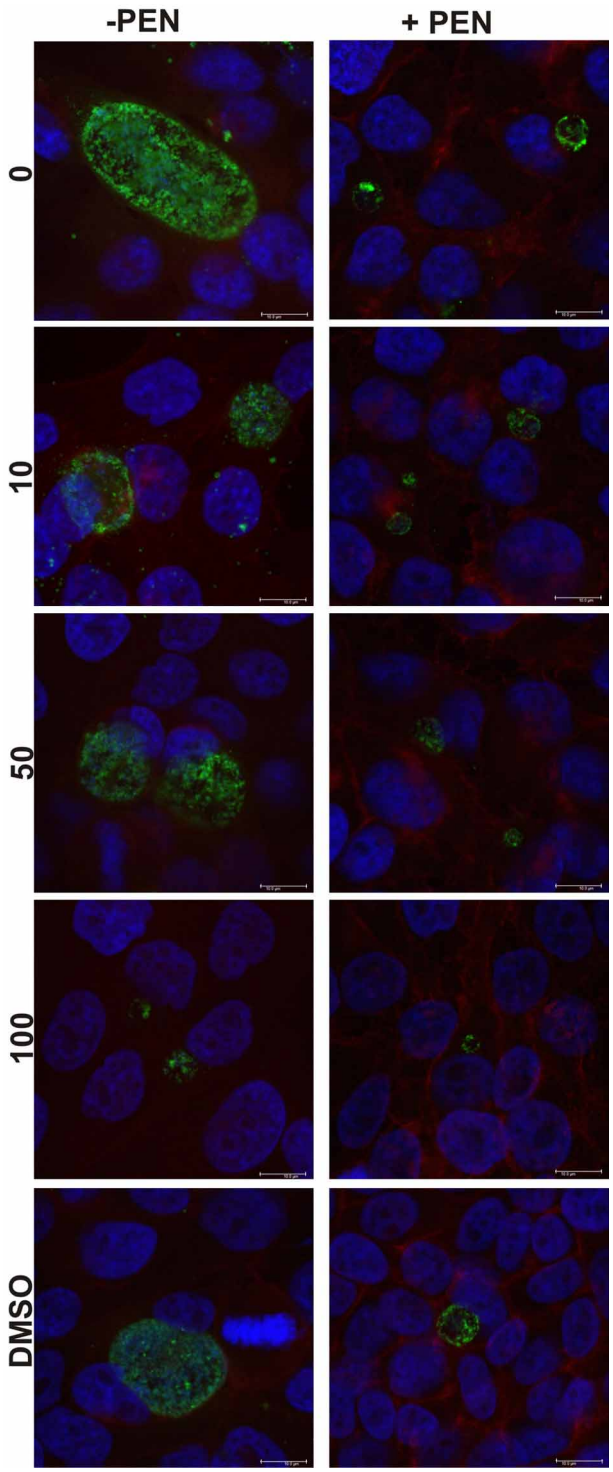

FIGURE 5 | Confocal microscopy images of *C. trachomatis* cultures at 68 h PI, or 38 h after penicillin reversion commenced. Cultures were treated with penicillin 100 U ml^{-1} (right column) at 4 h PI, and with JO146 at 16 h PI (concentrations indicated by the gray shading, legend to right of each graph). Penicillin was removed from the cultures at 30 h PI. The image colors are as follows, green: MOMP (major outer membrane protein) is green, blue: cell nucleus (DAPI), and red indicates β-actin. Scale bar (bottom right) indicates 10 μm.

RESULTS

JO146 ADDITION TO *C. trachomatis* HEp-2 CULTURES DURING PENICILLIN PERSISTENCE RESULTS IN A REDUCED VIABLE YIELD

Due to the bi-phasic nature of the chlamydial developmental cycle it is not possible to measure the immediate impact on viability during the replicative phase of growth. Therefore, for each of these experiments we have assessed the viability once elementary bodies are formed, either at the conclusion of the developmental cycle or once reversion from persistence and development of elementary bodies has occurred. This also means that we can confirm that the cultures are persistent by detection of loss of viability in the persistent cultures when control cultures are demonstrated to have viable elementary bodies (as long as viability was subsequently restored by removal of the persistence inducing agent). We first wanted to monitor the impact of JO146 addition during persistence when aberrant bodies are present, hence for this experiment we added JO146 during persistence at 16 h PI (penicillin was added at 4 h PI to induce persistence) and one set of cultures were harvested to measure viability and also fixed and examined by confocal microscopy at 44 h PI. A second set of cultures were media changed at 30 h PI to remove both the JO146 and penicillin and harvested at 68, 78, and 90 h PI (or 38, 48, 60 h after penicillin removal) to allow time for reversion from persistence and elementary body formation (as outlined in **Figure 1**). These cultures were tested for viability and morphology was examined using confocal laser scanning microscopy.

The cultures were firstly confirmed to be persistent at 44 h PI by monitoring viability and impact of JO146 treatment in the presence and absence of penicillin. As shown in **Figure 2**, the cultures treated with penicillin had much smaller inclusions compared to the controls at 44 h PI. The inclusions were also much less populated with cell shaped bodies consistent with a persistent phenotype (**Figure 2** right column penicillin compared to control left column). The increasing concentrations of JO146 resulted in a decreased inclusion vacuole size for both the penicillin treated and control cultures (**Figure 3A**). The penicillin treated cultures were confirmed to be persistent by a lack of viable EBs at 44 h PI (**Figure 3B**) and supported by restoration of viability in subsequent experiments.

The viable infectious yield during the reversion from persistence from the cultures with and without JO146 treatment was determined [during reversion at 68, 78, and 90 h PI, when we expect to see recovery to viable elementary bodies (Skilton et al., 2009)]. As expected, and consistent with our previously published data, JO146 was completely lethal on the control (non-persistent) culture at 50 and 100 μM, when viability was measured at 44 h PI (**Figure 3B**) with some recovery of viability observed at the later time points (extended culture conditions, **Figure 4B**), as consistent with our previous work (Gloeckl et al., 2013). In contrast, JO146 was not lethal when it was added at the same time point during penicillin induced persistence (**Figure 4A**). Cultures were treated with JO146 during persistence then subsequently rescued by media change (30 h PI) to allow the formation of EBs (measured as viable infectious yield). JO146 treatment during the persistence phase resulted in a relatively minor loss of detectable viability, with approximately a 1 log reduction of viable yield observed at 100 μM JO146 when EBs were able to be detected

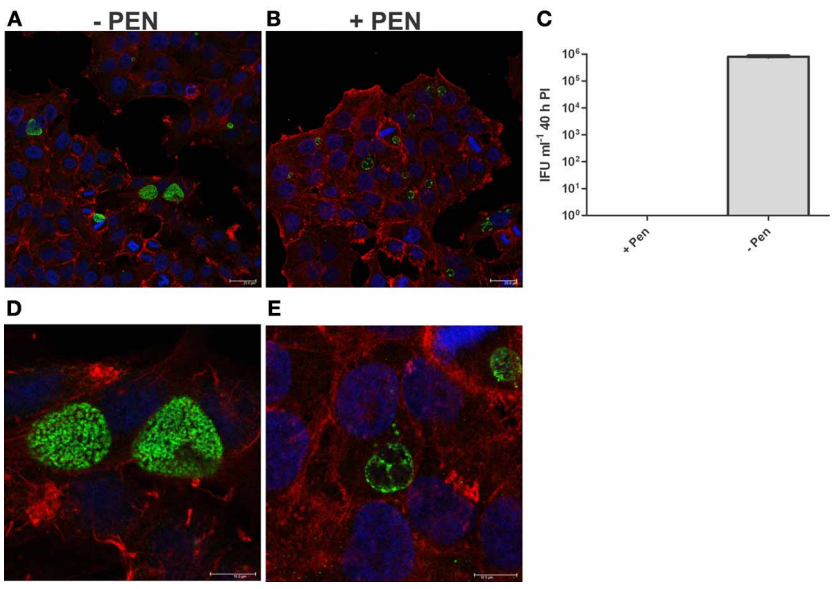

FIGURE 6 | Penicillin persistence cultures prior to commencement of reversion are not viable and are morphologically consistent with persistence. Cultures were treated with penicillin 100 U ml^{-1} at 4 h PI. **(A)** Confocal microscopy image of cultures at 40 h PI in the presence of penicillin (+PEN) **(B)** Confocal microscopy image of cultures at 40 h PI in the absence of penicillin (−PEN). The scale bar indicates 25 μm. **(C)** Viable infectious yield of the corresponding cultures ($n = 27$). **(D)** Enlarged area of **A**: confocal microscopy image of cultures at 40 h PI in the presence of penicillin (+PEN). **(E)** Enlarged area of **B**: confocal microscopy image of cultures at 40 h PI in the absence of penicillin (−PEN). The contrast has been equally adjusted on **D,E** to improve the visibility of the morphologies present.

at 68, 78, and 90 h PI (**Figure 4A**). The control cultures which were not persistent showed ∼2–3 log reductions in viability with 100 μM JO146 treatment at the extended culture times of 68–90 h PI (**Figure 4B**). This observation of reduced effectiveness of JO146 over extended culture conditions is consistent with our previous data (Gloeckl et al., 2013).

The cultures were monitored by immunofluorescence during the reversion period and representative images from 68 h PI are shown in **Figure 5** (left column control, right column penicillin). The penicillin treated cultures had smaller inclusions with different appearance (likely indicating there are still aberrant bodies present) at 68 h PI, with the inclusions generally appearing smaller in the presence of 100 μM JO146 (**Figure 5**, right column). The control cultures (no penicillin treatment) also showed a JO146 concentration dependent reduction of the inclusion sizes (**Figure 5**, left column). However, even though the inclusions appeared markedly smaller when recovering from penicillin persistence at 68 h PI, there was only ∼one log reduction in viable EB yield compared to the controls (**Figure 4A**).

JO146 ADDITION TO *C. trachomatis* HEp-2 CULTURES DURING REVERSION FROM PENICILLIN PERSISTENCE IS LETHAL

The mechanism of reversion from penicillin persistence has been described to be very asynchronous; with gradual budding of "normal" RBs from the aberrant persistent forms in the inclusion over 10–20 h after penicillin was removed (Skilton et al., 2009). These budded RBs are then thought to undergo replication by binary fission prior to conversion to the infectious elementary body form. Given the highly asynchronous nature of this reversion, at any one time there will be cells undergoing reversion and other cells undergoing replication at the same time, meaning that it is not possible to uncouple reversion from replication. We established penicillin persistent cultures and then commenced reversion by washing and media change at 40 h PI. 12 h (52 h PI) after reversion was commenced we added JO146 and monitored the formation of viable infectious elementary bodies at 84, 90, and 100 h PI.

Firstly, we confirmed that the cultures were persistent at 40 h PI by measuring viability and examining the morphology of the cultures using confocal laser scanning microscopy. As shown in **Figure 6**, the penicillin treated cultures were not viable at 40 h PI (**Figure 6C**) and morphologically the inclusions were smaller and appeared to have large cellular forms present inside each inclusion (**Figures 6A,B**). The penicillin persistent cultures were washed to remove penicillin to commence reversion at 40 h PI. JO146 was added to these cultures 12 h after commencement of reversion (i.e., 52 h PI). Viable infectious yield was then measured over time from the cultures. As shown in **Figure 7A** no viable *Chlamydia* were detected at 84 h PI from the persistence reversion cultures (44 h after penicillin was removed), however, at 90 and 100 h PI viable EBs were detected. The JO146 treatment of 100 μM JO146 was lethal to the cultures undergoing reversion (**Figure 7A**). In contrast, the cultures which were not penicillin persistent showed only minor reductions in viability due to the addition of JO146 (**Figure 7B**). These control cultures were likely either mainly in elementary body form or in the early stages of infection when JO146 was added (early or very late developmental cycle, or a mix of both), based on the morphological appearance of the inclusions and what we know about the timing of the chlamydial developmental cycle. We previously demonstrated that JO146 was less effective for both of these developmental phases (Gloeckl et al., 2013), so these results are consistent with what might be expected. Therefore, these data indicate that during reversion from penicillin persistence and recovery of viability, JO146 treatment is completely lethal for *Chlamydia*.

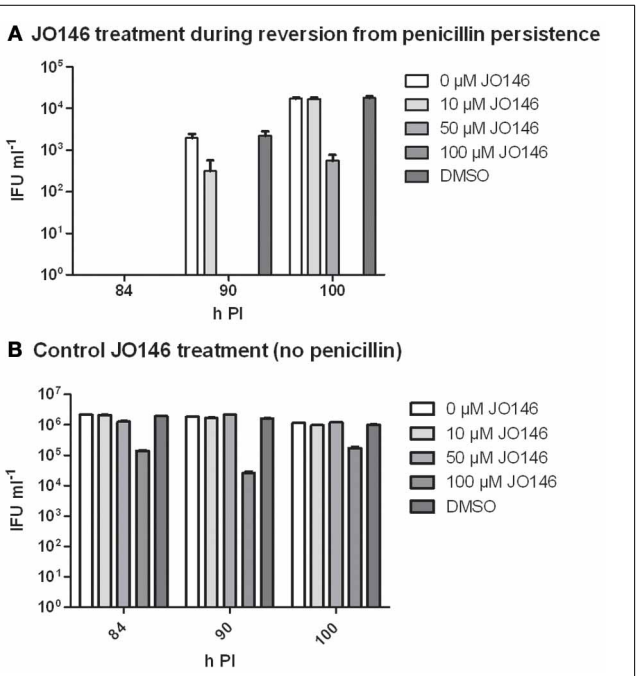

FIGURE 7 | Viable infectious yield when JO146 was added during the reversion from penicillin persistence. Cultures were treated with penicillin 100 U ml^{-1} at 4 h PI. Penicillin was removed at 40 h PI and JO146 treatment was commenced at 52 h PI. **(A)** JO146 was added 12 h after penicillin persistence reversion was commenced (i.e., at 52 h PI). **(B)** Control cultures that were not persistent with JO146 treatment also conducted at 52 h PI. The graphs show the viable infectious yield at several time points after reversion from persistence was commenced ($n = 27$). JO146 concentration is indicated by the gray shading (legend to the right of each graph).

We monitored the appearance of the cultures using immunocytochemistry and confocal laser scanning microscopy during the reversion from persistence to monitor the impact of JO146 on inclusion morphology at 64 and 70 h PI (12 and 18 h after JO146 addition). In this case we observed no obvious decrease in the inclusion size relating to JO146 treatment (**Figure 8**, second and fourth column), and as expected the inclusions from the persistent cultures were much smaller than those in the controls (**Figure 8** controls first and third column).

JO146 TREATMENT IS LETHAL DURING HEAT STRESS AND RECOVERY FROM HEAT STRESS FOR *Chlamydia*

In many bacteria, HtrA is widely acknowledged to be a stress response protease as well as being involved in crucial functions

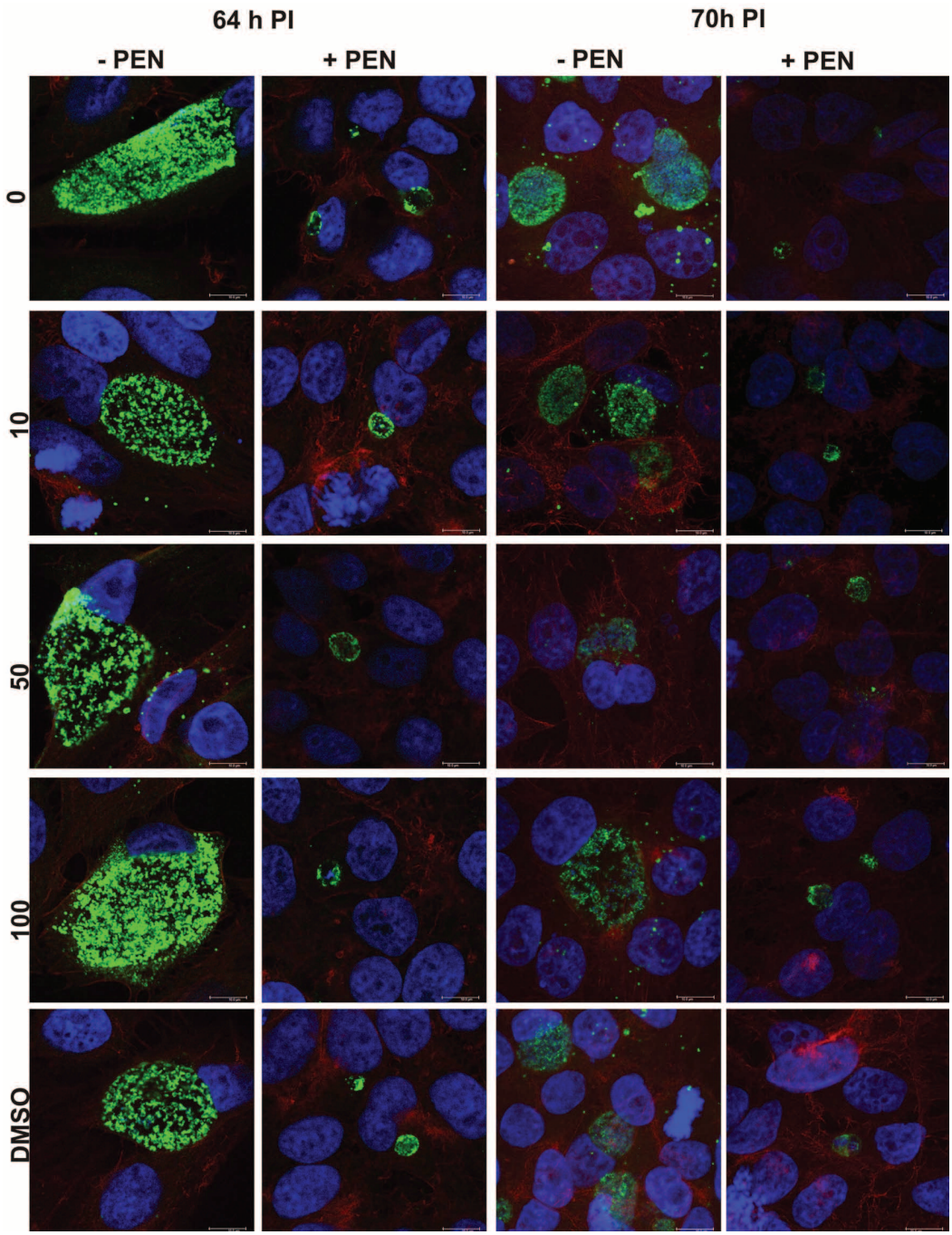

FIGURE 8 | Confocal microscopy images of penicillin persistent cultures and controls during reversion from persistence at 64 and 70 hPI. Cultures were treated with penicillin 100 U ml^{-1} at 4 h PI. Penicillin was removed at 40 h PI and JO146 treatment was commenced at 52 h PI. Controls with no penicillin first and third column, penicillin conditions second and fourth columns. JO146 concentrations are indicated to the right. The image colors are as follows, green: MOMP (major outer membrane protein) is green, blue: cell nucleus (DAPI), and red: β-actin. Scale bar (bottom right) indicates 10 μm.

in outer membrane protein assembly and general protein maintenance (reviewed, Clausen et al., 2011). Accordingly, we used JO146 to evaluate the role of CtHtrA during heat stress and also during recovery from heat stress. We had previously shown increased protein levels of CtHtrA during a heat stress model, at 20 h PI, and this is also the phase of the developmental cycle at which we already know JO146 is effective (Huston et al., 2008; Gloeckl et al., 2012). *C. trachomatis* cultures (20 h PI) were heat stressed for 4 h in a 42°C 5% CO_2 incubator prior to subsequent restoration to 37°C and completion of the developmental

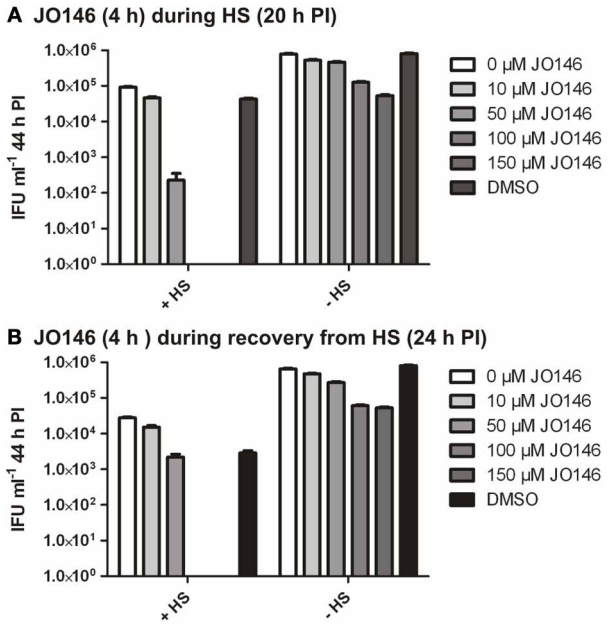

FIGURE 9 | *C. trachomatis* viable infectious yield at 44 h PI after 4 h heat shock with JO146 treatment (20–24 h PI) or JO146 treatment during recovery from heat shock (24–28 h PI). (A) The viable infectious yield after JO146 treatment for 4 h at 20 h PI with and without heat shock at 42°C. **(B)** Viable infectious yield after JO146 treatment for 4 h during recovery from heat shock (24–28 h PI) ($n = 27$). The concentration of JO146 is indicated by the gray scale bars, legend to the right of each graph.

cycle. JO146 was added either during the heat shock and subsequently removed, or during the 4 h of post heat shock recovery and subsequently removed. The impact of JO146 treatment and the heat shock conditions were evaluated by determining the viable infectious yield at 44 h PI. In our previous work using JO146 we demonstrated that the compound needed to be present for longer than 4 h for lethality, therefore, during this experiment we used a higher concentration of 150 µM. As shown in **Figure 9A**, the presence of JO146 during heat shock was completely lethal at 100 and 150 µM. 50 µM JO146 treatment during the 4 h heat shock also resulted in a marked loss of chlamydial viability (>2 log) (**Figure 9A**). Some JO146-induced reduction in viability was also observed in the controls which were not heat shocked (**Figure 9A**, indicated on *x* axis), and this is consistent with our previous observations that JO146 needs to be present throughout the replicative phase (not for 4 h only) to be completely lethal (Gloeckl et al., 2013).

JO146 treatment during the first 4 h of recovery from heat shock was also completely lethal at higher concentrations (100 µM and 150 µM), with a minor impact on viable yield observed with 50 µM JO146 (**Figure 9B**). There was a more noticeable reduction in viability for the heat shocked cultures compared to the controls during this experiment (**Figure 9B**, conditions indicated on *x* axis). However, in spite of these differences it is clear that JO146 treatment both during heat stress and recovery from was lethal for *Chlamydia*, with a more marked impact at lower concentrations during the heat stress.

DISCUSSION

HtrA is an essential maintenance protease and chaperone for many bacteria. The protein conducts diverse roles in bacterial pathogenesis from being essential for outer membrane protein assembly (Purdy et al., 2007), to stress response and survival (Lipinska et al., 1989), cleavage of host proteins (Hoy et al., 2010) and intracellular infection survival (Pedersen et al., 2001). We previously used a chemical inhibition strategy to establish that *Chlamydia* HtrA (CtHtrA) is essential during the replicative phase of intracellular development. Using JO146 we found that CtHtrA inhibition at 16 h PI resulted in the reduction of inclusion sizes and eventually the inclusions were lost from the host cells with no detectable production of viable elementary bodies (Gloeckl et al., 2013). This data supports an essential role for CtHtrA during the replicative phase of chlamydial development. However, given the multi-tasking nature of HtrA already described for many other bacteria (reviewed, Clausen et al., 2011), we set out to test the hypothesis that CtHtrA is essential for heat stress conditions and during persistence models.

The data we present demonstrates that CtHtrA is essential for the reversion and recovery to viability for penicillin persistence and is also essential during heat stress and recovery from heat stress. The heat stress model is clearly likely to involve extracytoplasmic protein stress which will require both the protease activity and chaperone activity of CtHtrA. Clearly CtHtrA is essential, either for stress response or restoration of protein biogenesis, during the reversion and recovery to EBs from penicillin persistence. The recovery from penicillin persistence is very asynchronous and so it was not possible to uncouple restoration from persistence and the subsequent replication of the restored RBs. Therefore, it is possible that the impact of JO146 in this experiment was on the replication of recovered RBs or the recovery from penicillin persistence to reticulate bodies, or both. However, 44 h after reversion was commenced no viable EBs were detectable but they were at 50 h, which does suggest that the JO146 treatment was during the time frame likely to correspond with the majority of the population still undergoing reversion from persistence.

Based on the lack of lethality in the first model when JO146 was added during persistence, it is tempting to suggest that penicillin persistence does not involve a detrimental level of extracytoplasmic protein stress. It is important to note that there is a possibility of some off-target impacts of JO146, however, given the marked phenotypes which correspond with very specific phases and conditions of chlamydial culture observed here, these impacts are likely minor. The absolute requirement for CtHtrA during recovery from penicillin persistence is an exciting finding, and to our knowledge is the first identification of an essential protein for this transition. In addition, JO146 treatment in the presence of heat stress in a time frame (4 h) was completely lethal. This is amazingly quick given 4 h is consistent with less than 2 rounds of binary fission for *C. trachomatis* serovar D [has been identified to take 2.4 h per round of binary fission (Miyairi et al., 2006)]. This suggests that CtHtrA is essential during certain stress conditions and does not necessarily relate to replication or binary fission. This data suggests that perhaps penicillin persistence is a strategy to reduce cellular and protein stress which may be an explanation for why CtHtrA was found not to be essential

during penicillin persistence. Heat stress is highly likely to be a strong inducer of protein stress and for many bacteria is the main *in vitro* condition during which *htrA*- or *degP*- mutants are lethal (Lipinska et al., 1989). Therefore, it is not surprising that CtHtrA was essential even in this relatively short time of heat treatment. In summary, the data presented here demonstrates that the CtHtrA inhibitor JO146 is lethal for chlamydial recovery from penicillin persistence and for heat stress conditions.

AUTHOR CONTRIBUTIONS

Vanissa A. Ong conducted the culture based experiments and analyzed data, James W. Marsh and Amba Lawrence conducted cultures experiments and analyzed data, Peter Timms contributed to experimental design and analyzed data. John A. Allan contributed to experimental design and analyzed data, Wilhelmina M. Huston contributed to experimental design and data analysis. All authors contributed to manuscript drafting.

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Host immune responses after hypoxic reactivation of IFN-γ induced persistent *Chlamydia trachomatis* infection

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Genital tract infections with *Chlamydia trachomatis* (*C. trachomatis*) are the most frequent sexually transmitted disease worldwide. Severe clinical sequelae such as pelvic inflammatory disease (PID), tubal occlusion, and tubal infertility are linked to inflammatory processes of chronically infected tissues. The oxygen concentrations in the female urogenital tract are physiologically low and further diminished (0.5–5% O_2, hypoxia) during an ongoing inflammation. However, little is known about the effect of a low oxygen environment on genital *C. trachomatis* infections. In this study, we investigated the host immune responses during reactivation of IFN-γ induced persistent *C. trachomatis* infection under hypoxia. For this purpose, the activation of the MAP-kinases p44/42 and p38 as well as the induction of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and MCP-1 were analyzed. Upon hypoxic reactivation of IFN-γ induced persistent *C. trachomatis* infection, the phosphorylation of the p44/42 but not of the p38 MAP-kinase was significantly diminished compared to IFN-γ induced chlamydial persistence under normoxic condition. In addition, significantly reduced IL-6 and IL-8 mRNA expression levels were observed for reactivated *Chlamydiae* under hypoxia compared to a persistent chlamydial infection under normoxia. Our findings indicate that hypoxia not only reactivates IFN-γ induced persistent *C. trachomatis* infections resulting in increased bacterial growth and progeny but also dampens inflammatory host immune signaling responses that are normally observed in a normoxic environment.

Keywords: *Chlamydia trachomatis*, persistence, hypoxia, reactivation, immune response

INTRODUCTION

Chlamydia trachomatis (*C. trachomatis*) is an obligate intracellular pathogen and the most frequent sexually transmitted bacterium worldwide. In the United States ∼1.5 million infections were reported in 2011 (Centers for Disease Control and Prevention, 2012). While most of the infections occur without symptoms, a symptomatic manifestation of urogenital chlamydial infection can be observed in ∼30% of the patients. In a subset of female patients, ascending genital tract infections cause disease such as salpingitis, pelvic inflammatory disease (PID), or tubal infertility (Peipert, 2003; Mardh, 2004). Chlamydial pathology is attributed to severe inflammatory processes leading to scarring and loss of functional epithelial tissue (Peipert, 2003; Mardh, 2004). During the infection, different pro-inflammatory cytokines such as Interleukin (IL)-1, IL-6, and IL-8 are induced and thought to affect the disease outcome (Rasmussen et al., 1997; Hanada et al., 2003; Buchholz and Stephens, 2007; Hvid et al., 2007). Reoccurrence or a chronic infection with *C. trachomatis* are discussed to be central mediators of disease progression and final outcome (Dean et al., 2000). However, it is not clear whether chlamydial reoccurrence after a symptomatic episode with/without antibiotic treatment occurs mainly due to reinfection transferred from the sexual partner or due to reactivation of persistent *C. trachomatis* from a silent state (Golden et al., 2005; Geisler, 2007). Persistence describes a non-infectious but viable developmental stage. In the *in vitro* chlamydial persistence models, the infection is characterized by an altered intracellular inclusion morphology that is accompanied by reduced chlamydial progeny and increased cell survival compared to actively replicating pathogens (Hogan et al., 2004). Chlamydial persistence can be induced through various stimuli including interferon-γ (IFN-γ), treatment with sub-inhibitory concentrations of antibiotics or iron depletion (Wyrick, 2010). Although the induction of persistent *Chlamydiae* has been extensively studied *in vitro*, data showing persistently infected urogenital tissues in diseased females are still missing, hence characteristics of persistent *in vivo* urogenital *C. trachomatis* infections in humans were not observed yet. Regarding this, persistence defining properties are only based on *in vitro* experiments and might be different *in vivo*. Persistent chlamydial infection of the urogenital tract *in vivo* have so far only been shown in a *C. muridarum* mice infection model (Phillips et al., 2012). Phillips et al. could show a reduced number of infectious *Chlamydiae* in persistent infection while pre-16s rRNA expression was not changed, indicating a viable but not infectious chlamydial form (Phillips et al., 2012). Further, they could show chlamydial inclusions with abnormal reticulate bodies but without elementary bodies (EBs) via transmission electron microscopy (Phillips et al., 2012). These findings were in line with the observations of *in vitro* persistence models.

We and others could show that persistent *C. trachomatis* are less susceptible to currently available first-line antimicrobials which presumably could result in clinical treatment failures (Reveneau et al., 2005; Phillips et al., 2012; Shima et al., 2013) and might favor *C. trachomatis* survival within its biological niche thereby inducing chronic infections. In this study, we focused on the IFN-γ induced persistence of *C. trachomatis* as the most extensively studied model in the past (Beatty et al., 1993, 1994; Roth et al., 2010). Based on a previous observation that anti-chlamydial activity of IFN-γ is reduced in a low oxygen environment allowing persistent *Chlamydiae* to reactivate and proliferate (Roth et al., 2010), we wondered about the host immune responses that are induced during this process.

METHODS

EPITHELIAL CELL CULTURE AND *C. TRACHOMATIS* INFECTION

A total of 2.5×10^5 HeLa-229 cells were cultured with 5 ml RPMI 1640 (PAA Laboratories, Cölbe, Germany) supplemented with 5% FBS (PAA Laboratories), 100 mg/L L-glutamine (PAA Laboratories), 1× non-essential amino acids (PAA Laboratories) with or without 5 U/ml IFN-γ (Peprotech, Hamburg, Germany) in a 6-well plate and incubated for 24 h under normoxia at 37°C, 20% O_2, 5% CO_2. IFN-γ was present over the whole experiment. After incubation, HeLa-229 cells were infected with 2 inclusion forming units (IFUs)/cell of *C. trachomatis* serovar D and centrifuged for 60 min at 700 × *g*. After 24 h incubation under normoxia, persistent *C. trachomatis* infected cells were further cultivated either in normoxic or hypoxic incubators (37°C, 2% O_2, 5% CO_2) (Toepffer Lab Systems, Goeppingen, Germany) for additional 2 and 3 days (d). The medium was exchanged every second day with medium containing 5 U/mL IFN-γ. Hypoxic samples were cultivated in preconditioned medium that was incubated under hypoxic conditions for 12 h before medium exchange.

CHLAMYDIAL RECOVERY

The burden of infectious *C. trachomatis* EBs after intracellular development under normoxic and hypoxic conditions was determined by titration experiments as described before (Beatty et al., 1993). In brief, infected cell monolayers were harvested and disrupted by glass beads. Disrupted cells including *C. trachomatis* were inoculated in serial dilutions on confluent HEp-2 cell monolayers in DMEM supplemented with 10% FBS, 100 mg/L L-glutamine, 1× non-essential amino acids under normoxia. Development of chlamydial inclusions was analyzed 48 h post infection (h p.i.) using an anti-*Chlamydia*-LPS antibody (kindly provided by H. Brade; Research Centre Borstel, Borstel, Germany) together with a secondary FITC-labeled anti-mouse antibody (Dako, Glostrup, Denmark). Recovered *C. trachomatis* were calculated by observation of 10 high-power fields (20× magnification). Infectious progeny under different conditions was determined by calculating the absolute IFUs. All data are the average from seven independent experiments and the error bars represent the standard error of the mean (s.e.m.).

FLUORESCENCE MICROSCOPY

To analyze *C. trachomatis* inclusion morphology after 2 and 3 d infection, *C. trachomatis* infected cells were grown on coverslips. After fixation with methanol, cells were stained with FITC-labeled monoclonal anti-*Chlamydia*-LPS antibody and evans blue (Oxoid, Cambridgeshire, UK) and morphology was analyzed by a fluorescence microscope (Keyence, Osaka, Japan). To determine the inclusion size 10 inclusions/condition from five different experiments (total 50 inclusions/condition) were measured by using the BZ-II-Analyzer (Keyence, Osaka, Japan).

WESTERN BLOT ANALYSIS

For determination of phospho-p38/-p44/42 (Cell Signaling, Danvers), cells were prepared with western blot lysis buffer (125 mM Tris-HCl pH 7.8, 20% glycerol, 4% SDS (sodium dodecyl sulfate), 0.1 M dithiothreitol, bromphenol blue, Sigma Aldrich, St. Louis) at the indicated time points. Samples were analyzed by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). Afterwards, proteins were transferred to nitrocellulose membranes (Whatman Inc., Florham Park, NJ). Membranes were blocked with TBS (0.1% Tween)/5% fat-free skimmed milk and incubated with the respective antibodies. For detection, a horseradish peroxidase-linked anti-mouse IgG antibody (Cell Signaling) and enhanced chemiluminescence substrate (Thermo Fisher Scientific, Rockford, IL) were used. Images were acquired by Fusion FX7 (Vilber Lourmat, Eberhardzell, Germany) and the density of each band was measured by Bio-1D software (Vilber Lourmat). Equal loading and blotting efficiency were verified by an anti-β-actin antibody and pre-stain marker (Cell Signaling). All data are the average from seven independent experiments and the error bars represent the standard error of the mean (s.e.m.).

ANALYSIS OF mRNA EXPRESSION

Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel, Dueren, Germany) and transcribed into cDNA by the First-Strand PCR kit (Roche, Basel, Switzerland). PCR amplification was performed by using the LightCycler Detection System (Roche). Relative quantification of IL-1β (forward TCCCCAGCCCTTTTGTTGA, reverse TTAGAACC AAATGTGGCCGTG), IL-6 (forward CCTTCCAAAGATGGC TGAAA, reverse CAGGGGTGGTTATTGCATCT), IL-8 (forward CCAGGAAGAAACCACCGGA, reverse GAAATCAGGA AGGCTGCCAAG), MCP-1 (forward CATTGTGGCCAAGGAGA TCTG, reverse CTTCGGAGTTTGGGTTTGCTT) mRNA expression was performed against 18S rRNA (forward TCAAGAACG AAAGTCGGAGG, reverse GGACATCTAAGGGCATCACA) and normalized to the respective mRNA expression levels in normoxic non-infected cells by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All data are the average from seven independent experiments and the error bars represent the standard error of the mean (s.e.m.).

STATISTICAL ANALYSIS

Data are indicated as mean ± s.e.m. Statistical analysis was performed with the tailed, unpaired Student *t*-test. *p*-values ≤ 0.05 were considered as statistically significant.

RESULTS

HYPOXIC REACTIVATION OF PERSISTENT *C. TRACHOMATIS*

To determine whether IFN-γ induced persistent *C. trachomatis* D could be reactivated in HeLa-229 cells under hypoxia, we

used the experimental setup displayed in **Figure 1**. After transfer of persistent *C. trachomatis* in a hypoxic environment (2% O_2), reactivation of chlamydial growth was observed by a significant increase in the inclusion size compared to cultivation under normoxic conditions (**Figures 2A–E**). In accordance, the amount of recoverable infectious *C. trachomatis* significantly increased after 3 days cultivation in hypoxia, whereas no increase was observed under normoxic conditions (**Figure 2F**). These observations confirm previous findings of reactivated *C. trachomatis* L2 in HEp-2 cells (Roth et al., 2010) and indicate that IFN-γ treatment under hypoxia is less effective to maintain *C. trachomatis* in a persistent state.

ANALYSIS OF MAP-KINASE p44/42 AND p38 PHOSPHORYLATION IN REACTIVATED *C. TRACHOMATIS* INFECTION UNDER HYPOXIA

To further investigate the influence of reactivated *C. trachomatis* on host cell immune responses under hypoxia, we analyzed the activation of the MAP-kinases p44/42 and p38 (**Figure 3**) which were described to be activated during productive infection under normoxic conditions. Under normoxic condition, we observed a significantly enhanced accumulation of the phosphorylated p44/42 MAP-kinase upon IFN-γ treatment which was not further enhanced in persistently infected cells. Interestingly, the accumulation of the phosphorylated p44/42 MAP-kinase was significantly reduced in IFN-γ treated cells with (**Figure 3**, 3 d) or without (w/o) (**Figure 3**, 2 and 3 d) *C. trachomatis* infection under hypoxic compared to normoxic conditions. Although hypoxic cultivation slightly enhanced the phosphorylation of p38 in all samples, no significant differences in the activation pattern were observed in IFN-γ treated cells w/o *C. trachomatis* infection. Furthermore, no accumulation of phosphorylated p38 could be observed in normoxic cells treated with IFN-γ w/o *C. trachomatis* infection.

REDUCED PRO-INFLAMMATORY CYTOKINE INDUCTION IN REACTIVATED *C. TRACHOMATIS* INFECTION UNDER HYPOXIA

To reveal whether hypoxic reactivation of persistent *C. trachomatis* is recognized by the host cell and translated into a pro-inflammatory cytokine response, mRNA expression levels of IL-1β, IL-6, IL-8, and MCP-1 were analyzed. Under normoxic conditions, IFN-γ treatment w/o *C. trachomatis* infection significantly up-regulated IL-6 and IL-8 mRNA expression after 2 and 3 d, respectively (**Figures 4A,B**). In contrast, IFN-γ alone did not (IL-8) or only moderately (IL-6) induce cytokine mRNA expression under hypoxic conditions. Besides, *C. trachomatis* infection of IFN-γ treated cells significantly up-regulated IL-6 (2 and 3 d) and IL-8 (3 d) expression compared to IFN-γ treated samples under hypoxic conditions. In all cases except for IL-8 in IFN-γ treated *C. trachomatis* infected cells after 3 d and the uninfected and untreated controls, the mRNA expression levels were significantly lower in cells that were incubated under hypoxia compared to normoxia (**Figures 4A,B**). For IL-1β and MCP-1 no induction of the mRNA expression levels was observed in persistently *C. trachomatis* infected cells under normoxia, nor reactivated *C. trachomatis* infection under hypoxia (**Figures S1A,B**). Our data indicate that in HeLa-229 cells reactivation of formerly persistent *C. trachomatis* in a hypoxic environment is accompanied by a dramatically less pronounced pro-inflammatory host cell immune response compared to persistent *Chlamydiae* under normoxic conditions.

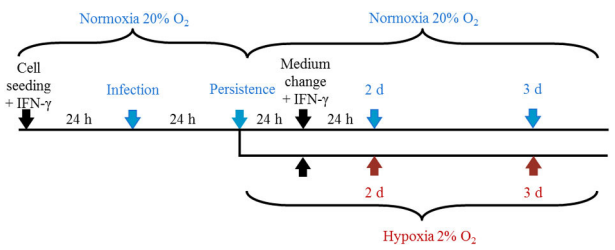

FIGURE 1 | Representation of the experimental setup. Cells were cultured under normoxia for 24 h in the presence of IFN-γ (5 U/mL) and were infected with *C. trachomatis* (2 IFU/cell) the following day. After 24 h, cells were further incubated under normoxia (20% O_2) or transferred to hypoxic (2% O_2) conditions with constant IFN-γ supplementation before samples were collected at the indicated time points.

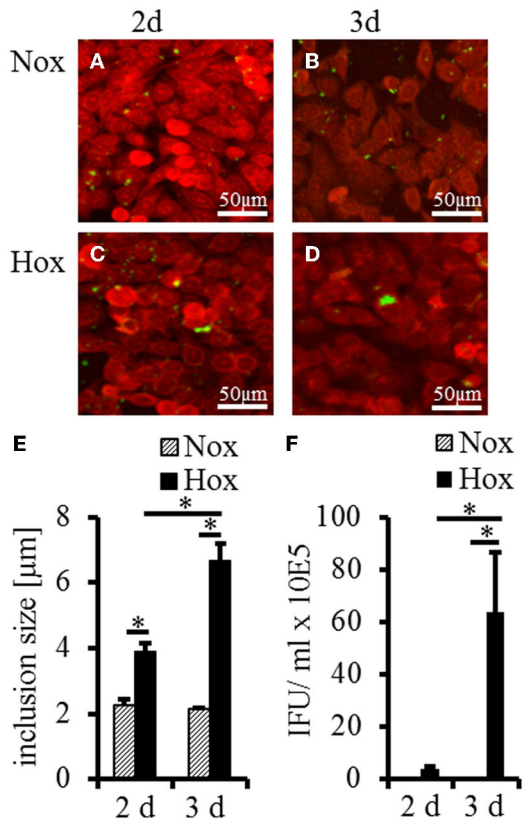

FIGURE 2 | Recovery analysis of IFN-γ treated *C. trachomatis* infected cells under normoxic and hypoxic conditions. Immunofluorescence staining shows reactivation of *C. trachomatis* (green) in HeLa-229 cells (red) under hypoxic (Hox) conditions **(C,D)** but not under normoxic (Nox) conditions **(A,B)** after 2 and 3 d, respectively. Quantitative analysis of the inclusion size showed significant larger inclusion after 2 and 3 d under hypoxic condition compared with normoxic condition **(E)**. Quantitative analysis of the infectious progeny of IFN-γ treated *C. trachomatis* after incubation for up to 3 d under normoxic and hypoxic conditions **(F)** ($n = 7$, mean ± s.e.m., $^*p \leq 0.05$).

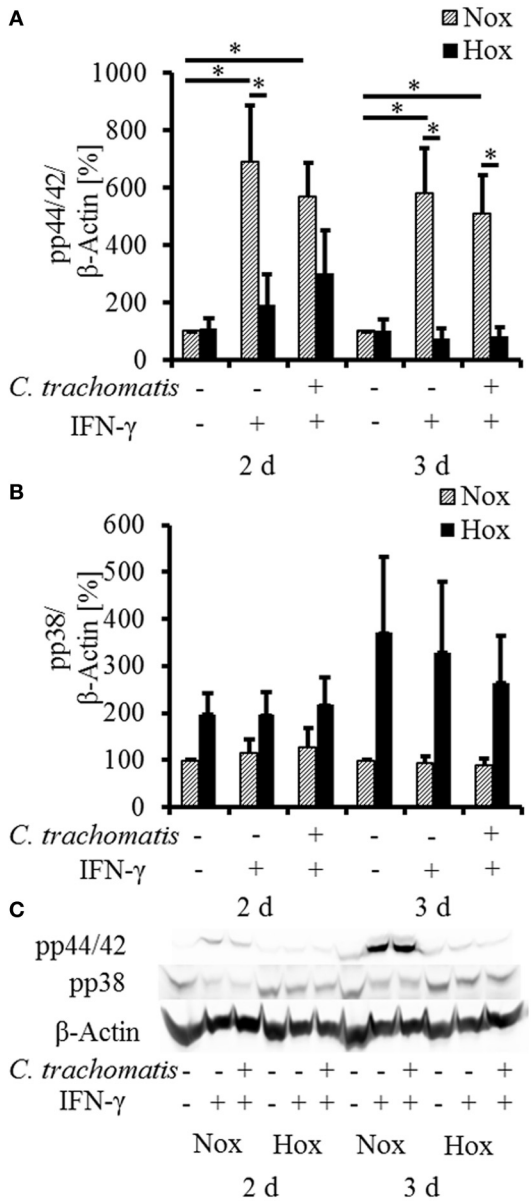

FIGURE 3 | Western blot analysis of MAP-kinase phosphorylation in IFN-γ treated *C. trachomatis* infected cells under normoxic and hypoxic conditions. Western blot and densitometric analysis of the phosphorylation of the MAP-kinases p44/42 **(A)** and p38 **(B)** in IFN-γ treated cells after 2 and 3 d cultivation under normoxic (Nox) and hypoxic (Hox) conditions ($n = 7$, mean ± s.e.m., $^*p \leq 0.05$). **(C)** displays a representative western blot of p38 and p44/42 phosphorylation under normoxic and hypoxic conditions.

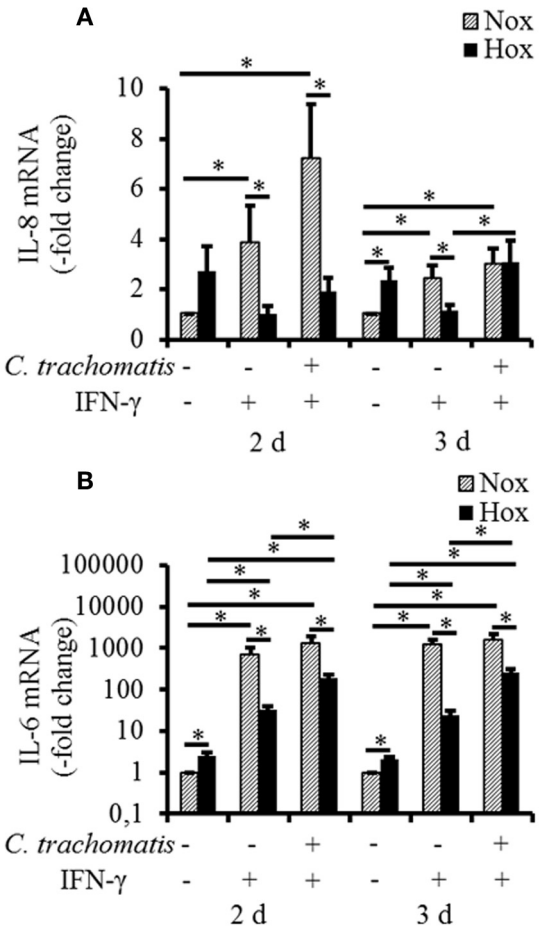

FIGURE 4 | mRNA expression of IL-6 and IL-8 in IFN-γ treated *C. trachomatis* infected cells under normoxic and hypoxic conditions. Quantitative analysis of IL-8 **(A)** and IL-6 **(B)** mRNA expression in IFN-γ treated *C. trachomatis* infected cells after 2 and 3 d cultivation under normoxic (Nox) and hypoxic (Hox) conditions ($n = 7$, mean ± s.e.m., $^*p \leq 0.05$).

DISCUSSION

Genital tract infections with the intracellular bacteria *C. trachomatis* are a frequent cause of PID, ectopic pregnancy, and tubal factor infertility (Peipert, 2003). Based on experimental data it is assumed that the infection leads via TLR or NOD1 signaling to an activation of host MAP-kinases (e.g., p44/42 and p38) as well as the NFκB pathway and subsequently to an induction of a pro-inflammatory immune response (Rasmussen et al., 1997; Hanada et al., 2003; Strober et al., 2006; Bastidas et al., 2013; Zhou et al., 2013). A mainly Th1 and NK-cell mediated release of IFN-γ protects against genital tract *C. trachomatis* infections in humans and mice (Cohen et al., 2000; Roan and Starnbach, 2006) but may also induce *C. trachomatis* persistence *in vivo* (Beatty et al., 1993). Impaired host immunity and micro- environmental conditions such as hypoxia have been shown to impair anti-chlamydial activity of IFN-γ leading to reactivation of *Chlamydiae* and finally productive infection (Roth et al., 2010).

However, nothing is known about the immune response in infected cells during reactivated *C. trachomatis* infection under hypoxic conditions. This is the first report showing that the activation of the MAP-kinase p44/42 and the expression of the pro-inflammatory cytokines IL-6 and IL-8 were diminished in reactivated *C. trachomatis* infection under hypoxia compared to persistently infected cells under normoxia. The underlying mechanisms are completely unknown but could either be attributed to oxygen-dependent host-cell signaling pathways or

linked to pathogen related factors. Under normoxic conditions IFN-γ was described to induce IL-6 via activation of p44/42 (Salmenpera et al., 2003). Besides, IFN-γ prolongs the activation of p44/42 (Valledor et al., 2008) which augments the induction of IL-6 and IL-8 expression in HeLa-229 cells under normoxic conditions (Yang et al., 2008). Although a strong IFN-γ mediated activation of p44/42 and an induction of IL-6 and IL-8 was detected under normoxic conditions, less phosphorylation or cytokine induction was observed under hypoxic conditions. In accordance with previous results we observed a diminished activity of IFN-γ under hypoxia (Roth et al., 2010), which can be explained by the altered p44/42 activation under hypoxic conditions finally leading to a significantly impaired IFN-γ driven induction of IL-6 and IL-8.

Furthermore, for productively *C. trachomatis* infected cells it is known that the pathogen directly attaches to the surface of the host cell thereby activating TLR2/4 (Darville et al., 2003; Bulut et al., 2009). In addition, *C. trachomatis* may induce IL-1β, IL-6, and IL-8 by inflammasome-dependent activation of caspase-1 or NOD1 recognition (Welter-Stahl et al., 2006; Buchholz and Stephens, 2008; Cheng et al., 2008). TLR- and NOD1-mediated signaling has been directly connected to the phosphorylation of MAP-kinase p44/42 (Buchholz and Stephens, 2008; Wortzel and Seger, 2011). Under hypoxic reactivation of persistent *Chlamydiae* no activation of the p44/42 MAP-kinase was observed, which could explain the diminished IL-6 and IL-8 induction under these conditions. It was previously reported that p44/42 could be inactivated by the mitogen-activated protein kinase phosphatase-1 (MKP-1), a dual specific phosphatase carrying two hypoxic response elements (HRE) in the promoter region (Liu et al., 2005). Therefore, it has to be further elucidated if hypoxia in general dampens the p44/42 phosphorylation by MKP-1 activation and thereby modulates the immune response and cell homeostasis in a so far unknown manner. There is a tight interconnection between the NFκB and HIF-1α (hypoxia-inducible factor 1-α) signaling pathways in the regulation of inflammatory host responses (Barnes, 1997; Taylor, 2008). Thus, NFκB is activated under hypoxia (Cummins et al., 2006) but in turn also induces transcriptional up-regulation of HIF-1α expression (Rius et al., 2008). NFκB activation and subsequent pro-inflammatory gene expression is supposed to be abrogated by an enhanced expression of IκB kinase-α (IKKα) under prolonged hypoxia (Lawrence et al., 2005; Cummins et al., 2006).

Besides the above mentioned host- related factors that may dampen immune responses under hypoxia, additional mechanisms are conceivable, which are directly induced by *C. trachomatis*. Possible mediators are the chlamydial protease CT441 which might interfere with the NFκB pathway, thereby modulating the immune response and inhibiting the IL-6 and IL-8 gene expression (Lad et al., 2007a,b). For other intracellular bacteria including *Chlamydia pneumoniae* (*C. pneumoniae*), *Mycobacterium tuberculosis* (*M. tuberculosis*), and *Ehrlichia chaffeensis* (*E. chaffeensis*) several other mechanisms for silencing host immune responses under normoxia have been described. Thus, it has been shown that these bacteria are able to regulate the host immune response by impairing the TLR signaling cascade, blocking the secretion of pro-inflammatory cytokines or induction of anti-inflammatory cytokines such as IL-10 (Ismail et al., 2002; Flynn and Chan, 2003). *M. tuberculosis* expresses a 19 kDa protein with immunmodulatory functions that directly binds to the TLR2 receptor and inhibits the IFN-γ induced MHC class II antigen presentation, whereas *E. chaffeensis* was described to interfere with cytokine mRNA stability by an unknown mechanism (Lee and Rikihisa, 1996; Flynn and Chan, 2003). Degradation of the TRAF3 signaling molecule, a downstream target of TLR3, which activates IFN-β secretion, was described in *C. pneumoniae* but not in *C. trachomatis* infection (Wolf and Fields, 2013). Nevertheless, *C. trachomatis* might have similar mechanisms that upon enhanced intracellular replication after hypoxic reactivation results in the down-regulation of host immune responses.

In conclusion, IFN-γ induced persistent *C. trachomatis* is reactivated under hypoxic condition and remains mostly unrecognized by the host cell. In further experiments the influence of the impaired activation of the host immune system under hypoxia has to be elucidated in the context of the cellular metabolism and apoptosis signaling. Furthermore, the influence of hypoxia on IFN-γ induced persistence has to be clarified *in vivo*.

AUTHOR CONTRIBUTIONS

Conception, design of the work: Stefan Jerchel, Jan Rupp; Acquisition of data: Stefan Jerchel, Larissa Schuchardt; Analysis and interpretation: Stefan Jerchel, Jan Rupp, Kensuke Shima, Inga Kaufhold, Larissa Schuchardt; Drafting the manuscript for important intellectual content: Stefan Jerchel, Jan Rupp, Kensuke Shima, Inga Kaufhold; Final approval of the version to be published: Stefan Jerchel, Jan Rupp, Kensuke Shima, Inga Kaufhold, Larissa Schuchardt; Agreement to be accountable for all aspects of the work: Stefan Jerchel, Jan Rupp, Inga Kaufhold, Kensuke Shima, Larissa Schuchardt.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00043/abstract

Figure S1 | mRNA expression of MCP-1 and IL-β in IFN-γ treated *C. trachomatis* infected cells under normoxic and hypoxic conditions. Quantitative analysis of MCP-1 **(A)** and IL-1β **(B)** mRNA expression in IFN-γ treated *C. trachomatis* infected cells after 2 and 3 d cultivation under normoxic (Nox) and hypoxic (Hox) conditions ($n = 7$, mean ± s.e.m., $^{*}p \leq 0.05$).

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Chlamydia exploit the mammalian tryptophan-depletion defense strategy as a counter-defensive cue to trigger a survival state of persistence

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We previously proposed that in *Chlamydiaceae* rapid vegetative growth and a quiescent state of survival (persistence) depend upon alternative protein translational profiles dictated by host tryptophan (Trp) availability. These alternative profiles correspond, respectively, with a set of chlamydial proteins having higher-than-predicted contents of Trp ("Up-Trp" selection), or with another set exhibiting lower-than-predicted contents of Trp ("Down-Trp" selection). A comparative evaluation of *Chlamydiaceae* proteomes for Trp content has now been extended to a number of other taxon families within the *Chlamydiales* Order. At the Order level, elevated Trp content occurs for transporters of nucleotides, S-adenosylmethionine (SAM), dicarboxylate substrates, and Trp itself. For Trp and nucleotide transporters, this is even more pronounced in other chlamydiae families (*Parachlamydiaceae*, *Waddliaceae*, and *Simkaniaceae)* due to extensive paralog expansion. This suggests that intracellular Trp availability served as an ancient survival cue for enhancement or restraint of chlamydial metabolism in the common *Chlamydiales* ancestor. The *Chlamydiaceae* Family further strengthened Up-Trp selection for proteins that function in cell division, lipopolysaccharide biosynthesis, and methyltransferase reactions. Some proteins that exhibit Up-Trp selection are uniquely present in the *Chlamydiaceae*, e.g., cytotoxin and the paralog families of polymorphic membrane proteins (Pmp's). A striking instance of Down-Trp selection in the *Chlamydiaceae* is the chorismate biosynthesis pathway and the connecting menaquinone pathway. The newly recognized 1,4-dihydroxy-6-napthoate pathway of menaquinone biosynthesis operates in *Chlamydiaceae*, whereas the classic 2-napthoate pathway is used in the other *Chlamydiales* families. Because of the extreme Down-Trp selection, it would appear that menaquinone biosynthesis is particularly important to the integrity of the persistent state maintained under conditions of severe Trp limitation, and may thus be critical for perpetuation of chronic disease states.

Keywords: tryptophan, chlamydiae, persistence, menaquinone biosynthesis, Up-Trp selection, Down-Trp selection, reductive evolution

INTRODUCTION

PERSISTENCE

One form of immune evasion is a developmental state of the *Chlamydiaceae* Family called "persistence" that is triggered as a response to stress stimuli that cue an impending immune response by the host (Beatty et al., 1994). Persistence is a sophisticated survival mode, whereby a state of reversible quiescence is implemented. Recent reviews have been published in which the nature of persistence has been discussed (Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). Beyond the general impact for pathogen survival, persistence can be equated with chronic disease states of the host, e.g., inflammatory arthritis in humans (Beatty et al., 1994; Hogan et al., 2004).

Up-Trp AND Down-Trp SETS OF PROTEINS

A chlamydial mechanism has evolved which mutes the expression of gene products necessary for the rapid pathogen proliferation associated with acute disease, but which is permissive to the expression of gene products that underlie the unique morphological and developmental characteristics of persistence. This switch from one translational profile to an alternative translational profile was proposed by Lo et al. (2012) to be accomplished by maximizing the tryptophan (Trp) content (Up-Trp selection) of some key proteins needed to sustain rapid proliferation, e.g., ADP/ATP translocase, hexose-phosphate transporter, phosphoenolpyruvate (PEP) carboxykinase, the Trp transporter, the polymorphic membrane protein (Pmp) superfamily for cell adhesion and antigenic variation, and components of the cell-division pathway—at the same time minimizing the Trp content (Down-Trp selection) of other proteins needed to maintain the state of persistence. A bioinformatic analysis of the Trp content of the proteomes of six *Chlamydiaceae* genomes was carried out (Lo et al., 2012) in which the Trp content of each protein was expressed as a "p/P ratio", i.e., (Trp content of a given protein); (Trp content of its Proteome).

Protochlamydia amoebophila (Pamo) was included as a phylogenetically near out-group proteome and *E. coli* (Ecol) was used as a phylogenetically distant out-group proteome. Trp content in proteomes increases with increase in genomic G/C content. Thus, p/P ratios were used to normalize the Trp-content data in order to facilitate the comparison of different organisms.

RATIONALE TO EXPLAIN FEASIBILITY OF Up-Trp SELECTION AS A PATHOGEN STRATEGY

The biosynthesis of Trp is particularly costly because of the energy-metabolite resources needed, which makes it understandable why chlamydiae (and many other pathogens and symbionts) have evolved the luxury of reliance upon host resources for preformed Trp to conserve energy. Since Trp is thus defined as a metabolite of particular value, a reasonable question arises as to how a pathogen strategy of selectively increased Trp usage to accommodate the translational profile of proteins important for rapid pathogen propagation could be feasible. In part, this is explained by the offsetting effect of Down-Trp selection for the set of proteins that is important for maintenance of the persistent state. Significantly, Up-Trp selection is further facilitated with minimal overall Trp usage by a number of innovative tactics: (i) A regionally dense concentration of Trp-residue placements can block translation of a given protein having an overall Trp content that is average or even low, (ii) Amplification of the Trp content of a single "master" protein required for expression or maturation of multiple "slave" proteins means that the suite of slave proteins remain sensitive to the controlling influence of Trp depletion, even though their Trp content might be low, and (iii) An elevated Trp content of just one or a few component enzymes in complex, multi-step pathways can create an Achilles-heel vulnerability of the overall pathway.

PHYLOGENETIC EXTENSION OF THE Trp-CONTENT ANALYSIS TO THE *Chlamydiales* ORDER

The *Chlamydiaceae* are well characterized obligate intracellular pathogens of humans and animals. In contrast, other families of the *Chlamydiales* Order have only recently come under the extensive scrutiny enabled by genome sequencing and bioinformatic analysis (Horn, 2008; Collingro et al., 2011). Three of these *Chlamydiae* families are the *Simkaniaceae*, *Parachlamydiaceae*, and *Waddliaceae*. The natural eukaryotic hosts for these families appear to be protozoans such as amoebae, although members of these chlamydial families exhibit a broad host range and have been associated with mammalian disease as emerging pathogens (Greub and Raoult, 2002). Phylogenetic trees indicate that of the four taxon families, the *Chlamydiaceae* branch at the deepest position (Collingro et al., 2011). This may seem surprising because their mammalian hosts, with which there are many co-evolved characteristics, appeared quite recently on the geologic timescale. It therefore seems likely that the *Chlamydiaceae* emerged recently as mammalian pathogens from an ancient, early-divergent lineage of the *Chlamydiales*, other descendents of which may yet be discovered in association with thus-far unknown hosts. The *Chlamydiaceae* possess the smallest, most evolutionarily reductive genome of the four taxon families, probably reflecting the niche specialization that is the relatively stable and homeostatic environment of the mammalian host. No free-living relatives have yet been described within the *Chlamydiales*. Thus far, all *Chlamydiales* have in common: (i) an obligate intracellular lifestyle as pathogens or endosymbionts, (ii) the targeting of eukaryotic organisms as hosts, and (iii) a similar developmental routine that transitions between infectious elementary bodies (EBs) and proliferative reticulate bodies (RBs). Since previous observations made with Pamo (Lo et al., 2012) had hinted that some events of Up-Trp and Down-Trp selection had occurred prior to the divergence of *Chlamydiaceae*, it was of interest to sort out which Up-Trp/Down-Trp selections were specific to the *Chlamydiaceae* and which exhibited a broader distribution among the *Chlamydiales*.

DIFFERENT MECHANISMS YIELD A COMMON OUTCOME OF Trp DEPLETION IN MAN AND MOUSE

The Trp starvation mechanism is best understood in the human/*Chlamydia trachomatis* host/pathogen relationship, but the similarity of Up-Trp and Down-Trp proteomic profiles in all pathogenic *Chlamydiaceae* implies that Trp availability is an underlying cue relied upon by this entire family of pathogens to trigger developmental transitions (Lo et al., 2012). However, the diversity of host organisms parasitized by the *Chlamydiaceae* deploy different immune-response tactics that do not necessarily implement the direct cytosolic degradation of Trp seen in *C. trachomatis*. How the same ultimate outcome of Trp depletion might have come to be is illustrated by a comparison of the scenarios of co-evolved features at work in the human/*C. trachomatis* and mouse/*C. muridarum* pairings of host and pathogen. Here replacement of an ancestral IFN-γ/GTPase/cytotoxin/Trp-depletion mechanism in the mammalian lineage by a contemporary IFN-γ/indoleamine dioxygenase/Trp-depletion mechanism in humans was proposed (Lo et al., 2012). The conclusion that the IFN-γ/GTPase/cytotoxin/Trp-depletion mechanism is the ancestral mechanism is the most parsimonious evolutionary possibility based upon the broadly distributed IFN-γ induced p47 GTPase/cytotoxin host/pathogen combination in mammals compared to the absence of the latter in primates which exhibit instead a phylogenetically narrow distribution of IFN-γ induced indoleamine dioxygenase (IDO).

THE MOUSE IFN-γ/GTPase/CYTOTOXIN/Trp-DEPLETION MECHANISM

As illustrated in **Figure 1**, four general steps are common to the generation of persistence in the two host/pathogen combinations. However, the specific events that intervene between steps (B) and (C) are quite different. The overall mechanism seen in mouse, in contrast with that of man, presumably resembles the mechanism present in the common ancestor of mammals. Here production of interferon-gamma (IFN-γ) induces p47 GTPase, which possesses membrane regulatory features that are effective against compartmented pathogens (Kim et al., 2011). The pathogen defense response, in turn, is to produce large, exportable cytotoxin molecules, virulence factors which target the p47 GTPase proteins (Bourne et al., 1990). The high-Trp cytotoxin molecules are very large (>3000 amino acids per monomer) and have been hypothesized (Lo et al., 2012) to act as Trp sinks within the inclusion, with cytotoxin export then completing the process creating a

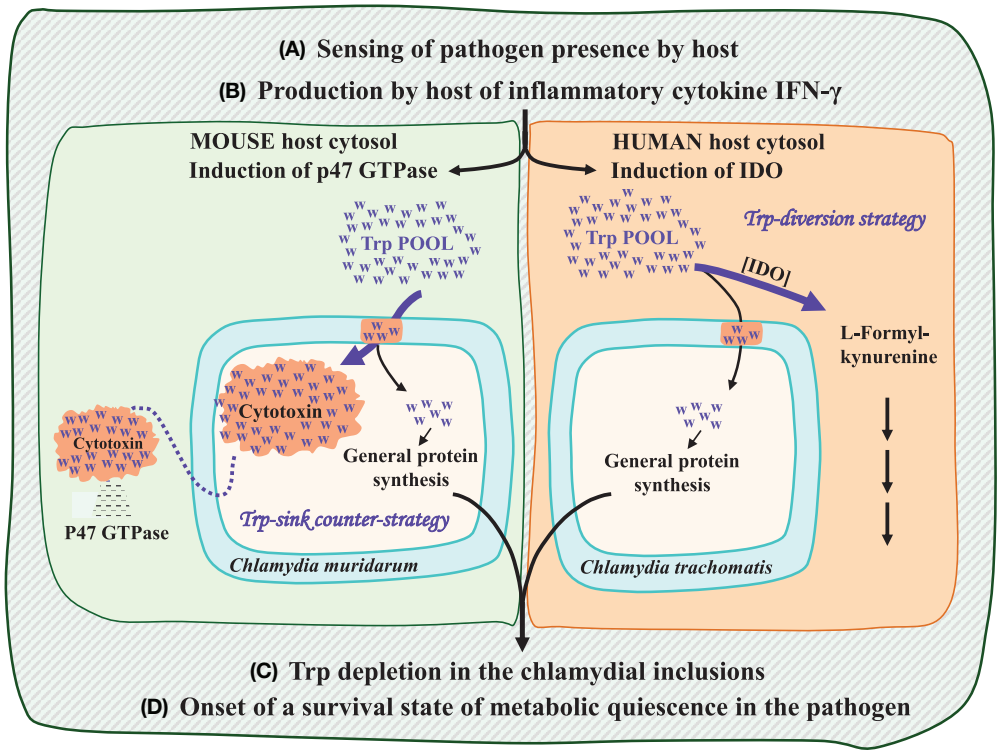

FIGURE 1 | Common and differential steps of the mammalian immune response: comparison of the mouse host (left side) with the human host (right side). Common steps are listed as **(A–D)**. Progression from step **(B)** to step **(C)** is mediated by different mechanisms. The human host deploys a strategy of Trp depletion via the action of an IFN-γ induced indoleamine dioxygenase (IDO). In the mouse host p47 GTPase production is the anti-chlamydial strategy that, in turn, is met with the chlamydial counter-strategy of cytotoxin production and its subsequent export from the chlamydial inclusion body. The cytotoxin molecules can be viewed as a Trp sink, with the exportation step effectively accomplishing Trp depletion. Free Trp molecules are depicted as blue "w"s, whereas proteinaceous Trp molecules are shown against bright orange backgrounds.

state of Trp depletion in the pathogen. The expenditure of Trp for cytotoxin translation is accentuated by the very large size of the protein and by its probable existence in a multimeric state (Voth et al., 2004). In the mouse pathogen, *C. muridarum*, flow of Trp to cytotoxin is at an even greater extreme because three paralog proteins are synthesized due to multiple gene duplications which generated three tandem paralog genes.

THE HUMAN IFN-γ/INDOLEAMINE DIOXYGENASE/Trp-DEPLETION MECHANISM

In man and other primates, IFN-γ manifests a quite different outcome, as visualized on the right side of **Figure 1**. Induction of the p47 GTPase family by IFN-γ does not occur in the primate lineage (Bekpen et al., 2005), instead being replaced by induction of IDO. The utilization of Trp as substrate by IDO directly creates a state of Trp depletion in the host cytosol. The ascension of IDO as a major player in immune surveillance in humans might be related to the increasing recognition that IDO induction and the consequent Trp depletion may be effective against some other intracellular pathogens, and even some extracellular pathogens. Indeed antiviral effects mediated by IDO have been reported as well (See Lo et al., 2012 and references therein). A very limited entry of Trp into the highly truncated fragments of the cytotoxin made by *C. trachomatis* is a consequence of the evolutionary disruption of the cytotoxin gene. Thus, the comparison given in **Figure 1** illustrates how the different effect of IFN-γ mobilization (step B) in mouse and man can unfold to give the same Trp-depletion result (step C). This occurs via direct exclusion of Trp from the pathogen inclusion in the first place (man) or indirectly, by generation of exportable cytotoxin (an effective Trp sink) to combat p47 GTPase (mouse).

OPPOSITE ADJUSTMENTS OF CYTOTOXIN IN *C. muridarum* AND *C. trachomatis*

In the absence of homologs from other *Chlamydiales* families, cytotoxin is concluded to have undergone Up-Trp selection by comparison of p/P Trp ratios with those of distant homologs available elsewhere (see Lo et al., 2012 for detailed comparisons). In the case of cytotoxin, an ancestral state of high Trp content produced by Up-Trp selection has been subject to very recent, and quite opposite adjustments in two species. Thus, Up-Trp selection has been further increased to a dramatic extent in the mouse pathogen (*Chlamydia muridarum*) via several rounds of paralog expansion, but drastically negated in the human pathogen (*C. trachomatis*) via frameshift mutations.

CRUCIAL FEATURES OF THE Trp-RESPONSIVE NETWORK SUPPORTING RAPID PATHOGEN PROLIFERATION

Figure 2 depicts alternative fates of Trp molecules in the human host cytosol: (i) as substrate for IDO with the consequence of Trp depletion (rightward arrow), or (ii) transport into the pathogen inclusion via two TyrP-family permeases (downward arrow). **Figure 2** is intended to display alternative (ii), i.e., the scenario that unfolds when immune surveillance has not yet been triggered to activate IDO catalysis. A selection of high-Trp proteins that are critical for rapid proliferation of *C. trachomatis* under conditions of acute disease is diagrammed in **Figure 2**. These include proteins that accomplish the import of essential nutrients from the host, proteins that accommodate the export of virulence factors that interact with the host biochemical network, and proteins that play key roles in basic metabolism.

PERMEASES

Membrane proteins generally exploit Trp for its unique physical properties, and Up-Trp selection has further increased the Trp content of a number of permeases. Noteworthy transporters of high Trp content shown across the top of **Figure 2** include a narrow-specificity ATP transporter, a broad-specificity nucleotide triphosphate (NTP) transporter, and transporters for glucose-6-phosphate, iron, S-adenosylmethionine (SAM), and dicarboxylate keto acids. Centrally, the import of Trp itself is mediated in *Chlamydiaceae* by one or two transporters of the TyrP family that have high-Trp content. Thus, import of Trp is self-limiting in the sense that any decrease of TyrP synthesis during starvation for Trp will tend to abort the entire pyramid of high-Trp proteins that depend upon TyrP for import of a Trp supply.

VIRULENCE FACTORS

Histone methylase is a high-Trp protein that well exemplifies an exported virulence factor that interacts with the host system under conditions of rapid growth. In *C. trachomatis* histone methylase (encoded by CT737) has been reported to methylate three different host proteins (Pennini et al., 2010). Not only its synthesis, but its export, is likely sensitive to Trp availability since its export depends upon the complex type III secretion system, some components of which exhibit high-Trp content. This methylase also undergoes self-methylation as a mechanism of increasing catalytic efficiency, a property of considerable interest in that the SAM substrate is expected to be of limited abundance under conditions of Trp starvation since the SAM transporter is a high-Trp protein.

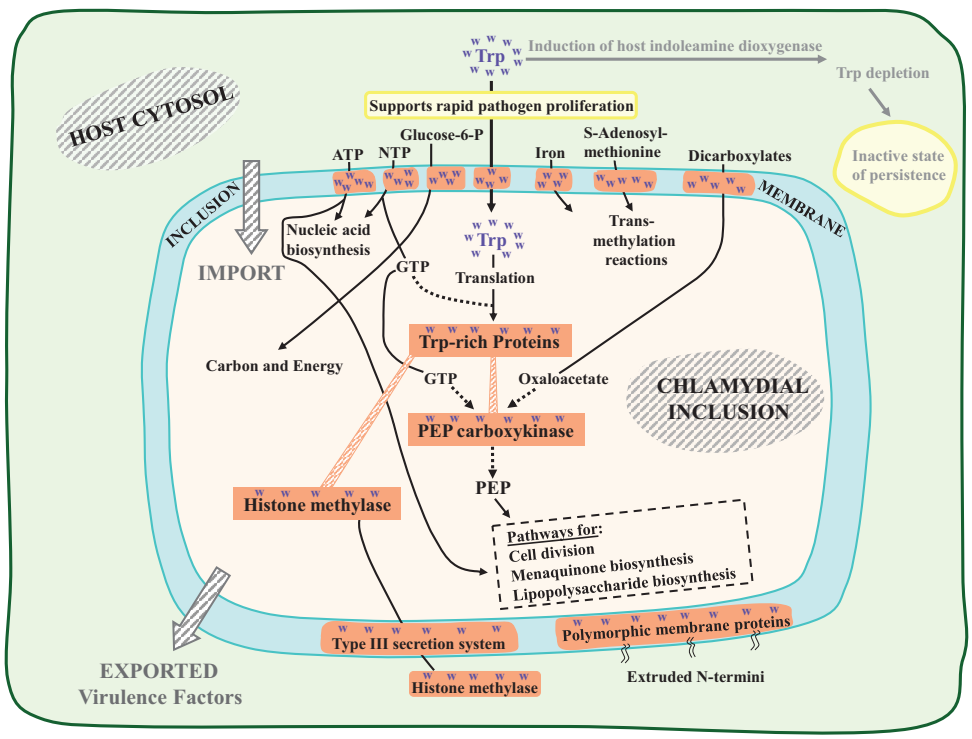

FIGURE 2 | Key elements in the *C. trachomatis* pathogen of a response network which is tuned to adequate Trp availability from human host cytosol. Two alternative flow routes for cytosolic Trp are indicated. The rightward flow route (dimmed) is initiated with the induction of indoleamine dioxygenase (IDO). When Trp is fully available prior to the triggering of any process accomplishing Trp depletion (downward bold arrow), a schedule of rapid pathogen proliferation is supported by expression of key Trp-rich proteins. Many of these are membrane proteins that promote transport into the inclusion body of important metabolites derived from the host cytosol, illustrated by those shown across the top of the diagrammed inclusion membrane. The *C. trachomatis* PEP carboxykinase is positioned at the center of an interactive assemblage of Trp-rich proteins, as discussed extensively in the text. The export (or partial export) of virulence factors is illustrated by histone methylase and by the family of polymorphic membrane proteins (Pmp's).

Multiple paralogs of Pmp's are *Chlamydiaceae*-specific proteins, whose extruded N-termini are important virulence factors. Although these N-termini extensions, in fact, have very low Trp content, their extrusion depends upon a C-terminal component that is a transmembrane barrel autotransporter of high Trp content (Henderson and Lam, 2001). The C-terminal portion of Pmp's illustrate how proteins that have Trp "hotspots" can be sensitive to Trp depletion without having an overall high Trp content. In the case of the multiple Pmp paralogs, which are specific to the *Chlamydiaceae* and therefore are not subject to extra-Family homolog comparisons, Up-Trp selection seems intuitively obvious in consideration of the unusual density of C-terminal Trp hotspots.

KEY METABOLIC STEPS

PEP carboxykinase is very much a key protein and is highlighted in **Figure 2**, not only because it is a conspicuous Trp-rich protein but because it is a touchstone element operating at the center of a complex and interactive chain of vulnerability to Trp depletion. Its GTP and oxaloacetate substrates require Trp-rich transporters. The PEP product of the enzyme reaction is a crucial substrate for multiple pathways that include cell division, lipopolysaccharide biosynthesis, and menaquinone biosynthesis. PckG was suggested to contribute strongly to an "Achilles-heel vulnerability" in the cell-division pathway at the level of MurA, the initial specific step of the Lipid II pathway of cell division (Lo et al., 2012). In addition to PEP, MurA requires N-acetyl-glucosamine as a co-substrate. N-acetyl-glucosamine, in turn, is a reaction product of GlmV, a Trp-rich enzyme that also utilizes UTP for catalysis. Furthermore, MurA is in competition with two other enzymes for N-acetyl-glucosamine, one being the initial step of LPS biosynthesis, and the other a downstream enzyme of cell division (MurG). Thus, even though MurA has a Trp content which is only average, the availability of its substrates depends upon a multiplicity of other proteins of very high Trp content.

The so-called "cell wall anomaly" in chlamydiae (Moulder, 1993), states that although the organism has the genetic capacity to produce peptidoglycan monomers, it has no detectable peptidoglycan cell wall. Further, it may not make a canonical peptidoglycan structure, given the absence of any annotated transglycosylase enzymes to link the sugar moieties. These observations have given rise to speculations that a peptidoglycan-like structure is produced transiently at the septum during cell division. Brown and Rockey (2000) identified an antigen in apparent chlamydial division planes that was not proteinaceous and may possibly have been the peptidoglycan-like structure. Nutrient availability and other environmental conditions impact cell growth and division in bacteria, as reviewed by Hill et al. (2013). Recent publications (Gaballah et al., 2011; Ouellette et al., 2012) have suggested a critical role for MreB as a functional substitute for FtsZ (which is absent in *Chlamydiales*) in its role of organizing the division plane. We had previously noted (Lo et al., 2012) that it might be meaningful that *mreB* and *pckG* (encoding PEP carboxykinase) co-exist as overlapping genes in an apparent operon. This could potentially be extended to *snf,* an apparent operon component which is upstream of *mreB*. In view of the important relationship of PckG to cell division discussed above, this new information about the role of MreB in cell division is quite interesting. Snf is a putative helicase that, although neither Up-Trp nor Down-Trp, contains a Trp hotspot at its C-terminus (including a tandem WW motif). This could potentially destabilize the putative three-gene operon to effect an overall synergism of impact upon cell division.

REDUCTIVE EVOLUTION OF Trp BIOSYNTHESIS IN *Chlamydiales*

EXTREME PHYLOGENETIC VARIATION OF REDUCTIVE EVOLUTION FOR *trp* GENES

The genes of Trp biosynthesis have generally undergone reductive evolution throughout most of the *Chlamydiales* Order. See **Figure 3** for the reactions of Trp biosynthesis, together with the acronyms that were originally formulated for *E. coli* shown in comparison with the logical set of replacement acronyms used here in which enzymes were named in the order of pathway steps (Xie et al., 2003). The *Chlamydiaceae* vary considerably in the extent to which genes encoding the enzymes of Trp biosynthesis have resisted reductive evolution. *C. abortus, C. pneumoniae*, and *C. psittaci*, have lost all *trp* genes. (Note that in accordance with the opinion of many chlamydiae experts (Stephens et al., 2009) that all known species so far belong to the single genus *Chlamydia*, we do not use the *Chlamydophila* genus designation in current use by NCBI). The genome of *C. muridarum* has retained only a *trpC* remnant that appears to have lost important catalytic residues (Xie et al., 2002). Some of the other chlamydiae have partial-pathway remnants that are no longer connected to chorismate, but which have evolved some fascinating functional specializations as detailed below. **Table 1** lists all of the *Chlamydiales* genes of *trp* biosynthesis and provides hyperlinks to the SEED database (Overbeek et al., 2005). This affords convenient scrolling among adjacent genes and quick access to SEED tools.

DOES *Simkania* RETAIN THE ANCESTRAL *trp* OPERON?

Only *Simkania negevensis* (Sneg) possesses a complete Trp pathway, in fact being in possession of the complete multi-branched pathway that extends to all three aromatic amino acids. Sneg deploys a very compact *trp* operon *trpR/trpAa/trpAb/trpB/trpD/trpC/trpEb/trpEa/aroA*. Only 18 nucleotides separate *trpR* and *trpAa*, and all other adjacent genes overlap indicating translational coupling. The inclusion of the *trpR* repressor gene within the operon indicates the existence of a form of self-regulation called autoregulation (Merino et al., 2008). The C-terminal *aroA* member of the operon encodes one of the three 2-keto,3-deoxy-**D**-*arabino*-heptulosonate-7-P (DAHP) synthase paralogs present in the genome, all belonging to the $AroA_{I\beta}$ superfamily (Jensen et al., 2002). The operonic DAHP synthase is probably specialized to ensure precursor provision to the Trp pathway, similar to the classic situation of partitioned AroA isoenzymes in *E. coli* (Ahmad et al., 1986). Although Sneg appears to sustain a complete, intact pathway of Trp biosynthesis, it shares a number of Up-Trp and Down-Trp selections that are common to the *Chlamydiales*, as elaborated later. This suggests that acquisition of Trp from host resources was important at a stage that preceded reductive evolution of *trp* genes.

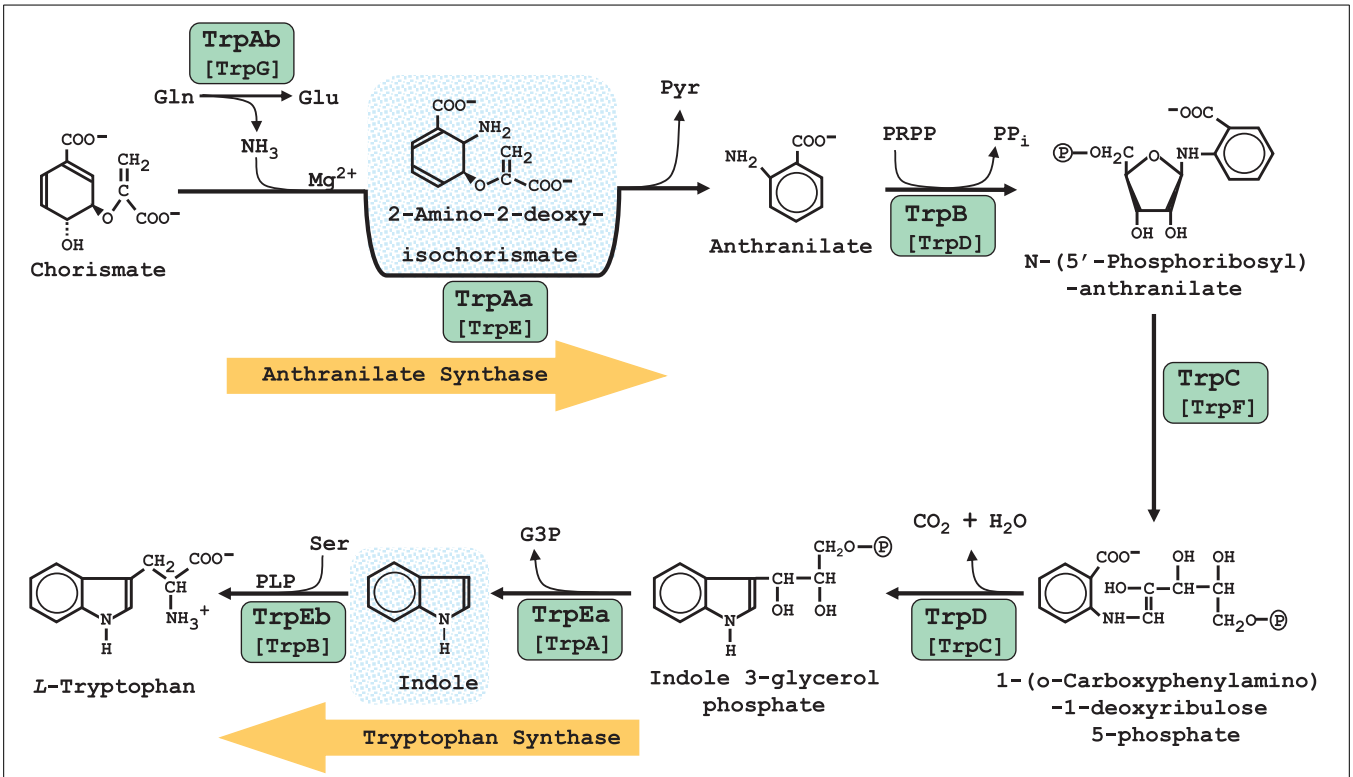

FIGURE 3 | Biochemical pathway of Trp biosynthesis. The green ovals encircle the logical acronym designations which are based upon the order of enzyme reactions (Xie et al., 2003). Below the latter are shown the classic acronym designations. Enzyme-bound intermediates for anthranilate synthase and tryptophan synthase are shown with blue shading. The initial pathway reaction is catalyzed by anthranilate synthase. The TrpAa subunit can catalyze the reaction as an aminase utilizing ammonia as a nitrogen donor, but the physiological reaction is carried out as an amidotransferase via the contribution of the TrpAb subunit which utilizes glutamine as the source of the ammonia reactant. Although tryptophan synthase is a two-subunit complex in which indole is an enzyme-bound intermediate, isolated TrpEb is capable of utilizing free indole.

VARIANT LINKAGES OF CHORISMATE TO MENAQUINONE AND AROMATIC AMINO ACIDS

Sneg is thus far unique among the chlamydiae in utilization of chorismate as a precursor that feeds into biosynthesis of Trp, tyrosine, and phenylalanine—as well as into menaquinone biosynthesis via the classic isochorismate (DH2N) pathway. *Waddlia* and Paca/Pamo have retained the chorismate-to-DH2N menaquinone pathway, but this now appears to be a linear pathway composite instead of a branched pathway since the Trp, tyrosine and phenylalanine branches have all been lost. Although the *Chlamydiaceae* also possess a linear chorismate-to-menaquinone pathway, the menaquinone pathway is the newly discovered DH6N variation as discussed fully in a later section.

THE Trp/KYNURENINE/Trp CYCLE

C. caviae, *C. felis*, and *C. pecorum* have a nearly complete Trp pathway that lacks the initial anthranilate synthase step. These species are able to implement an alternative synthesis of anthranilate from kynurenine, a host metabolite produced from host Trp in two steps following the IDO reaction. This was originally described by Xie et al. (2002) for *C. caviae* (at that time called *C. psittaci*). This group of organisms has a novel *trp* operon (*trpR/trpB/trpD/trpC/trpEb/trpEa/kynU/kprS*). This encodes all enzymes of Trp biosynthesis, except for the two subunits of anthranilate synthase (TrpAa and TrpAb). This discontinuity effectively disconnects the Trp pathway from the chorismate biosynthesis pathway and requires a different source of anthranilate. The intergenic spacing between *trpR* and *trpB* is much greater in these organisms (about 230 nucleotides) than that observed between *trpR* and *trpAa* in Sneg, indicating that the loss of trpAa/trpAb occurred in a way that moved *trpR* further away from the succeeding gene of the operon. This potentially provides space for regulatory features. Indeed, in C. *felis* a gene encoding a potential *trpL* leader peptide that has tandem Trp residues (MKINKADTFSTNALALLNNLCALYSSAFPFFFSL**WW**AFAQ) is located between *trpR* and *trpB* (see **Table 1**). Attenuator structures have not been reported for this region in the *caviae/felis/pecorum* grouping, and thus whether repression control by *trpR* may be integrated with attenuation control is a possibility that awaits experimental work. The last two genes of the operon are thus far not found elsewhere in the chlamydiae, nor are they known to comprise part of any other *trp* operon. *kynU* encodes kynureninase, catalyzing the formation of anthranilate from host kynurenine (a catabolite of Trp). This

Table 1 | Proteins of Tryptophan biosynthesis in *Chlamydiae*.

Organism[a]	Protein acronyms, Trp content[d], NCBI gene numbers, and SEED identifiers								
	TrpR	**TrpL**	**TrpAa**	**TrpAb**	**TrpB**	**TrpD**	**TrpC**	**TrpEb**	**TrpEa**
***Simkania negevensis* Z**	**1/91**	NP	**0/490**	**0/193**	**1/330**	**0/264**	**1/207**	**2/389**	**1/253**
NCBI gene number	SNE_A10150		SNE_A10160	SNE_A10170	SNE_A10180	SNE_A10190	SNE_A10200	SNE_A10210	SNE_A10220
SEED protein page	fig|331113.3.peg.1081		fig|331113.3.peg.1082	fig|331113.3.peg.1083	fig|331113.3.peg.1084	fig|331113.3.peg.1085	fig|331113.3.peg.1086	fig|331113.3.peg.1087	fig|331113.3.peg.1088
***Chlamydia caviae* GPIC**[b]	**1/102**	NP	NP	NP	**2/305**	**0/274**	**2/207**	**2/392**	**0/258**
NCBI gene number	CCA00562				CCA00563	CCA00564	CCA00565	CCA00566	CCA00567
SEED protein page	fig|227941.6.peg.606				fig|227941.6.peg.607	fig|227941.6.peg.608	fig|227941.6.peg.609	fig|227941.6.peg.610	fig|227941.6.peg.611
***Chlamydia felis* Fe/C-56**	**1/102**	**2/39**	NP	NP	**2/318**	**0/275**	**2/207**	**2/391**	**0/258**
NCBI gene number	CF0440	CF0441			CF0439	CF0438	CF0437	CF0436	CF0435
SEED protein page	fig|264202.11.peg.459	fig|264202.11.peg.458			fig|264202.11.peg.457	fig|264202.11.peg.456	fig|264202.11.peg.455	fig|264202.11.peg.454	fig|264202.11.peg.453
***Chlamydia pecorum* E58**	**1/93**	NP	NP	NP	**2/327**	**0/290**	**2/208**	**2/393**	**0/256**
NCBI gene number	G5S_1088				G5S_1087	G5S_1086	G5S_1085	G5S_1084	G5S_1083
SEED protein page	fig|331635.3.peg.973				fig|331635.3.peg.972	fig|331635.3.peg.971	fig|331635.3.peg.970	fig|331635.3.peg.969	fig|331635.3.peg.968
***Chlamydia trachomatis* D/UW-3/CX**	**1/95**	**3/50**	NP	NP	NP	NP	**3/208**	**2/392**	**0/253**
NCBI gene number	CT169	CT169a					CT327	CT170	CT171
SEED protein page	fig|272561.5.peg.182	fig|272561.5.peg.969					fig|272561.5.peg.352	fig|272561.5.peg.183	fig|272561.5.peg.184
***Chlamydia muridarum* Nigg**	NP	NP	NP	NP	NP	NP	**3/209**	NP	NP
NCBI gene number							TC065		
SEED protein page							fig|243161.6.peg.642		
***Coxiella burnetii* Dugway 5J108-111**[c]	**1/91**	NP	**0/493**	**0/121**	**0/334**	**0/258**	**1/601**	**1/601**	**0/268**
NCBI gene number	CBUD_1566		CBUD_1249	CBUD_1249a	CBUD_1251	CBUD_1252	CBUD_1253	CBUD_1253	CBUD_1255
SEED protein page	fig|434922.5.peg.1563		fig|434922.5.peg.1259	fig|434922.5.peg.1260	fig|434922.5.peg.1261	fig|434922.5.peg.1262	fig|434922.5.peg.1263	fig|434922.5.peg.1263	fig|434922.5.peg.1264

[a] *The following have no Trp pathway genes: Chlamydia abortus, C. pneumoniae, C. psittaci, Protochlamydia amoebophila, Parachlamydia acanthamoebae and Waddlia chondrophila.*

[b] *Chlamydia caviae has a gene (CCA00559) encoding a duplicate TrpEb (0/413) fig|227941.6.peg.600.*

[c] *Coxiella burnetii has the fusion TrpC·TrpEb.*

[d] *Number of Trp residues per amino-acid length shown as numbers with bold fonts.*

reaction allows host-diverted Trp to be recycled (via the interception of host kynurenine) back to Trp in the pathogen. PRPP synthase is the gene product of *prsA*. It is needed to produce PRPP, a co-substrate with anthranilate in the reaction catalyzed by TrpB. *prsA* is the other unique gene member of the operon and is closely related to *prsA* present in other chlamydiae genomes. In contrast, *kynU* is not present thus far in any other chlamydiae and may have originated via lateral gene transfer. Although a mammalian donor seemed feasible, detailed bioinformatic work did not confirm this possibility (Xie et al., 2002).

The traditional enzymes of Trp biosynthesis shown in **Table 1** all have a low Trp content, as indeed is generally expected because of selection for low cognate amino acid bias in amino-acid biosynthetic enzymes (Alves and Savageau, 2005). However, it has been suggested (Lo et al., 2012) that the recycling mechanism is unlikely to function for Trp production under conditions of persistence. Rather than being a seemingly obvious mechanism to thwart the host strategy of Trp depletion, the recycling mechanism may be geared to jump-start vegetative pathogen growth during the transitional phase where persistent cells encounter renewed availability of host Trp. This interpretation was based upon the fact that kynureninase is a protein of very high Trp content, as well as upon the consideration that PRPP synthase utilizes ATP (scarce under conditions of persistence) in a reaction in which two high-energy equivalents are consumed.

INDOLE UTILIZATION

Yet another Trp partial-pathway consisting only of gene products encoded by the operon assemblage *trpR/trpEb/trpEa* is maintained by *C. trachomatis* (Ctra). *C. trachomatis* also has a *trpC* pseudogene remnant, which likely is not functional (Xie et al., 2002). A single enzyme activity, the condensation of indole and serine to produce Trp, is a catalytic property of TrpEb. TrpEb is one of two subunits of Trp synthase, a protein/protein complex which catalyzes an overall reaction in which indole is an enzyme-bound intermediate produced by the TrpEa half-reaction and utilized by the TrpEb half-reaction (**Figure 3**). It is interesting that the source of indole is not the human host, but rather comes from indole-producing organisms that can co-exist in the tissue niche occupied by genital strains. Thus, indole can rescue genital strains of Ctra—but not ocular strains—from the Trp starvation caused by the host-mediated induction of IDO (Fehlner-Gardiner et al., 2002). Hence, strain-specific host tropism corresponds with niche-specific ability to scavenge indole from a given co-existing microbial community. It is curious that TrpEb function in Ctra requires the concomitant presence of a full-length, catalytically inactive TrpEa subunit (Fehlner-Gardiner et al., 2002). It seems likely that TrpEa may be required for stabilization of TrpEb since these subunits have well-established protein-protein interactions (Xie et al., 2002). This atypical interactive requirement for TrpEb function in Ctra is not a general characteristic of chlamydial TrpEb proteins, judging from the observed ability of isolated TrpEb from *C. caviae* to carry out the indole-plus-serine condensation in the absence of TrpEa (Wood et al., 2004).

Regulation by *trpR* in Ctra has been demonstrated by Akers and Tan (2006) in a publication that cites the report by Merino and Yanofsky (2005) of an attenuator just upstream of *trpEb*. The attenuation mechanism was included as an important aspect of the Akers and Tan model. In the same year Carlson et al. (2006) inexplicably asserted that no attenuator could be found upstream of *trpEb*. Subsequently, the existence of a predicted transcription attenuator between *trpR* and *trpEb* was affirmed (Merino et al., 2008). Indeed this attenuator was also found to be followed by a putative *trpL* gene encoding a leader peptide. The leader peptide has 59 residues in which the C-terminal segment has a Trp-rich hotspot of 3/12 Trp residues (MHALLMNKYSVLAVLVHKYSCSMPCKSAFQADCFQDIQKFI LLQRA**W**LSFES**W**RLST**W**R). The predicted secondary structure can be accessed at Merino's website http://cmgm.stanford.edu/%7Emerino/Chlamydia_trachomatis/15604889.html. Thus, there appears to be a strong basis for the TrpR repression mechanism to be supplemented by a transcription attenuation mechanism, as is the case in *E. coli*. In the latter case, attenuation increases the range of regulation mediated by TrpR by an additional order of magnitude (Merino et al., 2008).

Coxiella Burnetii: LATERAL GENE TRANSFER (LGT) RECIPIENT OF THE *Simkania trp* OPERON?

Coxiella organisms are *Gamma-proteobacteria* that are obligate intracellular pathogens of humans and other animals. It appears to have lost competence for Trp biosynthesis, judging from the pseudogene character of both *trpAb* and *trpB* (Xie et al., 2003). This appears to be a recent ongoing process of reductive evolution since most of the genes remain largely intact, and all of them are still recognizable. Interestingly, the structural genes and *trpR* are close homologs of those from *Simkania*, rather than of those from other *Gamma-proteobacteria*. The operon construction (see bottom of **Table 1**) differs in that (i) *trpR* (the initial operon gene of *Simkania*) has become separated from the *trp* operon in *Coxiella*, (ii) *trpC* and *trpEb* are fused in *Coxiella*, and (iii) the *Coxiella* operon does not contain *aroA* (the last operonic gene of *Simkania*). If this *aroA* gene was acquired by *Coxiella* via LGT, it was not retained since all *Coxiella aroA* paralogs are closely related to those of other *Gamma-proteobacteria*. Because the homology relationship of the *trp* genes of *Coxiella* are not phylogenetically congruent with those of other *Gamma-proteobacteria*, whole-pathway LGT from a *Chlamydiales* donor to a *Coxiella* recipient, is implicated—a phenomenon described for *trp* operons in a number of other cases (Xie et al., 2003).

WHICH EVENTS OF Up-Trp AND Down-Trp SELECTION PRECEDED DIVERGENCE OF *Chlamydiaceae*?

The list of proteins identified as Up-Trp or Down-Trp proteins can be sorted into groups that have undergone Trp-content selection at different evolutionary times. Those occurring at either the taxon level of the *Chlamydiaceae* or at the deeper taxon level of the *Chlamydiales* are enumerated in **Figure 4**.

Chlamydiales Up-Trp SELECTION

The *Chlamydiales* ancestor (yellow arrow) underwent Up-Trp selection for a number of important transporters. These include permeases for nucleotides, dicarboxylate substrates, S-adenosylmethionine (SAM), and for transport of Trp itself via TyrP, as listed in section A at the upper left of **Figure 4**. CT

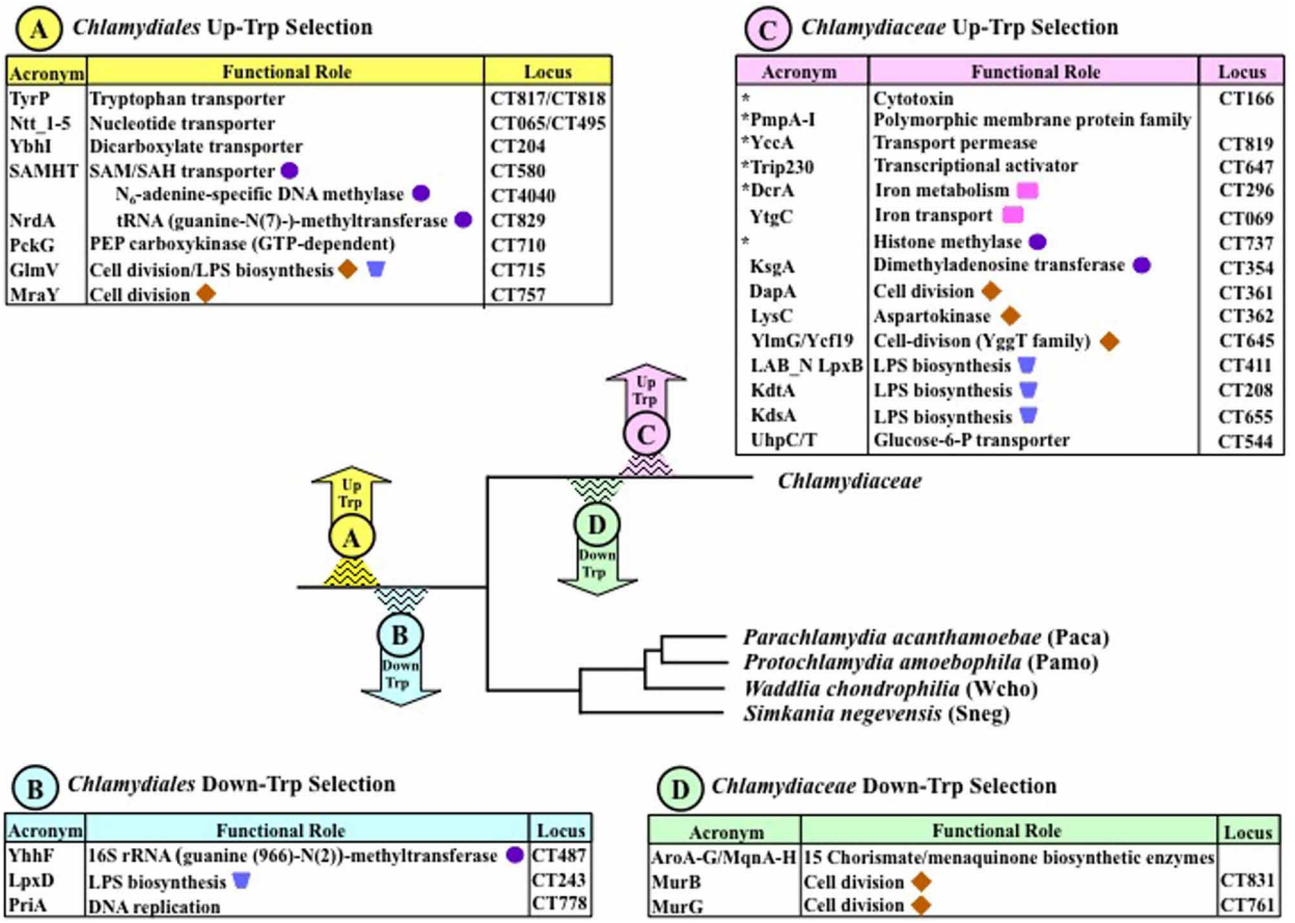

FIGURE 4 | Events of Up-Trp and Down-Trp selection in phylogenetic context. Early evolutionary events, i.e., ancestral to the *Chlamydiales* Order, are show in yellow (Up-Trp) and blue (Down-Trp). Later events specifically ancestral to the *Chlamydiaceae* are shown in pink and green. Even more recent events distinguish *C. trachomatis* from *C. muridarum* as previously exemplified in **Figure 1**. Different steps of several functional processes (cell division, LPS biosynthesis, and methylation) are marked with identifying symbols Asterisks within the box at the upper right indicate gene products that are expressed in the *Chlamydiaceae* but not elsewhere in the *Chlamydiales* Order.

numbers for the *C. trachomatis* genes are given at the right for convenient reference. Paralog expansion of TyrP, which occurred extensively throughout the *Chlamydiales* is discussed in detail in a later section. In the *Chlamydiaceae*, two paralogs accommodate nucleotide transport, as documented fully for the *Chlamydia* genus (Tjaden et al., 1999). One catalyzes ATP/ADP exchange, and the other facilitates the import of RNA nucleotides. A recent update of nucleotide parasitism at the deeper phylogenetic level of the *Chlamydiales* order highlights the individuality of the nucleotide transporters in the chlamydiae with respect to paralog number, transport specificity, and molecular mechanism—albeit against a general background of overall similarity (Knab et al., 2011). Families other than the *Chlamydiaceae* exhibit the most extensive paralog expansion. In one well-studied example, *P. amoebophila* (Pamo) possesses five nucleotide transporters. PamNTT1 is an ATP/ADP antiporter; PamNTT2 is a nucleoside triphosphate antiporter balancing the nucleotide pool; PamNTT3 is a UTP/H^+ symporter; PamNTT4 transports NAD^+ in exchange for ADP; and PamNTT5 is a GTP/ATP/H^+ symporter (see (Knab et al., 2011) and refs. therein). Sneg has four nucleotide transporters, the substrate specificity of one being uncertain. The two *C. trachomatis* (Ctra) nucleotide transporters utilize a cumulative 31 Trp residues for minimal monomer units. This is a very substantial Trp content, but the Trp burden associated with nucleotide transporters is even greater in the remaining Families of the *Chlamydiales*. Thus, the Trp burden is a cumulative 52 Trp residues for the five Pamo proteins, 62 cumulative Trp residues for the five *Waddlia chondrophila* (Wcho) proteins, and 47 cumulative Trp residues for the four Sneg proteins.

Up-Trp selection for the SAM-dependent DNA methylase and the SAM-dependent tRNA methyltransferase suggest a degree of synergistic sensitivity to Trp availability since not only these proteins *per se*, but the availability of their SAM substrates is reduced in the absence of Trp. This is because the SAM/SAH transporter (Binet et al., 2011) is conspicuously high in Trp content. This dependence of the methylase and the methyltransferase upon the

SAM transporter is indicated in **Figure 4** by indenting their names under that of the SAM/SAH transporter.

The intricate role of PEP carboxykinase in the overall metabolic network has been discussed above. The evolutionary choice of *pckG*, which uses GTP rather than of *pckA* which uses ATP, exemplifies the exercise of preference for one of two non-homologous functional equivalents that characteristically has a distinctly high Trp content. The function of PEP carboxykinase as the source of PEP is linked to several complex pathways that require the input of PEP. The high Trp content of several enzymes that participate in cell division (GlmV and MraY) or in LPS biosynthesis (GlmV) is one factor which should result in at least some restraint of these processes under conditions of Trp limitation. The additional impact of substrate limitation must be substantial. For example, the cell-division pathway requires 1 PEP, 1 UTP, and 5 ATP substrate inputs (see Figure 7 of Lo et al., 2012).

Chlamydiales Down-Trp SELECTION

The 16S rRNA methyltransferase (YhhF) and LpxD (shown at the lower left in **Figure 4**) are cases of Down-Trp selection that probably exemplify a compensatory Down-Trp selection in pathway functions of the *Chlamydiales* Order where one or more Up-Trp selections discussed above and listed at the upper left of **Figure 4** have probably created translation hurdles. Such a mechanism can help offset the great metabolic expense of using Trp. PriA is a very large molecule engaged in DNA replication. The extensive Down-Trp selection of PriA throughout the *Chlamydiales* supports its significance generally for survival under conditions of Trp limitation in the *Chlamydiales* and for maintenance of the persistent state in *Chlamydiaceae* in particular.

Chlamydiaceae Up-Trp SELECTION

The *Chlamydiaceae* have experienced a great intensification of Up-Trp selection that extends beyond those covered above for the *Chlamydiales*, as summarized in Box C at the upper right of **Figure 3**. Generally, these selections can be rationalized in a context of co-evolution with properties of the mammalian host. In addition to the two Up-Trp selections in the *Chlamydiales* that are relevant to cell division, three additional Up-Trp selections (DapA, LysC, and YlmG/Ycf19) are *Chlamydiaceae*-specific. Similarly, in addition to the two Up-Trp selections in the *Chlamydiales* that are relevant to LPS biosynthesis, three additional reinforcing Up-Trp selections (KdtA, KdsA, and LAB_N LpxB) are *Chlamydiaceae*-specific. (The appending of the Trp-rich LAB_N domain by fusion to LpxB exemplifies one mechanism for conferring increased vulnerability to Trp starvation.) The glucose-6-P transporter (UhpC/T) is a fusion protein that contains both receptor (UhpC) and transport (UhpT) functions, which are separate in other organisms. This protein is always Trp-rich in nature, but it was deemed to have been subject to Up-Trp selection in *Chlamydiaceae* because of further elevated Trp content with the addition of multiple Trp hotspots (Lo et al., 2012).

A number of important high-Trp proteins in the *Chlamydiaceae* are generally absent elsewhere in the *Chlamydiales* (shown with asterisks in **Figure 4**). These include the paralog family of Pmp's A-I, DcrA involved in iron metabolism, a histone methylase, an uncharacterized transport permease (YccA), a putative transcriptional activator (Trip230), and cytotoxin. The Pmp's, the histone methylase, and the cytotoxin were discussed earlier. The Trip230 activator is likely to be highly sensitive to Trp limitation since its Trp content scored the highest p/P Trp ratio in the *Chlamydiaceae* proteomes. As a probable transcriptional activator suggested to be involved in folate metabolism (Lo et al., 2012), it could be engaged in master/slave relationships in which effects of Trp limitation upon Trip230 may affect multiple other proteins, regardless of their individual Trp contents.

Chlamydiaceae Down-Trp SELECTION

The increased *Chlamydiaceae*-specific expenditure of Trp for the cell-division pathway shown in box C at the upper right of **Figure 4** is offset by the *Chlamydiaceae*-specific Down-Trp selection of MurB and MurG (box D). A most striking Down-Trp selection has occurred for the 15-step pathway that converts erythrose-4-P and PEP to menaquinone via chorismate. This suggests that function of this pathway is particularly crucial for maintenance of the persistent state. Menaquinone biosynthesis is covered comprehensively in a later section.

THE HIGHLY EXPANSIVE DISTRIBUTION OF TyrP ORTHOLOGS AND PARALOGS IN *Chlamydiales*

Chlamydiaceae

The TyrP family of permeases transport Trp and/or tyrosine with varying specificity (Sarsero et al., 1991). This is exemplified in *E. coli* where three permeases are utilized: TyrP being specific for tyrosine (Ecol_Wa_b1907 in **Figure 4**), Mtr having high affinity for Trp transport (Ecol_Wa_b3161), and TnaB having low affinity (but high capacity) for Trp transport (Ecol_Wc_b3709). Members of the *Chlamydiaceae* generally possess a single *tyrP* gene represented in Group A of **Figure 5**, but recent gene duplications have occasionally generated paralogs. Both *C. trachomatis* and *C. muridarum* possess paralog *tyrP* genes in a tandem configuration. It is interesting that *tyrP* copy number may be relevant to tissue tropism in *C. pneumoniae* at the strain level since respiratory strains, but not vascular strains, were found to have two or more paralogous copies of *tyrP* (Gieffers et al., 2003). It is very clear that induction of persistence occurs in *C. pneumoniae* as a result of IFN-γ-mediated activation of host cells (reviewed in Roulis et al., 2013), and although the regulation of Up- and Down-Trp protein expression has not been evaluated for *C. pneumoniae*, novel transcriptional patterns have been reported to be based on the method of persistence induction (Roulis et al., 2013). It was, however, noted in earlier work (Dairi, 2009) that a lesser capacity for Trp import, assumed to be associated with single *tyrP* copies in *C. pneumoniae*, may be tied to a greater tendency to exist in the persistent state. *tyrP* copy number might also be relevant to tissue tropism of *C. trachomatis* since an oculotropic trachoma isolate was found to have one disrupted copy of the tandem *tyrP* genes present in genitotropic strains (Carlson et al., 2005).

DISTRIBUTION OF TyrP GENES IN *Chlamydiales*

In contrast to the *Chlamydiaceae*, the *tyrP* family of genes exhibits extensive expansion in other Family taxa of the *Chlamydiales*, as illustrated by the radial tree shown in **Figure 5** where three

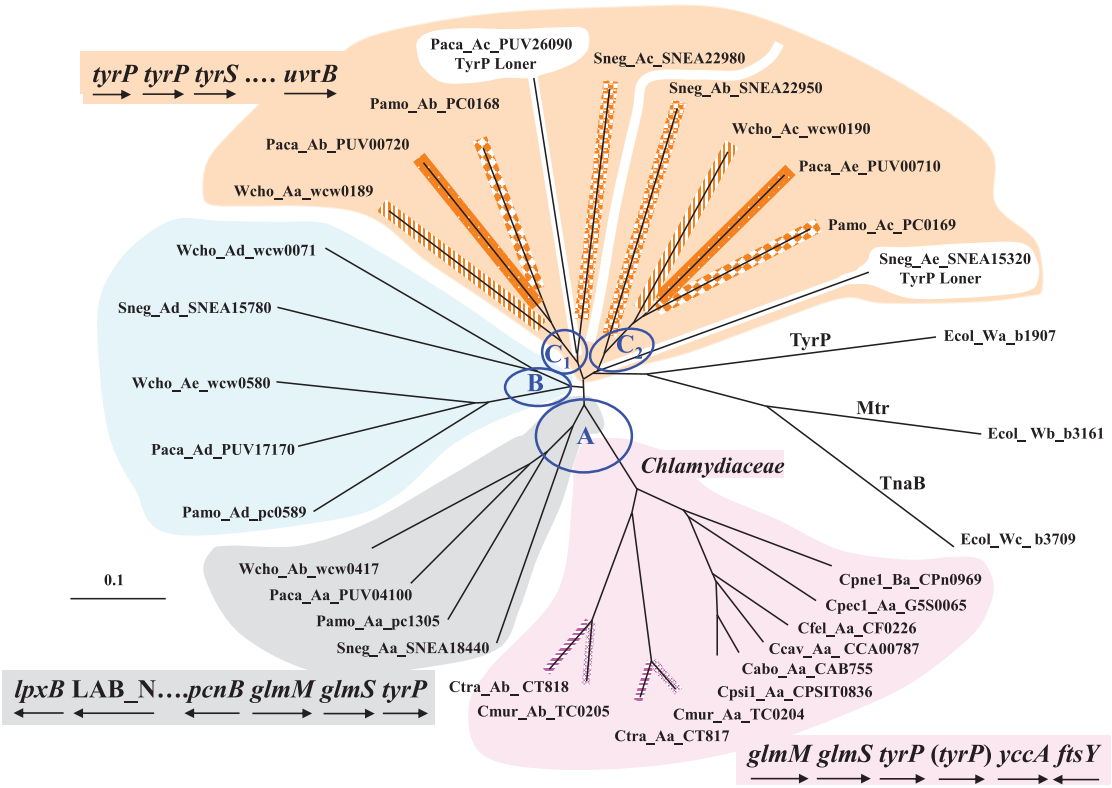

FIGURE 5 | Multiplicity of the TyrP transporter for Trp in the *Chlamydiales* Order. The three *E. coli* paralogs for Trp transport are shown at the right. Three main ortholog clusters of TyrP proteins (designated as A–C) are evident, together with an admixture of a few additional paralogs. Cluster A contains one TyrP protein from Wcho, Paca, Pamo, and Sneg – as well as the divergent set of proteins from the *Chlamydiaceae*. Ctra and Cmur each have two paralogs, which appear to have occurred independently via gene duplications that followed the speciation divergence. At the lower left is shown a suggested ancestral gene neighborhood which can be compared to the *Chlamydiaceae* gene neighborhood shown at the lower right. In the latter gene neighborhood inclusion of "(*tyrP*)" is relevant only to the gene duplicate present in the *Cmur/Ctra* pair. The *Chlamydiaceae* have no representation in ortholog clusters B and C. Cluster B possesses members from Wcho, Sneg, Paca, and Pamo – with Wcho having an additional paralog member. Cluster C is comprised of tandem *tyrP* genes (see the gene neighborhood at the upper left) which encode the four sets of ortholog pairs shown (with matched line patterns). Thus, duplication of the ancestral Group-C ortholog in the common ancestor of Wcho, Sneg, and Paca/Pamo generated the paralog sets that are positioned within Group C_1 and C_2. In addition Paca and Sneg possess a third paralog belonging to cluster C; the genes encoding these are unlinked to the aforementioned tandem genes (hence their gene products being designated as "TyrP Loners"). Abbreviations for organisms populating the *Chlamydiaceae*: Ctra, *Chlamydia trachomatis*; Cmur, C. *muridarum*; Cpsi, *C. psittaci*; Cabo, *C. abortus*; Ccav, *C. caviae*; Cfel, *C. felis*; Cpec, *C. pecorum*; and Cpne, *C. pneumoniae*. Other *Chlamydiales* organisms are abbreviated as given in **Figure 4**.

ortholog groups are designated as Groups A, B, and C. Groups A and C are associated with the conserved gene neighborhoods shown. Only Group-A TyrP proteins are found in the *Chlamydiaceae* and this is probably encoded by the ancestral gene, this ortholog being the only one that is present in each member of the four chlamydial families. The subdivision of Group A into two groups (*Chlamydiaceae* in pink on the right) and members of the other three families (Wcho, Paca, Pamo, and Sneg in gray on the left) corresponds with the variation of gene neighborhood shown. The gene order *glmM glmS tyrP* is absolutely conserved in all of the *Chlamydiales*. In the *Chlamydiaceae* gene neighborhood *yccA* encodes an uncharacterized protein that may participate in cell division since its homolog in *E. coli* has been reported to interact with FtsH, and it has a very high Trp content. *ftsY* encodes a signal recognition particle that is relevant to cell division. *glmM* and *glmS* encode initial enzymes leading to synthesis of UDP-N-acetylglucosamine, a crucial metabolite that is located at a branchpoint that diverges to LPS biosynthesis, on the one hand, and to the Lipid II pathway for cell division, on the other hand. The gene order on the lower left is probably very similar to the ancestral arrangement, perhaps including *yccA* and *ftsY* following *tyrP* since these genes can still be found in the vicinity of Group-A *tyrP* in Wcho, Paca, Pamo, and Sneg. Following the divergence of *Chlamydiaceae* from the other Families, *Lab_N* underwent fusion with the adjacent *lpxB* to give fused *Lab_N/lpxB* gene which is present in all *Chlamydiaceae*. This fusion event was associated with a translocation event that separated *Lab_N/lpxB* a substantial distance from the ancestral gene neighborhood.

The *Simkaniaceae*, *Parachlamydiaceae*, and *Waddliaceae* Families are all represented by at least one member in Groups B and C TyrP's. Wcho has two Group-B TyrP paralogs. The greatest paralog expansion has occurred in Group C. A gene duplication in the common ancestor of the three families has generated tandem paralog sets that are adjacent to *tyrS* and in the

vicinity of *uvrB*. In **Figure 4** Wcho, Paca/Pamo, and Sneg each are represented by the set of two paralogs, one in Group C_1 and the other in Group C_2. In addition Paca has an additional paralog that emerged from Group C_1, and Sneg has an additional paralog that emerged from Group C_2. The latter two are referred to as TyrP loners because the encoding genes have been translocated far from the parental paralog pairs. The Sneg gene neighborhood has been disrupted somewhat from the suggested ancestral arrangement in that the two paralog *tyrP* genes have been separated by two inserted hypothetical genes, and they are far separated from *tyrS*, as well. *uvrB* is still a close-neighbor gene.

LYSOSOMAL DEGRADATION AS A SOURCE OF Trp

It has recently come to be appreciated (Ouellette et al., 2011) that in order for Trp import to be fully understood, an evaluation of oligopeptide or Trp molecules obtained from ongoing degradation processes in host lysosomes must be included. Not only is the pathogen inclusion physically proximal to lysosomes, but a substantial multiplicity of oligopeptide and dipeptide transporters occurs in chlamydial genomes. For example, Ctra possesses, as just one of many illustrative cases, an *oppABC* operon (CT478-CT480) encoding three gene products having a total Trp burden of 30 Trp residues (for monomeric entities). Peptidase gene products are represented as well. This aspect of Trp acquisition awaits detailed bioinformatic analysis.

Chlamydiaceae ARE THE SOLE TAXON FAMILY WITHIN *Chlamydiales* TO DEPLOY THE RECENTLY RECOGNIZED DH6N PATHWAY OF MENAQUINONE BIOSYNTHESIS

In E. coli ubiquinone and menaquinone are essential components of the electron-transport chain under aerobic or anaerobic conditions, respectively. In many organisms only menaquinones are used (Bentley and Meganathan, 1982), and this appears to be the case in the *Chlamydiales.* The classic menaquinone pathway is illustrated with blue highlighting as part of the composite given in **Figure 6**. The thioesterase reaction of the classic pathway, previously thought to be perhaps a spontaneous, non-enzymatic transformation, has recently been documented as an enzymatic reaction (Widhalm et al., 2009) and is denoted as MenX in **Figure 6**.

COMPARISON OF THE CLASSIC DH2N PATHWAY WITH THE DH6N PATHWAY

Recently, an alternative pathway of menaquinone biosynthesis called the futalosine pathway by Dairi and his collaborators was reported (Hiratsuka et al., 2008; Dairi, 2009, 2012). Since futalosine has subsequently proven to not necessarily be an intermediate, this pathway is herein referred to as the DH6N pathway and the classic isochorismate pathway is referred to as the DH2N pathway. The *Chlamydiaceae* family is distinctive among the *Chlamydiales* in having the DH6N pathway. All other known *Chlamydiales* families use the classic DH2N pathway. As illustrated with yellow highlighting in **Figure 6**, DH6N and DH2N are closely related positional isomers. The newly recognized DH6N pathway itself exhibits variation in some of the early steps, such that futalosine is made directly following reaction of chorismate and inosine (denoted $MqnA_i$ in **Figure 6**) or indirectly in two steps by an initial reaction of chorismate and adenosine to form AFL ($MqnA_a$) followed by a deaminase reaction (MqnX). A third flow route takes AFL directly to DHFL ($MqnB_{af}$), thus by-passing futalosine altogether (Arakawa et al., 2011). Thus, a general MqnA reaction sorts into enzymes having specificity for adenosine ($MqnA_a$) or having specificity for inosine ($MqnA_i$). [Note that early tracer studies have indicated a likely role of PEP or pyruvate as a substrate reactant (Seto et al., 2008)]. Likewise, MqnB enzymes sort into those having specificity for aminodeoxyfutalosine ($MqnB_{af}$) or for futalosine ($MqnB_f$). Organisms such as *Thermus thermophilus* take the two-step futalosine pathway to DHFL, whereas organisms such as *Campylobacter*, *Helicobacter*, and *Chlamydia* use the two-step aminodeoxyfutalosine pathway to DHFL. Yet other organisms, such as *Streptomyces coelicolor* and *Acidothermus cellulolyticus* take the three-step pathway to DHFL, deploying $MqnA_a$, the MqnX deaminase, and $MqnB_f$ (Dairi, 2012). $MqnB_{af}$ has been identified recently as synonymous with 5'-methylthioadenosine nucleosidase (MTAN) in *Campylobacter jejuni* (Li et al., 2011).

FEATURES OF DH6N PATHWAY VARIATION IN *Chlamydiaceae*

The use of the adenosine-dependent step ($MqnA_a$), rather than of the inosine-dependent step ($MqnA_i$) by the *Chlamydiaceae* is consistent with the report (McClarty and Fan, 1993) that *C. psittaci* was able to utilize adenosine, but not inosine, from the host. Undoubtedly there are some interesting properties of the DH6N pathway that await discovery in the *Chlamydiaceae*. For example, CT263 is a gene of unknown function which is *Chlamydiaceae*-specific and which overlaps with CT262 encoding MqnD. CT261 encoding the epsilon subunit of DNA polymerase also overlaps CT262. In another operonic arrangement, CT427 encoding $MqnA_a$ and CT428 encoding MqnH are contiguous, these genes encoding the first and last steps of the pathway. Curiously, CT426, an apparent paralog of CT767 encoding MqnC, is also a member of the operon. The enzyme encoded by CT426 is not shown as a catalytic participant in **Figure 6**, but the co-existence of two *mqnC* paralogs in nature is highly conserved. For example, *S. coelicolor* possesses in addition to the SCO 4550 *mqnC*, a sister *mqnC* paralog SCO4494 (32% identity of the gene products). These paralogs are members of the Radical_SAM family. The role of the paralog might have some sort of functional relationship with the assertion that PEP or pyruvate must be utilized in the early part of the pathway (Seto et al., 2008). The CT219 and CT220 genes overlap by 3 bp, and probably comprise an additional operon, another relationship which appears to be conserved (e.g., the corresponding genes in *S. coelicolor* are SCO4491 and SCO4492). CT 219 and CT220 have been annotated as *ubiA* and *ubiX*, respectively. However, the prenyltransferase, methyltransferase, and decarboxylase enzymes that exist in the ubiquinone and menaquinone pathways are homologs and can easily be mis-annotated.

MENAQUINONE GENES LOCATED ON THE LAGGING STRAND OF REPLICATION

A bias is well-known to favor the location of *trp* codons on the leading strand of replication. The cumulative influence of multiple *trp* codons in genes that encode high-Trp proteins should

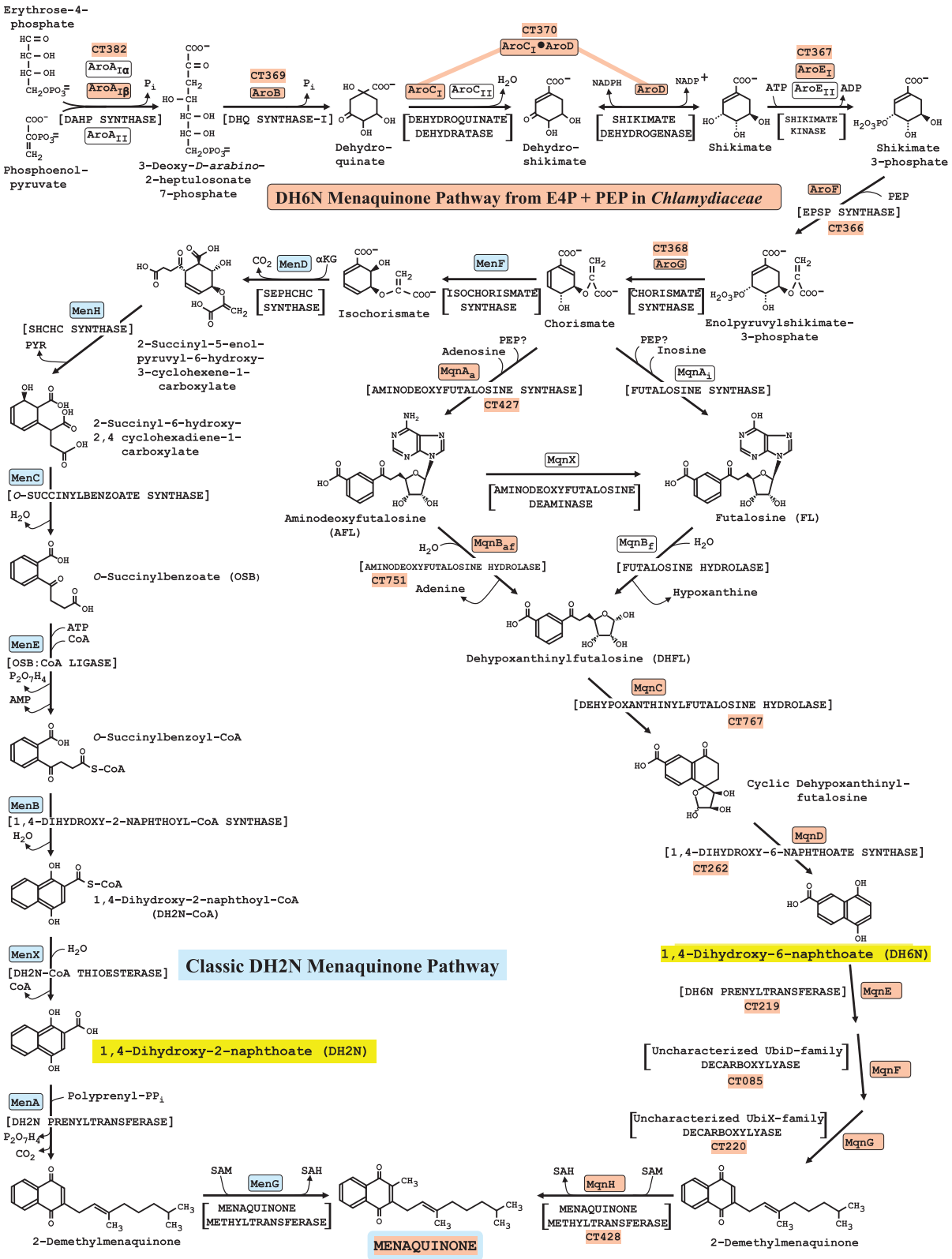

FIGURE 6 | Variant menaquinone pathways in nature. The composite diagram shows the biochemical variations to menaquinone that are so far known to exist in nature. The proposed 15-step *Chlamydiaceae* pathway, which includes the 7-step pathway to chorismate, is indicated with orange boxes surrounding the enzyme acronyms. For reference, the encoding CT gene numbers for *C. trachomatis* D/UW-3/CX are also shown. Multiple acronyms are indicated for the three enzyme steps in the chorismate

(Continued)

FIGURE 6 | Continued
pathway that can be performed by distinct sub-homolog types or by non-homologous isofunctional analogs (see http://www.aropath.lanl.gov/ for the logical system of acronym assignment used). AroC$_I$ and AroD are domain components of a single protein encoded by fused genes. The classic dihydroxy-2-naphthoate (DH2N) pathway from chorismate, generally known as the isochorismate pathway of menaquinone biosynthesis, is shown with acronyms in blue boxes at the left. The alternative DH6N pathway shown at the right was originally called the futalosine pathway (Dairi, 2009, 2012), but futalosine has proven to not necessarily be used as an intermediate because of alternative early-pathway steps that exist. Therefore, we refer to this as the DH6N pathway. The nine enzymes of the DH6N pathway in *S. coelicolor* are encoded by SCO4506, SCO4662, SCO4327, SCO4550, SCO4326, SCO4491, SCO4490, SCO4492, and SCO4556. The DH2N and DH6N structures, for which the pathways are named, are highlighted in yellow.

greatly increase the probability for the location of such genes to be on the leading strand. Location on the leading strand prevents head-to-head collisions of DNA polymerase engaged in DNA replication and RNA polymerase engaged in transcription. Of the 15 genes specifying the menaquinone pathway, 14 are located on the lagging strand of replication. This is consistent with the low *trp*-codon counts in these genes. The only exception is the initial *aroA* gene encoding DAHP synthase, a protein that does have a higher Trp content than other enzymes of the common aromatic pathway leading to chorismate (see **Figure 7**). Surprisingly, some crucial Ctra proteins of very high Trp content were observed to be encoded by genes located on the lagging strand of replication (Lo et al., 2012). These include the genes encoding glucose-6-P translocase, dicarboxylate transporter, and ADP/ATP translocase. It was suggested that perhaps transcripts of such genes might be stockpiled under conditions of persistence where their translation would not be favorable. During transition to rapid vegetative growth occasioned by the renewed presence of Trp, the availability of key transcripts might help jumpstart this developmental process. This idea is based upon the finding that transcription and translation are uncoupled in *Chlamydia* and that some transcripts made in the absence of translation can be very stable (Ouellette et al., 2006).

DRAMATIC Down-Trp SELECTION OF THE JOINED CHORISMATE/MENAQUINONE PATHWAY IN *Chlamydiaceae*

In the chlamydiae the seven-step pathway to chorismate and the connecting menaquinone pathway can be considered to be a lengthy but simple, unbranched pathway. The *Chlamydiaceae* members differ from members of other *Chlamydiales* families in utilizing the DH6N variation rather than the classic DH2N pathway. In contrast, in organisms such as *E. coli* and *Streptomyces coelicolor*, the menaquinone pathway is but one of many connecting branches—resulting in a highly branched, complex system of biosynthesis. **Figure 7** illustrates the Trp content of the enzymes of chorismate/menaquinone biosynthesis in *E. coli* (Ecol), *S. coelicolor* (Scoe), *C. trachomatis* (Ctra) as a representative of *Chlamydiaceae*, and in *P. amoebophila* (Pamo) as a representative of other *Chlamydiales*. Ecol and Pamo are similar in their utilization of the classic DH2N pathway (depicted with orange histogram bars in **Figure 7**), whereas Ctra and Scoe are similar in having the DH6N pathway routing (green bars). Extreme Down-Trp selection in Ctra (and all *Chlamydiaceae*) for enzymes of both the chorismate-pathway enzymes and the menaquinone pathway is apparent by examination of the summarized Trp content indicated by the horizontal bars at the bottom of **Figure 7**.

CONCLUDING PERSPECTIVE

The *Chlamydiaceae* exist in one of two alternative states. (i) A proliferative mode consists of two life-cycle phases: RBs are replicative bodies that exhibit high metabolic activity and are associated with acute disease; they parasitically exhaust the cellular resources and eventually cause lysis of the host cell in concert with the formation of EBs. The released EBs are infectious entities that find and infect new cell hosts. (ii) In the persistent mode, metabolic activity of the pathogen is greatly altered. Persistence is a survival mode that is proposed to be associated with environmental stress and subsequent survival in the absence of growth—a mode that may be reversed once the stress is relieved. It has been suggested that persistence, as defined by these terms, may be associated with a variety of chronic chlamydial infections (Gieffers et al., 2003; Seto et al., 2008; Ouellette et al., 2012), but this hypothesis requires vigorous *in vivo* validation (Byrne and Beatty, 2012).

There are surely unknown aspects of regulation in play, but at this time we can at least consider whether whole circuits of regulation might be activated or inactivated depending upon the Trp content of the regulator itself. An intriguing possibility is provided by Trip230, encoded by CT647 in *C. trachomatis*. It probably acts as a transcriptional activator and was suggested to function in folate metabolism (Lo et al., 2012). It has the highest p/P Trp ratio in the entire Ctra proteome. The menaquinone pathway of biosynthesis offers exciting research prospects because: (i) It is undoubtedly crucial for survival of either rapidly growing cells or cells in the persistent state of quiescence, and (ii) The menaquinone pathway of *Chlamydiaceae* is not present in the host or in typical beneficial flora, therefore providing multiple protein targets for new, specific antimicrobial agents.

The *Chlamydiales* are a group of highly specialized organisms evolved to survive in a unique environmental niche comprising a membrane-bound vacuole (inclusion) within the cytoplasm of the cells of the host. Survival within this niche requires that the chlamydiae be capable of sensing changes in host cell physiology that will evoke modulation of the chlamydial growth state (i.e., productive versus persistent growth). One key trigger for *Chlamydiales*, whether the host is a free-living amoeba or a human being, is the availability of a single metabolite. That metabolite is Trp. Since strong competition must exist for the limited amount of Trp available to the pathogen in the persistent mode, the protein assemblage supporting the persistent mode needs to compete advantageously under these conditions. Hence, evolutionary selection has occurred for Down-Trp proteins that characterize the persistent mode. In this light, the basis for such Down-Trp selection seems obvious. But why has Up-Trp selection

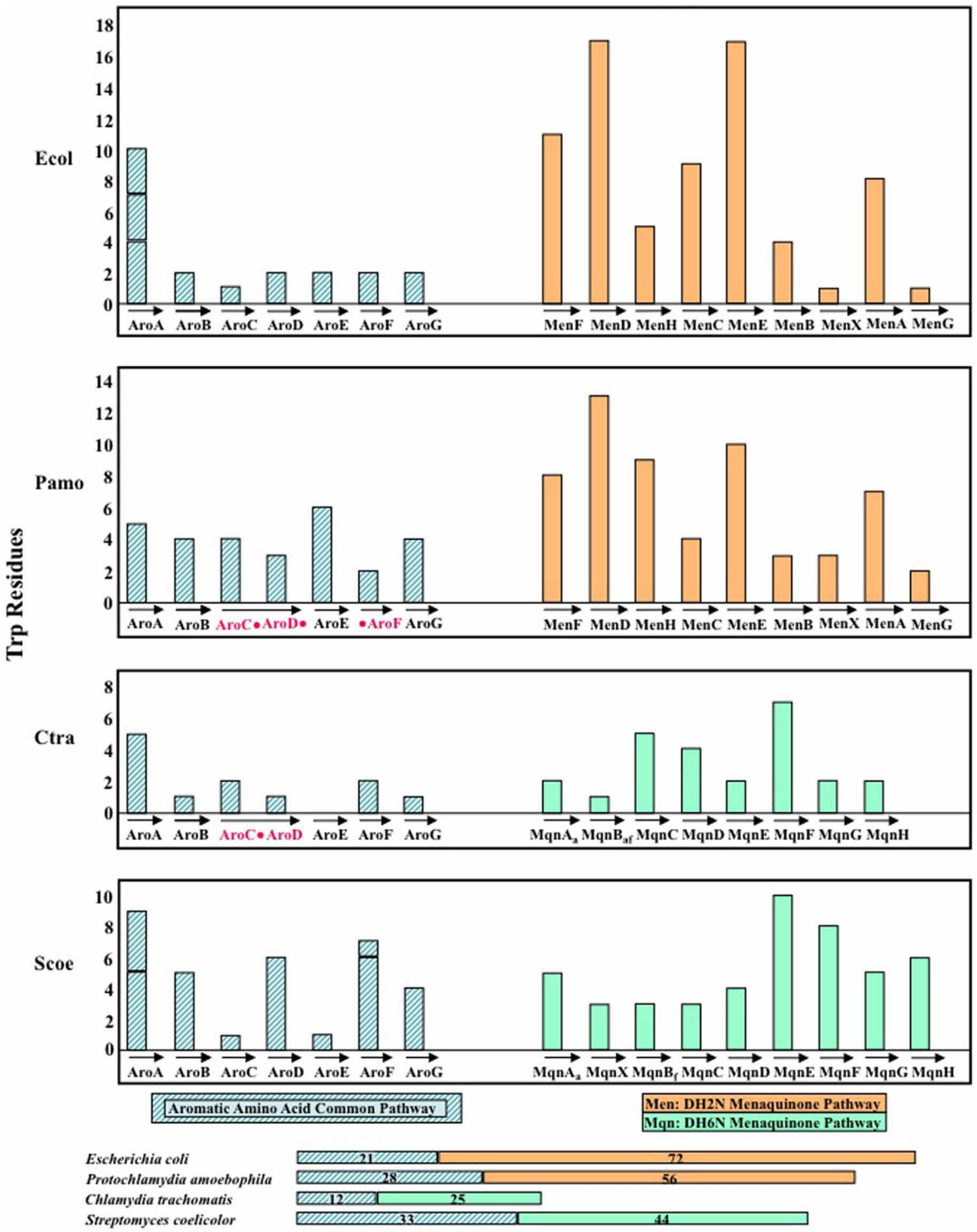

FIGURE 7 | Comparison of the Trp content of the chorismate/menaquinone pathway in *Chlamydia trachomatis* (Ctra), its close relative *Protochlamydia amoebophila* (Pamo), the classic bacterium *Escherichia coli* (Ecol), and *Streptomyces coelicolor* (Scoe). Pamo and Ecol both utilize the classic DH2N pathway for menaquinone biosynthesis, and this is represented by the orange histogram bars. On the other hand, Ctra and Scoe both utilize the DH6N pathway, and this is indicated by the green histogram bars. Note by relating the acronyms under the histogram bars to the pathway diagrams in **Figure 6** that Ctra and Scoe utilize different minor variations of the DH6N pathway, utilizing aminodeoxyfutalosine or futalosine, respectively, as unique intermediates. Ctra exhibits a fusion of AroC and AroD, whereas Pamo has a fusion of AroC, AroD, and AroF (as indicated in red). The cumulative total of Trp residues in the chorismate pathway and the connected menaquinone pathway are given within the color-coded horizontal bars displayed at the bottom of the figure.

occurred for proteins strongly engaged in the proliferative mode? We suggest that it is because this maximizes vulnerability to the completion of translation tasks whenever the shift to the persistent mode occurs. Lack of translational follow-through for the proliferative-mode set of proteins may not only facilitate the developmental transition but may also result in some proteolysis with release of small amounts of Trp then made available for the persistent-mode set.

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Influence of the tryptophan-indole-IFNγ axis on human genital *Chlamydia trachomatis* infection: role of vaginal co-infections

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The natural history of genital *Chlamydia trachomatis* infections can vary widely; infections can spontaneously resolve but can also last from months to years, potentially progressing to cause significant pathology. The host and bacterial factors underlying this wide variation are not completely understood, but emphasize the bacterium's capacity to evade/adapt to the genital immune response, and/or exploit local environmental conditions to survive this immune response. IFNγ is considered to be a primary host protective cytokine against endocervical *C. trachomatis* infections. IFNγ acts by inducing the host enzyme indoleamine 2,3-dioxgenase, which catabolizes tryptophan, thereby depriving the bacterium of this essential amino acid. *In vitro* studies have revealed that tryptophan deprivation causes *Chlamydia* to enter a viable but non-infectious growth pattern that is termed a persistent growth form, characterized by a unique morphology and gene expression pattern. Provision of tryptophan can reactivate the bacterium to the normal developmental cycle. There is a significant difference in the capacity of ocular and genital *C. trachomatis* serovars to counter tryptophan deprivation. The latter uniquely encode a functional tryptophan synthase to synthesize tryptophan via indole salvage, should indole be available in the infection microenvironment. *In vitro* studies have confirmed the capacity of indole to mitigate the effects of IFNγ; it has been suggested that a perturbed vaginal microbiome may provide a source of indole *in vivo*. Consistent with this hypothesis, the microbiome associated with bacterial vaginosis includes species that encode a tryptophanase to produce indole. In this review, we discuss the natural history of genital chlamydial infections, morphological and molecular changes imposed by IFNγ on *Chlamydia*, and finally, the microenvironmental conditions associated with vaginal co-infections that can ameliorate the effects of IFNγ on *C. trachomatis*.

Keywords: *Chlamydia trachomatis*, IFNγ, IDO1, tryptophan, persistence, indole, bacterial vaginosis, vaginal microbiome

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterium that has a unique biphasic developmental cycle. *C. trachomatis* serovars D through K are tropic for columnar epithelial cells of the urogenital tract; the endocervix is the most common site of infection in women, but organisms can ascend into the uterus and Fallopian tubes where they can cause pelvic inflammatory disease (PID) and the longer-term sequelae of tubal infertility and ectopic pregnancy. Infected, untreated women can also vertically transmit their infection to neonates, with consequences including pneumonia. Additionally, infected women have a significantly increased risk of acquiring, shedding and/or transmitting, human immunodeficiency virus-1 (HIV-1) (Wasserheit, 1992; Ghys et al., 1997; Fleming and Wasserheit, 1999; Chesson and Pinkerton, 2000; Farley et al., 2003). Despite extensive public health interventions including education, screening, and antibiotic treatment, *C. trachomatis* infections remain a significant global health problem, with reported U.S. cases reaching 1 million in 2006 and continuing to rise since (Centers for Disease Control, 2011). This burden of cases, together with the predominantly asymptomatic nature of the disease, have led the *C. trachomatis* infection to be called the "silent epidemic" (Wallis, 2001). The development of a vaccine is now considered *a priori* in the control of this infection.

The natural history of genital *C. trachomatis* infection can vary considerably. Untreated infections can be asymptomatic for substantial periods of time, progress to cause significant pathology, or spontaneously resolve without antibiotic treatment (Parks et al., 1997; Golden et al., 2000; Joyner et al., 2002; Morre et al., 2002; Molano et al., 2005; Geisler, 2010; Centers for Disease Control,

2011). It is not understood why some *C. trachomatis* infections can last from months to years; clearly, the organism has the ability to survive by exploiting, adapting to, or evading, certain genital immune and environmental conditions (Brunham and Rey-Ladino, 2005). Establishing the local genital environments that enable, enhance, or deter *C. trachomatis* survival should allow us to: (a) identify the women at risk for extended infections; (b) define the conditions needed for the genital immune system to resolve infection naturally, and (c) aid in designing targeted vaccination and therapeutic strategies. Pertinent to this, *C. trachomatis* is a tryptophan auxotroph; therefore, induction of the tryptophan-catabolizing enzyme, indoleamine-2,3-dioxygenase 1 (IDO1), by the cytokine interferon gamma (IFNγ) restricts chlamydial growth and development in human epithelial cells (Shemer and Sarov, 1985; Byrne et al., 1986, 1989; Carlin et al., 1989; Beatty et al., 1994a; Brunham and Rey-Ladino, 2005). For this reason, IFNγ is considered to be a major anti-chlamydial effector cytokine. *In vitro*, exposure of *C. trachomatis*-infected epithelial cells to IFNγ can result in bacterial death, or, can cause the organism to adopt a viable but non-infectious growth mode that is termed a persistent or abnormal growth form (reviewed by Wyrick, 2010). *In vitro,* dependent on the culture conditions, persistent growth forms can proceed to clearance upon prolonged starvation, or they can reactivate and replicate to produce infectious elementary bodies (EBs) (Byrne et al., 1986, 1989; Beatty et al., 1993, 1994a). Pertinent to IFNγ-induced tryptophan starvation, genital, but not ocular, serovars of *C. trachomatis* have retained a functional tryptophan synthase that enables them to synthesize tryptophan via indole salvage (Fehlner-Gardiner et al., 2002; Caldwell et al., 2003). Thus, if indole is present at a sufficient concentration in the infection microenvironment, genital serovars could circumvent the bactericidal/bacteriostatic effects of IFNγ (Beatty et al., 1993; Fehlner-Gardiner et al., 2002; Belland et al., 2003; Caldwell et al., 2003). This suggests the capacity to synthesize tryptophan in an IFNγ-rich infection microenvironment is an important virulence factor for genital *C. trachomatis* serovars. *In vivo*, the source of indole in the infection microenvironment remains unknown. However, perturbations in the vaginal flora that increase the prevalence of indole-producing bacteria during bacterial vaginosis (BV) may increase the susceptibility of women to extended infections even in the face of a robust IFNγ response, a hypothesis originally proposed by Caldwell and coworkers in a seminal Journal of Clinical Investigation study in 2003 (Caldwell et al., 2003). However, until recent work by others and us, the nature of a "real" clinical infection, the *in vivo* growth characteristics of *C. trachomatis*, and the composition of the genital milieu *in vivo* have remained almost completely uncharacterized, precluding a test of this hypothesis. The purpose of this review is to: (a) describe the recent advances made in the characterization of the normal and perturbed vaginal microbiome that are pertinent to the indole-rescue hypothesis; (b) describe recent *in vivo* data to support and extend this hypothesis, including evidence that *C. trachomatis* can adopt a persistent growth mode *in vivo*, and that BV provides an indole-rich genital environment; and (c) explicate the mechanism by which BV and/or the vaginal *Trichomonas vaginalis* (TV) co-infections could modulate the effect of IFNγ on *C. trachomatis* growth and clearance *in vivo*, including ramifications on clinical outcomes and choice of treatment.

IFNγ-MEDIATED IMMUNITY TO GENITAL *C. TRACHOMATIS* INFECTION

Several lines of evidence indicate genital *C. trachomatis* infection induces human immunity and that this immunity is variably protective, as reviewed recently (Batteiger et al., 2010). These include: (a) young age as a significant risk factor for infection acquisition (Arno et al., 1994); (b) reduced organism load with repeat infections (Barnes et al., 1990); (c) natural history studies documenting spontaneous resolution of infection (Parks et al., 1997; Golden et al., 2000; Joyner et al., 2002; Morre et al., 2002; Molano et al., 2005; Centers for Disease Control, 2010; Geisler, 2010); and (d) association of spontaneous resolution of infection with subsequent protection from incident disease (Geisler et al., 2013). Immunity in humans appears to develop slowly and protection from infection is generally thought to be robust only after multiple exposures and can be as short as several months (Katz et al., 1987; Molano et al., 2005), a finding corroborated in animal models (Rank and Whittum-Hudson, 2010). Further, chronic infection has been associated with pathology and the long-term sequelae of disease. Thus, significant current declines in PID are attributed to the aggressive "seek and treat" public health intervention strategies that are now in place in many developed countries (Brunham and Rekart, 2008, 2009). Paradoxically, this strategy has also been partially attributed to increasing rates of disease by blunting the development of protective immunity to this pathogen, thereby increasing the susceptibility of the population to disease (Brunham and Rekart, 2008). This has been termed the "arrested immunity" hypothesis and is corroborated by antibiotic intervention studies in mice (Su et al., 1999). Finally, in studies of sex workers who have a high risk of exposure to *C. trachomatis* via *C. trachomatis*-infected clients, the probability of incident *C. trachomatis* infection correlates inversely with duration of prostitution (Brunham et al., 1996). Finally, immune dysfunction, as indicated by HIV seropositive status, is a risk factor for incident *C. trachomatis* infection (*ibid*).

Evidence for an association of IFNγ with genital chlamydial infection is supported by multiple experimental studies in animals (reviewed in detail in Rank and Whittum-Hudson, 2010) and observational/correlative studies in humans. Pertinently, in the murine model of genital infection using *Chlamydia muridarum*, a species that shares substantial genomic synteny with *C. trachomatis*, T-cells and IFNγ are critical to the resolution of, and subsequent protection from, genital infection (Ramsey et al., 1988; Cain and Rank, 1995; Su et al., 1997; Johansson and Lycke, 2001). Thus, CD4, MHC class II, IFNγ and IFNγ-receptor depletion/knockout results in chronic infection, uncontrolled *C. muridarum* bacterial burden and/or lack of protection from reinfection (Cotter et al., 1997; Perry et al., 1997; Morrison et al., 2000; Li et al., 2008; Jupelli et al., 2010; Andrew et al., 2013). Interestingly, IDO1 is poorly induced by IFNγ in murine epithelial cells, and is not required for resolution of genital *C. muridarum* infection (McClarty et al., 2007). Rather, mice restrict *C. muridarum* though a cell-autonomous resistance mechanism by a

large family of IFNγ-inducible GTPases called immunity related GTPases (Nelson et al., 2005; Miyairi et al., 2007; Coers et al., 2008; Burian et al., 2010). Consistent with the molecular mechanisms underlying IFNγ-mediated restriction in mice differing from those operant in humans, subsequent elegant molecular and cross-species studies indicate the genes that are divergent in *C. muridarum* and *C. trachomatis* strongly correlate with the ability to evade species-specific IFNγ effector activities. Similarly comparisons of genital and ocular *C. trachomatis* serovars indicate a strong correlation with evasion of tissue-specific IFNγ effector activities (Morrison, 2003).

In human genital infections, local cervical T-cell infiltrates and genital IFNγ concentrations are significantly elevated during active infection, higher in women with recurrent vs. primary infection, and decreased upon resolution of infection (**Figure 1**), and (Arno et al., 1990; Loomis and Starnbach, 2002; Agrawal et al., 2007; Ficarra et al., 2008; Sperling et al., 2013). Local IFNγ-producing *C. trachomatis*-specific CD4 T-cells are found in the endometrium of women with a high risk of exposure to *C. trachomatis* (Ondondo et al., 2009). Systemically, anti-chlamydial IFNγ-producing T-cells generally peak 1–2 months after active infection in most antibiotic-treated women (Vicetti et al., 2012); however, in highly-exposed women, IFNγ-producing T-cells that recognize epitopes from *C. trachomatis* HSP60 correlate with protection against incident *C. trachomatis* (Cohen et al., 2005). Finally, in highly exposed HIV seropositive women, a low CD4 count is associated with low *C. trachomatis*-induced IFNγ production (Cohen et al., 2000), and increased the risk of infection spread to the upper reproductive tract and PID (Kimani et al., 1996). While human research is challenging, we believe that new studies and tools are critical to further delineate, and mechanistically dissect, the complex relationships that exist between host immunity and bacteria in the context of the local genital environment.

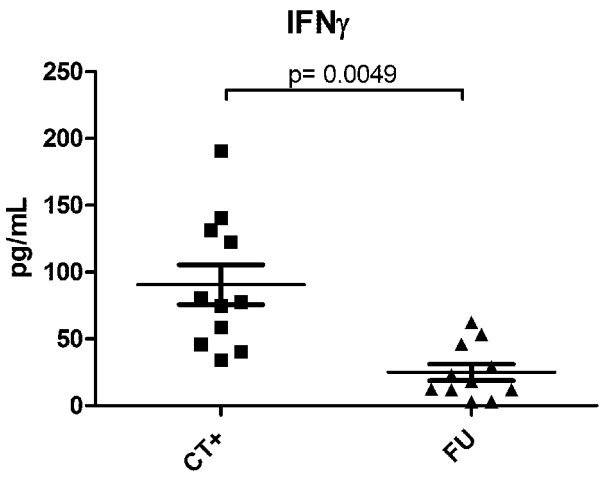

FIGURE 1 | IFNγ levels in paired endocervical secretion samples from women during, and post-antibiotic resolution, of *C. trachomatis* infection. Endocervical secretions, harvested as previously described (Gumbi et al., 2008), were collected from 11 women during an ongoing NAAT+ *C. trachomatis* infection, and again 3–6 weeks after azithromycin treatment and a confirmed NAAT− *C. trachomatis* test. Samples were quantified using multiplex cytokine analysis (Millipore), and comparisons between infected and non-infected states investigated by a Wilcoxon-Mann-Whitney test. A *p*-value of < 0.05 was considered significant. CT+ indicates samples from patients with an active infection and FU indicates post-antibiotic follow-up samples.

THE HUMAN EPITHELIAL CELL AND *C. TRACHOMATIS* RESPONSE TO IFNγ AND DEPLETION OF TRYPTOPHAN

Because *C. trachomatis* is a tryptophan auxotroph, IDO1-mediated depletion of tryptophan curtails its growth (Taylor and Feng, 1991; Beatty et al., 1994a). *C. trachomatis* development is initiated when an infectious, but largely metabolically inactive, EB attaches to and enters the host cell. The internalized EB differentiates and replicates by binary fission as reticulate bodies (RBs) within a cytoplasmic membrane-bound inclusion. The completion of the developmental cycle is marked by re-differentiation of RBs into EBs that egress from the host cell. However, under conditions of limited tryptophan availability, the developmental cycle is arrested in the RB stage in a manner that results in morphologically distinct, large aberrant, viable but non-cultivable, persistent growth forms (Beatty et al., 1994a). Restoration of tryptophan availability can reactivate these persistent forms to return to normal development (Byrne et al., 1986). Similarly, provision of indole can reactivate persistent forms from genital, but not ocular, serovars, because the former synthesize a functional tryptophan synthase in response to tryptophan starvation (Caldwell et al., 2003).

CHLAMYDIAL GENE EXPRESSION PATTERNS INDUCED BY IFNγ

The induction of persistent forms with IFNγ, and their subsequent reactivation, have been studied *in vitro* through microarray analysis. Persistent growth, characterized by large aberrant RBs, led to the up-regulation of genes involved in tryptophan utilization, DNA repair and recombination, phospholipid biosynthesis and translation. Up-regulation of the repressible *trp*BA operon (Belland et al., 2003) confirms the previous observations that IFNγ treatment reduces intracellular concentrations of tryptophan. In addition, a number of early genes were up-regulated, particularly the *euo* gene (30-fold increase), which encodes a DNA-binding protein that has been shown to bind to a late gene promoter region (i.e., *omc*AB Zhang et al., 1998) to repress expression. Euo has been shown to down-regulate multiple late genes (Rosario and Tan, 2012); down-regulation of *C. trachomatis* genes involved in RB to EB differentiation, proteolysis and peptide transport, and cell division were seen during persistent growth induced by IFNγ. These transcriptional analyses were consistent with the biological properties associated with aberrant RBs in that the RBs were blocked in cytokinesis, and the developmental cycle was arrested at a point preceding late gene expression. Indeed, EM analyses of persistent forms induced by IFNγ treatment, indicates that large aberrant RBs are arrested in cytokinesis. This morphology appears to contrast quite strikingly with persistent forms induced by exposure to penicillins or the danger signal adenosine (Pettengill et al., 2009; Skilton et al., 2009), but resemble the atypical forms observed previously upon IFNγ treatment (Beatty et al., 1993, 1994b).

Recently, we have developed protocols that allowed the parallel assessment of both *C. trachomatis* gene transcription (specifically the *euo*:*omcB* ratio), and ultrastructure of *C. trachomatis* growth forms found *in vivo* in human endocervical infection (Lewis et al., 2014). The sampling protocols also permitted the quantification of indole and IFNγ levels from the same environment (*ibid*). Using this multi-parameter sampling approach, we were able to visualize two strikingly different growth patterns of *C. trachomatis* growth in two patients. Specifically, one infection was characterized by morphologically normal late-stage inclusions, high viable bacterial numbers, and a low *euo*:*omcB* mRNA expression profile, together with the presence of a high concentration of indole and no IFNγ. In contrast, the second infection included morphologically aberrant forms similar in ultrastructure to *in vitro* persistence induced by IFNγ, a high *euo*:*omcB* mRNA expression profile, low infectious titer, and large bacterial DNA load together with a local IFNγ response. This approach, that may be expanded to include an even more detailed analysis of *C. trachomatis* and host gene expression, indicate the distinct possibility of understanding how *C. trachomatis* grows in the genital tract, along with the environmental stresses, including IFNγ, which it encounters.

TRYPTOPHAN SUPPLEMENTATION REVERSES TRANSCRIPTIONAL CHANGES INDUCED BY IFNγ

In the *in vitro* model of IFNγ-induced persistence, removal of IFNγ and supplementation with added tryptophan led to a rapid reactivation from persistent growth (Belland et al., 2003). During reactivation the expression differences rapidly returned to control levels, i.e., *euo* expression dropped 20-fold in 12 h. The transcriptional changes in the presence of IFNγ that result in persistent growth appear to constitute a persistence stimulon. Thus, we earlier postulated that this coordinated biological response is speculated to have evolved to allow the organism to rapidly respond to immunological pressure in a manner that allows for a period of resistance followed by rapid recovery after the waning of the host response.

GENITAL SEROVARS OF *C. TRACHOMATIS* CAN SYNTHESIZE A FUNCTIONAL TRYPTOPHAN SYNTHASE

Shaw *et al*, who reported that ocular serovars encoded a truncated TrpA protein (Shaw et al., 2000), initially described differences in tryptophan synthase between ocular and genital serovars of *C. trachomatis*. This analysis was extended by Fehlner-Gardiner et al., who reported that genital, but not ocular, *C. trachomatis* serovars encode a functional, tightly regulated, tryptophan synthase (*trpBA*) permitting the bacteria to synthesize tryptophan from indole (Fehlner-Gardiner et al., 2002; Akers and Tan, 2006; Carlson et al., 2006). Along with other genes that affect pathogenesis and distinguish ocular and genital serovars of *C. trachomatis* (Carlson et al., 2004; Nelson et al., 2006; Taylor et al., 2010), the enzymes required for the biosynthesis of tryptophan are also encoded by genes within the plasticity zone (McClarty et al., 2007). The complement of *trp* genes within this region varies between chlamydial species. *C. pneumoniae* and *C. muridarum* do not encode *trp* genes (Xie et al., 2002a). *C. psittaci* lacks the *trpE* gene and is therefore incapable of synthesizing the anthranilate, an essential precursor for tryptophan biosynthesis. *C. trachomatis* urogenital isolates only have a subset of *trp* genes, encoded in the plasticity zone: *trpR*, encoding a *trp* repressor; and *trpA* and *trpB*, encoding homologs of the α (TrpA) and β (TrpB) subunits of tryptophan synthase. As reviewed recently (Raboni et al., 2009; Miles, 2013), tryptophan synthase is a tetramer consisting of two α subunits and two β subunits (α2β2). Functional and sequence analyses indicate that in most bacterial species that express a α2β2 tetramer, the enzyme is predicted to be bi-functional and catalyze the cleavage of indole glycerol-3-phosphate (IGP) to indole and glyceraldehyde-3-phosphate (TrpA catalyzed α reaction), followed by the reaction of indole with serine to form tryptophan (TrpB catalyzed β-replacement reaction) (Xie et al., 2002b; Raboni et al., 2009; Miles, 2013). However, sequence analysis, and complementation studies conducted in *E. coli*, together indicate that the TrpA subunit in *C. trachomatis* appears to function structurally but not enzymatically (Fehlner-Gardiner et al., 2002; Xie et al., 2002b). As a consequence, the TrpA/TrpB tetramer expressed by *C. trachomatis* during tryptophan starvation cannot use IGP as a substrate for tryptophan biosynthesis; rather, it requires indole (Fehlner-Gardiner et al., 2002). Therefore, indole, and not IGP, may be an important molecule in chlamydial growth by modulating the effectiveness of the IFNγ response in bacterial clearance. Subsequent studies by Caldwell et al. verified the absolute correlation between tissue tropism and *trpBA* expression in >200 clinical isolates (Caldwell et al., 2003). All urogenital isolates had an intact *trpRBA* operon, while >90 ocular isolates invariably had frame-shift-inducing deletions within the *trpA* or *trpB* genes or had deleted the entire operon (*ibid*). This provided a molecular basis for the observation that only genital isolates could synthesize tryptophan from indole. While the enzymatic capacity of the chlamydial tryptophan synthase to salvage indole has been assessed only in *E. coli*, pioneering genetic studies reveal that a *C. trachomatis* null mutant in *trpB* has lost the capacity to escape IFNγ-mediated tryptophan depletion via indole salvage (Kari et al., 2011). Together, these data reveal a strong selective pressure for genital *C. trachomatis* strains to use indole salvage as a mechanism to escape IFNγ-mediated eradication by the host. Therefore, the ability to overcome tryptophan starvation may provide a mechanism through which *C. trachomatis* could cause extended and chronic infections in some women despite a robust induction of IFNγ. Critically, the availability of indole within the infection microenvironment is predicted to modulate the effect of IFNγ. Genital chlamydial infections occur in the context of the genital microbiome, of which the vaginal microbiome has been characterized in depth. The vaginal microbiome, particularly when perturbed, has been postulated to be the source of indole (Caldwell et al., 2003).

AN INDOLE-POOR OCULAR MICROENVIRONMENT MAY SELECT AGAINST A FUNCTIONAL TRYPTOPHAN SYNTHASE

Although not the focus of this review, the stark contrast in the ability of urogenital and ocular *C. trachomatis* isolates to express a functional tryptophan synthase is intriguing to us. Unlike the vaginal microbiome, which as described below has been characterized under a variety of conditions, the normal

ocular microbiome is far less characterized. The two most prominent phyla in the ocular microbiome from four healthy subjects were *Pseudomonas* and *Bradyrhizobium*, neither of which produce indole (Dong et al., 2011). We note that the absence of indole in the conjunctival microenvironment is insufficient to explain why ocular chlamydial isolates have uniformly lost the capacity to express a functional tryptophan synthase, most often as a consequence of point mutations. This apparent negative selection may result from an alternative enzymatic reaction catalyzed by tryptophan synthase when indole is absent; specifically, the β-elimination reaction in which L-serine is deaminated to produce pyruvate and ammonia (Kumagai and Miles, 1971; Miles and McPhie, 1974; Xie et al., 2002b; Raboni et al., 2009). While initially described as a function of β2 dimers, detailed studies examining the functions of the α2β2 tetrameric enzyme from *S. typhimurium* have revealed that it also catalyzes the β-elimination reaction necessary to produce ammonia (Ahmed et al., 1991; Raboni et al., 2005). While this catalysis is allosterically curtailed in the tetrameric enzyme by the glyceraldehyde-3-phosphate product of the α reaction, the *C. trachomatis* TrpA sequence, and functional analyses, indicate that it cannot bind IGP and catalyze the α reaction (Fehlner-Gardiner et al., 2002; Xie et al., 2002b). Further, sequence changes in the loop 6 of the *C. trachomatis* α subunit are predicted to prevent the inter-subunit interactions necessary for allosteric control (Schneider et al., 1998), consistent with the outcome of mutational analyses of the *S. typhimurium* enzyme (Yang and Miles, 1992; Brzovic et al., 1993; Kulik et al., 2002; Raboni et al., 2005). Pertinently, the active-site residues necessary for β-elimination catalysis to produce ammonia remain highly conserved in TrpB from *C. trachomatis*. Therefore, the α2β2 tetrameric enzyme from *C. trachomatis* is predicted to catalyze the generation of ammonia from serine when indole is absent. In addition to its anti-microbial effects (Rideal, 1895), ammonia is also known to induce apoptosis of epithelial cells that express the NMDA receptor (Suzuki et al., 2002; Sachs et al., 2011). In this context, the production of ammonia is currently proposed to underlie apoptosis of gastric epithelial cells induced by *H. pylori* (Seo et al., 2011). It may be of relevance that although their expression in the normal conjunctiva has not been examined, NMDA receptors are expressed in some ocular epithelial cell-lines (Oswald et al., 2012). As reviewed recently, cellular infiltrates associated with human conjunctival *C. trachomatis* infections include T-cells, which are capable of producing IFNγ (Hu et al., 2013). The consequential IFNγ-induced expression of a functional tryptophan synthase in an indole-limiting environment is predicted to generate ammonia by deaminating serine, with ensuing effects that may directly select against bacterial replication or cause apoptosis of infected cells prior to completion of the normal *C. trachomatis* developmental cycle.

Regardless of the pressure against the expression of a functional tryptophan synthase in trachoma, the strong selective pressure on urogenital isolates to retain the capacity to synthesize tryptophan via indole salvage is striking. It is likely this pressure reflects the influence of the urogenital microbiome on *C. trachomatis* replication in the face of a protective immune response.

THE VAGINAL MICROBIOME AND ITS EFFECTS

It is now well appreciated that the vaginal microbiome can significantly impact the reproductive health of women, their fetuses and their newborns (Hillier et al., 1995; Zhou et al., 2010; Ma et al., 2012). Bacterial vaginosis (BV), which affects 29% of reproductive age women in the US, is characterized by the loss of *Lactobacillus* species and a concomitant overgrowth of diverse anaerobes (Koumans et al., 2001, 2007). BV can be diagnosed by a gram stain-based scoring system termed the Nugent score and is calculated by assessing for the presence of large Gram-positive rods (chiefly *Lactobacillus* species), small Gram-variable rods and curved gram-variable rods; scores range from 1–10 and a Nugent of 7–10 is considered a diagnosis of BV and represents a sharp decrease in the number of Gram-positive rods with a simultaneous increase in the latter two morphotypes (Nugent et al., 1991; Delaney and Onderdonk, 2001). BV increases susceptibility to various STDs and PID (Haggerty et al., 2004) as well as the acquisition of HIV (Taha et al., 1998). Recent studies also indicate that the abnormal vaginal microbiome during BV affects the natural history of cervical human papillomavirus (HPV) and the development of cervical intraepithelial neoplasia (CIN) (Rodriguez-Cerdeira et al., 2012; Gao et al., 2013; Wheeler, 2013). Such observations indicate a dynamic relationship between the vaginal microbiome and the host; colonization by "normal" microbiomes may protect by preventing colonization by potential pathogens or by creating conditions that do not favor survival of the latter (Brotman et al., 2013). By disrupting this equilibrium, abnormal microbiomes create an environment that favors infection or colonization by various pathogens.

NORMAL AND BV-ASSOCIATED VAGINAL MICROBIOMES

Advances in high-throughput sequencing have recently permitted the development of culture-independent methods to determine the composition of vaginal microbial communities (Ravel et al., 2011). They have also permitted the evaluation of the effect of various perturbations such as antibiotics, contraceptives and sexual activity on this microbiome (Gajer et al., 2012; Brotman et al., 2013; Ravel et al., 2013). Further, the altered microbiomes present in clinically defined conditions such as BV have been characterized (Ravel et al., 2011, 2013; Datcu et al., 2013). Cross-sectional surveys using such culture-independent methods have revealed the existence of several types of vaginal communities in normal, healthy, women with distinct bacterial species compositions (Ravel et al., 2011; Ma et al., 2012; Brotman et al., 2013). Four of these communities are dominated by *Lactobacillus* species: Type I—*L. crispatus*; Type II—*L. gasseri*; Type III—*L. iners*; and Type V—*L. jensenii*. The fifth community type, labeled Type IV, is dominated by facultative and strict anaerobes combined with insignificant numbers of lactobacilli. The Type IV community type is most closely associated with BV, as defined by a Nugent score of 7–10. A study examining the cervical microbiome has largely recapitulated the vaginal findings (Smith et al., 2012).

EFFECTS OF THE VAGINAL MICROBIOME ON *C. TRACHOMATIS*

Several studies indicate the vaginal microbiome can influence *C. trachomatis* infection. For example, pregnant women with flora

in which H_2O_2-positive *Lactobacillus* spp. predominate are less likely to be infected by *C. trachomatis* (Hillier et al., 1992). In contrast, studies using an American cohort, aged 15–30, indicated a strong correlation between BV, as defined by Nugent scores ranging from 7–10, and *Chlamydia* infection (odds ratio 3.4) (Wiesenfeld et al., 2003). These results were recapitulated in studies examining a Japanese cohort of similarly aged women. The latter studies found an association between BV (NS 7–10) and *Chlamydia* infection with an odds ratio of 3.5 (Yoshimura et al., 2009).

Differences between the normal and BV microbiome could influence the normal development of *Chlamydia*, and the effect of IFNγ on normal development in multiple ways. First, we know that some members of the BV microbiome can express a functional tryptophanase to produce indole from tryptophan (Sasaki-Imamura et al., 2011), thus providing genital serovars of *C. trachomatis* with a means to obtain tryptophan via indole salvage. We also know from our recent studies that indole is present in the vaginal secretions of patients with BV (Lewis et al., 2014). Therefore, BV microbiome-produced indole could ameliorate the effect of IFNγ-induced tryptophan depletion on *Chlamydia* development. Indeed, tryptophan synthesis through indole salvage is likely to be desirable for *C. trachomatis* for several reasons. First, under hypoxic conditions, IDO1 can catabolize tryptophan but not indole. Second, IDO1-mediated catabolism depletes tryptophan levels within the chlamydial inclusion by decreasing extracellular and cytoplasmic tryptophan, and not by directly acting upon tryptophan within the inclusion. Third, mammalian cells lack a tryptophan synthase activity; therefore, tryptophan biosynthesis through indole salvage occurs solely within the chlamydial inclusion, providing only the bacterium with this essential amino acid with no competition from the host cell. Finally, because IDO1 catabolizes only extracellular and cytoplasmic tryptophan, it will not affect tryptophan synthesized within the inclusion.

The BV microbiome might also limit the effect of IFNγ on *C. trachomatis* by other mechanisms. The *Lactobacillus* sp. that predominate in vaginal microbiome types I, II, III, and V do not synthesize indole. In addition, they create a highly acidic H_2O_2-rich environment (pH < 4.2) that is also not conducive to chlamydial growth and development (Das et al., 2005; Haggerty et al., 2009). In contrast, anaerobes present during BV (vaginal microbiome type IV) raise the pH to >4.6 and simultaneously produce a hypoxic microenvironment. It is pertinent to note that the normal vaginal pH ranges from 3.8 to 4.5. Chlamydial re-infection is favored at higher pH, and hypoxia reduces the restrictive effect of IFNγ on chlamydial growth in two ways: (1) Low oxygen partial pressure conditions (pO_2) limit both IFNγ-dependent signaling pathways (Roth et al., 2010); and (2) The enzymatic capacity of IDO1 to catabolize tryptophan by dioxygenation is significantly hampered by low pO_2 (Herbert et al., 2011).

POSSIBLE SOURCES OF INDOLE IN THE GENITAL TRACT

Although the normal and BV microbiomes display variations between patients, there are several commonalities in their composition. The normal vagina (Nugent scores 0–3) contains $\sim 10^7$ bacteria/10 ng of DNA recovered from vaginal swabs, in which *Lactobacillus* spp. predominate. During BV (Nugent score 7–10), the bacterial load is increased to $\sim 10^9$ bacteria/10 ng DNA, in which combinations of *Prevotella* spp. ($>10^8$/10 ng), *Gardnerella vaginalis* (10^7/10 ng), *Atopobium vaginae* (10^5/10 ng), and/or *Megasphaera* spp. (10^7/10 ng) become abundant. A large increase in the fastidious bacterium of the order *Clostridiales* (BVAB1—10^8/10 ng) is also observed. *Lactobacillus* spp. do not produce indole, however many *Prevotella* spp. and strains can express a tryptophanase (*tnaA*) to produce indole (Sasaki-Imamura et al., 2011). Many sequenced members of the order *Clostridiales* also encode a tryptophanase gene. BV infections are occasionally coincident with infections by the protozoan pathogen *Trichomonas vaginalis* that can also express a tryptophanase to produce indole (Lloyd et al., 1991; Zubacova et al., 2011). Thus *T. vaginalis* co-infections may represent another mechanism by which vaginal co-infections impact the effect of IFNγ on *C. trachomatis*.

These results clearly indicate that indole-producing bacteria are present within patients, with the levels of indole increasing with higher Nugent scores. Several recent studies examining the BV microbiome indicate that the increased representation of *Prevotella* spp. is the strongest correlate of BV (NS 7–10) (Datcu et al., 2013, 2014). Consistent with these, a metagenomic study examining mRNA expressed by the BV microbiome indicated that *Prevotella* spp. mRNA accounted for approximately 30% of all the mRNA expressed by the BV microbiome (Twin et al., 2013). The high representation of *Prevotella* spp. mRNA, coupled with the observation that some oral strains of *Prevotella* can express a tryptophanase, led us to test whether genital isolates of *Prevotella* from BV patients could also synthesize indole. For this, residual speculum fluid from three BV patients was used to isolate anaerobes using laked blood agar kanamycin/vancomycin media. Kanamycin/vancomycin-resistant constitutive anaerobes were isolated from all three samples and tested for indole production. Multiple isolates from two patients produced indole robustly, while only a single isolate from the third patient did. Partial 16S rRNA sequences (GenBank accession numbers KJ435311—KJ435324) confirmed all but one of the isolates (indole producing and non-producing) to be *Prevotella* spp. These results indicate that indole-producing bacteria can be found in the microbiome from the female genital tract. Further, there are variations between patients, such that even within the same genus (i.e., *Prevotella*), genetic differences between isolates can alter indole availability in a patient-specific manner.

A GENERAL ROLE FOR INDOLE AVAILABILITY IN THE GENITAL TRACT

Genomic and functional analyses of other bacteria indicate that indole is available in the genital tract. For example, while environmental and nosocomial infection-associated isolates of *Staphylococcus aureus* retain the capacity to synthesize tryptophan *de novo* from chorismic acid, 80% of the Toxic Shock Syndrome Toxin (TSST) producing *S. aureus* isolates have lost this capacity, typically due to a deletion within the *trpD* gene (McGavin et al., 2012). However, these TSS-associated isolates continue to encode a functional tryptophan synthase, suggesting that indole and/or IGP is likely available within the genital microenvironment, permitting bacterial growth even during tryptophan starvation (*ibid*).

Similarly, several members of the normal and BV microbiome can synthesize tryptophan *de novo* from chorismic acid, others, such as *G. vaginalis*, resemble CT, in that they can only synthesize tryptophan by indole salvage. An analysis of the *Gardnerella vaginalis* reference genome (GenBank accession NC_013721) indicates it encodes a TrpB subunit that is 54% identical to the TrpB subunit from *Escherichia coli*, with no additional domains. However, no sequenced isolate of *G. vaginalis* encodes a TrpA subunit, implying that akin to CT, *Gardnerella* can use indole, but not IGP, in the environment to synthesize tryptophan. Therefore, it is likely that during tryptophan starvation, *C. trachomatis* and *Gardnerella* both rely on indole produced by tryptophan autotrophs that also encode a tryptophanase. For this reason, the development and use of small molecule therapeutics that target tryptophanase will not only promote IFNγ-mediated *C. trachomatis* clearance, but also aid in the clearance of indole-dependent BV bacteria such as *G. vaginalis*.

CONCLUDING REMARKS

In this review, we have described the mechanism by which IFNγ could act as a protective cytokine against chlamydial infections. IFNγ's protective effects result from catabolism of the essential amino-acid tryptophan by the enzyme IDO1. Depletion of tryptophan can induce a viable but not cultivable persistent growth phenotype in *Chlamydia* that can be reactivated when the IFNγ-response wanes and/or tryptophan is made available in the environment. Consistent with this, IFNγ exposure induces a chlamydial pattern of gene expression that causes an up-regulation of genes that can synthesize tryptophan through indole salvage and a down-regulation of genes necessary for later stages of the chlamydial normal development cycle. Genomic analyses indicate that a selective pressure to maintain tryptophan synthesis via indole salvage has been applied strictly to every genital *Chlamydia* serovar. In contrast, no ocular serovars can salvage indole. Given that the genes necessary for indole salvage (*trp*BA) are present in a genetic plasticity zone, it is likely that indole availability in the infection microenvironment has been the selective factor to maintain the capacity to salvage indole. Consistent with this, we have found indole in vaginal secretions from patients that have BV, and have isolated indole-producing bacteria from patients that have BV. Therefore, it is likely that natural immunity against chlamydial infections driven by a protective IFNγ response will be attenuated in patients that have BV, dependent on the bacterial representation within individual patients. For these reasons, further studies that examine the correlates between spontaneous clearance of chlamydial infections, the host cytokine response, the host microbiome, and metabolites such as indole, are essential for the successful development of a protective vaccine against *Chlamydia*. Further, understanding the nature and contribution of BV-associated bacteria and their indole-producing capacity and how this relates to the effectiveness of an IFNγ-mediated resolution of *C. trachomatis* infection may also guide the management of BV in *C. trachomatis*-infected patients in the future.

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Morphologic and molecular evaluation of *Chlamydia trachomatis* growth in human endocervix reveals distinct growth patterns

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In vitro models of *Chlamydia trachomatis* growth have long been studied to predict growth *in vivo*. Alternative or persistent growth modes *in vitro* have been shown to occur under the influence of numerous stressors but have not been studied *in vivo*. Here, we report the development of methods for sampling human infections from the endocervix in a manner that permits a multifaceted analysis of the bacteria, host and the endocervical environment. Our approach permits evaluating total bacterial load, transcriptional patterns, morphology by immunofluorescence and electron microscopy, and levels of cytokines and nutrients in the infection microenvironment. By applying this approach to two pilot patients with disparate infections, we have determined that their contrasting growth patterns correlate with strikingly distinct transcriptional biomarkers, and are associated with differences in local levels of IFNγ. Our multifaceted approach will be useful to dissect infections in the human host and be useful in identifying patients at risk for chronic disease. Importantly, the molecular and morphological analyses described here indicate that persistent growth forms can be isolated from the human endocervix when the infection microenvironment resembles the *in vitro* model of IFNγ-induced persistence.

Keywords: bacterial persistence, *Chlamydia trachomatis*, endocervix, human, interferon gamma, indole

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterium and serovars D-K are tropic for the columnar and transitional epithelial cells of the genital tract. Chlamydial infections in women are generally asymptomatic and therefore often go undetected and untreated (Brunham and Rey-Ladino, 2005). Natural history studies indicate untreated infections can be asymptomatic for substantial periods of time, can spontaneously resolve or can progress to cause complications (Parks et al., 1997; Golden et al., 2000; Joyner et al., 2002; Morre et al., 2002; Molano et al., 2005; Geisler et al., 2008). Infection most commonly occurs in the endocervix and can result in cervicitis. If bacteria ascend into the endometrium and Fallopian tubes chronic infection can lead to pelvic inflammatory disease (PID). Approximately 11% of women with PID will subsequently develop tubal factor infertility, but, as many of these infections are also clinically silent, they also remain undiscovered until reproductive consequences ensue (Cohen and Brunham, 1999).

Why so many chlamydial infections are so extended in their duration is not well understood, but does indicate the organism is capable of adapting to, or evading, specific immune and environmental conditions (Brunham and Rey-Ladino, 2005). One strategy documented *in vitro* for immune evasion or adaptation in the human host is the ability of *C. trachomatis* to enter into a persistent growth form (Beatty et al., 1994b; Belland et al., 2003a). This bacterial form is viable but non-cultivable and results in an extended relationship between the pathogen and its host cell (*ibid*). Compelling, but thus far indirect evidence, for this alternative mode of growth *in vivo* includes documentation of recurrent disease when re-infection is unlikely, and the detection of chlamydial antigen or nucleic acid in the absence of cultivability (Nagasaki, 1987; Patton et al., 1994; Dean et al., 2000).

Classic *in vitro* studies have shown *C. trachomatis* has a unique developmental cycle that normally alternates between an infectious elementary body (EB) and a non-infectious reticulate body (RB) (Abdelrahman and Belland, 2005). EBs attach to, and invade, susceptible cells where they are internalized in membrane bound vacuoles termed inclusions (*ibid*). EBs, now known to possess some metabolic activity (Omsland et al., 2012), then differentiate into highly metabolically active RBs, undergo repeated cycles of binary fission and then differentiate back to EBs whence they are released from the host cell by lysis or extrusion to infect neighboring cells (Abdelrahman and Belland, 2005;

Hybiske and Stephens, 2007). The entire developmental cycle may take 30–48 h, dependent on the serovar, with temporally distinct patterns of gene expression categorized as early, mid-cycle and late that correlate with *C. trachomatis* growth stages (Belland et al., 2003b). Stressful growth conditions that are also likely to be encountered *in vivo* can induce an alternate, persistent growth mode *in vitro* (Wyrick, 2010). These stressors include, nutrient and iron deprivation (Raulston, 1997; Igietseme et al., 1998), specific antibiotics (Matsumoto and Manire, 1970; Clark et al., 1982), co-infection with herpes simplex virus (HSV) (Vanover et al., 2008), exposure of infected cells to the danger signal adenosine (Pettengill et al., 2009), and interferon gamma (IFNγ) (Beatty et al., 1993), the latter of which, under optimal conditions, is believed to be a key immune mediator in resolution of, and subsequent protection from, infection(Rank and Whittum-Hudson, 2010; Aiyar et al., 2014). Persistent bacterial forms, induced by IFNγ, are morphologically characterized as large, atypical, or aberrant RBs in which binary fission appears to be arrested (Byrne et al., 1986; Beatty et al., 1993, 1994b; Wyrick, 2010). Molecularly, gene expression profiles associated with persistent forms are consistent with RBs blocked in binary fission and arrest of the developmental cycle at the stage just preceding late gene expression (Belland et al., 2003a). Removal of IFNγ generally reverses these changes such that aberrant RB re-enter the developmental cycle and differentiate into infectious EBs. Well-characterized *in vitro* models indicate that IFNγ acts against *C. trachomatis* via nutrient deprivation (Beatty et al., 1993). Specifically, IFNγ induces the tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO1), thereby depriving *C. trachomatis*, a tryptophan auxotroph, of this essential amino-acid (Byrne et al., 1986; Taylor and Feng, 1991; Beatty et al., 1994a,b). Depending on the concentration and duration of exposure to IFNγ, and consequently the environmental tryptophan levels, *C. trachomatis* either enters into a persistent state of growth, or can be eradicated (Byrne et al., 1989). Importantly, genital serovars of *C. trachomatis* can uniquely synthesize tryptophan through indole salvage (Fehlner-Gardiner et al., 2002), suggesting that exogenous sources of indole, likely microbial-derived in the natural environment, may extend or permit the survival of *C. trachomatis* in the presence of IFNγ (Fehlner-Gardiner et al., 2002; Caldwell et al., 2003; Aiyar et al., 2014).

While *in vitro* models have proven very insightful in elucidating chlamydial growth modes under highly controlled conditions, there have been no definitive studies that directly establish whether persistent growth forms as described above are an *in vivo* survival mechanism for *C. trachomatis* (Wyrick, 2010). In fact, there is a paucity of information describing *C. trachomatis* growth in the human genital tract milieu, the composition of this milieu, how endogenous and exogenous co-factors alter the composition, and the resultant effects on *C. trachomatis*. Variability in these co-factors may be critical in determining whether *C. trachomatis* survives or is eradicated by host immune responses. Addressing this gap in our knowledge will likely reveal the mechanisms by which *C. trachomatis* maintains *in vivo* reservoirs of infection. It will also provide crucial normative data to aid in the design of diagnostics, vaccines, and adjunct therapies that could classify, target, and eliminate human *C. trachomatis* infections. Therefore, the objective of the study described here was to develop methodology to harvest, preserve, and analyze cells and secretions from the human endocervix that would permit parallel molecular and morphological analyses of *C. trachomatis*, along with analyses of the immune and environmental milieu in which it survives. Using this novel multifaceted approach, we were able to identify highly contrasting patterns of bacterial growth, and associated cervicovaginal environment factors, in two pilot patients. These preliminary results indicate that this approach may pave the way to the establishment of a biomarker panel suitable for assessing chlamydial growth patterns and disease outcomes of *C. trachomatis* infections in women. Importantly, the contrasting molecular and morphological characteristics observed in these two patients provide the first evidence for the existence of persistent growth forms in the human genital tract.

MATERIALS AND METHODS

STUDY POPULATION AND CLINIC PROCEDURES

Institutional Review Board approval for this study was obtained from LSU Health Sciences Center. Women aged 18–28 years and attending the Delgado STD Clinic were asked to participate and then enrolled in this study if they had a high probability of chlamydial infection based on the following criteria: a recent positive NAAT screening test for *C. trachomatis*; recent sexual contact with a male suspected of chlamydial infection; or clinical evidence of cervicitis. Exclusion criteria were as follows: pregnancy; underlying chronic disease; use of steroids or antibiotics within the last 2 weeks; sexual intercourse within the last 12 h; current menstrual bleeding; documented infection with human immunodeficiency virus; or a history of genital herpes. Samples were excluded from the analyses if women were *C. trachomatis* NAAT or culture negative at the enrollment visit. All *Chlamydia trachomatis*-infected women were treated with azithromycin at this treatment/enrollment visit. Two groups of women were sequentially enrolled into the study; variations in the cytobrush and/or endocervical culture swab collection fluid or processing was the only difference in the two groups.

SAMPLE COLLECTION

Pelvic samples were taken in the following order: (i) vaginal swab for a wet mount and a Gram stain preparation; (ii) vaginal swab placed in an InPouch (BioMed Diagnostics Inc.) for *Trichomonas vaginalis* culture; (iii) two sequential cervical cytobrush samplings, each one as a gentle 360° sweep of the cervical os, immersed in 1.5 ml collection fluid; (iv) endocervical swab immersed in endocervical transport medium (Ficarra et al., 2008) in a one dram vial with glass beads for *C. trachomatis* culture and genotyping; and (v) an endocervical swab for NAAT testing (*ibid*). In study 1 (the inclusion identification study), cytobrushes were immediately placed in a commercial transport fluid for liquid-based Papanicalou (PAP) testing (SurePath by BD Diagnostics or CytoLyt by Hologic). In study 2 (the ultrastructure and growth biomarker study), three changes were made to the protocol as follows: (1) cytobrushes were immediately immersed in a modified Electron Microscopy (EM) buffer (4% paraformeldehyde-1% glutaraldehyde); (2) the endocervical swab in endocervical

transport medium from patients in study 2 was immediately vortexed in clinic and 25% of the sample was removed and placed in an equal volume of MasterPure tissue and cell lysis solution (Epicentre, Illumina); and (3) Merocel ophthalmic sponges (Medtronic Xomed Inc.) were placed in the posterior fornix of the vagina for 2 min to absorb secretions (Kozlowski et al., 2000). Sponges were immediately placed in cryovials and transported to the laboratory on ice, after which they were stored at -80°C until vaginal fluid was extracted.

STD DIAGNOSTICS

Endocervical swab specimens were used to determine the presence of *C. trachomatis* and *N. gonorrhoeae* using the Aptima Combo 2 test as instructed by the manufacturer (Genprobe). A vaginal wet preparation was made in the clinic, and bacterial vaginosis (BV) was later diagnosed by Gram stain, with a Nugent's score of $\geq$7 being positive (Nugent et al., 1991). InPouch culture for *T. vaginalis* screening was read at baseline, 48 and 72 h (Ficarra et al., 2008). Swab specimens in endocervical medium were immediately frozen until they were processed for semi-quantitative culture for inclusion forming units (IFU) and for genotyping of *C. trachomatis*, as previously described (*ibid*).

CYTOBRUSH PROCESSING

Cytobrushes from study 1 were centrifuged, resuspended in 500 µl PBS, and concentrated endocervical cell preparations were dropped into PAP-pen circumferenced circles on glass slides, dried, fixed in 90% methanol for 10 min and stored at −20°C until immunofluoresecent analyses (IFA) were performed. Cytobrushes in modified EM buffer were vortexed, washed twice, and resuspended in PBS, then cells were enumerated. Twenty percent of the sample was used to make slides as described above, and the remainder was further processed for EM analysis if inclusions were noted on an IFA screen.

IMMUNOFLUORESCENT STAINING, COUNTING OF INCLUSIONS, AND DECONVOLUTION MICROSCOPY

Cells on slides were rehydrated in PBS and incubated overnight at 4°C with a blocking agent (Background Sniper, Biocare Medical). Between 50 and 100% of each sample was used to count inclusions in the inclusion identification study, depending on the number of cells retrieved from the patient, and 100% was used in the EM/Biomarker study. An anti-chlamydial LPS antibody conjugated to fluorescein isothiocyanate (FITC) and which contains Evans Blue as a counterstain (Merifluor, Meridian) was used to visualize *C. trachomatis* forms; 4',6 diamidino-2-phenylindole (DAPI) was also applied to visualize nucleic acid (Molecular Probes). Slides were cover-slipped with Prolong Gold antifade reagent (Invitrogen) prior to the examination of inclusions. In samples with larger numbers of cells, we also investigated the expression of the chlamydial proteins OmcA and CT223. In brief, following rehydration and blocking, samples were incubated with Image-iT R FX Signal Enhancer followed by a mouse monoclonal antibody to OmcA (1:750, B12K, a kind gift of Dr. Li Shen) (Zhang et al., 1987) or a rabbit polyclonal anti-CT223 at (1:500, CT223 186, a kind gift of Dr. Dan Rockey) (Alzhanov et al., 2009). Samples were incubated with a secondary antibody (Alexa568 conjugated anti-mouse antibody or Alexa 594 conjugated anti-rabbit antibody, 1:500, Molecular Probes) followed by an anti-chlamydial LPS-FITC antibody (1:20, YVS 1683, Accurate). Dual stained slides were finally counterstained and cover-slipped as described above. All images were obtained with a Leica DMRXA automated upright epifluorescent microscope (Leica Microsystems); a Sensicam QE CCD (Cooke Corporation); and filter sets optimized for Alexa 488 (exciter HQ480/20, dichroic Q495LP, and emitter HQ510/20m), Alexa 568/594 (exciter HQ560/55x, dichroic Q595LP, and emitter HQ645/75m) and DAPI (exciter 360/40x, dichroic 400DCLP, and emitter GG420LP). Images were captured with a 63X objective (NA = 1.42) and a 2× zoom. Z-axis plane capture, deconvolution, and analysis were performed with Slidebook™ Deconvolution Software (Intelligent Imaging Innovations, Denver, CO).

ULTRASTRUCTURAL ANALYSES

Cervical cell samples were post-fixed in 1% osmium tetroxide (Polysciences Inc.) for 1 h as previously described (Belland et al., 2003a). Samples were then rinsed extensively in deionized water (dH_20) prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc.) for 1 h. Following several rinses in dH_20, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Thick sections (200–300 nm) obtained with a Leica Ultracut UCT ultramicrotome were stained with toluidine blue for initial screening of tissue for inclusions at the light microscopy level. Ultrathin sections of 95 nm were stained with uranyl acetate and lead citrate for detailed ultrastructural analyses of bacteria and inclusions on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc.). The bacterial forms in identified inclusions were subsequently evaluated using the scientific image processing and analysis program, Fiji (Schindelin et al., 2012), as described here. After appropriately setting the scale, the freehand selection tool was used to create a rough outline of each inclusion. Image thresholding was used to remove background pixel density within the inclusion. The particle analysis function was used to enumerate the number of EBs per inclusion. Particles that were greater than 0.05 μm^2 with a circularity between 0.45 and 1.0 were enumerated. These parameters identified immature EBs with an electron-dense core, electron dense mature EBs, circular RBs, RBs undergoing binary fission, and atypical RBs that were larger in size and displayed multiple septum formation. For those inclusions in which the background density did not permit a single threshold to be applied, an alternative procedure was used. Image thresholding was applied to the outlined inclusions to reveal immature and mature EBs that were sized for their areas. The following procedure was used to enumerate and size the RBs and atypical RBs. First, the thresholded image containing mature/immature EBs was used to create a mask image in which all the EBs were outlined. This mask was subtracted from the original micrograph to remove all EBs. A new image threshold was now applied to subtract the background and enumerate and size RBs and atypical RBs.

qPCR FOR *C. TRACHOMATIS* GENES AND GENOME COPY NUMBER QUANTITATION

Endocervical swab samples in MasterPure lysis solution (Epicenter, Illumina) were processed for total nucleic acid

according to the manufacturers instructions and stored at −80°C until use. For each patient, 30% of each sample was used to determine *C. trachomatis* genome copy number. RNA was removed by digestion with RNaseA, the DNA was re-purified by isopropanol precipitation and samples (150 ng/well) were then analyzed by Taqman™ qPCR in triplicate, including a no-template control, using an Applied Biosystems PRISM 7700 Sequence Detection System. The remaining total nucleic acid was treated with DNase and re-precipitated. Each of the samples was subjected to qRT-PCR using Taqman primer/probe sets specific for the mRNAs encoded by the genes *euo* and *omcB*. Equivalent amounts of RNA (20 ng) were used to measure expression levels in triplicate and a no-RT control was included for each primer/probe set. Normalization was performed using *C. trachomatis* genome copy numbers, as previously described (Ibana et al., 2011).

DETECTION OF IFNγ AND INDOLES IN VAGINAL SECRETIONS

Vaginal fluid was eluted from Merocel sponges in a spin assembly apparatus, and secretion volumes and dilution factors were calculated as previously described for Weck-Cel sponges (Kozlowski et al., 2000). Total protein was assayed using a Pierce BCA protein assay kit (Thermo Fisher Scientific). IFNγ was quantified by a cytometric bead array assay (MILLIPLEX MAP Immunology Multiplex Assay). Millipore, Billerica, MA) as previously described (Buckner et al., 2011, 2013). Total indoles were quantified using Salkowski's test (Salkowski, 1885), modified as described by Szkop et al. (2012). In brief, an equimolar mixture of multiple indoles (Sigma-Aldrich, Inc., St. Louis, MO, USA) was used to generate a standard curve by measuring absorbance at 530 nm after incubation with Salkowski's reagent. Indoles that can be detected by this test include indole, 3-methyl indole, indolic acids, and indolic alcohols. Samples were processed in parallel, following which the standard curve was used to determine the total concentration of indoles in the sample. Concentrations were corrected for the sample dilution factor.

RESULTS

STUDY POPULATION AND CHARACTERISTICS OF ENDOCERVICAL INFECTION

Seventy-five women were recruited into the study and samples from 37 women were included in the final analyses; 29 women were excluded because they were *C. trachomatis* NAAT and/or culture negative, and 9 were excluded because samples were insufficient or could not be processed in a timely fashion. The median age of the women with samples included in the study was 22 years (range 18–30 years). Fourteen of the women had mucopurulent cervicitis (MPC) (38%). Semi-quantitative culture of *C. trachomatis* indicated infectious burden in the endocervix varied considerably, with IFU per endocervical swab ranging from 7 to 336,168 IFU (median 4802). None of the women were positive for *Neisseria gonorrhoeae,* but 3 (8.1%) were co-infected with *T. vaginalis*. Twenty-one women (56.8%) were diagnosed with BV (Nugent score 7–10) and 7 (18.9%) had an intermediate (4–6) Nugent score (Nugent et al., 1991).

IDENTIFICATION OF INTACT CHLAMYDIAL INCLUSIONS IN EPITHELIAL CELLS HARVESTED FROM CERVICAL CYTOBRUSH SPECIMENS

We previously described the utility of cytobrushes for the non-invasive and longitudinal retrieval of cervical lymphocytes (Ficarra et al., 2008). Here, we established that cytobrush sampling can be used to harvest and enumerate *C. trachomatis*-infected cervical epithelial cells. The original protocol was modified such that cytobrushes were immersed immediately upon collection into liquid-based Pap test collection fluid. Bright-field microscopy revealed epithelial cells in these samples retained their characteristic morphology, permitting the detection of chlamydial antigen and nucleic acid by fluorescence. DAPI staining for DNA and immunofluorescent staining for chlamydial LPS detected inclusions in 79.2% of samples (**Figures 1A–C**). The median number of cells with inclusions in positive samples was 11 (range 2–50), with no correlation observed between inclusion number and IFU ($R^2 = 0.08$). Clusters of multiple infected cells were often observed, and neutrophils frequently appeared to be associated with the infected cells (**Figure 2B**). However, we note that samples always included infected and uninfected "bystander" cells (**Figures 1C**, **2B**). In samples that had larger numbers of cells, dual staining was performed using antibodies recognizing chlamydial LPS and OmcA, a late cycle-expressed protein, or chlamydial LPS with CT223, an inclusion membrane protein. We observed that our results paralleled *in vitro* studies, with only EB-size forms staining for OmcA (**Figure 2A**) and CT223 irregularly distributed in a patchy "dash-like" distribution at the inclusion surface (**Figures 2B,C**) (Alzhanov et al., 2009).

ULTRASTRUCTURAL ANALYSES OF CERVICAL INCLUSIONS PROVIDE EVIDENCE FOR ABERRANT FORMS

The finding that immunofluorescence could be used to screen for inclusion frequency in cervical samples encouraged us to next develop a protocol that permitted a detailed ultrastructural and molecular examination of these bacterial forms, along with molecular studies of the cervicovaginal milieu. There were three modifications of the sample collection protocol for the 13 women enrolled into this component of the study. First, cytobrushes were collected directly into a modified EM fixative; the formulation of this fixative maintained the antigenic integrity of chlamydial LPS and allowed visualization by standard IFA in samples processed within 6 h of collection. This enabled us to screen samples by IFA prior to processing for EM. Second, 25% of the endocervical swab sample was immediately stabilized in MasterPure buffer in the clinic. This enabled parallel analysis of chlamydial DNA and RNA from the same sample used for determination of the IFU count. Third, vaginal fluid was collected using an ophthalmic sponge for quantification of IFNγ and indoles in these secretions.

A fraction (20%) of each cytobrush sample was screened for the presence of inclusions by immunofluorescence using an anti-chlamydial LPS antibody. Inclusions were detected by this method in 5 of 13 samples, two of which had multiple (>10) inclusions and were hence selected for further analyses. The remaining 80% of the cytobrush for each of these two samples were processed for EM and scanned for inclusions. Using this protocol, we were able to identify and examine three inclusions in Patient 1 and five inclusions in Patient 2. There was a

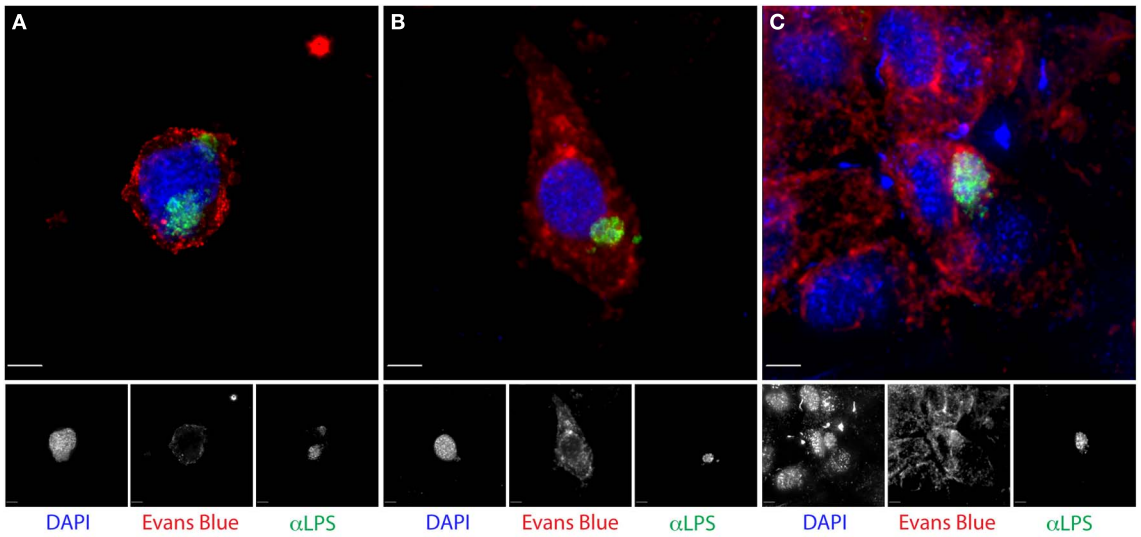

FIGURE 1 | Identification of chlamydial inclusions in endocervical cells retrieved by cytobrush from *C. trachomatis*-infected women. Cytobrush specimens immediately placed in **(A)** Surepath or **(B,C)** Cytolite were processed as described in the methods, stained with anti-chlamydial LPS-FITC (green), Evans blue (red) and DAPI (blue) and visualized by fluorescent deconvolution microscopy. Note the morphology and staining pattern in **(A)** suggests a single inclusion that is wrapped around the nucleus. Scale bar is 5 μm.

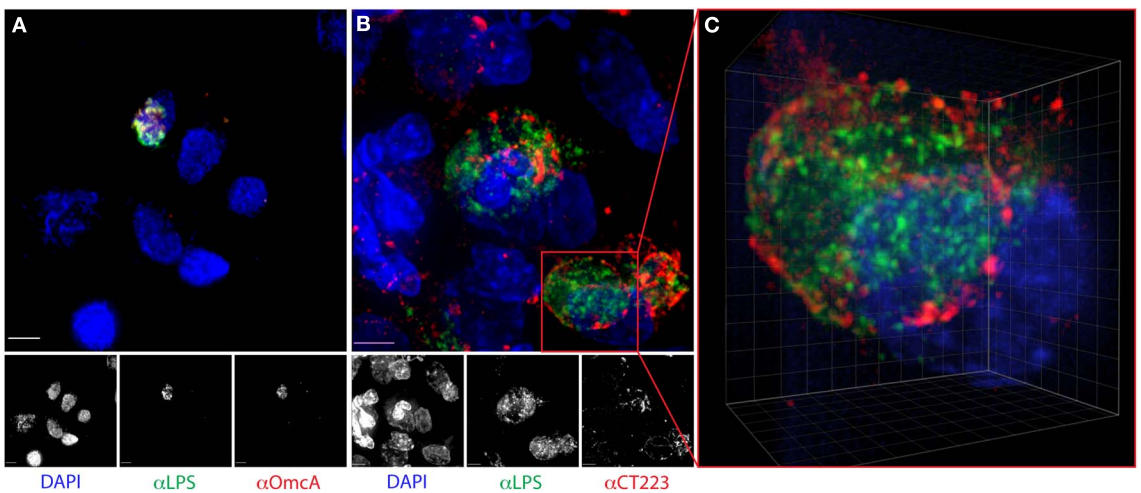

FIGURE 2 | Distribution of chlamydial LPS, OmcA, and CT223 in *C. trachomatis*-infected epithelial cells harvested from human endocervix. Cervical cells were dual-labeled with anti-chlamydial LPS (green) and **(A)** OmcA (red) or **(B)** CT223 (red), counterstained with DAPI (blue) and visualized by fluorescence deconvolution microscopy, as described in the methods. Colocalization of antigen is visualized in yellow. Scale bar is 5 μm. Image **(C)** is a 3-dimensional maximum intensity projection extracted from a deconvolved stack of an infected cell in image **(B)**. Note the irregular "dashed line" membranous staining of CT223. Grid is 1 μm.

striking difference in the morphology of bacterial forms in the inclusions from these two subjects. Two EM images from Patient 1 are shown in **Figures 3A,B**. The morphological characteristics in the third inclusion were similar to these two inclusions. Each image displays the inclusion cross-sectional area, within regions indicated by the green arrowheads magnified. A graph indicating the distribution of particle sizes (as area) is also shown. Inclusions from Patient 1 had an area of approximately 13–14 μm^2, with 25–40 "particles" within them. The average area for each particle was approximately 0.15 μm^2. Particles were sized by area rather than diameter because some of them were shaped irregularly. The area of an EB (diameter 0.2–0.3 μm) is anticipated to be in 0.07–0.12 μm^2. The area within an RB (diameter 0.7–1 μm) is expected to be about 0.4–0.8 μm^2 (Ward, 1983; Wyrick, 2010). Particle analyses indicated that the particles within inclusions from Patient 1 were skewed toward the area of an EB. The morphology of the particles within these inclusions is indicated in the particles highlighted by the green

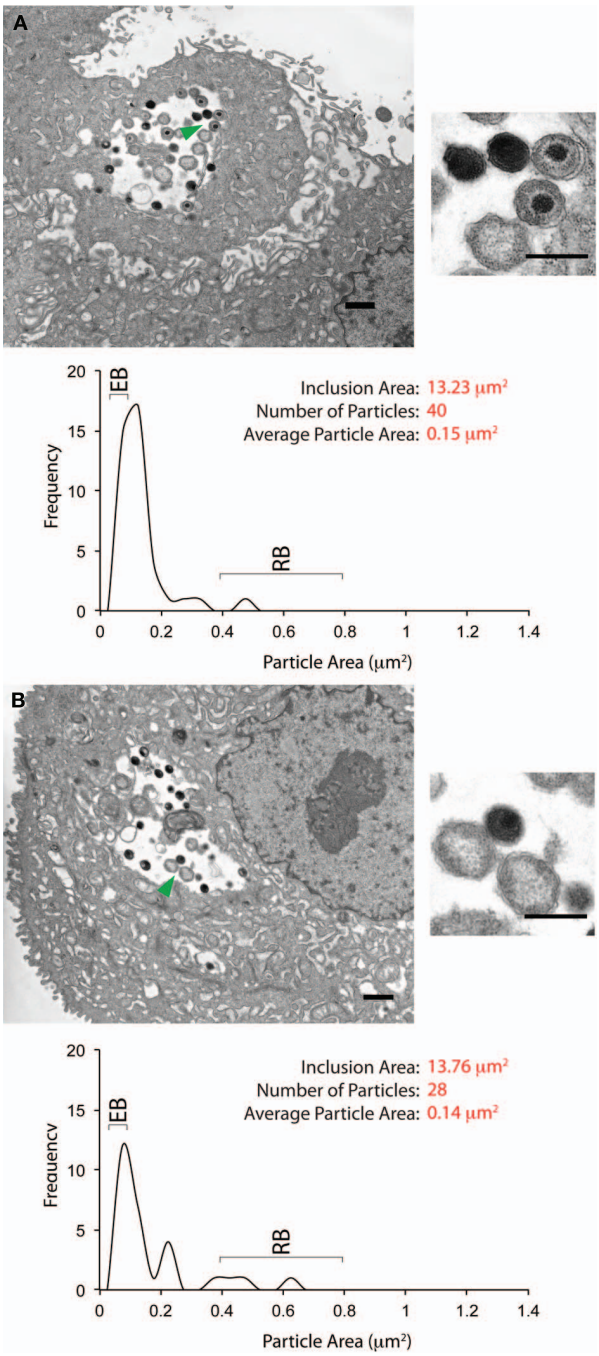

FIGURE 3 | Ultrastructural images of inclusions from the cervix of a single *C. trachomatis* patient. (A) An inclusion-containing electron micrograph from Patient 1, processed and analyzed as described in the Materials and Methods section. The entire micrograph is shown in the large image on the left, with the scale bar indicating a distance of 1 μm. The smaller image on the right is a magnified view of the features indicated by the green arrow. For this image, the scale bar indicates a distance of 0.5 μm. Mature and immature EBs can be observed in the magnified view. Quantification of the inclusion and its contents is indicated below the image. The inclusion area was 13.23 μm^2. Forty EBs, RBs, and atypical RBs were counted as particles within the inclusion, with an average particle area of 0.14 μm^2. The plot indicates the frequency distribution of particles sized *(Continued)*

FIGURE 3 | Continued
by their cross-sectional areas. The anticipated cross-sectional areas for EBs and RBs are indicated in the frequency plot. The plot indicates the frequency distribution of particles sized by their areas. **(B)** A second inclusion-containing electron micrograph from Patient 1, processed and analyzed as described above. The smaller image on the right indicates a magnified view of the area highlighted by the green arrowhead on the larger image. Two EBs and two RBs can be observed in the magnified view. Quantification of inclusion area, particle area, and particle area frequency distribution indicates the characteristics of this inclusion to be very similar to those in **(A)**.

arrowheads. Electron dense EBs, immature EBs, and RBs were observed.

EM images from five unique inclusions were obtained from Patient 2. The entire inclusion cross-sectional area was not obtained for the first inclusion by EM, preventing calculation of all parameters used for the other inclusions. EB morphotypes predominated in this inclusion (**Supplementary Figure 1**). The second inclusion is shown in **Figure 4A**. The inclusion membrane was difficult to visualize for this inclusion; therefore inclusion area was calculated by subtracting a mask created using cytoplasmic/nuclear pixel density. The area of this inclusion, 26 mm^2, was larger than the areas for inclusions from Patient 1. Particles within this inclusion displayed the morphological characteristics of EBs, immature EBs, and RBs. Some atypical/abnormal RBs (ABs) displaying unequal binary fission were also observed. The average particle area within this inclusion was 0.21 μm^2, reflecting a larger number of RBs than the inclusions shown in **Figure 3**. The other three inclusions from this patient (**Figures 4B–D**) displayed similar characteristics to each other. They contained a large number of RBs/ABs, with very few EBs, reflected in an average particle area of 0.68 μm^2. The green arrowhead in **Figure 4B** indicates particles with RB and EB morphology. While many RBs displayed equal binary fission, some abnormal binary fission events were also observed. The micrograph shown in **Figure 4C** contains three inclusions indicated by the large black arrowheads, two of which are within the same cell. The third inclusion may also be within that cell. While inclusion areas for the first two inclusions were determined by automatic thresholding, the area for the third inclusion was determined by manually outlining the inclusion. The largest inclusion contained EBs, RBs, and several ABs, as evaluated by particle area. No EB sized particles were observed in the two smaller inclusions. The average area for particles within these three inclusions was skewed toward RBs. As classified by area, several atypical RBs were observed in all three inclusions. The fifth inclusion from this patient is shown in **Figure 4D**. The morphological characteristics of this inclusion resemble those observed for the inclusions shown in **Figures 4B,C**. This inclusion contained a few EB-sized particles (green arrowhead), although these particles did not have the electron density of mature EBs, or the electron dense core observed in immature EBs. Multiple RBs, as exemplified by the red arrowhead were also observed. The average particle area was skewed toward RBs (0.58 μm^2), and several ABs, as defined by large area and unequal binary fission events were observed. While this analysis was performed with limited numbers of inclusions, we note there is a statistically significant

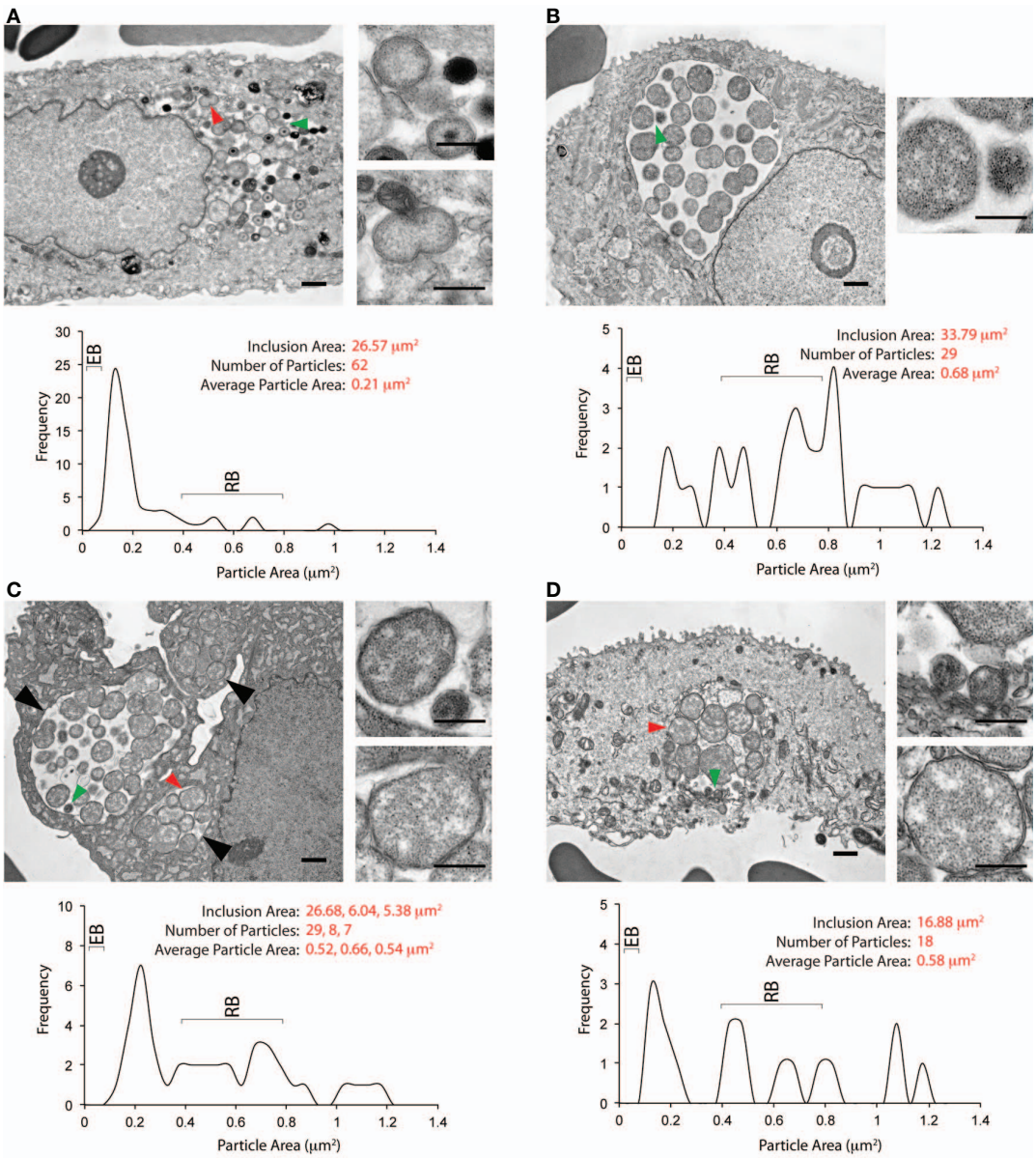

FIGURE 4 | Ultrastructural images from the cervix of a second *C.trachomatis* infected patient. (A) An inclusion-containing electron micrograph from Patient 2, processed and analyzed as described for **Figure 3**. The entire micrograph is shown in the large image on the left, with the scale bar indicating a distance of 1 μm. The smaller images on the right are magnified views of the features indicated by the green (upper image) and red (lower image) arrows. For these images, the scale bar indicates a distance of 0.5 μm. The area indicated by the green arrowhead contains an EB, and an RB. The area indicated by the red arrowhead contains an RB undergoing binary fission. Quantification of the inclusion and its contents is shown below the images. The inclusion area was 26.57 μm^2. Sixty-two EBs, RBs, and atypical RBs were counted as particles within the inclusion, with an average particle area of 0.21 μm^2. The plot indicates the frequency distribution of particles sized by their cross-sectional areas. The anticipated cross-sectional areas for EBs and RBs are indicated in the frequency plot. **(B)** A second inclusion-containing electron micrograph from Patient 2. The smaller image on the right indicates a magnified view of the area highlighted by the green arrowhead on the larger image. A RB and an EB-sized particle can be observed in the magnified view. Quantification of inclusion area, particle area, and particle area frequency distribution indicates the characteristics of this inclusion to differ significantly the inclusion shown in **(A)** or **Figure 3**. Very few particles with the area of an EB were observed. The average area, 0.68 μm^2, was skewed toward the anticipated area of a RB, with several larger particles also observed. **(C)** A third inclusion-containing electron micrograph from Patient 2. Three inclusions were observed in this micrograph, indicated by the large black arrows, at least two of which are within the same cell. The largest inclusion contained particles with the characteristic area of RBs and EBs, as highlighted by the green arrow, and displayed in the upper image on the right. The two smaller images contained particles with areas corresponding to those of RBs, as highlighted by the red arrow, and displayed in the lower image on the right. Quantification of the three inclusions is indicated below. The largest inclusion had an area of 26.68 μm^2, whereas the smaller inclusions had areas of 5–6 μm^2. The average area of particles within these three inclusions was similar to that observed in **(B)**, and distinct from the observations in **(A)**. **(D)** A fourth inclusion-containing micrograph from Patient 2. The characteristics of this inclusion are close to those observed for the inclusions in **(B,C)**. The green arrowhead indicates particles with the area of EBs (upper magnified image on the right), while the red arrowhead indicates a particle with the area of an RB (lower magnified image on the right). Similar to the inclusions seen in **(B,C)**, the particles within this inclusion were skewed toward the size of RBs, with an average area of 0.58 μm^2.

difference in intra-inclusion particle size between Patients 1 and 2 (**Supplementary Figure 2**). The mixture of a few EBs, RBs, and ABs observed in **Figures 4B–D** are reminiscent of inclusion morphology observed *in vitro* when infected cells are exposed to IFNγ. They also differ strikingly from inclusions observed after penicillin exposure, which contain a single large RB (Skilton et al., 2009), or after adenosine exposure, the latter being largely empty with a few EBs (Pettengill et al., 2009).

Interestingly, particles with greater than EB-sized cross-sectional areas observed within inclusions from Patient 2 appear to display two types of binary fission characteristics (**Figure 5**). Some of them (**Figures 5A–C**) displayed roughly equal binary fission. However, several others (**Figures 5D–F**) displayed apparently unequal binary fission. Some fission events with apparently multi-septated ABs were also observed (**Figures 5G,H**). Because a decreased expression of chlamydial genes required for cytokinesis is observed during IFNγ -induced persistence *in vitro* (Byrne et al., 2001; Belland et al., 2003a), it is possible that the latter events may result from the effects of IFNγ.

PARALLEL MOLECULAR ANALYSIS OF CERVICAL SPECIMENS WITH ULTRASTRUCTURAL DATA IDENTIFIES A PATIENT WITH A BACTERIAL PROFILE CONSISTENT WITH PERSISTENT GROWTH FORMS

A substantial body of indirect evidence supports the possibility that *C. trachomatis* adapts, and survives long-term, during human infection (Hogan et al., 2004; Brunham and Rey-Ladino, 2005; Wyrick, 2010). However, direct proof that the bacterium can enter into a persistent mode of growth in human infections requires morphologic evidence of aberrant forms to be substantiated by molecular evaluation of bacterial growth patterns to determine if the latter coincide with patterns observed *in vitro*. As our second study protocol was designed to harvest, and immediately preserve, parallel *ex vivo* endocervical samples for EM, nucleic acid, and infectious particle (IFU) analyses, we next examined multiple parameters of chlamydial growth in the 2 patients for whom we had ultrastructural inclusion data. First, we observed the two patients greatly contrasted in their IFU burden; Patient 1 had an IFU of greater than 336,168 and Patient 2 an IFU of 67 (**Table 1**). The median IFU in this patient population was 4802; thus, these two patients represent cases at the very highest, and at the lower end, of the spectrum of young women with NAAT and culture-positive infections. Second, using total nucleic acid derived from the same endocervical sample used to generate IFU values, we enumerated genome copy numbers. Importantly, both patients had high genome copy numbers despite the strikingly lower IFU counts in Patient 2 whose genome copy number actually exceeded that of Patient 1 (**Table 1**). These data indicate that, relative to Patient 1, a significantly smaller proportion of the total bacterial burden were infectious EB particles in Patient 2. Third, we evaluated the relative RNA expression levels of *euo,* an early-expressed chlamydial gene that codes for Euo (Zhang et al., 1998), and *omcB,* a gene expressed later in the developmental cycle that is repressed by Euo (Rosario and Tan, 2012). The ratio of *euo* to *omcB* mRNA expression also indicated contrasting patterns of growth in the two patients, with a significant predominance of *omcB* transcripts in Patient 1 and a significant predominance of *euo* transcripts in Patient 2 (**Table 1**). These data taken together with the morphological evidence, suggests that endocervical infection in both patients is characterized by a heavy bacterial genome burden, but, in contrast to the highly productive growth of *Chlamydia* in patient 1, the chlamydial growth profile of patient 2 is relatively asynchronous, and predominated by aberrant and early stage bacterial forms.

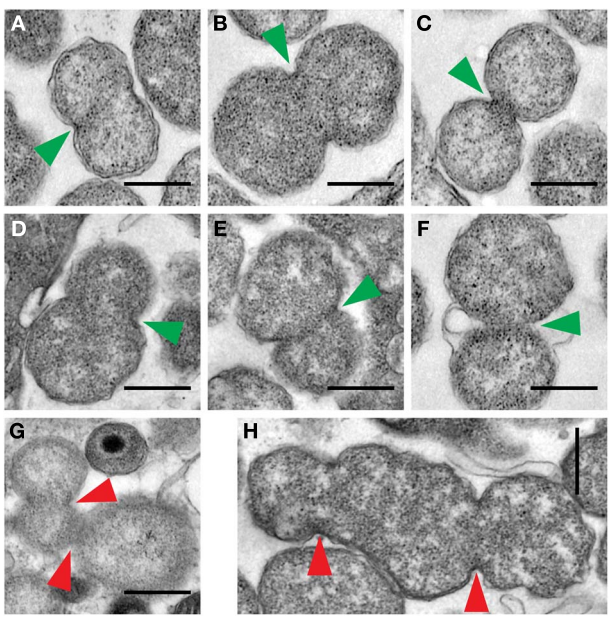

FIGURE 5 | Examples of RB binary fission detected in electron micrographs from Patient 2. Three types of binary fission were observed, including, septum formation where the plane of division, indicated by a green arrowhead, is predicted to result in equal division **(A–C)**, or unequal division **(D–F)**, or where multiple septum formation events, indicated by the red arrowheads, were apparently present **(G–H)**. The scale bar indicates a distance of 0.5 μm.

IFNγ AND INDOLES IN SECRETIONS IDENTIFY POTENTIAL CORRELATES FOR DIFFERING CHLAMYDIAL GROWTH PATTERNS BETWEEN PATIENTS

Next, we examined the infecting *ompA* genotype, clinical history, and key immune and environmental elements that might explain the contrasting growth patterns seen in Patient 1 and Patient 2. We determined that both patients were infected with an identical E *ompA* genotype (data not shown), neither had a

Table 1 | Measurement of multiple parameters of growth of *C. trachomatis* isolated from the cervix of two infected patients.

Growth parameter	Patient 1	Patient 2
IFU	336,168	67
Genome copy number	359,002	895,313
Genome copy number: IFU	1.067924	13,362.88
Euo: omcB	1.865	10.0

Shown are total cervical infectious forming units (IFU), genome copy numbers, ratio of IFU to genome copy number and ratio of Euo to omcB mRNA recovered from an endocervical swab for each patient.

documented history of *C. trachomatis* infection and both had a mucopurulent cervical discharge but no vaginal discharge. Both were negative for *N. gonorrhoea* and *T. vaginalis* but had a positive Nugent score (8 for Patient 1 and 7 for Patient 2, respectively) indicating BV. Finally, we assayed genital secretions to reveal the local concentration of indoles, and IFNγ. Indoles were detected in both patients, with the levels in Patient 1 being approximately twice as high as those in Patient 2 (278 vs. 159 mM). Importantly, while Patient 1 had a very low local concentration of IFNγ, this response was robust in Patient 2 (1.45 vs. 68.95 pg/mg protein, 35.48 vs. 613.09 pg/ml) (Aiyar et al., 2014).

DISCUSSION

In this study, we sought to develop techniques to identify the growth forms of *C. trachomatis* present in the human endocervix, the most common site of infection in women, with the concurrent evaluation of other biomarkers that could aid in classifying the spectrum of infections that occur at this site. During the course of this study, we established methodology to preserve bacteria and host epithelial cells for detailed morphologic and molecular analyses. This novel approach allowed us to document the presence of inclusions containing normal, morphologically aberrant, or a combination of these chlamydial forms, in epithelial cells. Importantly, in a pilot study, we could identify two patients with high numbers of bacterial genomes in their cervices, but, with contrasting bacterial growth patterns as revealed by parallel measurements of IFU, inclusion morphology, and transcriptional analyses of key early and late expressed chlamydial genes. Specifically, one patient (Patient 1) had a chlamydial growth pattern predominated by high numbers of infectious particles, whereas the other (Patient 2) displayed a very low IFU with inclusions that predominantly included both aberrant and normal RB forms. Coincident with these observations, a minimal IFNγ response was detected in Patient 1 secretions, in contrast to a more robust IFNγ response in Patient 2. A significant level of indoles was detected in both patients, likely consistent with being BV-positive.

Relatively few studies have described the morphology and abundance of chlamydial inclusions in the human cervix. In 1938, Braley examined cervical biopsies and conjunctival smears from respective mother-infant pairs in which the infant was diagnosed with inclusion blennorrhea (Braley, 1938). Inclusions were readily identified by Giemsa staining in infant conjunctival epithelial cells, but were only found in the cervix of a small proportion of women and were few in number (*ibid*). Three decades later, using EM on biopsied transition zone and adjacent cervix in two women co-infected with *C. trachomatis* and *N. gonorrhoeae*, Swanson identified inclusions in the transitional zone and in the columnar epithelium (Swanson et al., 1975). Of note, only classical inclusions were investigated and described, and antigen-specific staining procedures were not undertaken. Subsequent endocervical infection studies predominantly focused on diagnostics (presence/absence of EB). Exceptions to this were a study by Dunlop et al. who report observing chlamydiae in 7/159 patients by EM and present a micrograph of a single normal inclusion (Dunlop et al., 1989), and a study by Bragina et al., who reported the presence of small, aberrant bacterial forms in a cervical smear of a patient after inappropriate antibiotic treatment (Bragina et al., 2001). *In vivo* identification of human infections with persistent *C. trachomatis* forms is clinically important as these could: (1) provide a reservoir of persistent bacteria that reactivate when conditions in the local micro-environment are permissive; (2) evade key pathways involved in immune clearance; (3) contribute to the chronic inflammatory process; (4) underlie the recalcitrance of chlamydiae to clearance by certain antibiotics; and (5) avoid elimination by vaccine-induced immunity optimized to eradicate replicating forms.

In the study described here, we were able to demonstrate the utility of cytobrushes to sample intact infected cells from endocervix, finding similar numbers of inclusions to those observed in adult conjunctival smears (Braley, 1938). This methodology is important as cytobrushes (i) are non-invasive, unlike biopsies that are extremely difficult to obtain at this site, (ii) provide a method for longitudinally sampling, and (iii) can be immediately placed in a medium of choice for *ex vivo* analysis. Thus, by immediate immersion of cytobrushes into a modified EM buffer, we could preserve bacterial growth forms *ex vivo* for analyses by widefield or EM. The subsequent EM studies provide the first collection of micrographs of multiple *in vivo* inclusions. In one patient examined, inclusions were relatively homogeneous, predominantly contained EBs, and are therefore late in the developmental cycle. In contrast, in the second patient, we identified inclusions with predominantly RBs, predominantly ABs, a mix of RB and aberrant forms, a heterogeneous mix of mid-stage forms, and a classical late stage inclusion containing EBs. This wide spectrum of inclusion morphology suggests the possibility of different microenvironments in the same tissue in which factor/s driving bacterial development or persistence could be spatially and/or temporally variable.

The IFNγ-mediated host response to *C. trachomatis* infection has been studied in depth. In human cells, IFNγ induces the tryptophan-catabolizing enzyme IDO1 that catabolizes tryptophan to kynurenine (Shemer and Sarov, 1985; Byrne et al., 1986, 1989; Carlin et al., 1989; Beatty et al., 1994a; Brunham and Rey-Ladino, 2005). This depletion interferes with the growth of *C. trachomatis,* which is a tryptophan auxotroph (*ibid*). At sufficient and sustained concentrations of IFNγ *in vitro*, *C. trachomatis* can be eliminated from human host cells establishing this cytokine as a key component in local host defense (Byrne et al., 1989). In tryptophan-limiting but sub-inhibitory concentrations of IFNγ *in vitro* however, a scenario likely often encountered *in vivo*, chlamydiae enter into the persistent mode of growth (Beatty et al., 1993, 1994a). Transcriptome analysis of this altered state reveals a gene expression profile consistent with continued expression of genes governing DNA replication but not with those genes involved in bacterial cell division (Belland et al., 2003a). This includes upregulation of genes involved in tryptophan utilization, DNA repair and recombination, phospholipid biosynthesis and translation (*ibid*). A number of early genes are also upregulated, and in particular, *euo* (30-fold increase), which encodes a DNA-binding protein that binds to a late gene promoter region and is the transcriptional regulator of *omcB* (Zhang et al., 1998, 2000; Rosario and Tan, 2012). Down-regulation of genes involved

in RB to EB differentiation (such as *omc*AB), proteolysis, peptide transport, and cell division are also noted (Belland et al., 2003a). Removal of IFNγ leads to a rapid reactivation with gene expression rapidly returning to control levels, for example, *euo* expression drops 20-fold in 12 h (*ibid*). The knowledge that *euo* and *omcB* are differentially expressed in active and IFNγ-driven persistent growth, the commonality of this expression pattern to other persistence inducers, their lack of co-expression and the known stability of both mRNAs, all suggested the utility of choosing *euo/omcB* expression ratio as a putative biomarker of *in vivo* growth. By calculating this ratio, we were able to corroborate morphological, IFU, and genome data that the two patients described in the study had contrasting patterns of chlamydial growth. Future studies should confirm the utility of this combination of biomarkers to detect and determine the consequences of a spectrum of *C. trachomatis* infection seen in the human endocervix. Transcriptional analyses of a wider panel of genes should also reveal the common inducers of persistence in the genital tract. IFNγ is likely to be one of these stressors, and our laboratory and others have identified a clear association with, but spectrum of, IFNγ concentration in genital secretions during *C. trachomatis* infection that wanes after resolution of infection (Ficarra et al., 2008; Aiyar et al., 2014). Certainly the transcriptional changes that occur in the presence of IFNγ and result in persistent growth appear to constitute a persistence stimulon and suggest a coordinated biological response that has evolved to allow the organism to rapidly respond to, and survive, immunological pressure by a period of resistance followed by a rapid recovery after waning of the host response or by supplementation of tryptophan (Belland et al., 2003a). Importantly, by expressing tryptophan synthase during tryptophan starvation genital *C. trachomatis* serovars can uniquely salvage indole to supplement tryptophan, and indole-producing organisms such as bacteria associated with BV and *T. vaginalis* have been suggested to be the enabling co-factors (Caldwell et al., 2003; Morrison, 2003; Aiyar et al., 2014). In support of this, both patients in this pilot study were diagnosed with BV and indoles were present in their vaginal secretions. We speculate that the weak IFNγ response noted in Patient 1 may have been further compromised by indoles. In contrast, the lower levels of indoles detected in Patient 2 may be insufficient to overcome the restriction imposed by a robust IFNγ response.

While undoubtedly challenging, human studies targeted at elucidating if, when and how *C. trachomatis* enters into a persistent growth mode *in vivo* has been repeatedly stressed as *a priori* since these will likely significantly deepen our knowledge of the pathogenesis of this disease and hence stimulate new targeted treatment and prevention strategies (Wyrick, 2010; Schoborg, 2011). Because BV and *T. vaginalis* co-infections are so prevalent in *C. trachomatis* infected women (Koumans et al., 2007; Sutton et al., 2007), the role they play in supplementing local tryptophan, compromising IFNγ-mediated immunity and aiding establishment of chronic infections is also critical (Caldwell et al., 2003; Aiyar et al., 2014). The approaches described here could be applied to evaluate chlamydial infections at other sites *in vivo*. Further, as the commitment to a vaccine grows, we must also consider the multiple implications of our, and other recent, findings. Importantly, if the community is unable to develop a sterilizing vaccine, then we will likely need to formulate a vaccine to include antigens that are predominantly expressed by persistent growth forms. In addition we will need to determine the type of, and local level, of immunity and environmental milieu that will be most effective at eradicating, and least likely to drive bacteria into, a persistent growth mode, or to cause tissue damage. We believe these studies, and the potential biomarker panel we have investigated, form a strong platform for beginning to answer these questions. They also provide an avenue to explore the effect, and consequences of genital co-infections *on C. trachomatis* survival *in vivo*.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00071/abstract

Supplementary Figure 1 | Ultrastructural image of an inclusion from Patient 2. The entire inclusion cross-sectional area was not obtained for this inclusion and therefore quantitative measurements were not performed. EB morphotypes predominated in this inclusion.

Supplementary Figure 2 | Upper Panel: Particle cross-sectional area (Y-axis) for every particle detected in the two inclusions from Patient-1 (Figure 3 in the manuscript). Particle identity was assigned in numerical sequence by Fiji (X-axis). Particle cross-sectional area was calculated as described in the Materials and Methods section. The light green shading indicates the anticipated area for EBs, while the anticipated area for RBs is shaded in light blue. Lower Panel: Particle cross-sectional area for particles from the four inclusions from Patient-2 shown in **Figure 4** of the manuscript. The average cross-sectional area for particles from Patient-1 was $0.126 \pm 0.109\,\mu m^2$, while the average cross-sectional area for particles from Patient-2 was $0.431 \pm 0.373\,\mu m^2$. The difference in particle cross-sectional areas within inclusions from Patient-1 and Patient-2 was determined to be statistically significant by the Wilcoxon rank-sum test: P(two-sided) = 10e-16.

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Membrane vesicle production by *Chlamydia trachomatis* as an adaptive response

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Bacteria have evolved specific adaptive responses to cope with changing environments. These adaptations include stress response phenotypes with dynamic modifications of the bacterial cell envelope and generation of membrane vesicles (MVs). The obligate intracellular bacterium, *Chlamydia trachomatis*, typically has a biphasic lifestyle, but can enter into an altered growth state typified by morphologically aberrant chlamydial forms, termed persistent growth forms, when induced by stress *in vitro*. How *C. trachomatis* can adapt to a persistent growth state in host epithelial cells *in vivo* is not well understood, but is an important question, since it extends the host-bacterial relationship *in vitro* and has thus been indicated as a survival mechanism in chronic chlamydial infections. Here, we review recent findings on the mechanistic aspects of bacterial adaptation to stress with a focus on how *C. trachomatis* remodels its envelope, produces MVs, and the potential important consequences of MV production with respect to host-pathogen interactions. Emerging data suggest that the generation of MVs may be an important mechanism for *C. trachomatis* intracellular survival of stress, and thus may aid in the establishment of a chronic infection in human genital epithelial cells.

Keywords: *Chlamydia trachomatis*, membrane vesicles, adaptive response, persistent growth state, stress

INTRODUCTION

Chlamydia trachomatis serovars D-K account for the most prevalent bacterial sexually transmitted infections (STIs) worldwide. Despite aggressive control efforts, *C. trachomatis* infections have continued to constitute a serious public health risk (World Health Organization, 2011; Rekart et al., 2013). Infection may result in cervicitis, and in some women, *C. trachomatis* may ascend into the endometrium and Fallopian tubes, where it can establish a chronic infection leading to diseases such as pelvic inflammatory disease (PID), ectopic pregnancy, and infertility. *C. trachomatis* infections of women also pose a risk to infants, as infants born from mothers with *C. trachomatis* infections can develop conjunctivitis and/or pneumonia. Finally, epidemiological evidence indicates that *C. trachomatis* infection of the reproductive tract also may increase the risk of HIV transmission, making the study and understanding of the pathogenicity of this bacterium imperative.

In adapting to an intracellular niche, *C. trachomatis* has evolved a notably reduced genome of ∼1 million base pairs that supports a unique developmental cycle (Stephens et al., 1998). This cycle typically involves two forms: infectious elementary bodies (EBs) and dividing, metabolically active reticulate bodies (RBs) (Moulder, 1991). Under stressful conditions *in vitro*, however, *C. trachomatis* can enter into an alternative viable but non-dividing growth form, termed a persistent growth form (Beatty et al., 1994b). This persistent growth state extends the host-bacterial relationship *in vitro* and has thus been proposed to be linked to chronic infection and adverse outcomes, including elicitation of tissue-damaging host responses, *in vivo* (Hogan et al., 2004; Darville and Hiltke, 2010). Persistent forms are also less responsive to antimicrobial therapy *in vitro* (Wyrick and Knight, 2004; Reveneau et al., 2005) and *in vivo* (Byrne, 2001; Phillips-Campbell et al., 2014). Several excellent reviews provide extensively insightful descriptions of the chlamydial persistent growth state and the potential to establish a chronic relationship with the host (Beatty et al., 1994b; Hogan et al., 2004; McClarty et al., 2007; Wyrick, 2010; Schoborg, 2011; Lo et al., 2012).

C. trachomatis growth exclusively takes place within an inclusion, a membrane-bound vacuole. Despite the presence of this barrier, *C. trachomatis* actively communicates with the host cells. One method of interaction is the coordination of trafficking specific subsets of host vesicles to and from the inclusion, enabling delivery of the inclusion components and nutrients required for infection (Fields and Hackstadt, 2002). *C. trachomatis* also secretes numerous effectors with host cell-modulating activities across the complex membranes of the bacterium and the inclusion or host cytoplasm to host cell compartments. Although diverse secretory pathways, including the type II (Sec), type III, and type V (autotransporter) secretion systems, have been shown to play a role in the translocation of protein effectors (Crane et al., 2006; Valdivia, 2008; Chen et al., 2010a; Mueller et al., 2014), a mechanism for robust delivery of complex bacterial

components to host cells is likely to be mediated by membrane vesicles (MVs) that emerge from the envelope of growing bacteria (Giles et al., 2006; Giles and Wyrick, 2008; Wang et al., 2011a; Frohlich et al., 2012). As part of bacterial growth and/or envelope stress responses, the formation of MVs is a universal feature found in all Gram-negative bacteria (Beveridge, 1999; Kulp and Kuehn, 2010), *Mycobacterium* spp. (Prados-Rosales et al., 2011), and Gram-positive bacteria such as *Bacillus* spp. (Dubey and Ben-Yehuda, 2011). Herein, we review recent findings on the mechanistic aspects of bacterial adaptation to stress with a focus on how *C. trachomatis* remodels its envelope, produces MVs, and the potential important consequences of MV production with respect to host-pathogen interactions.

MOLECULAR ARCHITECTURE OF THE CELL ENVELOPE DURING THE CHLAMYDIAL DEVELOPMENTAL CYCLE

One of the critical developmental events common to *Chlamydia* spp. is the ability of the organisms to adapt their envelopes for the purpose of interacting with the host cell. Like other Gram-negative bacteria, the *C. trachomatis* envelope consists of an outer membrane (OM), an inner membrane (IM), and a periplasm (**Figure 1A**). Recent elegant studies by several groups have revealed that chlamydiae possess functional peptidoglycan necessary for bacterial division, despite the lack of the cytoskeletal protein FtsZ (McCoy and Maurelli, 2006; Ouellette et al., 2012; Pilhofer et al., 2013; Liechti et al., 2014). Nevertheless, a unique feature of the chlamydial envelope, distinguishing them from other Gram-negative bacteria, is the presence of a disulfide bond cross-linked major outer membrane protein (MOMP) with the periplasmic localized OmcB and the lipoprotein OmcA only in EBs (Hackstadt et al., 1985; Hatch, 1996). Both OmcB and OmcA contain abundant cysteines. This cross-linkage is believed to contribute to cell wall rigidity and osmotic stability of the EBs.

Proteins of the OM are important for various purposes including envelope architecture, virulence, transport, cell division, induction of inflammatory cytokine production, and immune evasion (Hatch, 1996; Stephens and Lammel, 2001; Abdelsamed et al., 2013). All of these tasks are presumably related to the generation and function of MVs that will be discussed in the later sections of this review. MOMP is the most abundant surface-exposed protein of both RBs and EBs (Caldwell et al., 1981; Hatch, 1996) and it functions as an adherin and a porin with its β-barrel structure (Wang et al., 2006; Sun et al., 2007). MOMP also has alternative conformations that may adapt to specific chlamydial growth stages (Feher et al., 2013) and may impart different levels of immunogenicity. Also located in the OM is a family of polymorphic membrane proteins (Pmps) or autotransporters unique to *Chlamydia* spp. (Stephens et al., 1998). *C. trachomatis* encodes nine Pmps (PmpA-I) that are either temporally or constitutively expressed (Tanzer and Hatch, 2001; Tan et al., 2010). Such temporal expression may promote antigenic variation, tissue tropism, and differential disease severity (Gomes et al., 2006; Tan et al., 2009; Taylor et al., 2011; Abdelsamed et al., 2013). Caldwell's group has reported that PmpD is a species-common, pan-neutralizing target (Crane et al., 2006). Other OM proteins studied include plasmid encoded Pgp3 (Chen et al., 2010b), PorB (Kubo and Stephens, 2000), HSP70 (DnaK) (Raulston, 1995),

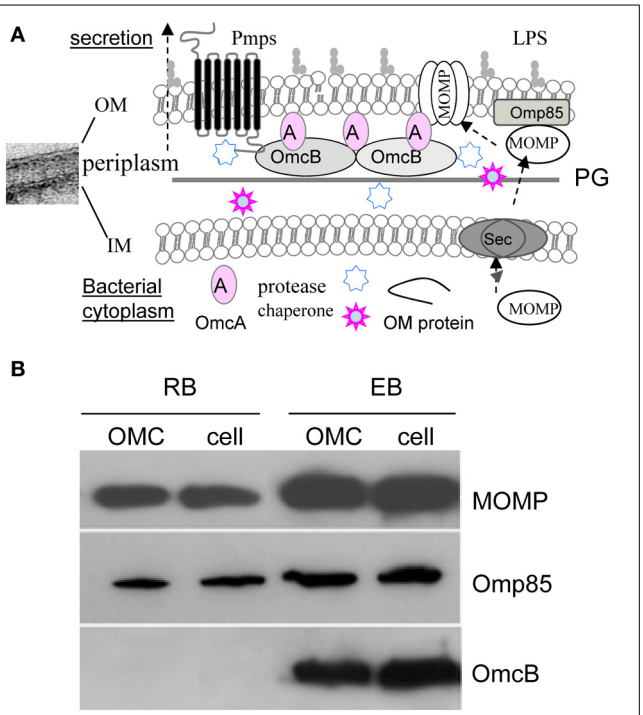


FIGURE 1 | (A) A schematic diagram of the envelope structure of *C. trachomatis*. Showing are the representative OM associated components [Lipopolysaccharide (LPS), peptidoglycan (PG), MOMP, Pmps, OmcA, OmcB, and Omp85], and putative chaperone and protease in the periplasm in EBs. The potential process of MOMP or other OM protein transport across the inner membrane via the Sec pathway, and insertion and assembly into the OM is indicated. **(B)** Immunoblot of *C. trachomatis* serovar F Omp85. Similar to MOMP, Omp85 is present in both EBs and RBs as a component of the OM complex (OMC), however, OmcB exists only in EBs. The OMCs were prepared from the insoluble fraction of isolated EBs or RBs in phosphate buffered saline containing 2% Sarkysol as described previously (Caldwell et al., 1981). The lysates of EBs and RBs, and their OMCs were dissolved in Laemmli sample buffer (1:1, v:v) supplemented with 5% 2-mercaptoethanol, 10 mM dithiothreitol and heated for 10 min at 100°C, isolated on 10% SDS-PAGE, followed by immunoblotting using polyclonal antibodies to Omp85 or OmcB, and a monoclonal antibody to *C. trachomatis* serovar F MOMP.

and Omp85 (YaeT) (Stephens and Lammel, 2001) that exists in both EBs and RBs (**Figure 1B**) (Liu et al., 2010). In bacteria, Omp85 facilitates the insertion of assembly intermediates from the periplasm to the OM (Ricci and Silhavy, 2012).

Many chlamydial envelope components are ligands recognized by host pattern-recognition receptors that induce inflammatory cytokine production and generate adaptive immune responses (Wang et al., 2010; Taylor et al., 2011; Abdelsamed et al., 2013). Some envelope components may also contribute to immune evasion. To this end, several chlamydial envelope components have been considered vaccine candidates (Crane et al., 2006; Schautteet et al., 2011; Hafner et al., 2014). *Chlamydia* spp. may quantitatively or qualitatively change their envelope structures as a result of adaptation to developmental signals or environmental cues (Beatty et al., 1994b; Hatch, 1996; Carrasco et al., 2011). The plasticity of the envelope has important implications for the

design of control strategies against bacterial infection because of its importance in determining susceptibility to host defenses and antibiotics (Tan et al., 2009; Hurdle et al., 2011).

STRESS INDUCES DRAMATIC CHANGES IN CHLAMYDIA SPP. *IN VITRO*

Matsumoto and Manire described the first ultrastructure of an aberrant RB form induced by penicillin in *C. psittaci*-infected L929 cells as viewed by transmission electron microscopy (TEM) (Matsumoto and Manire, 1970). They found that, in the presence of penicillin, RBs no longer divided, the RB to EB transition was disrupted, and aberrant multinucleated RBs accumulated. Concurrently, abundant vesicles were pinched off the aberrant RBs. Removal of penicillin allowed for the chlamydiae to reenter a normal growth state. Many elegant studies since have revealed that the viable but nondividing altered persistent form occurs when *Chlamydia* spp. are exposed to a variety of stress conditions. These include IFNγ (Beatty et al., 1994a), sub-inhibitory concentrations of antibiotics (Engel, 1992; Wyrick and Knight, 2004), nutrient deprivation (Beatty et al., 1994a; Raulston, 1997; Harper et al., 2000), co-infection with either herpes simplex virus (Vanover et al., 2008; Prusty et al., 2012) or *Toxoplasma gondii* (Romano et al., 2013) *in vitro*, and infection with *Chlamydia*-phage (Hsia et al., 2000). These stressors are often encountered by pathogens during infection *in vivo* (Wyrick, 2010). IFNγ, one of the stressors commonly studied, is a key component of immunity to intracellular pathogens. IFNγ induces expression of indoleamine-2, 3dioxygenase (IDO) that catalyzes the initial step in the degradation of L-tryptophan to *N*-formylkynurenine and kynurenine in eukaryotic cells (Beatty et al., 1994a). Such tryptophan depletion profoundly interferes with chlamydial growth, which, depending on the IFNγ concentration and exposure time, induces the bacteria to enter into a persistent growth form or results in bacterial eradication. Exposure to β-lactam antibiotics is another well-studied stressor that causes chlamydiae to enter into a persistent growth state and is used in simulating an inadequate antimicrobial treatment of chlamydiae infection (Matsumoto and Manire, 1970; Gerard et al., 2001; Giles and Wyrick, 2008; Carrasco et al., 2011; Wang et al., 2011a; Ouellette et al., 2012; Phillips-Campbell et al., 2014). *Chlamydia* spp. have a wide range of responses to stress. A clear shift of gene expression profiles specific to each stressor has been consistently found in many host cell types (Beatty et al., 1993, 1994b; Gerard et al., 2001, 2013; Molestina et al., 2002; Belland et al., 2003a,b; Ouellette et al., 2006; Lo et al., 2012). Changes in gene expression induced by stress at the levels of transcription and translation are consistent with the nature of a persistent growth form that undergoes DNA replication, but not division by binary fission. Interestingly, IFNγ exposure resulted in a global transcriptional upregulation lacking increased translation in chlamydiae (Ouellette et al., 2006). In contrast, penicillin exposure induced an alteration in transcription coupled to a change in translation (Ouellette et al., 2006). These data support that the impact of each stressor on bacteria is mediated by different mechanisms, and the bacterial response to each stress may be distinct.

To explore the mechanisms by which *C. trachomatis* survives under persistence inducing conditions, we utilized human primary endocervical epithelial cells, the main site of *C. trachomatis* infection *in vivo*. We found that normal *C. trachomatis* forms could develop in cultured primary cells similarly to HeLa cells (Wang et al., 2011a). **Figures 2A–C** show that exposure to ampicillin or IFNγ led to aberrant RB phenotypes and an accumulation of abundant MVs generated by *C. trachomatis* in cultured cells. Additionally, confocal microscopy and cell fractionation analyses demonstrated that the secreted chlamydial T3S effectors, CopN and Tarp, and the secreted protease, CPAF (Zhong et al., 2001) were decreased in ampicillin-induced persistent forms (Wang et al., 2011a), the latter of which is consistent with previous observations using IFNγ exposure or iron-deprivation persistence models (Shaw et al., 2002; Heuer et al., 2003). Changes in the spectrum of host cytosolic chlamydial proteins may underlie the host-pathogen relationship because of their importance in modulating host cell signaling (Valdivia, 2008; Zhong, 2011; Mueller et al., 2014).

MV PRODUCED DURING BACTERIAL INFECTION ARE PRESENT IN *C. TRACHOMATIS* INFECTED CELLS *IN VIVO* IN HUMANS

We have been particularly intrigued by the observed vesicular structures that are derived from both pathogen and host cells during *C. trachomatis* infection in culture, as they may serve as a vital element for host-pathogen interactions. *C. trachomatis* is likely to encounter numerous known and not-yet-identified conditions *in vivo*. Of these, varying levels of IFNγ in the endocervix during chlamydial infection *in vivo* are likely crucial as demonstrated by studies *in vitro*, in animal models, and in observational studies in humans (Arno et al., 1990; Beatty et al., 1994a; Byrne, 2001; Aiyar et al., 2014; Lewis et al., 2014). The direct involvement of persistent growth forms in pathogenesis *in vivo* in humans is challenging to prove, but several TEM studies have visualized atypical pleomorphic RBs and aberrant *C. trachomatis* forms in individuals with chronic infections, in Fallopian tube tissues, and in the synovium of reactive arthritis patients (Patton et al., 1994; Nanagara et al., 1995; Mazzoli et al., 2000; Bragina et al., 2001). Very recently, the Quayle laboratory developed methodology to sample endocervical cells and components of the endocervical environment in *C. trachomatis* infected women. By using a cytobrush to retrieve cells followed by immediate placement of these brushes in modified TEM fixative, the ultrastructure of "*in vivo*" chlamydial growth forms in endocervical epithelial cells could later be visualized by TEM (Lewis et al., 2014). These TEM analyses revealed that chlamydial infection in the human endocervix could result in a variety of inclusion types, containing normal forms, a mixture of relatively "normal" and aberrant forms, or inclusions with highly aberrant forms, and this varies between patients (Lewis et al., 2014). Since MVs are so small in size and lack unique biological markers for identification by standard immunostaining techniques, we have begun to take advantage of these samples to examine MV production *in vivo*. High magnification TEM images were obtained from cross sections of several inclusions and indicated the *in vivo* presence of *C. trachomatis* MVs in inclusions sampled from two different patients (**Figures 3A–D**). MVs appeared to be single membrane structures and apparently associated with the OM of chlamydial organisms

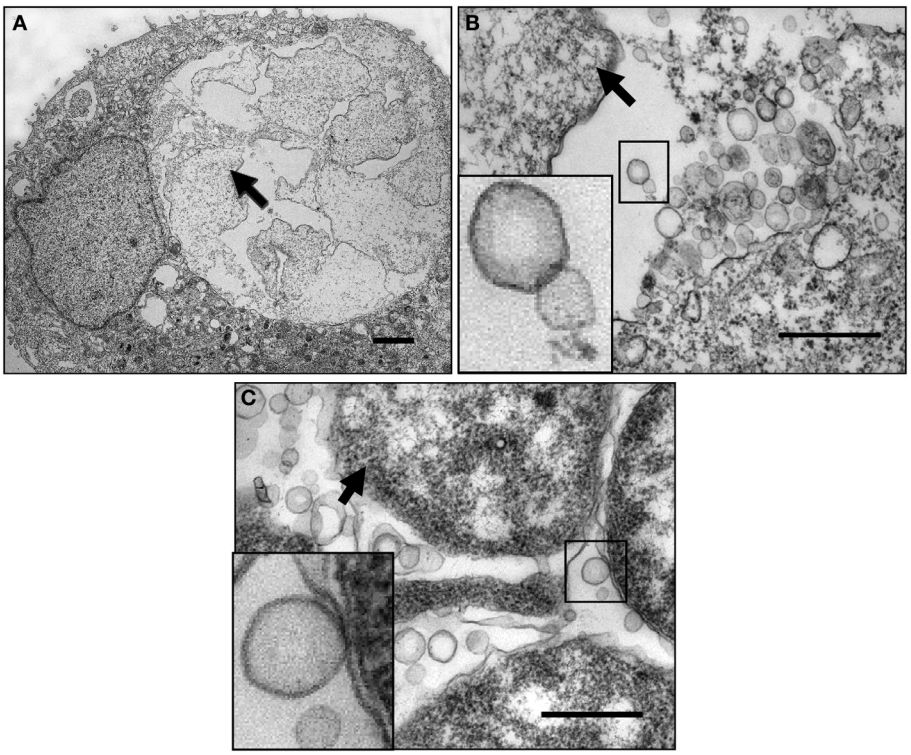


FIGURE 2 | Micrograph of chlamydial MVs in cultured human cervical epithelial cells. (A) Ampicillin-induced persistent growth forms in infected human primary endocervical epithelial cells. **(B)** A high magnification image showing ampicillin-induced *C. trachomatis* MVs. The boxed MVs are enlarged in the insert. **(C)** IFNγ-induced *C. trachomatis* MVs in infected HeLa cells. The boxed bacterial membranes and the MVs are enlarged in the insert. Note: the vesicles appear to be single-membrane structures different from the double membrane structures of intact chlamydial organisms. They are associated with the bacterial surface, clustered or scattered within the inclusion lumen. Arrows indicate persistent forms. The primary cell cultures were established from endocervical tissue explants as previously described (Herbst-Kralovetz et al., 2008). Cells were infected with serovar F EBs resulting in a 30% infection rate. Ampicillin (10 μg/ml) was added to the culture at 16 h post-infection. For the IFNγ exposure model, HeLa cells were exposed to RPMI 1640 medium containing 50 units/ml of IFNγ for 24 h prior to infection. Fresh IFNγ-containing medium was added after infection. Cells were harvested at 36 h post-infection, fixed and processed for TEM as described previously (Belland et al., 2003a). Scale bars: **(A)** 500 nm. **(B,C)** 200 nm.

similar to those observed *in vitro* (**Figure 2**). The double membrane structures of *C. trachomatis* forms are intact, which is an indication of faithful sample preservation. This preliminary but novel observation provides an important link to those observations made *in vitro*, indicating that MVs are a component of *C. trachomatis* infection *in vivo* in humans. Further examination of samples using this methodology is in progress to determine the relationship between MV formation and the bacterial growth state in natural human infections.

THE IMPORTANCE OF MVs DURING *C. TRACHOMATIS* INFECTION

The best-studied mechanism that is related to OM changes in Gram-negative bacteria is the formation of MVs during both physiological adaptation and responses to envelope stress. Bacterial MVs are not only compositionally similar to the OM, containing LPS, phospholipids, and OM proteins, but also contain selective periplasmic or cytoplasmic components, such as toxins, DNAs, and RNAs, depending on the strain (Kadurugamuwa and Beveridge, 1999; Beveridge, 1999; Kuehn and Kesty, 2005). Research demonstrates the potential of MVs as vehicles of pathogenicity, as they can deliver complex bacterial molecules to target cells (Kuehn and Kesty, 2005; Bomberger et al., 2009; Amano et al., 2010; Elmi et al., 2012). The MVs also involve immune activation or suppression, stress responses, and attachment and internalization of the bacteria. Because of the cargo carrier nature and potent built-in adjuvanticity of most MVs studied to date, they are being utilized as vaccines (Collins, 2011; Unal et al., 2011). Engineered MVs in *E. coli* have exhibited a promising robust and tunable platform for the development of recombinant multivalent vaccines (Chen et al., 2010c; Bartolini et al., 2013).

Although chlamydial MVs were first observed over four decades ago (Matsumoto and Manire, 1970; Stirling and Richmond, 1980), the implications of these multi-functional MVs are only beginning to be elucidated (Giles et al., 2006; Giles and Wyrick, 2008; Wang et al., 2011a; Frohlich et al., 2012). A puzzle remains with regard to what role chlamydial MVs play during infection. Research data, including our own, suggest that the generation of chlamydial protein containing MVs occurs during productive infection and is enhanced by stress (**Figures 2**, **3**) (Matsumoto and Manire, 1970; Giles et al., 2006;

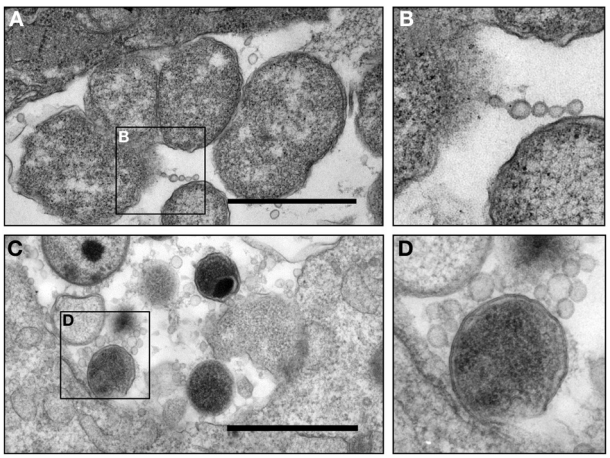
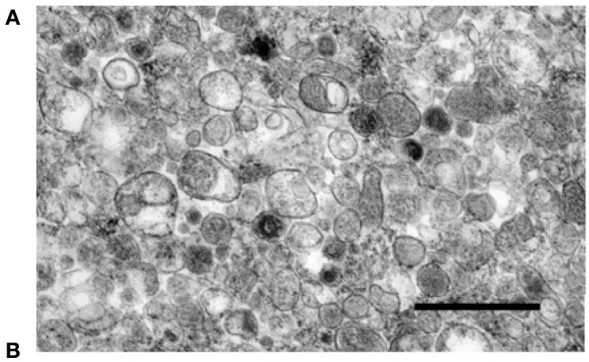


FIGURE 3 | Micrograph of chlamydial MVs in cervical epithelial cells from *C. trachomatis* infected patients. (A,B) Presence of *C. trachomatis* vesicles associated with an aberrant *C. trachomatis* form in an inclusion. Vesicles appeared in the form of a chain or single round vesicular structures. This sample was obtained from a patient (patient 2) presented in Lewis et al. in this journal (Lewis et al., 2014); **(C,D)** presence of *C. trachomatis* MVs associated with a normal *C. trachomatis* form in an inclusion obtained from an epithelial cell from a second *C. trachomatis* infected patient. *C. trachomatis* infected human epithelial cells were collected by cytobrush from patients with an NAAT and culture positive *C. trachomatis* infection, and were immediately placed in a modified TEM fixative (Lewis et al., 2014) (currently under review for this Journal) and processed as previously described (Belland et al., 2003a). Scale bar = 500 nm.



B

Proteins detected	Infected Cell	Purified EB	Ct MVs	Mock vesicle
TGN38	+	-	-	+
LAMP-2	+	-	-	+
Calnexin	+	-	+	+
β-actin	+	+	+	+
MOMP	+	+	+	-
CPAF	+	-	+	-
Pgp3	+	+	+	-
CT159	+	+	+	-
CT166	+	+	+	-
OmcA	+	+	-	-
RpoB	+	+	-	-

FIGURE 4 | Compositional analysis of *C. trachomatis* MV fractions. (A) Micrographs of isolated MV fractions from *C. trachomatis* infected L929 cells by method described previously (Frohlich et al., 2012). At the ultrastructural level, the MVs show heterogeneity in their size and morphology. Scale bar = 500 nm; **(B)** *C. trachomatis* (Ct) or host cell proteins detected by immunoblotting with the lysates of infected cells, EBs, chlamydial MVs, and the control vesicles from mock infected cells. Note: coexistence of host ER marker (calnexin) with selective chamydial proteins (MOMP, CPAF, CT159, and CT160). Proteins were separated by 12% SDS-PAGE, followed by immunoblotting with antibodies to the protein of interest as described previously (Frohlich et al., 2012).

Giles and Wyrick, 2008; Wang et al., 2011a; Frohlich et al., 2012). The presence of MVs, not only during the β-lactam induced aberrant state, but also during both tryptophan starvation via IFNγ exposure and *in vivo* infection demonstrate a universal mechanism that appears to be responsive to stress rather than merely a by-product of dysfunctional membrane biogenesis during β-lactam exposure.

PROTEIN COMPOSITION OF CHLAMYDIAL MVs

A challenge to studying chlamydial MVs is the obligate intracellular lifestyle of *C. trachomatis* and the complexity of isolating vesicle populations derived from the pathogen as opposed to the host cells. To more comprehensively understand the properties of chlamydial MVs, we developed a method that permits efficient isolation and enrichment of intact chlamydial MVs for biochemical analysis (Frohlich et al., 2012). This approach uses a combination of nitrogen cavitation, magnetic bead immunological capture, and isopycnic centrifugation of *C. trachomatis* infected L929 cells. This method not only allows for the study of MVs, but also isolation of chlamydial forms, host organelles, and host cytoplasm. Therefore, a "snapshot" of the total host and chlamydial environments can be obtained from a single experiment.

The integrity of chlamydial MVs isolated by this approach was confirmed by TEM analysis (**Figure 4**) (Frohlich et al., 2012). MOMP and several important *C. trachomatis* cytotoxic and/or secreted proteins (CPAF, Pgp3, CT159, and CT166) are found to be associated with MVs induced by ampicillin as determined by immunoblotting analyses (**Figure 4**) (Frohlich et al., 2012). These data support that MVs are generated by *C. trachomatis* as a means of carrying and delivering chlamydial proteins or antigens. Since those factors identified as being associated with chlamydial MVs often also stimulate traditional secretion pathways, it is possible that MV delivery provides an alternative and specific mode of protein delivery. It has been proposed that translocation of chlamydial proteases, CPAF and HtrA, from the bacterial cytoplasm to the periplasm is Sec-dependent (Wu et al., 2011; Zhong, 2011), but secretion of these proteins to an extrabacterial location or the host cytosol is likely mediated by MVs. One

could envision a stress or developmental cycle-specific alternative protein delivery system employed to further circumvent host processes designed to limit chlamydial growth. Interestingly, evidence supports the association of chlamydial protein-containing MVs with the endoplasmic reticulum (Giles and Wyrick, 2008; Frohlich et al., 2012). Considering the widespread strategy of chlamydial exploitation of host cellular machinery, it is likely that the delivery of chlamydial proteins to the host by MVs, at least in some cases, hijacks the host cells' own protein delivery systems to provide a targeted and specific localization of chlamydial proteins. Further experimentation is needed to confirm and understand these processes.

POTENTIAL FUNCTIONS OF CHLAMYDIAL MVs IN INFECTED HOST CELLS—CARGO DELIVERY

Although the exact destination of MVs is difficult to determine, TEM in combination with biochemical and immunodetection analyses suggest that MVs can connect with bacterial cells, accumulate in the inclusion lumen, associate with the inclusion membrane, evert from but still associate with the inclusion, and be found beyond the confines of the inclusion (Giles et al., 2006; Giles and Wyrick, 2008). Previous studies have suggested that a subset of these vesicles are antigen-containing structures that emerge from the inclusion membrane to release antigens from the inclusion without the need for inclusion disruption (Richmond and Stirling, 1981; Giles et al., 2006). These vesicles appeared to contain inclusion membrane proteins (Incs) co-localized with *C. trachomatis* antigens, including MOMP, LPS, GroEL2, and GroEL3, but not GroEL1 (Giles et al., 2006). We found that the isolated MV fraction was rich in CPAF, Pgp3, CT159, and CT166 (Frohlich et al., 2012). The functions of these proteins relating to chlamydial virulence or modulating host cellular functions have been studied (Belland et al., 2001; Zhong et al., 2001; Chen et al., 2010b; Thalmann et al., 2010). It is possible that distinct subpopulations are trafficked through cellular machinery alongside host vesicles. Presumably, the release of bioactive contents mediated by MVs influences the fate of host cells. How does MV translocation across the inclusion membrane and cytosol and/or surface of the host cell occur? How do these chlamydial antigen-containing vesicles influence host cell signaling and antigen presentation? Is the generation of MVs eliciting a variety of specific and highly regulated adaptive responses to protect *C. trachomatis* from the offending stress, or do they modulate innate and/or adaptive immunity? Do MVs produced by *C. trachomatis* relate to exosome formation by cervical epithelial cells with *C. trachomatis* infection? Certainly, any one of these events alone or in concert would confer a selective advantage in adaptation to and survival in the ever-changing intracellular niche.

THE POTENTIAL ROLE OF MVs IN THE INNATE IMMUNE RESPONSE

The MVs derived from many Gram-negative bacteria are heavily laden with complexes of pathogen-associated molecular patterns, such as LPS, flagellin, CpG DNA, and virulence factors. These MVs are strongly recognized by the host immune system, resulting in the upregulation of pro-inflammatory cytokine secretion (Amano et al., 2010; Kulp and Kuehn, 2010). To determine if chlamydial MVs, induced by ampicillin, elicit an innate immune response, we investigated the impact of isolated MVs on the secretion of cytokines by epithelial cells. An immortalized human endocervical epithelial cell line (A2EN) infected with *C. trachomatis* was used as our model of infection. A2EN cells retain site appropriate expression of hormone receptors, responsiveness to exogenous hormone stimulation, expression of TLRs, and responsiveness to TLR agonists by secreting cytokines, chemokines, and anti-microbial peptides (Buckner et al., 2013).MVs isolated from *C. trachomatis* infected cell cultures exposed to ampicillin were added to monolayers of A2EN cells. Purified host cell vesicles without *C. trachomatis* infection, heat-killed *Pseudomonas aeroginosa* PAO1, and A2EN cells productively infected with chlamydial organisms were used as controls. Culture supernatants were collected at 24 and 48 h. Chlamydial MVs induced a modest, albeit statistically significant ($p = 0.0230$ based on performing a One Way ANOVA with a Bonferroni post-test), 2-fold increase in CXCL-8 at 24 h compared to the mock MV control, although, no significant increase of IL-6, TNF-α, or GM-CSF was observed (**Figure 5**). At 48 h, however, the differences observed were not significant as both chlamydial MVs and the mock vesicle control induced a similar increase in CXCL-8 and GM-CSF levels, perhaps due to the species differences between the mouse L929 derived vesicles and the human A2EN cells. As previously published, *C. trachomatis* infection in A2EN cells failed to upregulate secretion of CXCL8 (Buckner et al., 2013). Productive infection with *C. trachomatis* conducted at the same time as the MV experiments recapitulated these published observations and provided an additional control lending to the consistent response of A2EN cells across different laboratories (data not shown). These data suggest that MVs have more capacity to elicit an innate immune response than productive chlamydial infection, and that the molecules on the surface or contained within these vesicles may be structurally distinct compared to those present on or within whole chlamydial particles. The significance of such an early (at 24 h) but weak stimulatory effect on endocervical cells by chlamydial MVs remains to be determined. These results are clearly different from previously reported observations with MVs from other Gram-negative bacteria, as MVs derived from these other bacteria induce a robust and significant increase in pro-inflammatory cytokines and chemokines and induce antibody production (Amano et al., 2010; Nakao et al., 2011; Sharpe et al., 2011; Zhao et al., 2013). However, given the obligate intracellular nature of *C. trachomatis*, a robust immune response would not likely be favorable to continued or long-term infection. These results support other observations of immune-evasion tactics used by chlamydiae, even in the delivery of MVs.

THE POTENTIAL ROLE OF MVs IN EXCHANGE OF GENETIC MATERIALS

It has been demonstrated that bacterial MV production provides a general advantage for survival and social networks in many bacteria species (Kuehn and Kesty, 2005; Amano et al., 2010; Bielig et al., 2011; Unal et al., 2011). Of great interest to the study of MVs is their participation in inter- and intra-species exchange of bacterial material in addition to their immuomodulatory functions (Kadurugamuwa and Beveridge, 1999; Renelli et al., 2004; Chiura et al., 2011; Barteneva et al., 2013). MVs may bind to bacterial

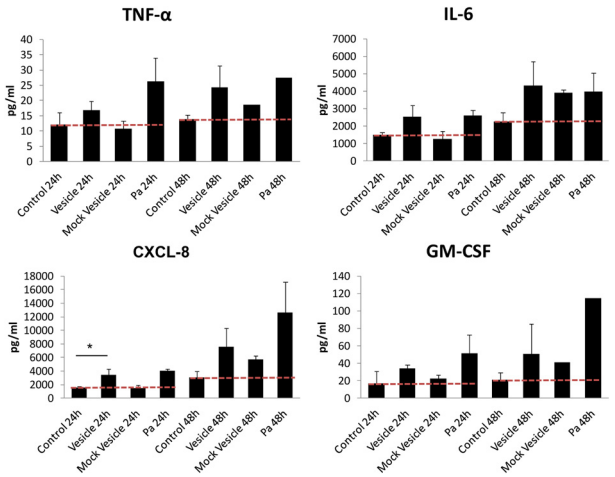


FIGURE 5 | Cytokine response of A2EN cells to vesicles isolated from a *C. trachomatis* infection of L929 cells. MVs isolated from *C. trachomatis* infected cell cultures exposed to ampicillin (10 μg/ml) were added to a monolayer of A2EN cells. Purified host cell vesicles from a mock infection, heat-killed *Pseudomonas aeroginosa* PAO1 (Pa), and A2EN cells productively infected with chlamydial organisms were used as controls. Cytokine measurements were made using multiplex analyses as previously described (Buckner et al., 2013). $*p = 0.0230$. The red dashed line represents baseline control values.

or host cells, fuse with the target cell membrane, and deliver their cargo to the cytosol of the target cells. Recent evidence that protein and DNA transfer between distantly related species raises the prospect of a widely distributed mechanism of bacterial communication (Dubey and Ben-Yehuda, 2011). This poses an interesting potential for *C. trachomatis* given the vast sampling of both normal and pathogenic microorganisms potentially inhabiting the same niche in the genital tract and could potentially provide a significant adaptation and survival advantage.

While there is no evidence that chlamydial MVs deliver products to other species, it is possible that MVs could be used to deliver not only proteins but also genetic materials among chlamydiae. Recently, it has become evident that *Chlamydia* spp. have all the necessary recombination machinery despite an inclusion-confined lifestyle (Zhang et al., 1995; Demars et al., 2007; Joseph et al., 2011; Harris et al., 2012). Recombination has been documented between strains with different tissues tropisms, the lymphogranuloma venereum with the urogenital biovars, and within different genital strains *in vitro* and *in vivo* (Jeffrey et al., 2010, 2013; Harris et al., 2012). Whole genome sequencing analyses with a collection of diverse clinical isolates and laboratory strains have revealed that the recombination or mutation events often occur in several regions of the chromosome encoding surface-exposed proteins, such as MOMP, Pmps and the T3S system effector Tarp (Gomes et al., 2004; Brunelle and Sensabaugh, 2006; Gomes et al., 2006; Joseph et al., 2011; Harris et al., 2012). Different *C. trachomatis* strains can recombine following mixed infection for a relatively short time period *in vitro* (Jeffrey et al., 2013). Their potential capabilities for DNA exchange might explain, at least in part, the acquisition of virulence genes and fitness traits, as well as variations in these surface exposed proteins. Whether or not MVs directly contribute to the horizontal gene transfer between chlamydial organisms, as is observed in other bacteria, is unknown, but it begs the question: what impact does MV-mediated cell-to-cell communication have on pathogenesis and bacterial survival within the host? How might these MVs directly or indirectly affect the genital tract microbiome? Further addressing these questions will help in understanding the occurrence of antigen variation and diversity in *C. trachomatis* during infection and promote the development of novel genetic tools to study genetics and pathogenesis of *C. trachomatis*.

MOLECULAR MECHANISMS CONTROLLING MV FORMATION IN *C. TRACHOMATIS*

Despite tremendous efforts, mechanisms by which bacteria produce MVs while sustaining their viability remain unclear. Based on studies in model organisms, including *P. aeruginosa* and *E. coli*, several mechanisms of MV formation have been proposed. First, the loss of OM-PG cross-links may allow excess lipid formation to induce MV formation. Second, accumulated periplasmic proteins simply push out the OM, trapping large amounts of protein inside the resulting vesicle. Third, the involvement of integral membrane proteins or signal molecules [such as *Pseudomonas* quorum sensing (PQS) molecules] induces membrane curvature and vesiculation (Schertzer and Whiteley, 2012). Fourth, the presence of rough LPS in bacteria contributes directly and indirectly to the formation of MVs (Kadurugamuwa and Beveridge, 1995; Sabra et al., 2003). Finally, genetic mechanisms may exist to regulate MV formation (McBroom et al., 2006; Schwechheimer et al., 2013). These MV inducing factors that have been proposed may not be mutually exclusive, and multiple dynamic surface components may contribute to MV production in bacteria (Kulp and Kuehn, 2010; Schwechheimer et al., 2013).

We know surprisingly little about OM biogenesis, and even less is known about the related vesicle formation in *C. trachomatis*. It is likely that after synthesis in the bacterial cytoplasm, chlamydial protein translocation to the OM involves complex steps conserved in Gram-negative bacteria (Hagan et al., 2010; Silhavy et al., 2010). These include: (i) translocation across the IM via the Sec pathway; (ii) folding facilitated by periplasmic chaperones/proteases, and (iii) assembly and insertion into the lipid phase of the OM, a process facilitated by a β-barrel assembly machine (BAM) complex that consists of Omp85 and its interacting lipoproteins (Ricci and Silhavy, 2012). Also necessary for maintenance of EB envelope integrity is the disulfide bond cross-linking of MOMP with OmcA and OmcB (Hatch, 1996).

We hypothesize that both bacterial and host factors contribute to the generation and release of MVs from *C. trachomatis*. We envision different mechanistic scenarios involved in MV formation during productive infection or the persistent growth state induced by ampicillin. In the case of productive infection, MV generation may reflect local OM deformation and/or a physiological turnover of envelope components because of envelope remodeling, a critical process in chlamydial development. In fact, RB multiplication and division require a rapid surface expansion or alteration achieved by the synthesis, assembly, and insertion of lipid or non-lipid OM components, while transition from a

large RB to a small EB undergoes the opposite. In contrast, ampicillin induced hyper-vesiculation phenotypes may be the result of an envelope stress response. Exposure to ampicillin blocks RB division, although DNA replication remains. These can, in turn, result in attenuation of OM protein expression, accumulation of misfolded proteins in the periplasm, activation of proteolytic processes, and interference with the correct assembly and insertion of proteins in the OM. All of these are likely to induce heightened vesiculation. MV formation may offer a means to remove stress caused by the accumulated "toxic" waste or unfolded proteins. Chlamydial HtrA, a key player in envelope stress response (Huston et al., 2007, 2008; Zhong, 2011), is likely to play a role in controlling the formation of MVs, as is observed in other bacteria (McBroom et al., 2006). Given the strong capacity of chlamydial exploitation of host cellular machinery, MV cargo delivery may partially depend on a yet to be defined host trafficking pathway. Whether or not a potential host factor contributes to the formation of chlamydial MVs remains to be determined. Nevertheless, studying molecular mechanisms underlying envelope adaptation to development or stress offers a powerful tool and new route to understanding how *C. trachomatis* adapts to and survives in nutrient rich but hostile intracellular niches.

SUMMARY AND PERSPECTIVE

The production of MVs in bacteria is a universal mechanism whereby virulence factors, signaling molecules, and genetic materials can be packaged and effectively transported to target cells. Major challenges in the field of vesicle research with obligate intracellular bacteria include (i) developing new comprehensive approaches for vesicle isolation and characterization, (ii) isolating pure populations from bacteria or host cells, and (iii) monitoring vesicle dynamics under physiologic relevant conditions. Nevertheless, the information already available indicates that MVs produced during chlamydial infection are present in cultured cell lines, primary human endocervical epithelial cells infected with *C. trachomatis,* and more importantly, in clinical specimens from *C. trachomatis* infected patients. These data provide preliminary evidence that the MV is a component of chlamydial infection *in vivo*. We hypothesize that *C. trachomatis* varies OM organization and produces MVs during the developmental cycle and in response to stress. Many mysteries remain with regard to how MVs contribute to host-pathogen interactions, the relationship of developmental envelope biogenesis within the context of MV formation and regulation, and the modes of MV cellular targeting and delivery. Improved understanding of the mechanisms of MV mediated mass material exchanges and the effects on *C. trachomatis* survival may have relevance to several important aspects of chlamydial biology, including the *C. trachomatis* persistent growth state *in vivo*. Recent progresses in chlamydial genetics may have great potential to inspire significant advance in MV studies in *C. trachomatis* (Wang et al., 2011b; Gérard et al., 2013; Gong et al., 2013; Song et al., 2013; Bauler and Hackstadt, 2014). Since global control of *C. trachomatis* infection will best be achieved with a vaccine, developing a greater understanding of the functional role and the mechanisms of envelope modification and MV production may open new therapeutic avenues against this medically important intracellular bacterium.

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Characterization of serine hydroxymethyltransferase GlyA as a potential source of D-alanine in *Chlamydia pneumoniae*

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For intracellular *Chlamydiaceae*, there is no need to withstand osmotic challenges, and a functional cell wall has not been detected in these pathogens so far. Nevertheless, penicillin inhibits cell division in *Chlamydiaceae* resulting in enlarged aberrant bodies, a phenomenon known as chlamydial anomaly. D-alanine is a unique and essential component in the biosynthesis of bacterial cell walls. In free-living bacteria like *Escherichia coli*, penicillin-binding proteins such as monofunctional transpeptidases PBP2 and PBP3, the putative targets of penicillin in *Chlamydiaceae,* cross-link adjacent peptidoglycan strands via meso-diaminopimelic acid and D-Ala-D-Ala moieties of pentapeptide side chains. In the absence of genes coding for alanine racemase Alr and DadX homologs, the source of D-Ala and thus the presence of substrates for PBP2 and PBP3 activity in *Chlamydiaceae* has puzzled researchers for years. Interestingly, *Chlamydiaceae* genomes encode GlyA, a serine hydroxymethyltransferase that has been shown to exhibit slow racemization of D- and L-alanine as a side reaction in *E. coli*. We show that GlyA from *Chlamydia pneumoniae* can serve as a source of D-Ala. GlyA partially reversed the D-Ala auxotrophic phenotype of an *E. coli* racemase double mutant. Moreover, purified chlamydial GlyA had racemase activity on L-Ala *in vitro* and was inhibited by D-cycloserine, identifying GlyA, besides D-Ala ligase MurC/Ddl, as an additional target of this competitive inhibitor in *Chlamydiaceae*. Proof of D-Ala biosynthesis in *Chlamydiaceae* helps to clarify the structure of cell wall precursor lipid II and the role of chlamydial penicillin-binding proteins in the development of non-dividing aberrant chlamydial bodies and persistence in the presence of penicillin.

Keywords: chlamydial anomaly, persistence, aberrant bodies, D-alanine, alanine racemase, GlyA, penicillin, D-cycloserine

INTRODUCTION

Acute and chronic diseases caused by *Chlamydiaceae* are a global health problem. The Gram-negative obligate intracellular pathogens depend on eukaryotic host cells to maintain their unique biphasic developmental cycle. One elusive phenomenon of the chlamydial biology has fascinated researches for two decades: for endobacteria, such as *Chlamydiaceae*, there is no need to resist osmotic challenges and a functional cell wall has not been detected in these pathogens so far (McCoy and Maurelli, 2006). Nevertheless, in the evolutionary process of adaptation to the host environment, *Chlamydiaceae* species conserved in their reduced genomes a nearly complete cell wall precursor biosynthesis pathway (**Figure 1**) and antibiotics that target cell wall biosynthesis are active (McCoy and Maurelli, 2006). Penicillin has no bactericidal effect, as seen in free-living bacteria, but induces a reversible state of persistence in *Chlamydiaceae* that is characterized by the formation of viable, enlarged, reticulate bodies. These persisting cells are called aberrant bodies (AB) and show resistance to azithromycin (Wyrick and Knight, 2004), the first-line treatment for chlamydial infections (CDC, 2010). Beta-lactam induced formation of non-dividing ABs has been observed in cell culture (Skilton et al., 2009) as well as *in vivo* (Phillips Campbell et al., 2012).

In free-living bacteria, cell division must be highly coordinated with cell wall biosynthesis to maintain cell integrity. The need for tightly interconnecting both cell biological processes may be reflected by the partial overlap of components from both multi-protein machineries; e.g., the transpeptidase PBP3 (FtsI) is essential for the incorporation of cell wall building blocks at the septal cell wall and for cell division.

The bacterial cell wall consists of peptidoglycan, a polymer of long chains with alternating sugar units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), which are cross-linked via flexible peptide bridges. Peptidoglycan is found in all eubacteria with the exception of some obligate intracellular species. Biosynthesis of peptidoglycan takes place in three stages (**Figure 1**). In the cytoplasm, six enzymes (MurA to MurF) catalyze the formation of the soluble precursor UDP-MurNAc-pentapeptide. Notably, the precursor contains D-Ala in positions 4 and 5 of the pentapeptide moiety. D-alanine is a unique and

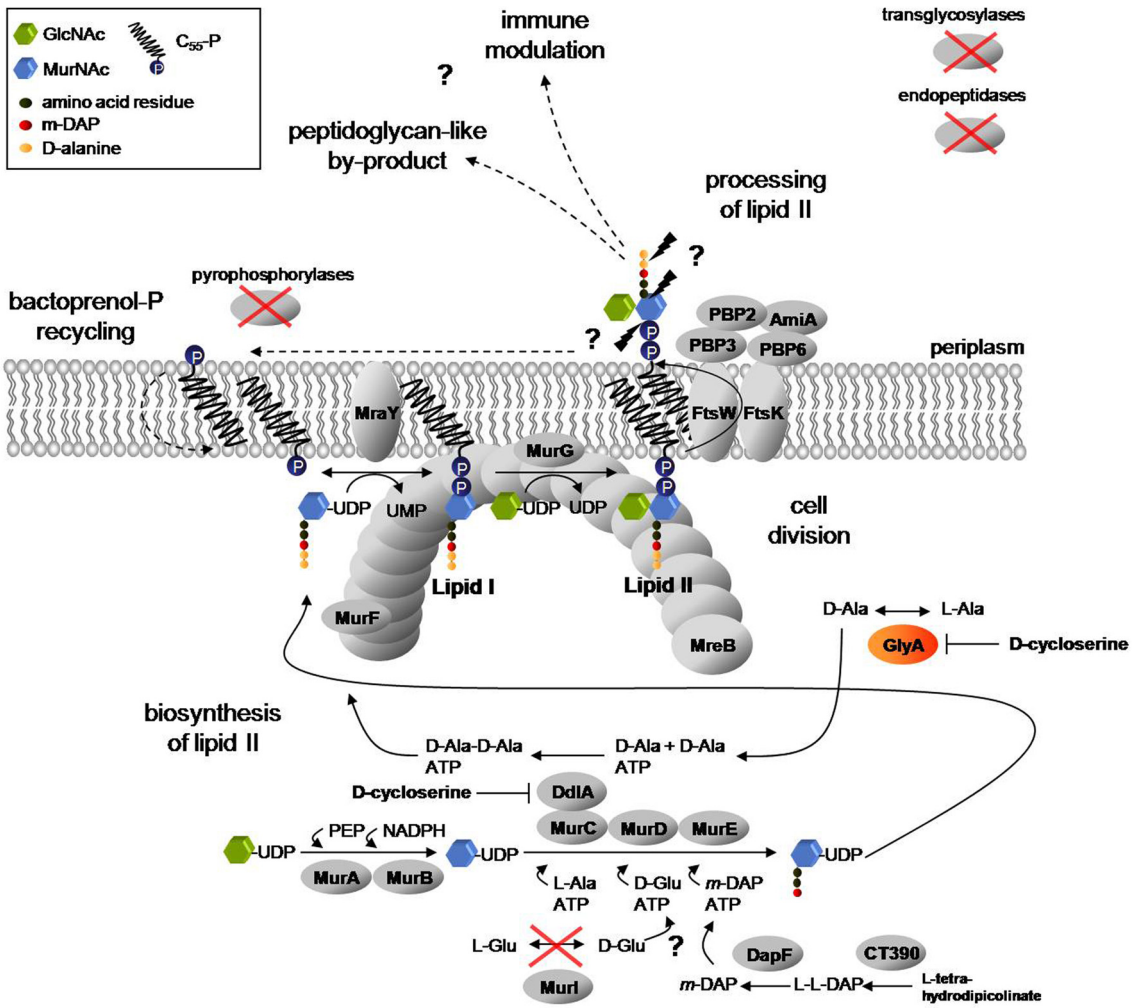

FIGURE 1 | Proposed lipid II pathway in *Chlamydiaceae*. A complete cycle of lipid II biosynthesis, including translocation to the periplasm, processing and bactoprenol carrier recycling is required for coordinated function of the divisome machinery and modulation of Nod1 and Nod2 mediated host immune response to chlamydial muropeptides. Biosynthesis of lipid II takes place in the cytoplasm and at the inner leaflet of the cytoplasmic membrane. In two consecutive biosynthesis steps of L-Ala racemization and D-Ala ligation, catalyzed by GlyA and the MurC/Ddl fusion protein, the D-Ala-D-Ala dipeptide is produced. MurF adds the dipeptide to the nascent peptide chain to complete synthesis of the soluble precursor UDP-MurNAc-pentapeptide and to provide D-Ala-D-Ala moieties in the cell wall precursors for transpeptidation activity of the penicillin-binding proteins PBP2 and PBP3. Actin-ortholog MreB functionally organizes MurF, MraY, and MurG (Gaballah et al., 2011), the last three enzymes in lipid II biosynthesis, at the septum. The synthesized precursor is translocated to the periplasm and processed by the concerted activity of the PBP enzymes and amidase AmiA to allow for bactoprenol-P recycling. In the process, the rudimentary by-product found by Liechti et al. (2013), in which the peptide side chains are cross-linked by peptide bonds, might result (Ghuysen and Goffin, 1999) and released muropeptides might contribute to modulation of the host immune response (McCoy and Maurelli, 2006). *Chlamydiaceae* lack transglycosylases as well as endopeptidases and pyrophosphorylases described so far to link lipid II sugar units to form glycan chains, to cleave peptide bridges between cross-linked glycan chains and to dephosphorylate bactoprenol-PP, respectively. Moreover, the L-Glu racemase MurI is absent. Question marks and dashed arrows highlight steps of the pathway that remain to be clarified. (GlcNAc: N-acetylglucosamine; MurNAc: N-acetylmuramic acid).

essential component in the biosynthesis of bacterial cell walls. The non-proteinogenic amino acid is synthesized by alanine racemases Alr and DadX and ligated by Ddl to form D-Ala-D-Ala. The dipeptide is attached to the amino acid in position 3 by the action of MurF to complete the pentapeptide side chain. In the first membrane-linked step, MraY catalyzes the synthesis of lipid I by transferring UDP-MurNAc-pentapeptide to the lipid carrier bactoprenol-phosphate (undecaprenyl-P). With the addition of UDP-GlcNAc, MurG synthesizes lipid II, the completed peptidoglycan cell wall building block. Lipid II is then translocated by the flippase FtsW to the outside of the cell and incorporated into the peptidoglycan network by the action of penicillin-binding proteins (PBPs) which exhibit transglycosylase and DD-transpeptidase activities.

The human cytosolic Pattern Recognition Receptors, Nod1 and Nod2, sensing bacterial cell wall fragments, recognize intracellular *C. pneumoniae* and subsequently mediate activation of the transcription factor NFkB which plays a key role in regulating

the immune response to infection (McCoy and Maurelli, 2006). Nod1 and Nod2 receptor mediated recognition, together with the susceptibilty to penicillin, suggests that cell wall precursors/peptidoglycan fragments are synthesized by *Chlamydiaceae* during infection.

A nearly complete lipid II biosynthesis pathway has been found in genomes of *Chlamydiaceae* (**Figure 1**) (McCoy and Maurelli, 2006), and functional conservation of enzymes catalyzing cytoplasmic steps (MurA, MurC/Ddl, CT390, DapF, MurE, MurF) and the two membrane-linked steps (MraY and MurG) of cell wall precursor biosynthesis has been demonstrated (McCoy and Maurelli, 2006; McCoy et al., 2006; Henrichfreise et al., 2009; Patin et al., 2009, 2012). *Chlamydiaceae* genomes code for only two PBPs that serve as DD-transpeptidases in free-living bacteria. PBP2 and PBP3 are the putative targets of penicillin in *Chlamydiaceae* and cross-link adjacent peptidoglycan strands via meso-diaminopimelic acid and D-Ala-D-Ala moieties in *E. coli*. AmiA is the only ortholog of septal peptidoglycan hydrolyzing amidases found in chlamydial genomes. Moreover, *Chlamydiaceae* harbor a rudimentary set of cell division proteins, lacking the central organizer FtsZ, but comprising FtsW, FtsI (PBP3) and FtsK, and possess, despite their spherical shape, the cytoskeletal protein MreB (Gaballah et al., 2011). Chlamydial MreB was shown to interact with key components in lipid II biosynthesis and FtsK (Gaballah et al., 2011; Ouellette et al., 2012).

We proposed that retaining biosynthesis of lipid II in cell wall-lacking "minimal bacteria," like *Chlamydiaceae,* may reflect a vital role of the lipid II pathway in prokaryotic cell division (Henrichfreise et al., 2009). Moreover, we discussed a model for the role of the conserved lipid II pathway in maintaining a functional divisome and contributing to modulation of host response in *Chlamydiaceae* (**Figure 1**). A recent study revealed the presence of cell wall sacculi in *Protochlamydia,* a genus of evolutionary older amoeba symbionts with less reduced genomes as compared to *Chlamydiaceae* (Pilhofer et al., 2013). In the pathogenic *Chlamydiaceae*, however, no functional cell wall but ring-like shaped structures were found and supposed to contain peptidoglycan-like material and localize to the division plane (Liechti et al., 2013). These findings are consistent with our model described above which implicates a crucial function of the PBP2 and PBP3 DD-transpeptidase activity in lipid II processing and sustaining a complete cycle of lipid II biosynthesis and recycling. PBP catalyzed DD-transpeptidation depends on the presence of the D-Ala-D-Ala terminus in the pentapeptide side chain of cell wall building blocks. Genomes of *Chlamydiaceae* do not encode homologs of the pyridoxal-5′-phosphate (PLP) cofactor requiring alanine racemases Alr and DadX. Therefore, the source of D-Ala and thus the presence of substrates for PBP2 and PBP3 activity in *Chlamydiaceae* have remained unclear for years.

We searched chlamydial genomes for genes encoding other PLP dependent proteins and found serine hydroxymethyltransferase GlyA to be conserved in all chlamydial genera. Serine hydroxymethyltransferases are found in eu- and prokaryotes and are well known for their function in reversible interconversion of serine and glycine using tetrahydrofolate as the one-carbon carrier. In addition, the enzymes show a particularly broad reaction specificity and catalyze other side reactions typical for PLP dependent enzymes, such as decarboxylation, transamination and retroaldol cleavage (Contestabile et al., 2001). Moreover, an alanine racemase co-activity was proven *in vitro* for GlyA from *E. coli* (Shostak and Schirch, 1988).

The aim of this study was to analyze GlyA as a potential source of D-Ala in *Chlamydiaceae*.

Here, we demonstrate that GlyA from *C. pneumoniae* is capable of the racemization of alanine *in vivo* and *in vitro* implicating that the enzyme can substitute for the absent alanine racemases and that D-Ala is self-synthesized in *Chlamydiaceae.*

RESULTS

RACEMIZATION OF ALANINE IN CHLAMYDIAE

Using BLAST alignments, we searched *Chlamydiaceae* and enviromental chlamydiae genomes to identify genes coding for orthologs of *E. coli* PLP cofactor-requiring enzymes known to confer alanine racemization activity (**Table 1**). In contrast to the *Chlamydiaceae* and *Simkania,* the three environmental chlamydiae genera *Parachlamydia, Protochlamydia*, and *Waddlia* harbored one ortholog of the Alr or DadX alanine racemases. GlyA

Table 1 | PLP cofactor-requiring enzymes involved in biosynthesis of D-Ala.

E. coli	Cpn	Ctr	Pac	Pam	Wch	Sne
Alr (alanine racemase)	–	–	–	pc0631 (3e–34)	wcw_0679 (1e–32)	–
DadX (alanine racemase 2)	–	–	PUV_23750 (2e–28)	–	–	–
GlyA (serine hydroxymethyltransferase)	CPn0521 (2e–107)	CT432 (2e–108)	PUV_05830 (6e–118)	pc0444 (3e–107)	wcw_1457 (2e–117)	SNE_A20270 (1e–114)
MetC (cystathionine beta-lyase)	–	–	PUV_18690 (2e–40)	–	wcw_1145 (4e–40)	–

Locus tags of genes coding for enzymes involved in biosynthesis of D-Ala are shown for two exemplary Chlamydiaceae and for environmental chlamydiae species. The expected (E) values of BLAST P alignments are listed in brackets. E. coli, Escherichia coli W3110 (NC_007779.1); Cpn, Chlamydia pneumoniae CWL029 (NC_000922); Ctr, Chlamydia trachomatis D/UW-3/Cx (NC_000117); Pac, Parachlamydia acanthamoebae UV-7 (NC_015702.1); Pam, Protochlamydia amoebophila UWE25 (NC_005861.1); Wch, Waddlia chondrophila WSU 86-1044 (NC_014225.1); Sne, Simkania negevensis Z (NC_015713.1).

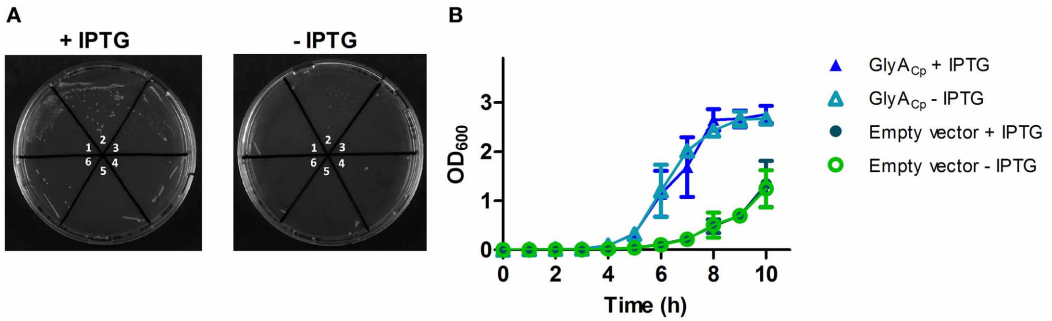

FIGURE 2 | GlyA$_{Cp}$ exhibits *in vivo* activity in an *E. coli* racemase double mutant. A temperature sensitive Δ*alr*Δ*dadX E. coli* double mutant was transformed with pET21b-*glyA*$_{Cp}$ to allow for the expression of GlyA$_{Cp}$ in the cytoplasm. Independently generated transformants (1–3 containing pET21b-*glyA*$_{Cp}$ and 4–6 containing the empty vector) were grown on solid **(A)** or in liquid **(B)** LB medium under limited D-Ala growth conditions at 42°C. LB medium was supplemented with 5 mg/L D-Ala, 50 μM of cofactor PLP, 25 μg/ml thymine and 50 μg/ml ampicillin. Expression of GlyA$_{Cp}$ was induced by the addition of 0.1 mM IPTG.

was the only enzyme to be encoded in *Chlamydiaceae* and in all enviromental chlamydiae.

IN VIVO ACTIVITY OF GlyA FROM *C. pneumoniae*

In the absence of a tractable system to genetically manipulate *C. pneumoniae* we tested whether heterologous expressed *C. pneumoniae* GlyA (GlyA$_{Cp}$) shows an effect on the D-Ala auxotrophic phenotype of an *E. coli* Δ*alr*Δ*dadX* racemase double mutant strain. Our experiments in liquid and solid culture revealed that chlamydial GlyA did not completely reverse the need of exogenous D-Ala of the racemase mutant strain but favored its growth under D-Ala limited conditions (**Figure 2**). These findings suggest that GlyA$_{Cp}$ is a functional alanine racemase and capable of generating D-Ala in *E. coli*.

RECOMBINANT GlyA$_{Cp}$ HAS L-ALANINE RACEMASE ACTIVITY

To investigate the potential alanine racemase activity of the serine hydroxymethyltransferase GlyA from *C. pneumoniae*, we overexpressed recombinant GlyA$_{Cp}$ in *E. coli* and purified the Strep-tagged protein. *In vitro* activity of GlyA$_{Cp}$ was tested in a D-amino acid oxidase coupled enzymatic assay containing L-Ala and cofactor PLP. Alanine racemase from *Bacillus stearothermophilus* served as positive control. D-Ala that was produced by GlyA was converted to pyruvate by the activity of D-amino acid oxidase (DAAO) and colorimetrically quantified. The chlamydial GlyA converted L-Ala to D-Ala *in vitro* exhibiting weak racemase activity in comparision to the enzyme from *B. stearothermophilus* (**Figure 3**).

GlyA$_{Cp}$ RACEMASE ACTIVITY IS SENSITIVE TO D-CYCLOSERINE

D-cycloserine is a structural analog of D-Ala and competitively inhibits activity of alanine racemases and D-Ala ligases from free-living bacteria (Strominger et al., 1960; Lambert and Neuhaus, 1972). The inhibitor has anti-chlamydial activity in chick embryo yolk sac infection (Moulder et al., 1963) which can be reversed by the addition of D-Ala. For the *C. trachomatis* D-Ala ligase Ddl which is encoded as a fusion with MurC, as typical for *Chlamydiaceae*, sensitivity to D-cycloserine has been proven

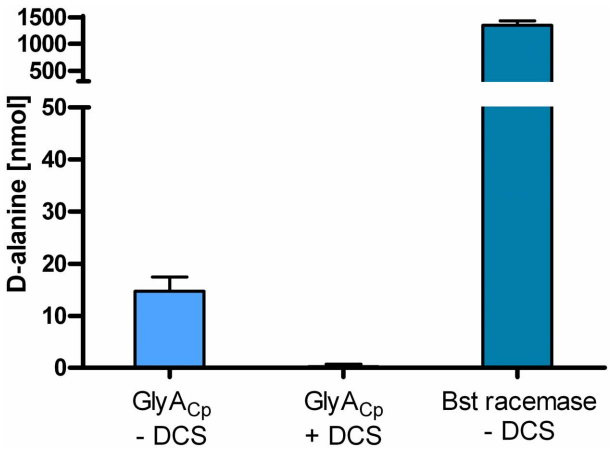

FIGURE 3 | The purified serine hydroxymethyltransferase GlyA$_{Cp}$ has alanine racemase activity. *In vitro* activity of recombinant GlyA$_{Cp}$ was tested in a coupled enzymatic assay containing L-Ala and cofactor PLP. D-Ala that was produced by 1 μg GlyA or Bst racemase was converted to pyruvate by the activity of DAAO and colorimetrically quantified. (DCS: D-cycloserine; Bst racemase: alanine racemase from *B. stearothermophilus*).

before (McCoy and Maurelli, 2005). We performed activity assays for GlyA$_{Cp}$ in the presence of D-cycloserine and identified the enzyme to be a second target of D-cycloserine in *Chlamydiaceae* (**Figure 3**).

DISCUSSION

The source of D-Ala in *Chlamydiaceae* and thus the presence of the transpeptidation substrates for PBPs is a crucial aspect of the chlamydial anomaly that will help to gain understanding of the penicillin induced persistence in these human pathogens. Penicillin and other beta-lactams structurally mimic the D-Ala-D-Ala terminus of the pentapeptide side chain of the lipid II cell wall building blocks and are recognized by the active sites of DD-transpeptidase PBPs. Beta-lactams are active against *Chlamydiaceae* in cell culture (McCoy and Maurelli, 2006; Skilton

et al., 2009) and *in vivo* (Phillips Campbell et al., 2012) and the chlamydial monofunctional DD-transpeptidases PBP2 and PBP3, recovered in detergent-soluble fractions from whole cell preparations, bind [H^3]-penicillin (Barbour et al., 1982). Moreover, the recombinant MurC/Ddl protein from *C. trachomatis* has been shown to specifically ligate D-Ala but not the L-Ala enantiomer to form alanine dipeptides and MurF was demonstrated to add these D-Ala-D-Ala dipeptides to the lipid II peptide side chain (McCoy and Maurelli, 2006; Patin et al., 2012). Moulder et al. (1963) proved anti-chlamydial activity of D-cycloserine in chick embryo yolk sac infection models and demonstrated the specific reversal of this effect by the addition of D-alanine (Moulder et al., 1963). D-cycloserine is a structural analog of D-Ala and well known as competitive inhibitor of alanine racemases and the D-Ala ligase Ddl in other bacteria (Strominger et al., 1960; Lambert and Neuhaus, 1972). Finally, feeding of replicating *Chlamydia trachomatis* with D-Ala-D-Ala probes revealed evidence for the incorporation of D-Ala into ring-like shaped peptidoglycan-like structures (Liechti et al., 2013). All in all, these data strongly indicate that D-Ala is present in the cells of *Chlamydiaceae* and plays an essential role in chlamydial cell biology. In the past, the mammalian host was discussed as a potential source of D-Ala (McCoy and Maurelli, 2006) and in *Chlamydiaceae* genomes a D-alanine permease (DagA_2) was annotated (Stephens et al., 1998) that could allow for the passive transport of D-Ala over the chlamydial cytoplasmic membrane. Though D-Ala is found almost exclusively in the microbial world and is not synthesized in mammalian cells, D-Ala was detected in trace quantities in mammalian urine and tissues, apparently due to breakdown products from intestinal and food bacteria (Guoyao, 2013). Nevertheless, experiments with *Listeria monocytogenes* and *Shigella flexneri* indicated that mammalian host cells cannot serve as source of D-Ala. Alanine racemase knockout mutants of both facultative intracellular species failed to survive within mammalian host cells unless exogenous D-Ala was added to the cell culture medium (McCoy and Maurelli, 2006). Based on these data, self-biosynthesis of D-Ala by alternative racemase activity is more likely the source of D-Ala in *Chlamydiaceae*.

For *E. coli*, besides the constitutive expressed Alr racemase and the catabolic DadX racemase, two other PLP dependent enzymes involved in the methionine pathway have been shown to confer L-Ala racemization as a side reaction. Overexpressed cystathionine beta-lyase MetC completely reversed the D-Ala auxotrophic phenotype of an *E. coli* racemase double mutant whereas racemase co-activity of serine hydroxymethyltransferase GlyA was not sufficient to allow for growth on D-Ala lacking medium (Kang et al., 2011). Recently, *in vitro* activity of MetC from *Wolbachia* indicated that the enzyme substitute for the absent alanine racemases in these endosymbionts of many arthropods and filarial nematodes. *Wolbachia* are excellent targets for anti-filarial therapy. Similar to *Chlamydiaceae*, these obligate intracellular bacteria lack a cell wall but treatment with lipid II biosynthesis blocking fosfomycin, results in enlarged *Wolbachia* cells (Vollmer et al., 2013). Among the *Chlamydiales* only the environmental *Parachlamydia* and *Waddlia* possess a MetC ortholog but all chlamydial genera harbor GlyA. The serine hydroxymethyltransferase is the only component of the methionine pathway that is encoded by *Chlamydiaceae* genomes and phylogenetic analysis indicated lateral transfer of the *glyA* gene from Actinobacteria to the common ancestor of *Chlamydiales* (Griffiths and Gupta, 2006). Moreover, transcription profiles revealed overlapping expression of the genes encoding GlyA, enzymes for lipid II biosynthesis (MurA to MurF, MraY, and MurG) as well as processing (PBP2, PBP3, and AmiA), the structural protein MreB, and the cell division proteins FtsW and FtsK (Belland et al., 2003). These data suggest an essential function of GlyA in chlamydial biology and correlation with the cellular processes of lipid II biosynthesis and cytokinesis.

We demonstrated L-Ala racemase activity for the GlyA enzyme from *C. pneumoniae in vivo* in an *E. coli* racemase double mutant and characterized the purified protein *in vitro*. Moreover, we identified GlyA as a second target of D-cycloserine besides MurC/Ddl in *Chlamydiaceae*.

Our results implicate that the enzyme can substitute for the absent alanine racemases and that D-Ala is present and self-synthesized in *Chlamydiaceae*. The observed weak alanine racemase activity of $GlyA_{Cp}$ cannot completely compensate D-Ala requirements of the *E. coli* racemase mutant to build a functional cell wall but might be sufficient to produce D-Ala in amounts that maintain the lipid II biosynthesis pathway in the cell wall-lacking *Chlamydiaceae* for the proposed functions of the cell wall precursor in co-ordination of cell division and modulation of the host immune response. Future research toward the elucidation of the chlamydial anomaly will include the isolation and structural characterization of lipid II building blocks and biochemical analysis of the penicillin binding proteins PBP2 and PBP3, the putative targets of penicillin whose activity depends on the presence of D-Ala-D-Ala in the pentapeptide side chain of lipid II.

Like other effectors, such as interferon-γ and tumor necrosis factor-α, penicillin can be used to induce persistence as an experimental tool to study chlamydiae/host interactions. Knowledge of the underlying mechanisms of penicillin induced formation of ABs will help to assess these results on the pathogenicity of *Chlamydiaceae*.

Moreover, analysis of the molecular biology of penicillin induced persistence is important to improve understanding of long-term infection in patients in particular as to the role of chlamydial cell wall precursors in immune modulation and refractory of ABs to anti-chlamydial agents.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

E. coli JM83 harboring the $GlyA_{Cp}$ expression vector was grown on Luria Bertani (LB) agar plates containing 30 μg/ml chloramphenicol and 100 μg/ml ampicillin, respectively. The temperature sensitive *E. coli* Δ*alr*Δ*dadX* racemase double mutant TKL-10 was maintained on LB agar plates containing 25 μg/ml thymine.

IN VIVO COMPLEMENTATION

E. coli TKL-10 Δ*alr*Δ*dadX* was transformed with pET21b-$glyA_{Cp}$ and grown in liquid or on solid LB medium supplemented with 5 mg/L D-Ala, 50 μM PLP, 25 μg/ml thymine, 50 μg/ml ampicillin and 0.1 mM IPTG at 42°C. For each experiment three independently generated transformants were used and controls with

E. coli TKL-10 Δ*alr*Δ*dadX* harboring the empty pET21b vector were carried out.

CLONING OF *glyA*

The *glyA* gene from *C. pneumoniae* GiD was amplified by PCR using primer glyACp_f (5′-ATGGTAGGTCTCAGGCCTT GCTAAAAGTTTTTGAGAAATTTAAGA-3′) and glyACp_r (5′-ATGGTAGGTCTCAGCGCTAACTAAAGCTTCTAAATCAAT TTCAGG-3′) and cloned into pASK-IBA2c (IBA, Germany) using the BsaI restriction site to generate an N-terminal OmpA-leader peptide fused, C-terminal Strep-tagged protein for periplasmic overproduction. For cytoplasmic expression in complementation assays, *glyA* was amplified with primers glyACp_pET21_f (5′-CGTCTTTAGAAGCATATGCTAAAAG-3′) and glyACp_pET21_r (5′-GTCTCTGCGGCCGCAACTAAAG CTTC-3′) and cloned into pET21b (Novagen, VWR, Germany) using NdeI and NotI restriction sites.

OVERPRODUCTION AND PURIFICATION OF $GlyA_{Cp}$

E. coli JM83 cells, transformed with pASK-IBA2c-*glyA*$_{Cp}$, were grown in no salt LB in presence of 30 μg/ml of chloramphenicol, 250 mM sucrose and 50 mM L-serine at 30°C. After induction at an OD_{600} of 1.2 with 200 ng/ml anhydrotetracyline (AHT), 50 μM PLP and 200 μM of folinic acid were added and the cells were incubated for 4 h at 25°C. The purification of $GlyA_{Cp}$ was performed using the protocol for cleared lysates recommended by the manufacturer (IBA, Germany) with small modifications: the buffers contained 2% N-lauroylsarcosine (or 0.1% N-lauroylsarcosine in the washing and elution buffer), 2 mM 1,4-dithiothreitol (DTT) and 50 μM PLP. Purity of the protein was controlled using SDS-PAGE.

IN VITRO $GlyA_{Cp}$ ACTIVITY ASSAY

Racemase activity of $GlyA_{Cp}$ was determined in a DAAO coupled enzymatic assay system as described previously with slight modifications (Francois and Kappock, 2007). Briefly, 1 μg $GlyA_{Cp}$ or DAAO were incubated with 50 mM L-alanine in a final volume of 60 μl for 16 h at 37°C in 50 mM KH_2PO_4, pH 8, 100 mM KCl, 80 μM PLP and 2 mM DTT. D-Ala that was derived from $GlyA_{Cp}$ racemization was deaminated into pyruvate by the activity of DAAO and indirectly quantified by determining the amount of produced pyruvate with a colorimetric assay using 2,4-dinitrophenylhydrazine (DNPH) as described before (Milner and Wood, 1976). Alanine racemase from *B. stearothermophilus* (Sigma-Aldrich, Germany) was used as positive control. PLP containing enzymes have been described to show weak transamination activity converting alanine to pyruvate. As a control for potential L-Ala transamination activity of $GlyA_{Cp}$, we ran experiments in the absence of DAAO. No $GlyA_{Cp}$ catalyzed production of pyruvate was detected. For $GlyA_{Cp}$ inhibition assays, 10 mM D-cycloserine was added and both enzymatic steps were carried out consecutively with a step of heat deactivation in between as DAAO is sensitive to D-cycloserine inhibition.

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Porcine epidemic diarrhea virus (PEDV) co-infection induced chlamydial persistence/stress does not require viral replication

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Chlamydiae may exist at the site of infection in an alternative replicative form, called the aberrant body (AB). ABs are produced during a viable but non-infectious developmental state termed "persistence" or "chlamydial stress." As persistent/stressed chlamydiae: (i) may contribute to chronic inflammation observed in diseases like trachoma; and (ii) are more resistant to current anti-chlamydial drugs of choice, it is critical to better understand this developmental stage. We previously demonstrated that porcine epidemic diarrhea virus (PEDV) co-infection induced *Chlamydia pecorum* persistence/stress in culture. One critical characteristic of persistence/stress is that the chlamydiae remain viable and can reenter the normal developmental cycle when the stressor is removed. Thus, we hypothesized that PEDV-induced persistence would be reversible if viral replication was inhibited. Therefore, we performed time course experiments in which Vero cells were *C. pecorum*/PEDV infected in the presence of cycloheximide (CHX), which inhibits viral but not chlamydial protein synthesis. CHX-exposure inhibited PEDV replication, but did not inhibit induction of *C. pecorum* persistence at 24 h post-PEDV infection, as indicated by AB formation and reduced production of infectious EBs. Interestingly, production of infectious EBs resumed when CHX-exposed, co-infected cells were incubated 48–72 h post-PEDV co-infection. These data demonstrate that PEDV co-infection-induced chlamydial persistence/stress is reversible and suggest that this induction (i) does not require viral replication in host cells; and (ii) does not require *de novo* host or viral protein synthesis. These data also suggest that viral binding and/or entry may be required for this effect. Because the PEDV host cell receptor (CD13 or aminopeptidase N) stimulates cellular signaling pathways in the absence of PEDV infection, we suspect that PEDV co-infection might alter CD13 function and induce the chlamydiae to enter the persistent state.

Keywords: *Chlamydia pecorum*, chlamydial persistence, chlamydial stress response, stressed chlamydiae, porcine epidemic diarrhea virus

INTRODUCTION

The *Chlamydiaceae* are Gram-negative, obligate intracellular bacteria that cause a large spectrum of diseases in both humans and agriculturally important animals. For example, *Chlamydia suis*, *C. abortus*, *C. pecorum* and *C. psittaci* cause syndromes in swine ranging from conjunctivitis to abortion (Pospischil et al., 2010). Asymptomatic chlamydial infections are also common in pigs and can render them more susceptible to other infections (reviewed in Schautteet and Vanrompay, 2011). Related chlamydial species, such as *C. trachomatis*, also cause medically important conditions, like trachoma, in humans. Though chlamydial infections can cause acute symptoms, they are most associated with chronic inflammation and scarring, which can result in significant host tissue damage (Schachter, 1999). However, to play a causative role in chronic diseases, chlamydiae would need to persist within infected cells/tissues for extended periods of time. How the organisms maintain long-term host infection is a central question in chamydial biology.

Chlamydiae are characterized by a complex developmental cycle, in which they alternate between a metabolically less-active, infectious form (the elementary body or EB), and a more metabolically active, replicative form (the reticulate body or RB). Upon host cell infection, the EB converts into an RB, which grow and divide within a cytoplasmic, membrane-bound inclusion. After several rounds of division, RBs then convert back into infectious EBs, which are released from the host cell (reviewed in Wyrick, 2000). The third developmental stage, variously termed persistence or the chlamydial stress response, is defined as a viable but non-cultivable state. Persistent/stressed RBs are enlarged, irregularly shaped and non-dividing; these altered developmental forms are called aberrant bodies (ABs). A variety of stressors induce chlamydial persistence/stress: these include IFN-γ exposure; glucose, iron, and amino acid depravation; penicillin G exposure; and heat shock (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). Interestingly, chlamydiae can remain in the persistent/stressed

state in culture for up to 9 months (Galasso and Manire, 1961). Once the stressor is removed, persistent/stressed chlamydiae can reenter normal development and produce infectious EBs, which suggests that persistent/stressed chlamydiae may serve as a long-term reservoir for pro-inflammatory chlamydial antigens and/or infectious organisms (reviewed in Hogan et al., 2004; Wyrick, 2010). Although this hypothesis has not yet been directly tested, there is significant evidence that the persistent/stressed state can occur during *in vivo* infection. For example, ABs have been observed in tissues isolated from *C. suis*-infected swine (Pospischil et al., 2009), *C. muridarum*-infected mice (Rank et al., 2011; Phillips-Campbell et al., 2012) and *C. trachomatis*-infected humans (Nanagara et al., 1995).

Mixed infections are prevalent in both humans and other animals and may alter pathogenesis of one, or more, of the agents involved (recently reviewed in Debiaggi et al., 2012; Stelekati and Wherry, 2012; Alizon et al., 2013). Unfortunately, typical experimental systems exploring the interaction between a single pathogen and cell type do not accurately reflect host-multiple pathogen interplay observed *in vivo*. Therefore, it seems worthwhile to test interactions between multiple pathogenic microorganisms in simplified cell culture systems. In one such system, chlamydiae within Herpes Simplex Virus (HSV) super-infected genital epithelial cells entered the persistent/stressed state (Deka et al., 2006, 2007) via a mechanism distinct from previously characterized models of chlamydial persistence (Vanover et al., 2008). More recent data indicate that HSV glycoprotein D/host nectin-1 interaction restricts *C. trachomatis* development (Vanover et al., 2010) by an as yet incompletely characterized mechanism involving increased host cellular oxidative stress (Prusty et al., 2012). Both HSV (Deka et al., 2006, 2007; Vanover et al., 2010) and Human Herpes Virus 6 (Prusty et al., 2012) induce persistence by mechanisms that are independent of productive virus infection, but require host cell attachment and/or uptake of the virus by the host cell. As we are interested in chlamydial and viral swine pathogens, our group established a culture model of porcine epidemic diarrhea virus (PEDV)/*C. pecorum* co-infection (Stuedli et al., 2005). Both *C. pecorum* and PEDV (a coronavirus) cause economically-important gastrointestinal infections in swine (Pensaert and de Bouck, 1978; Pospischil et al., 2010). PEDV super-infection of *C. pecorum*-infected Vero cells: (i) induced AB formation; and (ii) reduced chlamydial infectivity, both of which are consistent with induction of the persistence/stress response (Borel et al., 2010). Notably, herpesviruses (which are double-stranded DNA viruses) and coronaviruses (which are single-stranded RNA viruses) use different attachment/entry mechanisms, replicate in different cellular compartments, replicate their genomes via different mechanisms, and infect different host cell types. Thus, it seems unlikely that HSV and PEDV induce chlamydial persistence/stress by the same mechanism. As a first step in dissecting the mechanism by which PEDV super-infection alters chlamydial development, we tested the hypothesis that viral replication is required for the PEDV-mediated *C. pecorum* persistence/stress response.

MATERIALS AND METHODS

HOST CELLS, CHLAMYDIAE, AND VIRUSES

Vero 76 cells (African green monkey kidney cells, CRL 1587, American Type Culture Collection) were propagated in growth medium: Minimal Essential Medium (MEM) with Earle's salts, 25 mM HEPES, without L-glutamine (GIBCO, Invitrogen, Carlsbad, CA) but with 10% fetal calf serum (FCS) (BioConcept, Allschwil, Switzerland), 4 mM GlutaMAX-I (200 mM, GIBCO) and 0.2 mg/ml gentamycin (50 mg/ml, GIBCO). For infection experiments, Vero cells were seeded on round glass coverslips (13 mm diameter, Thermo Fisher Scientific, Cambridge, UK) at 2×10^5/well in growth medium without gentamycin. *Chlamydia pecorum* 1710S (an intestinal swine isolate kindly provided by J. Storz, Baton Rouge, Louisiana, LA, USA) was used in this study. Stocks of *C. pecorum* were propagated in HEp-2 cell monolayers, purified and stored at −80°C in sucrose-phosphate-glutamate (SPG) medium as described (Borel et al., 2010). An MOI of 1 of *C. pecorum* was used for all mono-infection and mixed-infection experiments. Both *C. pecorum* and *C. abortus* development is altered by PEDV co-infection (Stuedli et al., 2005; Borel et al., 2010), but *C. pecorum* was chosen for this study because it is more sensitive to PEDV co-infection than is *C. abortus* (Borel et al., 2010). Ca-PEDV strain CV777 (kindly provided by M. Ackermann, Institute of Virology, University of Zurich) was propagated as previously described (Hofmann and Wyler, 1988), but without antibiotics for culturing the cells and for viral stock preparation. The virus stock ($1 \times 10^{5.5}$ TCID50/ml) was used undiluted for mixed-infections.

MIXED-INFECTION PROTOCOL

Mixed-infections were performed essentially as described (Borel et al., 2010). Briefly, replicate Vero cells on coverslips were divided into four groups: mock-infected, *C. pecorum*-infected, PEDV-infected, and *C. pecorum*/PEDV co-infected. For co-infections, cell monolayers were first infected with *C. pecorum* at 1 MOI. After centrifugation for 1 h at 1000 × g and 25°C, the infected monolayers were subsequently incubated for 14 h at 37°C in growth medium without gentamycin. At time 0 (T0), all cell monolayers used for either mixed-infection or PEDV mono-infection were PEDV-infected ($1 \times 10^{5.5}$ TCID50), whereas for chlamydial mono-infections and mock-infections, only growth medium was applied. In some experiments, an equal volume of UV-inactivated PEDV ($PEDV_{UV}$) was used. After viral infection, all cells were centrifuged again, after which the inoculum was removed, the cells refed with growth medium without gentamycin, and incubated for an additional 24, 48, 72, or 96 h, depending upon the experiment (**Figure 1A**). Replicate samples were then subjected to immunofluorescence (IF), transmission electron microscopy (TEM), or infectious titer analysis, as appropriate. In some experiments, cycloheximide (CHX), which inhibits host cellular (Obrig et al., 1971), coronaviral (van den Worm et al., 2011) but not chlamydial (Ripa and Mårdh, 1977) protein synthesis, was added to the culture medium 1 h before PEDV infection. In these experiments, all cultures were incubated in growth medium plus either 1 or 5 ug/ml CHX from the addition time until the end of the experiment. Since both 1

and 5 μg/ml CHX inhibit PEDV replication (**Figure 1**) but not *C. pecorum* development (**Figure 2**), 5 μg/ml CHX was used in time course/recovery experiments to suppress PEDV replication.

UV INACTIVATION OF PEDV

Five hundred microliter aliquots of PEDV stock were UV-inactivated using a UV 500 crosslinker (Amersham Biosciences, Little Chalfont, UK), as described (Deka et al., 2007), using a total UV dose of 4 J/cm^2. Similar UV doses have been used to inactivate other coronaviruses (Darnell et al., 2004). UV-inactivated PEDV stocks were unable to induce PEDV M protein positive staining syncytium formation when inoculated into Vero cell monolayers, even after a 48 h incubation period (data not shown). In contrast, control cultures infected with replication competent PEDV showed PEDV positively staining single cells and syncytia in this same time period. These data indicate that the PEDV stocks were successfully UV-inactivated.

IMMUNOFLUORESCENCE ASSAYS (IFA)

Infected monolayers on coverslips were methanol fixed and IFA stained immediately post-fixation as described (Borel et al., 2010). PEDV-infected single cells (and syncytia) were detected using a mouse monoclonal antibody against the viral 27 kD integral membrane M protein (mcAb 204, kindly provided by M. Ackermann, Institute of Virology, University of Zurich), diluted 1:4 in PBS plus 1% BSA, and a 1:500 diluted Alexa Fluor 594-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, USA). Chlamydial inclusions were labeled with a *Chlamydiaceae* family-specific mouse monoclonal antibody directed against lipopolysaccharide (LPS, Clone ACI-P, Progen, Heidelberg, Germany), and a 1:500 diluted Alexa Fluor 488-conjugated secondary goat anti-mouse antibody (Molecular Probes). Host and chlamydial DNA were labeled using 1 μg/ml 4′,6-Diamidin-2′-phenylindoldihydrochlorid (DAPI, Molecular Probes). As both primary antibodies were of mouse origin, PEDV and chlamydia-specific labeling were performed on separate, duplicate coverslips. Coverslips were mounted inverted on glass slides using Immumount (Shandon, Pittsburgh, USA). Samples were examined under oil immersion at 1000× magnification using a Leica DMLB fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Fluorescence photomicrographs were captured with the BonTec measuring and archiving software (BonTec, Bonn, Germany).

TRANSMISSION ELECTRON MICROSCOPY (TEM)

Coverslips were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, USA) for 1 h, and processed for embedding in epoxy resin (Borel et al., 2010). Ultrathin sections (80 nm) were mounted on gold grids (Merck Eurolab AG, Dietlikon, Switzerland), contrasted with uranyl acetate dihydrate (Fluka), and lead citrate (lead nitrate and tri-natrium dihydrate; Merck Eurolab AG). Fixed and counterstained gold thin sections were examined at 7000× magnification with a Tecnai 10 (FEI) transmission electron microscope at 60–80 kV in the Quillen College of Medicine TEM Core Facility.

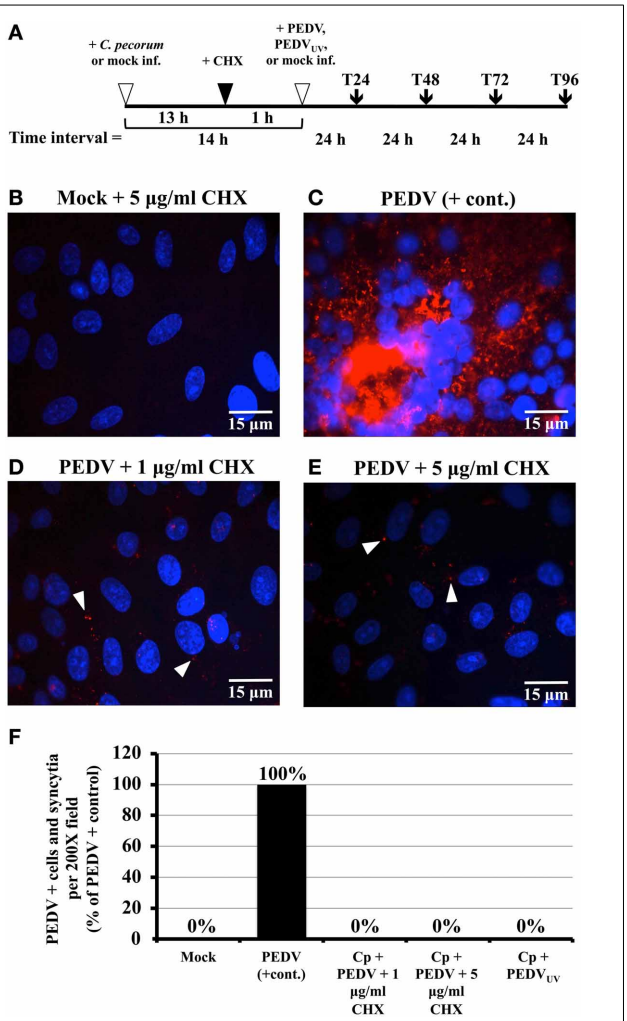

FIGURE 1 | Exposure to cycloheximide (CHX) and UV inactivation inhibits PEDV replication in Vero cells. (A) Simplified diagram of the co-infection procedure. At the start of each experiment, replicate Vero cell cultures on coverslips were either mock- or *C. pecorum*-infected. Fourteen hours later, they were mock-, PEDV- or PEDV$_{UV}$-infected, as appropriate. In some experiments, cells were CHX-exposed from 1 h prior to viral infection to the end of the experiment. Regardless, all cells were refed with fresh growth medium (+ or − CHX as appropriate) after PEDV infection (T0). Samples were harvested for various analyses at 24 (T24), 48 (T48), 72 (T72) or 96 (T96) hpvi. **(B–E)** Vero cells were pre-exposed to either 1 μg/ml CHX or 5 μg/ml CHX, as indicated. One hour later, cultures were either PEDV-infected (PEDV) or mock-infected (Mock). Cultures were incubated for 48 hpvi, fixed, and immunolabeled with anti-PEDV M (red) and DAPI (blue). Representative fields at 1000× magnification are shown with 15 μm scale bars. White arrows indicate anti-PEDV M punctate staining, as discussed in the text. **(F)** Vero cells were mock-infected (Mock), mono-infected with PEDV (PEDV + cont.), infected with *C. pecorum* and PEDV (Cp + PEDV), or infected with *C. pecorum* and PEDV$_{UV}$ (Cp + PEDV$_{UV}$) as in **(A)**. In some cases, the cells were pre-exposed to 1 μg/ml CHX or 5 μg/ml CHX, as indicated. At 24 hpvi, cells were fixed, and labeled with anti-PEDV M (red) and DAPI (blue). Twenty random 200× magnification fields were examined on each coverslip; PEDV M protein positive single cells and syncytia were counted for each field, added together, and the average total number/field calculated. The PEDV positive control was set at 100% and the average counts obtained for the other samples were used to calculate % of the positive control. The % positive control values obtained were then plotted on the Y-axis; sample identity is shown below the X-axis.

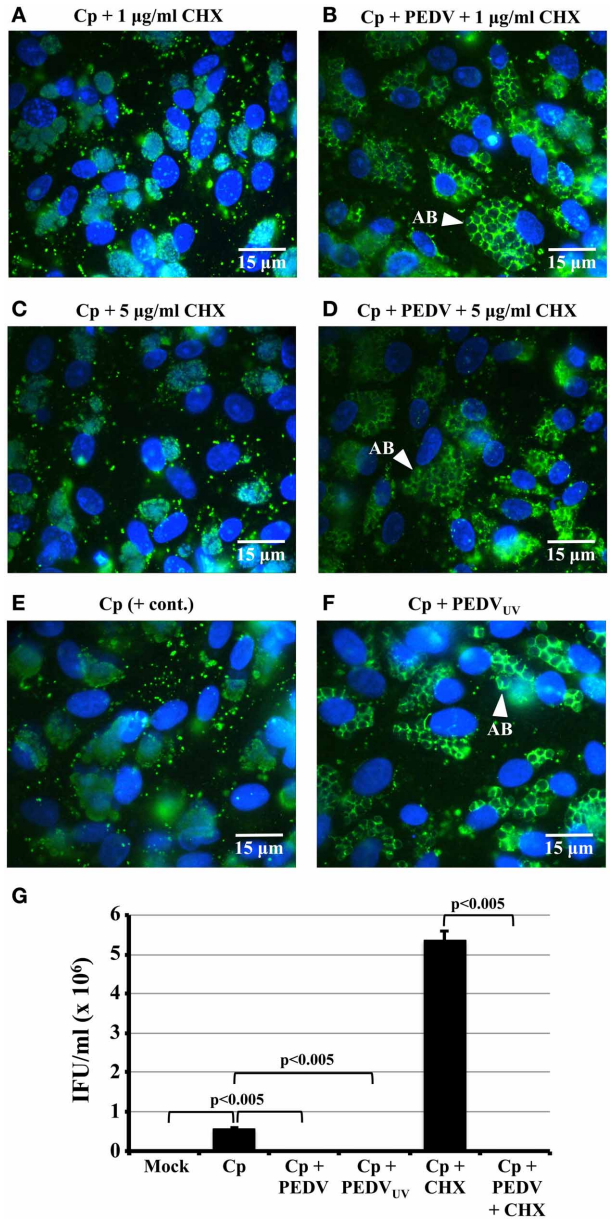

FIGURE 2 | PEDV viral replication is not required for PEDV interference with *C. pecorum* development. (A–F) Vero cells were mock-infected (Mock), mono-infected with *C. pecorum* (Cp), infected with *C. pecorum* and PEDV (Cp + PEDV), or infected with *C. pecorum* and PEDV$_{UV}$ (Cp + PEDV$_{UV}$) as diagrammed in **Figure 1A**. In some cases, the cells were pre-exposed to either 1 or 5 μg/ml CHX to prevent PEDV replication, as indicated. At 24 hpvi, replicate coverslips were fixed, and labeled with anti-chlamydial LPS (green) and DAPI (blue) or anti-PDV M protein and DAPI. Quantification of PEDV positive cells and syncytia from this experiment is shown in **Figure 1F**. Representative fields at 1000× magnification are shown with 15 μm scale bars. White arrows indicate anti-LPS-staining AB. **(G)** Replicate cultures from the co-infections in **(A–F)** were subjected to sub-passage titer analysis. Some monolayers were pre-exposed to 5 μg/ml CHX (+ CHX samples) as previously described. Inclusion counts were used to calculate inclusion forming units (IFU)/mL (Y-axis). The average from three biologic replicates ± s.e.m. is shown and the data are representative of three independent experiments. Statistical comparisons are indicated by brackets and were all significant to $p \leq 0.005$.

CHLAMYDIAL TITRATION BY SUBPASSAGE

Depending upon the experiment, monolayers were scraped into 1 ml of cold growth medium at 24, 48, or 72 h post viral infection (hpvi). Infected host cell lysates were harvested and sub-passaged on fresh Vero cell monolayers in triplicate as described (Borel et al., 2010). Fixation and staining with DAPI and anti-chlamydial LPS was performed as described above. The number of inclusions in 20 random microscopic fields per sample was determined using a Leica fluorescence microscope at 200× magnification. Triplicate coverslips were counted and the counts averaged for each coverslip. The number of inclusion-forming units (IFU) in the undiluted inoculum was then calculated and expressed as IFU per ml inoculum as described (Deka et al., 2006).

STATISTICAL ANALYSES

With the exception of the TEM experiments, all experiments were repeated three times independently. TEM experiments were performed twice. Statistical analyses for chlamydial titrations were performed using Microsoft Excel. The IFU/ml value for each biologic replicate is the mean of three determinations (3 replicate titer coverslips). All plotted IFU/ml values are averages of three biologic replicates ± standard error of the mean (SEM). These were compared using a 2-sample *t*-test for independent samples and *p* values of ≤ 0.05 were considered significant.

RESULTS

PEDV REPLICATION IN VERO CELLS IS INHIBITED BY UV-INACTIVATION AND CHX EXPOSURE

Although there is a newly developed system for PEDV RNA recombination and gene replacement (Li et al., 2013a), there is currently no available system for complementing/propagating PEDV replication-deficient mutants. As a result, we could not use such mutants to address our hypothesis. Therefore, we used UV inactivation and host cell CHX pre-treatment to inhibit PEDV replication in co-infected cells. A similar approach was used to determine that HSV replication was required for chlamydial persistence/stress induction (Deka et al., 2007). CTX was chosen because it inhibits host mammalian cell (Obrig et al., 1971) and coronaviral (van den Worm et al., 2011) protein synthesis, but not that of chlamydiae (Ripa and Mårdh, 1977). UV light has been used to inactivate other coronaviruses (Darnell et al., 2004) and is widely used to inactivate virions without altering their ability to bind and enter host cells. To test the efficacy of these inactivation methods for PEDV, Vero monolayers were pre-exposed for 1 h to growth medium plus: (i) 1 μg/ml CHX; or (ii) 5 μg/ml CHX. CHX was also added to the PEDV inoculum and to the culture medium after infection. Cultures were incubated 48 h post-infection (hpi) and then fixed, IFA stained to detect PEDV antigens and examined microscopically for PEDV-positive cells and syncytia (**Figures 1B–E**). During viral replication, PEDV antigen-positive cells and syncytia with up to 50–100 nuclei are observed (Hofmann and Wyler, 1988; Borel et al., 2010) and, because coronaviruses replicate in the host cell cytoplasm, anti-M protein staining is primarily cytoplasmic (**Figure 1C**; Borel et al., 2010). Thus, these characteristics can be used to determine whether PEDV productive replication has occurred during an experiment. As expected, positively

staining single infected cells and syncytia were readily detectable in PEDV-infected cultures (**Figure 1C**). Addition of either 1 or 5 μg/ml CHX prior to PEDV infection eliminated PEDV antigen-positive single cells and syncytium formation (**Figures 1D,E**). Likewise, Vero monolayers infected with UV-inactivated PEDV ($PEDV_{UV}$) contained neither M protein cytoplasmically-positive cells nor syncytia (data not shown). These data indicate that host cell pre-exposure to CHX and UV-inactivation both inhibit PEDV replication. Though strong cytoplasmic anti-M protein staining is not observed in $PEDV_{UV}$-infected or CHX-pre-exposed cultures, small dots of anti-M protein immunostaining are observed in these cultures (**Figures 1D,E**, white arrows). These anti-M foci are not observed in uninfected cultures (**Figure 1B**) and are unlikely to be background staining. As the M protein is an abundant structural component of coronavirus particles, these foci are most likely immunolabeled PEDV virions that have bound to and/or entered into host cells. These data suggest that CHX and UV inactivation inhibit PEDV replication but not viral attachment/entry.

PEDV REPLICATION IS NOT REQUIRED TO ALTER *C. pecorum* DEVELOPMENT

To determine whether PEDV replication is required to alter *C. pecorum* development, Vero monolayers were infected first with *C. pecorum* and later with PEDV, or mock-infected, as described above. In some replicates (**Figures 2A–D**), cells were pre-exposed to 1 or 5 μg/ml CHX before viral infection. In others, cells were co-infected with $PEDV_{UV}$ in the absence of CHX (**Figure 2F**). As expected, neither mock- nor PEDV singly-infected cells stained with anti-LPS (**Figures S1A,D**). IFA staining with anti-LPS revealed normal inclusions in *C. pecorum*-infected control cells (**Figure 2E**, **Figures S1B,C**). In contrast, inclusions within *C. pecorum*/PEDV co-infected cells contained anti-LPS tagged, greatly enlarged AB (**Figures S1E,F**, white arrows), as previously reported (Borel et al., 2010). Exposure to either 1 μg/ml (**Figure 2A**) or 5 μg/ml CHX (**Figure 2C**) had no effect on inclusion size, morphology or anti-LPS staining intensity compared to a *C. pecorum*-infected control in the absence of CHX (**Figure 2E**). Notably, AB were readily apparent in *C. pecorum*/PEDV co-infected cultures in the presence of CHX (**Figures 2B,D**, white arrows). Co-infection with $PEDV_{UV}$ similarly induced AB formation in the absence of CHX (**Figure 2F**, white arrow). Infectious titer analysis on replicate cultures indicated that co-infection with PEDV, $PEDV_{UV}$ or PEDV in the presence of CHX significantly decreased infectious titer compared to either the *C. pecorum* alone or *C. pecorum* + CHX controls, as appropriate (**Figure 2G**). Finally, anti-PEDV IFA of replicate coverslips indicated that the UV inactivation and CHX-exposure completely eliminated PEDV (+) single cells and syncytia (**Figure 1F**), as previously observed (**Figures 1D,E**). These data indicate that PEDV replication is not required for co-infection induced persistence/stress induction and suggest that PEDV binding/entry may be sufficient to induce this effect.

THE PEDV-INDUCED *C. pecorum* DEVELOPMENTAL CYCLE ALTERATION IS REVERSIBLE

One hallmark of the non-infectious but viable state is that it is reversible—if the stressor is removed, the chlamydiae re-enter normal development and infectious progeny are produced (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). Since CHX prevented PEDV replication in host cells but did not interfere with persistence/stress induction, we reasoned that any PEDV particle components responsible for this effect might eventually be degraded (and persistence/stress subsequently "reversed") if co-infected cells were kept under continuous CHX exposure to prevent viral replication. A similar approach demonstrated that HSV-induced persistence was also reversible (Vanover et al., 2010). Therefore, we co-infected and CHX-exposed replicate Vero cultures as previously described, except that coverslips were collected at 24, 48, 72, and 96 hpvi (**Figures 3A–F**). IFA revealed anti-LPS staining ABs in CHX-exposed, PEDV co-infected cultures out to 96 hpvi (**Figures 3C–F**). In contrast, inclusions in *C. pecorum* + CHX cultures did not contain visible ABs (**Figure 3B**). Notably, at 72 and 96 hpvi (**Figures 3E,F**), co-infected cultures contained fewer host cell nuclei (and inclusions) than did cultures harvested at earlier times (**Figures 3A–D**). Because reentry into normal development and production of infectious EBs is one possible explanation for the observed host cell and inclusion loss, we performed infectious titer assays on replicate samples (**Figure 4A**). As previously observed, PEDV co-infection significantly reduces infectivity at 24 hpvi, compared to that in *C. pecorum* control cultures at the same time. Importantly, production of infectious EBs from PEDV co-infected, CHX-exposed cultures is significantly increased at 48 and 72 hpvi, compared to co-infected samples collected at 24 hpvi. These data indicate that the PEDV-induced loss of chlamydial infectivity is reversible within 48 hpvi if continued viral replication is inhibited.

PEDV CO-INFECTION INDUCES *C. pecorum* AB MORPHOLOGICALLY INDISTINGUISHABLE FROM PERSISTENT/STRESSED ORGANISMS

Persistent/stressed chlamydiae have a striking ultrastructural appearance (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). The enlarged organisms observed by IFA in co-infected cells resemble ABs, but the AB morphology is best observed by TEM. Therefore, we performed electron microscopy on replicate samples from the time course infection/CHX-exposure experiment described above. Unsurprisingly, inclusions in *C. pecorum*-infected cells exposed to CHX contained normal RBs and EBs (**Figure 4B**, black arrows). Greatly enlarged, misshapen RBs (i.e., ABs similar to those observed in other persistence/stress tissue culture models) were present in co-infected cells (**Figure 4C**) at 24 hpvi as previously observed (Borel et al., 2010). Co-infected, CHX-exposed samples at both 24 and 72 hpvi (**Figures 4D,E**) and in cells co-infected with $PEDV_{UV}$ at 24 hpvi (**Figure 4F**) also contain primarily ABs. Interestingly, smaller ABs with condensed, darkly staining nucleoids, were observed at later times post-PEDV infection (**Figure 4E**, white asterisk), some of which appeared to be in the process of "budding" from larger ABs (**Figure 4E**, white arrows). Thus, chlamydiae within PEDV co-infected cells have the typical persistent/stressed AB ultrastructure regardless of whether or not viral replication is prevented by CHX-exposure.

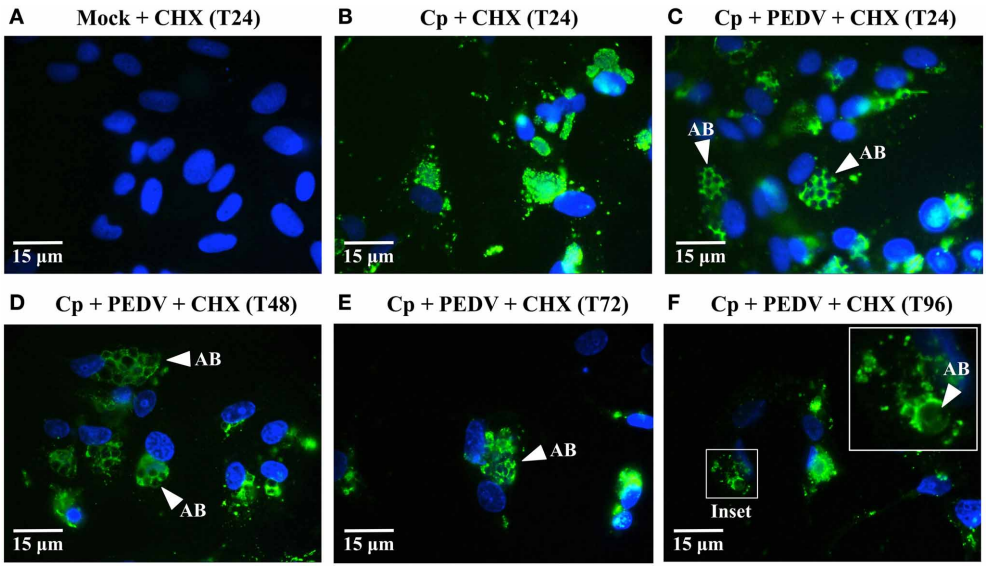

FIGURE 3 | ABs are present in co-infected cells for up to 96 hpvi when PEDV replication is blocked. (A–F) Vero cells were mock-infected (Mock), mono-infected with *C. pecorum* (Cp), or co-infected with *C. pecorum* and PEDV (Cp + PEDV) as diagrammed in **Figure 1A**. All cultures were pre-exposed to 5 μg/ml CHX starting at 1 h before viral infection to prevent PEDV replication, as indicated. At 24 hpvi (T24), 48 hpvi (T48), 72 hpvi (T72), and 96 hpvi (T96), replicate coverslips were fixed and labeled with anti-chlamydial LPS (green) and DAPI (blue). Replicate coverslips were also stained for PEDV M protein to confirm suppression of viral replication; results similar to those in **Figure 1F** were obtained (data not shown). Representative fields from anti-LPS/DAPI stained coverslips are shown at 1000× magnification with 15 μm scale bars. White arrows indicate anti-LPS-stained AB. In **(F)**, the inset shows a higher magnification view of an inclusion at 96 hpvi (white box).

DISCUSSION

Taken together, these and previously published (Borel et al., 2010) data definitively demonstrate that PEDV co-infection induces the *C. pecorum* persistence/stress response. Both anti-LPS IFA and electron microscopic examination indicates the presence of grossly enlarged, electronlucent ABs (**Figure S1**, **Figure 4** and Borel et al., 2010), which is consistent with the interrupted RB cytokinesis observed during persistence/stress (Matsumoto and Manire, 1970). Chlamydiae within co-infected cells are viable (as shown by the ability to recover infectivity by 48 hpvi if viral replication is inhibited by CHX-exposure; **Figure 4A**), but non-infectious (as shown by reduced chlamydial titer immediately post-co-infection; **Figure 2G** and Borel et al., 2010). Published data indicate that: (i) recovery from penicillin-exposure takes 10–20 h after drug removal; and (ii) replicative RBs may "bud" from ABs to reenter the productive developmental cycle (Skilton et al., 2009). We observe similar recovery timing, in that infectious EBs are not observed until 48 h after PEDV infection and CHX addition (**Figure 4A**). Interestingly, at late times post-PEDV/CHX addition, we detect smaller ABs with condensed nucleoids, some of which appear to be budding from larger ABs (**Figure 4E**). However, the presence (or absence) of replicative RBs "budding" from ABs in co-infected cells can only be confirmed by time lapse photography, similar to that published by Skilton et al. Though we do not currently have access to the necessary equipment, it might prove interesting to perform such analyses in the future to determine whether AB to RB "budding" is a general characteristic of the transition from persistent/stress to normal development regardless of the stressor used.

Chlamydiae in co-infected cells enter the persistent/stressed state regardless of whether PEDV replication is inhibited by CHX-exposure or UV-inactivated virions are used for co-infection (**Figures 2**, **4D–F**). Control experiments show that PEDV replication ceases under these conditions (**Figures 1B–F**), demonstrating that PEDV replication is not required to induce the persistence/stress response. These data have several important implications. First, PEDV-induced *C. pecorum* persistence is unlikely to be a byproduct of host resource consumption by the replicating virus. This is an important issue because host cellular nutrient deprivation can cause developing chlamydiae to enter the persistent/stressed state (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011). Second, UV light inactivates RNA viruses by damaging the genome, which prevents genomic replication and subsequent events (like viral gene expression and assembly). As a result, it seems more likely that an early event in the PEDV replication cycle, such as host cell attachment or entry, triggers this response. If so, the initiating molecule is most likely to be a physical component of the PEDV particle. Third, these data also suggest that host proteins synthesized in response to PEDV co-infection, like cytokines or type 1 interferons, are also unlikely to be involved. Notably, Luminex bead-based ELISA experiments indicate that neither TNF-α, nor IFN-γ are detected in co-infected culture supernatants, though IL-6 is observed (data not shown). Thus, PEDV-induced *C. pecorum* persistence/stress is unlikely to be mediated by the cytokines currently known to induce this response (reviewed in Hogan et al., 2004; Wyrick, 2010; Schoborg, 2011).

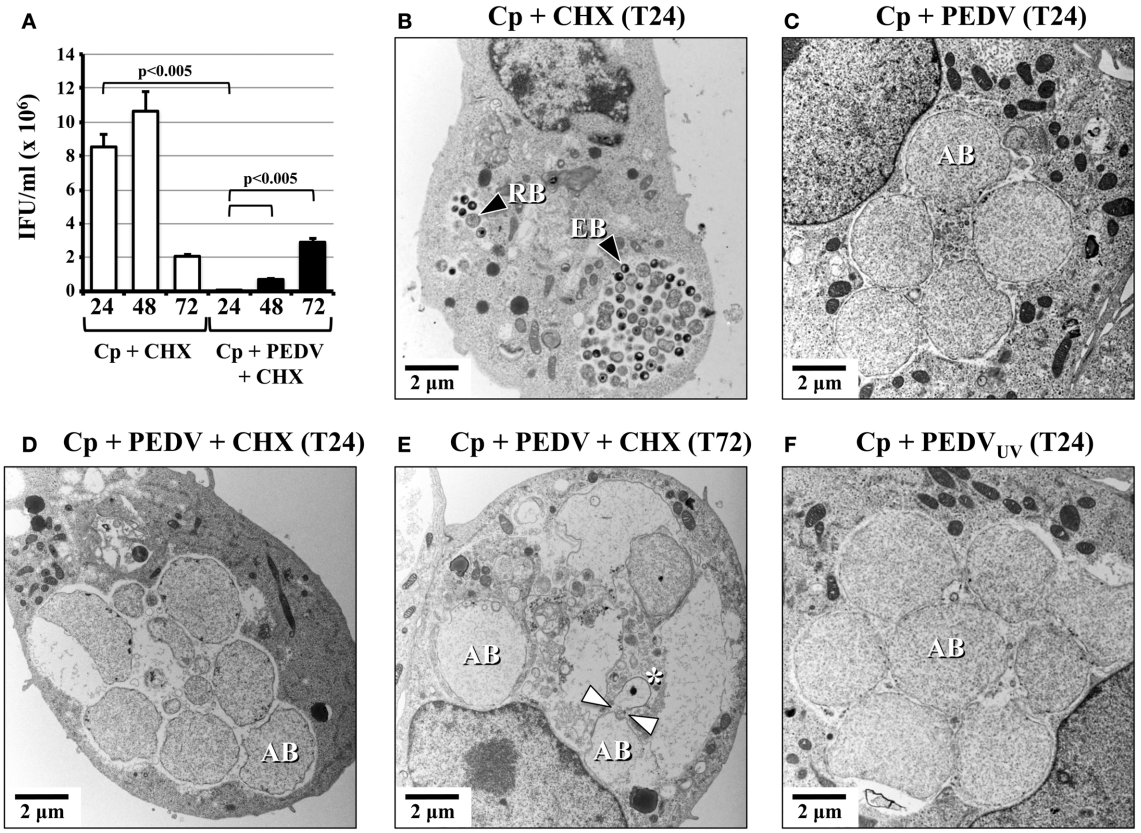

FIGURE 4 | The PEDV-induced developmental cycle disruption is reversible. (A) Vero cells were mock-infected (Mock; data not shown), mono-infected with *C. pecorum* (Cp), or co-infected with *C. pecorum* and PEDV (Cp + PEDV) as diagrammed in **Figure 1A**. Cultures (+CHX) were pre-exposed to 5 μg/ml CHX starting at 1 h before viral infection to prevent PEDV replication, as described in **Figure 1A**. At 24 hpvi (T24), 48 hpvi (T48), and 72 hpvi (T72), replicate coverslips were used for sub-passage titer analysis. Inclusion counts were used to calculate inclusion forming units (IFU)/mL (Y-axis). The time hpvi and sample are shown below the X-axis. The average from three biologic replicates ± s.e.m. is shown; these data are representative of three independent experiments. Statistical comparisons are indicated by brackets and all were significant to $p < 0.005$. **(B–F)** Vero cells were mock-infected (Mock; not shown), infected with *C. pecorum* (Cp), infected with *C. pecorum* and PEDV (Cp + PEDV), or infected with *C. pecorum* and PEDV$_{UV}$ (Cp + PEDV$_{UV}$), as shown in **Figure 1A**. Some cultures (+CHX) were pre-exposed to 5 μg/ml CHX starting at 1 h before viral infection. At 24 hpvi (T24) and 72 hpvi (T72), replicate coverslips were fixed and processed for transmission electron microscopy. Representative photomicrographs at 7000× magnification are shown, scale bars are 2 μm. RB (RB-black arrow), EB (EB-black arrow), and AB are indicated. A white asterisk and double white arrows indicate a small AB with a condensed nucleoid that may be "budding" from an adjacent AB **(E)**.

If a PEDV virion component does, indeed, influence the *C. pecorum* developmental cycle, which is the most likely component involved? The coronavirus literature suggests several likely candidates, one of which is the viral envelope S glycoprotein. PEDV attachment and entry are initiated when the S protein binds to aminopeptidase N (APN or CD13) on the host cell surface (Li et al., 2007; Nam and Lee, 2010). CD13 is a 150- to 160-kDa type II glycoprotein that has peptidase activity and is expressed by epithelial cells in the kidney, intestine and respiratory tract (Wentworth and Holmes, 2001). CD13 is a known modulator of signal transduction and cell motility (Petrovic et al., 2007), and regulates TNF-α-induced apoptosis in neutrophils by inhibiting TNFRI shedding (Cowburn et al., 2006). CD13 also co-localizes with FcγRI (a receptor for immunoglobulin Fc) on the monocytic cell membrane, suggesting it may act as a regulator of FcγRI signaling (Mina-Osorio and Ortega, 2005). The SARS coronavirus (SARS CoV) S protein is also a pathogen associated molecular pattern (PAMP) that signals through the host Toll-like receptor 2 (TLR-2) to stimulate IL-8 production from human macrophages (Dosch et al., 2009). Finally, mouse hepatitis virus (MHV) and SARS-CoV S proteins increase endoplasmic reticulum (ER) stress in murine L fibroblasts (Versteeg et al., 2007).

Another known "bio-active" coronavirus virion component is the single-stranded RNA (ssRNA) genome, which like S protein, would be present in cells that are either infected with PEDV$_{UV}$ or infected with replication-competent PEDV but pre-exposed to CHX. Single-stranded viral genomic RNAs (ssRNAs) are strong activators of TLR7 and TLR8, which subsequently activate diverse cellular processes, including pro-inflammatory and regulatory cytokine production (reviewed in Cervantes et al., 2012). Recently, GU-rich RNA fragments derived from the SARS-CoV ssRNA genome were shown to activate TNF-α, IL-6, and IL-12 release from murine RAW264.7 cells in culture via TLR7

and TLR8 activation. These RNAs can also cause fatal acute lung injury in mice in the absence of infectious virions (Li et al., 2013b). Thus, contact with PEDV S protein and/or genomic RNA from incoming virions can profoundly alter host cell physiologic processes. Whether or not such perturbations subsequently influence chlamydial development is currently unknown, but is a question we are very interested in answering.

As mentioned above, CHX-exposure and $PEDV_{UV}$ infection experiments both suggest that neither *de novo* synthesized host proteins nor *de novo* produced viral components are required for PEDV-induced *C. pecorum* persistence/stress induction. However, a recent study suggests that low amounts of SARS-CoV and murine hepatitis virus (MHV) RNA synthesis can occur even when viral protein synthesis is inhibited with CHX (van den Worm et al., 2011). It is also possible that low level viral RNA expression and/or synthesis of PEDV proteins occurs in our system even when replication is inhibited by CHX or by virion UV inactivation. Though unlikely, we also have to consider the possibility that a PEDV product produced during replication (rather than a viral particle component) might initiate the observed effects on chlamydial development. The PEDV ORF3 protein is one candidate with the potential to profoundly influence the host cellular internal environment. PEDV ORF3 is a member of an increasingly large group of viral proteins called "viroporins" and has potassium channel activity when etopically expressed in either *Xenopus* oocytes or *Sacchromyces cerevisiae* (Wang et al., 2012). Another coronaviral replication product with significant host cell effects is double-stranded RNA (dsRNA), which is produced during viral genome replication (reviewed in Hagemeijer et al., 2012). Double-stranded RNA is a potent activator of both TLR3 and cytoplasmic Rig-like receptors (RLRs), which can activate IFN-β production and anti-viral host cellular responses (reviewed in Kawai and Akira, 2008). It is, therefore, possible that low-levels of dsRNA could alter chlamydial development by activating other host anti-pathogen responses. Alternatively, toxic effects of a PEDV protein, like ORF3, on the host cell could produce a similar result. Because our data indicate that PEDV replication proteins/RNA are less likely candidates, we will first examine the possible contribution of PEDV virion components, like S protein and genomic ssRNA.

While the CHX-exposure data suggest that *de novo* synthesis of host proteins in response to PEDV infection is not required to induce *C. pecorum* persistence/stress, host cells can also release preformed mediators in response to damage or infection. These molecules are called DAMPs (damage or danger associated molecular patterns) and include host proteins (like heat shock protein 60) and non-proteins [like uric acid and extracellular ATP (ATPe)] (reviewed in Piccinini and Midwood, 2010; Miyake and Yamasaki, 2012). Exposure of chlamydia-infected host cells to ATPe or adenosine (Ado) arrests the developmental cycle and reduces *C. trachomatis* infectivity, as observed during persistence/stress. However, AB formation is not observed in response to Ado (Pettengill et al., 2009). Since PEDV co-infection is a strong inducer of AB formation (**Figures 2**, **4**, **Figure S1**), it is unlikely to be mediated by Ado release from co-infected cells. However, other DAMPs released from PEDV-infected cells could abort normal chlamydial development—a possibility that should be examined in the future.

Since the specific viral and/or host inducer(s) molecule is unknown, it is difficult to speculate on the mechanism by which PEDV co-infection induces *C. pecorum* persistence/stress. Prusty et al. suggest that HHV-6-induced host cellular oxidative stress activates *C. trachomatis* persistence in co-infected cells. However, antioxidant-exposure only partially reverses the observed effect, indicating that other mechanisms may also be involved (Prusty et al., 2012). Notably, SARS CoV infection increases transcription of host oxygen stress-related genes, suggesting that coronaviral infection may increase host cell oxidative stress (Hu et al., 2012). Thus, comparison of oxidative stress markers in *C. pecorum* mono-infected and co-infected cultures may also be warranted. Although we do not yet know the inducers involved or the molecular mechanism, our current data are essential to guide future studies. Regardless of the mediator involved (host or viral), its identification is likely to reveal interesting new facets of the host/chlamydial interaction and the means by which chlamydial entry into and exit from persistence/stress is regulated.

AUTHOR CONTRIBUTIONS

Robert V. Schoborg and Nicole Borel designed the experiments, conducted all experiments and analyzed the data. Both authors contributed to drafting the manuscript and figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcimb.2014.00020/abstract

Figure S1 | PEDV co-infection induces *C. pecorum* AB formation at 24 hpvi. **(A–F)** Vero cells were mock-infected (Mock), mono-infected with PEDV (PEDV), mono-infected with *C. pecorum* (*C. pecorum*), or co-infected with *C. pecorum* and PEDV (Cp + PEDV) as diagrammed in **Figure 1A**. At 24 hpvi, replicate coverslips were fixed and labeled with anti-chlamydial LPS (green) and DAPI (blue). Representative fields at 1000× magnification are shown with 15 μm scale bars in **(A,B,D,E)**. **(C,F)** are higher magnification photos of the boxed areas in **(B,E)**. The scale bars in **(A,B,D,E)** are 15 μm; those in **(C)** and **(F)** are 5 μm. White arrows indicate anti-LPS-staining AB.

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