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REVIEW ARTICLE

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Pathogenicity and virulence of *Chlamydia trachomatis*: Insights into host interactions, immune evasion, and intracellular survival

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ABSTRACT

Chlamydia trachomatis is an obligate intracellular pathogen and the leading cause of bacterial sexually transmitted infections and infectious blindness worldwide. All *Chlamydia* species share a unique biphasic developmental cycle, alternating between infectious elementary bodies (EBs) and replicative reticulate bodies (RBs). The pathogenesis of *C. trachomatis* is driven by a sophisticated arsenal of adhesins, conventional type III secretion system effector proteins, and inclusion membrane proteins that subvert host cellular processes to establish infection and promote survival. In this review, we highlight the molecular mechanisms underlying *C. trachomatis* infection, focusing on key stages of its developmental cycle, including adhesion, invasion, replication, and egress. We delve into its interactions with host cytoskeletal structures, immune signaling pathways, and intracellular trafficking systems, as well as its strategies for immune evasion and persistence. Understanding these mechanisms offers critical insights into *C. trachomatis* pathogenesis and identifies promising avenues for therapeutic and vaccine development.

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Introduction

Bacteria of the *Chlamydia* genus are Gram-negative, obligate intracellular pathogens that are responsible for a variety of human and veterinary diseases [1]. Among them, *Chlamydia trachomatis* (*C. trachomatis*) and *Chlamydia pneumoniae* (*C. pneumoniae*) are significant human pathogens. While *C. pneumoniae* has both medical and veterinary relevance, *C. trachomatis* is a human-specific pathogen [1] and the leading cause of bacterial sexually transmitted infections (STIs) and infectious blindness (trachoma) [2]. Most *C. trachomatis* infections are asymptomatic, contributing to underdiagnosis and increased transmission. In women, untreated infections can lead to severe reproductive complications, including pelvic inflammatory disease, ectopic pregnancy, and infertility [3–6]. Furthermore, infections are associated with an elevated risk of cervical and ovarian cancer, as well as increased susceptibility to other STIs, such as *Neisseria gonorrhoeae* and HIV [6–14].

C. Trachomatis is comprised of 15 serovars classified into two distinct biovars: the trachoma biovar (serovars A-K) and the lymphogranuloma venereum (LGV) biovar (serovars L1-L3) [15,16]. Serovars A-C are primarily associated with ocular trachoma, while serovars D-K are typically responsible for oculogenital infections [6,15,16]. LGV serovars cause invasive infections that originate in the genital or rectal mucosa, potentially infecting macrophages and disseminating to proximal lymph nodes, leading to lymphadenopathy and, in later stages, anogenital strictures [15–17].

C. Trachomatis exhibits a unique biphasic developmental cycle, alternating between an infectious elementary body (EB) and a replicative reticulate body (RB) [18] (Figure 1). Upon contact with a host cell, the chlamydial EB delivers pre-packaged type III secretion system (T3SS) effector proteins into the host cell to facilitate endocytosis of the EB [19,20]. The resulting membrane-bound compartment, termed the inclusion, is modified to evade lysosomal fusion and traffics to the peri-Golgi region where the bacteria establish its replicative niche and differentiates into RBs [21,22]. Throughout the infection cycle, the bacteria, residing within the inclusion, interacts with host organelles and cellular structures to acquire essential nutrients for replication while evading the host's innate immune response [23]. After several rounds of replication, RBs asynchronously convert back into EBs and exit the host cell either through cell lysis or extrusion, initiating a new infection cycle [24]. As an obligate intracellular bacterium, *C. trachomatis* relies on the successful completion of this developmental cycle for pathogenesis.

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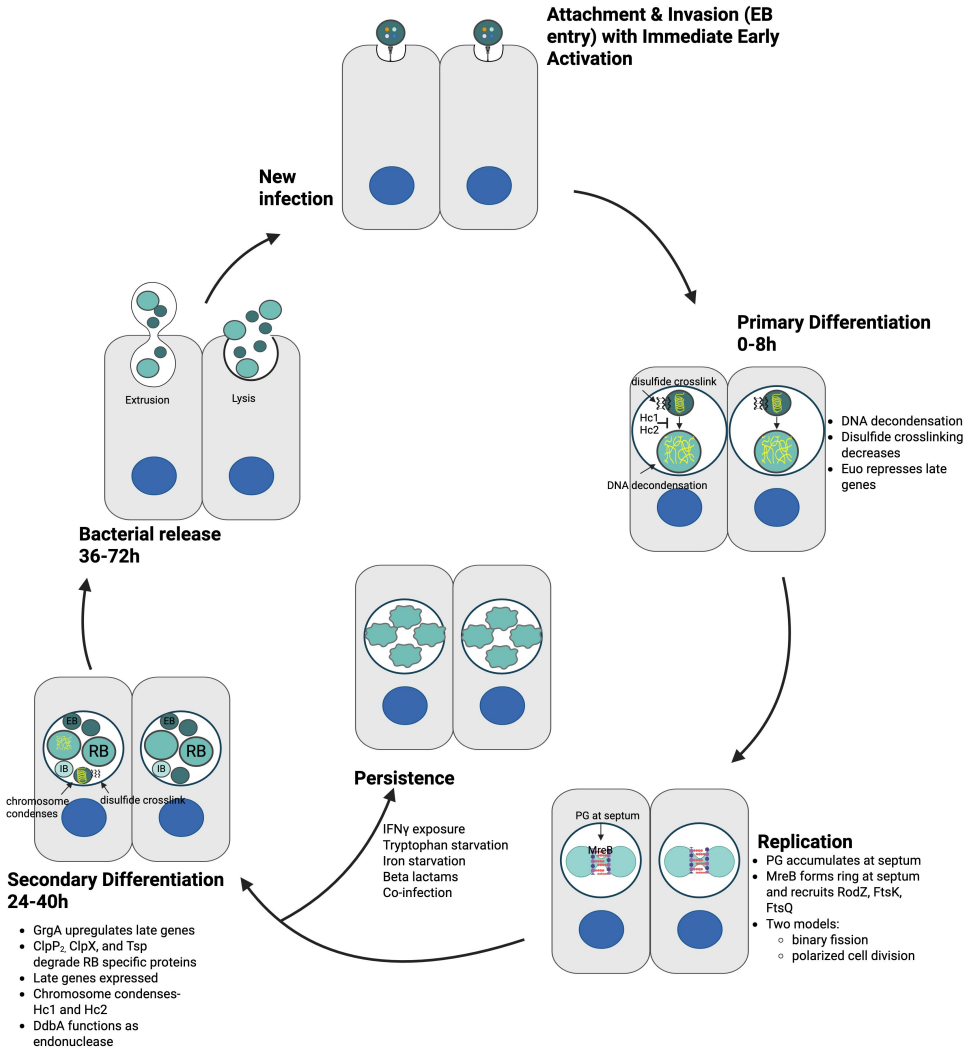

Figure 1. *C. trachomatis* developmental cycle. Infectious elementary bodies (EBs) secrete type III secretion system (T3SS) effector proteins to facilitate host cell invasion. Once inside the host cell, EBs undergo primary differentiation to convert into replicative reticulate bodies (RBs). After multiple rounds of replication, RBs asynchronously convert back into EBs (secondary differentiation) and are released from the host cell via lysis or extrusion. The newly infectious EBs then infect additional cells, perpetuating the infection cycle. In response to stress during replication, RBs can convert to persistent aberrant bodies (ABs), which exhibit reduced immune recognition and increased resilience. ABs are capable of generating infectious progeny through budding after removal of the stressor.

Adhesins

EB adhesion, an essential step for host cell invasion, occurs in two stages: a reversible stage, in which the EB binds to heparan sulfate glycosaminoglycan (GAG) receptors [25] or other host cell receptors [26], and an irreversible stage, where the EB attaches to the cell surface via membrane proteins in a temperature-sensitive manner (Figure 2a) [27]. *C. trachomatis* employs a repertoire of adhesins, chlamydial outer membrane proteins (COMPs), and other outer membrane components, to facilitate its adhesion.

Outer membrane complex protein B (OmcB) is localized on the outer membrane of EBs and surface-exposed regions of the protein bind to host heparan sulfate, which then interacts with host GAG receptors (Figure 2a) [25,28]. Notably, while OmcB and heparan sulfate are important for the adhesion of LGV serovars to various types of epithelial cell, they are not essential for trachoma or urogenital serovars. However, all serovars exhibit sensitivity to heparin inhibition during invasion [28–32]. This LGV-specific difference in OmcB's binding to heparan sulfate GAGs is attributed to variations in the OmcB GAG binding domain. Specifically, three positions within the variable region confer the ability to bind [32,33], preventing GAG binding in non-LGV serovars. In the heparan sulfate-dependent serovar L2, host membrane-associated vesicular trafficking complex COPI has also been implicated in *C. trachomatis* adhesion and invasion [34]. Depletion of

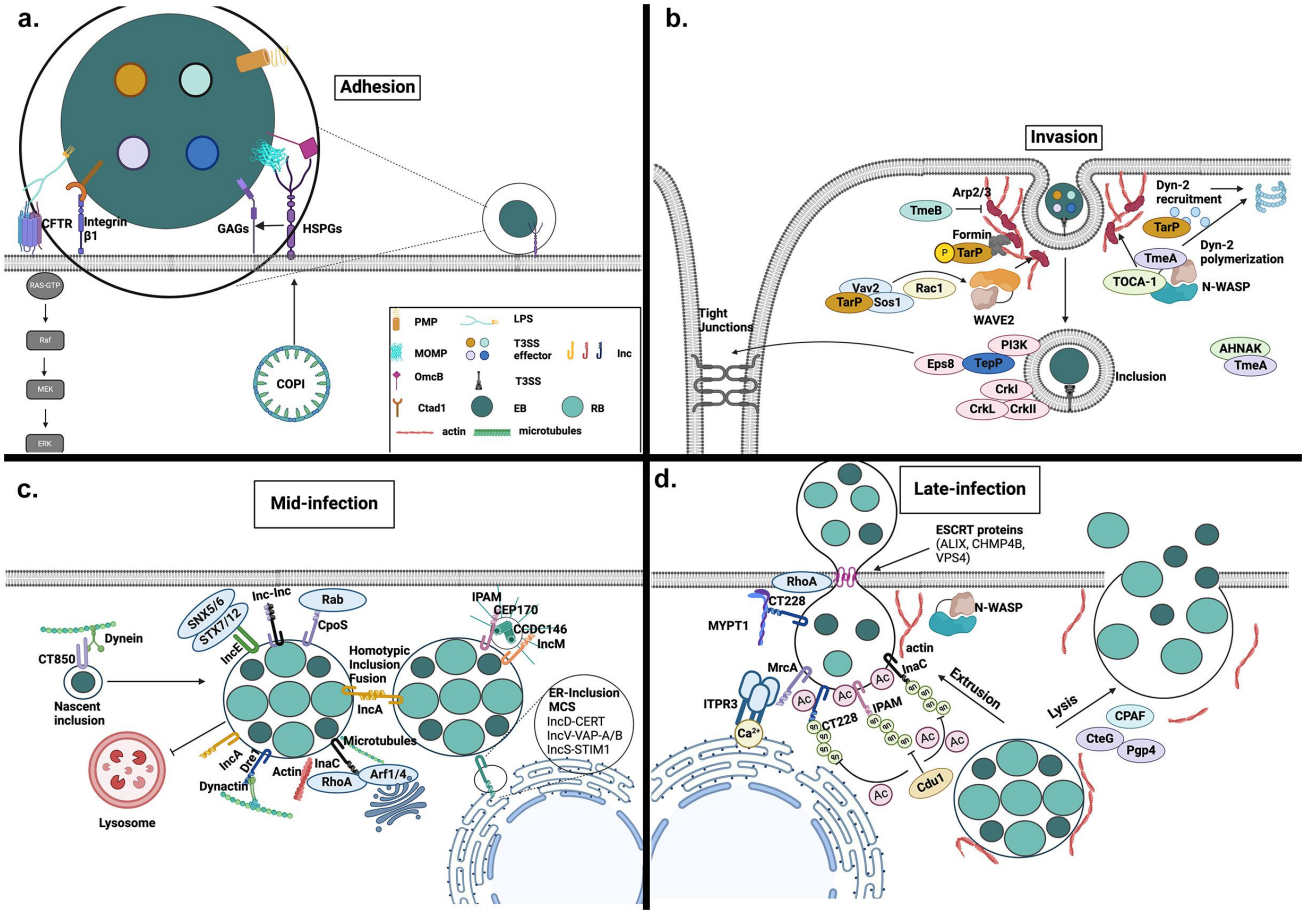

Figure 2. Role of *C. trachomatis* effectors and adhesins in manipulating host cell processes. (a) Key adhesins and host receptors facilitating *C. trachomatis* attachment. MOMP and OmcB mediate interaction with host heparan sulfate (HSPG), while LPS interacts with CFTR and Ctad1 interacts with integrin β1, stimulating ERK signaling. (b) Early secreted effectors TarP and TmeA orchestrate cytoskeletal rearrangements to promote bacterial uptake and manipulate host cellular dynamics. TarP activates the Arp2/3 complex via Rac1 signaling and Wave2, while TmeA directly activates Arp2/3 through N-WASP binding and promotes pedestal formation via TOCA-1. Dyn2 is recruited by TarP and polymerized by TmeA. TmeB antagonizes Arp2/3, likely reversing cytoskeletal changes post-invasion. TepP recruits crk, CrkL, PI3K, and Eps8 to the inclusion, with Eps8 contributing to the breakdown of epithelial tight junctions. (c) During mid-cycle, *C. trachomatis* establishes ER-inclusion contact sites via IncD, IncV, and IncS. It co-opts small GTPases such as arfs, Rabs, and RhoA through Inc proteins like CpoS and InaC, which contributes to inclusion growth, stability, and nutrient acquisition. IncE manipulates Snx5/6 and STX7/12 to recruit hybrid vesicles, while IncA facilitates homotypic inclusion fusion. Centrosome repositioning near the inclusion is mediated by IncM, IPAM, and Dre1. (d) Late in the infection cycle, bacterial release occurs via extrusion or lysis. Extrusion is driven by post-translational modifications of Inc proteins CT228, InaC, and IPAM by Cdu1. CT228 and MrcA regulate extrusion by interacting with MYPT1 and ITPR3, respectively. *C. trachomatis* effectors CteG, Pgp4, and CPAF contribute to host cell egress via lysis.

COPI reduces the availability of heparan sulfate on the host cell membrane, likely through a trafficking defect, which subsequently diminishes *C. trachomatis* serovar L2 adhesion [34]. While this interaction does not directly involve binding to a *C. trachomatis* adhesin, it underscores the importance of endogenous host heparan sulfate for LGV adhesion. Additionally, the *C. trachomatis* major outer membrane protein (MOMP) interacts with GAG receptors, whereas recombinant MOMP is able to bind heparan sulfate receptors directly (Figure 2a) [35–38]. The combined roles of MOMP and OmcB in heparan sulfate-mediated adhesion and invasion have not yet been fully elucidated. However, heparan-sulfate mediated adhesion appears to be specific to LGV serovars, while adhesion of other *C. trachomatis* serovars is heparan-sulfate independent. This has led to an intriguing hypothesis for future investigation: that heparan sulfate–dependent adhesion may contribute to the increased invasiveness of LGV serovars [30].

C. trachomatis, along with other *Chlamydia* species (*C. spp.*), possesses a unique lipopolysaccharide (LPS) characterized by a 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) trisaccharide structure with alpha-Kdo -(2→8)-alpha-Kdo-(2→4)-alpha-Kdo linkages which is

conserved within the genus [39,40]. LPS is essential for secondary differentiation [41], as further described below. In *C. trachomatis* serovar D, it has been shown that LPS binds to the host cell cystic fibrosis transmembrane conductance regulator (CFTR), and this interaction is important for *C. trachomatis* uptake (Figure 2a) [42]. Furthermore, *C. trachomatis* LPS-CFTR interactions may play a role in clathrin-mediated endocytosis of the bacterium, as CFTR is internalized via this pathway [43]. This presents an interesting area for follow-up study.

Polymorphic membrane proteins (Pmps), expressed by *C. trachomatis* and other *Chlamydia spp*., also mediate host cell adhesion [26,44,45]. *C. trachomatis* encodes nine Pmps, which are present across all serovars but exhibit limited overall sequence homology [46]. Notably, subtle differences in Pmp sequence – particularly between serovars and biovars – correlate with tissue tropism [46], suggesting that even minor sequence variations may contribute to serovar-specific pathogenesis. Pmps are catagorized as autotransporters based on sequence and structural predictions, as well as functional data indicating that PmpD is an autotransporter [47–49]. The Pmp passenger domains contain tetrapeptide repeats (GGA[I,L,V] followed by FxxN) [47] which studies in *C. pneumoniae* indicate are necessary for host cell adhesion. It has been hypothesized that these motifs contribute to *C. trachomatis* adhesion by promoting β-barrel formation within the passenger domain, rather than directly binding host cells. The passenger domains of *C. trachomatis* Pmps form heterotropic and homotropic structures on the EB membrane that may interact with yet unidentified host receptors [48,50,51]. Although their adhesion capacities vary, all nine Pmps of serovar E are capable of mediating adhesion to host cells, and pre-incubation of soluble Pmps with host cells inhibits EB adhesion [26]. Furthermore, antisera against serovar L2 PmpD blocks adhesion of LGV, ocular, and urogenital serovars, emphasizing the pan-serovar importance of Pmps in adhesion to host cells [52]. However, it remains unclear how redundant these nine Pmps are, or whether all nine must be depleted to abolish *C. trachomatis* adhesion to host cells.

Host receptors involved in Pmp-mediated adhesion have been identified in other *C. spp*.. Human epidermal growth factor receptor (EGFR), which interacts with *C. pneumoniae* Pmp21 [53] and *C. psittacii* Pmp17 [54], has been shown to play a role in *C. trachomatis* EB adhesion [55]. Notably, EGFR does play a role in *C. trachomatis* adhesion [55], though whether EGFR similarly interacts with *C. trachomatis* Pmps or another *C. trachomatis* factor remains unknown. Blocking EGFR does not completely prevent *C. trachomatis* adhesion [55], indicating that other receptors can compensate in its absence.

C. trachomatis adhesin 1 (Ctad1) (CT017) is a conserved adhesion found across *C. trachomatis* serovars and plays a role in both adhesion and host cell invasion [56]. In serovar E, Ctad1 localizes to the surface of EBs [56]. Pre-incubation of HEp-2 cells with soluble recombinant Ctad1 significantly inhibits EB attachment and invasion [56]. The SH3 domains and the N-terminus of Ctad1 is required for binding to the host integrin β1 subunit and activation of the MAP kinase Erk1/2 signaling pathway (Figure 2a) [56]. Notably, Ctad1-coated latex beads (1 μm) are internalized by HEp-2 cells, suggesting that the Ctad1-integrin interaction is sufficient to mediate uptake of particles approximating or exceeding the size of EBs (0.3 μm) [56]. Furthermore, EB internalization is reduced both by pre-treatment of HEp-2 cells with soluble Ctad1 and by infection of GD25 cells lacking integrin β1, further supporting a role for Ctad1-integrin interactions invasion [56]. However, it remains unclear whether Ctad1-mediated activation of integrin β1 alone is sufficient to trigger downstream signaling events such as Rac1 activation and cytoskeletal remodeling, or if additional *C. trachomatis* effectors are required to facilitate full EB entry.

Other host proteins involved in EB adhesion but lacking identified *C. trachomatis* adhesin partners include platelet derived growth factor receptor-β (PDGFR-β) [57] and protein disulfide isomerase (PDI). PDI localized on the host cell surface is critical for adhesion of serovars E, D, and L2 [58,59]. Its enzymatic activity is not required for adhesion, and it does not appear to bind directly to a *C. trachomatis* adhesin but instead acts as a structural interactor with other host receptors [60]. PDI's enzymatic activity appears to play a role in *C. trachomatis* invasion, though the exact mechanism remains unclear [58,60]. Similarly, EphrinA2 receptor (EphA2) plays a role in both adhesion and uptake of serovars L2 and D EBs [61] but lacks an identified *C. trachomatis* interacting partner. It is also unknown whether EphA2 binding alone can stimulate cytoskeletal modulation and uptake independently of other invasion mechanisms.

Invasion

Similar to adhesion, invasion represents a potential target for therapeutic intervention due to its essential role in establishing infection. *C. trachomatis* invasion involves multiple host-pathogen interactions, including contributions from T3SS effector proteins that are

preloaded into EBs prior to their release from the previous host cell. Upon host cell contact, the T3SS delivers a repertoire of effector proteins that manipulate host signaling pathways and cytoskeletal architecture to facilitate internalization and promote the establishment of a replicative niche [19,20] (reviewed in [62]). While several pre-loaded effectors have been implicated in promoting invasion, genetic studies suggest that most individual mutants display only modest or context-dependent phenotypes, indicating functional redundancy or compensation among effectors [63]. Thus, rather than relying on a small, dedicated subset, invasion likely involves the coordinated action of multiple effectors acting in concert with host factors [63].

Upon host cell adherence, four effectors are secreted with the aid of the T3SS chaperone Slc1: TarP [64], TmeA and TmeB, which are encoded on the same operon [65], and TepP [66]. TepP has been shown to modulate the host cell immune response during early infection and dismantle tight junctions through interactions with Eps8 [66,67], whereas the other three effectors play varying roles in invasion (Figure 2b).

The first *C. trachomatis* protein that was shown to be secreted upon host cell attachment was Translocated actin-recruiting Phosphoprotein (TarP) (CT456) (Figure 2b). TarP is secreted via the T3SS upon host cell contact and contains domains that interact with actin at the plasma membrane of the host cell [68]. It is capable of binding and bundling F-actin via its FAB1 and FAB2 domains [69]. Additionally, TarP has a proline-rich domain that binds and nucleates G-actin to form new actin filaments [70]. TarP is phosphorylated by host Src and Abl family tyrosine kinases [71], as well as by tyrosine kinase Syk [72]. Phosphorylation is species-specific and unique to *C. trachomatis* TarP, as orthologs in *C. pneumoniae, C. muridarum*, and *C. caviae* are not phosphorylated but retain actin recruitment activity [73]. Upon phosphorylation, *C. trachomatis* TarP binds guanine nucleotide exchange factors (GEFs) Vav2 and Sos1 with adaptor proteins Abi1 and Eps8 to recruit Rac1, inducing Rac signaling pathways and stimulating actin polymerization during invasion [74]. Assembly of the Rac signaling complex leads to the recruitment of WAVE2 and Abi-1 to activate the Arp2/3 complex, resulting in actin branching [75,76]. TarP additionally recruits Fmn1 to the site of invasion, indicating it also interacts with host proteins involved in actin filament elongation [77]. Collectively, these findings show that TarP plays a role in rapid modulation of host cell actin during *C. trachomatis* uptake.

However, TarP is not the only bacterial effector involved in the invasion of host cells. Translocated membrane-associated effector A (TmeA) (CT694) and TmeB (CT695) are secreted during invasion and interact with various host proteins to stimulate bacterial uptake (Figure 2b) [78,79]. TmeA was first described as interacting with host AHNAK, which was hypothesized to modulate the host cytoskeleton during infection via its actin bundling activity, however AHNAK is dispensable for *C. trachomatis* invasion [78,79]. Whether this interaction plays a role in establishment of infection after invasion has yet to be determined. More recently, TmeA was shown to recruit and activate N-WASP during invasion, leading to activation of the Arp2/3 complex and host cell invasion through a mechanism that is distinct from TarP [80,81]. TmeA also binds to TOCA-1 to promote pedestal formation [82]. Additionally, both TarP and TmeA interact with host Dynamin-2 (Dyn2), whereby TarP recruits and TmeA oligomerizes Dyn2 to stimulate *C. trachomatis* uptake through rapid disassembly of *C. trachomatis*-induced actin structures, allowing for efficient engulfment of the bacterium [83]. Unlike TarP and TmeA, TmeB does not upregulate invasion, but it weakly binds to and inhibits Arp2/3 activity [84]. This leads to the hypothesis that TmeB plays a role in the disassembly of actin structures during the final steps of the invasion process (Figure 2b) [84]. It is interesting to speculate that the roles of Dyn2 and TmeB are similar, inducing disassembly of actin structures to complete uptake, and thus the lack of an invasion defect in the TmeB mutant could be due to compensation by *C. trachomatis* manipulation of host proteins, including Dyn2.

While there are models for both independent and interdependent mechanisms of *C. trachomatis* uptake mediated by TarP and TmeA, the successful generation of a double mutant [80] shows that there are additional *C. trachomatis* proteins yet to be identified that are capable of stimulating uptake of the bacterium. Whether this is due to additional secreted effectors or receptor-mediated uptake utilizing adhesins such as Ctad1 remains to be tested. Additionally, while modulation of host cell actin is distinctly important, the structures generated by this modulation have not been fully characterized. Pedestal-like structures [68,85], filopodial capture [86], macropinocytosis-like phagocytic cups [24,86], clathrin-coated pits [24], phagocytosis [24], and caveola-mediated endocytosis [24] have all been implicated as potential mechanisms by which *C. trachomatis* mediates its uptake.

Trafficking and establishment of replicative niche

Upon uptake, EB-containing, plasma-membrane derived vacuoles, known as the chlamydial inclusion,

are decorated with T3SS proteins known as inclusion membrane (Inc) proteins, as first identified in *C. psittaci* [87,88]. These secreted proteins integrate into the vacuole membrane, where they perform various functions to aid in localization [89], fusion of EB-containing vacuoles [90], nutrient acquisition through recruitment of Golgi and endoplasmic reticulum vesciles [91,92], make contact sites with host organelles [91,93–96], and evasion of the host immune response [97].

Nascent inclusions associate with microtubules and traffic to the microtubule organizing center (MTOC) in a dynein-dependent manner [98]. The host cell cytoskeleton is composed of polar microtubules, with the minus end located at the MTOC [99]. This configuration allows the dynein motor complex to transport endosomal cargo along microtubules toward this central site [99]. EB-containing vacuoles engage dynein and the dynactin subunit p150$^{(Glued)}$ during trafficking, although other dynactin components typically required for dynein-cargo binding appear dispensable in this context [21]. This is due to a direct interaction between the dynein light chain and the *C. trachomatis* Inc protein CT850 [89] (Figure 2c), which is expressed early during infection [100]. Src family kinases are also required for trafficking [101], and are similarly enriched at inclusion-associated microdomains with CT850 [102]. Together, these findings suggest that CT850 may coordinate both cytoskeletal and signaling machinery to promote efficient inclusion trafficking.

When multiple EBs infect the same host cell, their nascent inclusions converge at the MTOC and subsequently fuse into a single inclusion [103] while simultaneously avoiding fusion with lysosomes [104,105] (Figure 2c). Homotypic fusion of inclusions at the MTOC is dependent on the cytosolic core of IncA [90,106]. The cytosolic core contains a THATCH-like domain necessary for these interactions [107]. This core consists of two coiled-coil SNARE-like domains (SLDs) that mediate both inclusion fusion and inhibition of lysosome fusion. While either SLD can inhibit lysosome fusion, both are required for homotypic fusion [108,109]. Although inclusion fusion is not essential for pathogen survival or infectivity, clinical isolates lacking IncA are associated with decreased infections burdens and fewer clinical symptoms [110], suggesting that it does play a role in pathogenicity.

Positioning the inclusion at the MTOC facilitates access to host-derived lipids, including sphingomyelin. Within two hours post-infection, the *C. trachomatis* inclusion avoids fusion with endocytic vesicles [111] and instead redirects exocytic vesicles from the trans-Golgi network to the inclusions acquire sphingomyelin [22]. Distinct mechanisms are used for early and late sphingomyelin acquisition, but in both cases, sphingomyelin is essential for *C. trachomatis* growth. A detailed discussion of sphingomyelin acquisition throughout the developmental cycle can be found in [112].

At the MTOC, the inclusion protein for actin assembly (InaC) (CT813) acts as an important regulator of microtubule and Golgi positioning around the inclusion [91,93]. Loss of InaC results in the production of fewer infectious progeny [93]. InaC binds and activates host ARF GTPases 1 and 4, leading to the formation of microtubule cages, as well as host RhoA to generate actin scaffolds (Figure 2c) [91,93,113,114]. This is notable, as microtubules and actin scaffolds are regulated by different mechanisms. During early infection, microtubules are stabilized through detyrosylation, which is essential for Golgi fragmentation and repositioning [115]. In the absence of InaC or ARFs 1 or 4, microtubule cages are no longer post-translationally modified (PTM), suggesting a previously uncharacterized role of ARFs in microtubule PTM [93]. Repositioning of Golgi stacks around the inclusion is necessary for acquisition of essential lipids [116]. Late in the infection, InaC recruits F-actin crosslinking proteins (e.g. α-actinins), stabilizing RhoA-generated actin scaffolds that support the inclusion [113,114].

The inclusion protein acting on MTs (IPAM) (CT223) promotes inclusion stability by hijacking host microtubule organization through its interactions with the centriole component protein CEP170 [117] (Figure 2c). Additional Inc proteins, such as the dynactin recruiting effector 1 (Dre1) (CT192) and Inc mediating multinucleation (IncM)(CT288) (Figure 2c) also contribute [118,119]. Notably, an IncM mutant exhibits increased sensitivity to microtubule depolymerization, which correlates with altered inclusion morphology [119]. These findings support a role for IncM in stabilizing microtubules to maintain inclusion structure and facilitate organelle rearrangement. Advances in *C. trachomatis* genetic manipulation will offer opportunities to generate multi-knockout mutants that will help us better understand functional overlaps between these proteins.

In addition to modifying microtubules and recruiting fragmented Golgi, *C. trachomatis* recruits the endoplasmic reticulum (ER) to the inclusion, forming close membrane contact sites (MCS) [92]. Two Inc proteins, IncD [92,94,120] and IncV [95,96], have been identified as important for this interaction (Figure 2c). IncD interacts directly with the host protein CERT [92,94,120], which is responsible for non-vesicular transport of the sphingomyelin precursor ceramide from the ER to the Golgi at ER-Golgi MCS

[121]. In uninfected cells, CERT binds to phosphatidylinositol-4 monophosphate (PI4P) on the *trans*-Golgi [121] via its PH domain and VAP-A and VAP-B on the ER via its FFAT motif [122]. These interactions generate MCS between the ER and Golgi [121,122]. In infected cells, IncD binds the PH domain of CERT, acting as a tether at ER-inclusion membrane MCS [94]. CERT recruitment allows *C.t.* to generate the sphingomyelin necessary for replication in a host sphingomyelin synthase independent manner [123]. Due to the lack of IncD mutant this has not been directly linked to IncD, but given the direct IncD-CERT interactions, it is likely that these ER-inclusion MCS are necessary for ceramide acquisition in an IncD-dependent manner.

IncV in contrast directly binds VAP-A/B though two FFAT motifs and aids in the formation of ER-inclusion MCS [95] (Figure 2c). IncV's tethering activity depends on phosphorylation of its FFAT motifs by host kinase CK2 at the inclusion membrane [96]. IncV can also recruit VAP-A/B to the MCS, though it is not essential for recruitment or formation of ER-inclusion MCS [95,96]. It is interesting to note that despite apparent redundancies in their tethering abilities, the IncV mutant lacks a growth defect, while an IncD mutant has yet to be created, implying that IncD is essential for *C. trachomatis* survival. This is likely due to IncD's contribution to CERT interactions and potential role in ceramide and thus sphingomyelin acquisition, but this role has yet to be fully elucidated.

Other host proteins, including STIM1, are recruited to ER-inclusion MCS through interactions with IncS [124,125] (Figure 2c). Loss of IncS, but not STIM1, results in severe growth defects during the early stages of the developmental cycle and early inclusion lysis [125,126], suggesting IncS plays additional, important roles beyond interacting with STIM1. One hypothesis is that STIM1 interactions suppress store-operated Ca^{2+} entry (SOCE), where low calcium stores within the ER trigger uptake of extracellular calcium [127]. STIM1 is a host cell ER-associated protein typically found at ER-plasma membrane (PM) MCS, where it binds to and activates Ca^{2+} channel protein Orai1 when calcium stores are low [127]. This triggers calcineurin/NFAT signaling, leading to NFAT translocations to the nucleus where it upregulates genes associated with the immune response, apoptosis, and cell cycle [128]. During *C. trachomatis* infection, SOCE is impaired, resulting in decreased localization of NFAT to the nucleus [129]. It has been hypothesized that this impairment is due to STIM1 recruitment to ER-inclusion MCS. Interestingly, in the initial experiments that uncovered this localization pattern, Ca^{2+} depletion led to increased STIM1 recruitment to ER-PM MCS [124]. Further studies utilizing endogenous STIM1 and measuring NFAT signaling in cells infected with the IncS mutant are needed to elucidate the physiological roles of STIM-IncS interactions.

Rab GTPases regulate vesicle transport within the cell through reversible associations with membranes, defining vesicle identity and directing trafficking by recruiting specific host cell effectors [130]. As *C. trachomatis* resides within a parasitophorous vacuole, it relies on the recruitment of Rabs to the inclusion membrane to maintain proper localization within the cell and facilitate the delivery of vesicles essential for its growth and development. The inclusion membrane protein Chlamydia promoter of survival (CpoS)(CT229), binds and recruits multiple Rab GTPases [97,131–133] via its coiled coil (CC) domain 1 (CC1) (Figure 2c) [133,134]. CpoS recruits Rab4A, an early endosomal Rab associated with dynein recruitment and other trafficking functions, shortly after *C. trachomatis* uptake [131]. However, Rab4A is not essential for trafficking along microtubules to the MTOC [131]. Additionally, CpoS-mediated recruitment of Rab35, plays a critical role in clathrin-coated vesicle trafficking and transferrin delivery, suggesting a role for CpoS in *C. trachomatis* iron acquisition [133]. The CpoS-Rab35 interaction also suppresses IFN-signaling through a STING-independent mechanism [134]. Notably, other Rabs, such as Rab11, are recruited independently of CpoS, though their contributions to *C. trachomatis* niche formation remain unclear [135]. Mutations in CpoS result in premature host cell death at 24 hours post infection [97,134,136], underscoring the diverse roles of CpoS-Rab interactions in promoting *C. trachomatis* survival and immune invasion. Since many of these interactions remain uncharacterized, they present significant opportunities for further investigation.

In addition to its interaction with Rabs, CpoS also binds to other Inc proteins. Bacterial-two hybrid screens have shown CpoS interacts with CT222, IPAM, and IncD [137] and CpoS has been confirmed to bind to IPAM, CT226, IncA, IncC, and InaC via its CC2 domain during infection [134,138]. CpoS appears to play a critical role in the generation of microdomains on the inclusion membrane, as IPAM loses its microdomain localization in the absence of CpoS [134]. Previous studies have highlighted the importance of Inc-Inc interactions in organizing the inclusion membrane [139], with over- or under-expression of certain Incs altering the overall Inc protein composition of the inclusion [140]. This raises the intriguing possibility that CpoS-Inc interactions organize an Inc-Inc network

that maintains microdomains and mediates crucial interactions with the host.

IncE (CT116) also mediates vesicular trafficking in the host cell through interactions with syntaxin (STX) 7 and 12, as well as sorting nexin (SNX) 5 and 6 (Figure 2c) [132,141–144]. The interactions with SNX5 and 6 disrupt retromer trafficking; IncE displaces endogenous SNX interactors, thereby preventing inhibition of *C.t.* growth [141–144]. IncE also binds STX7 and STX12 to support the inclusion. STX7 is important for the production of infectious progeny, as its knockdown leads to a modest ~ 1-log reduction in progeny production. STX12 plays a role in inclusion fusion, with knockdown resulting in a 1.5× increase in the number of inclusions at high MOIs [144]. How STX7 and STX12 promote these modest but statistically significant effects remains to be determined.

Primary differentiation

By 8 hours post-infection, the infectious EBs complete conversion into non-infectious, replicative RBs (Figure 1). These two forms exhibit significant morphological differences: EBs are smaller (~300 nm) and contain electron-dense nucleoids, while RBs are larger (1 µm) and lack DNA condensation [18]. Although previously considered metabolically inactive, recent studies have shown that EBs retain limited metabolic activity [145,146], which aids in maintaining infectivity [147,148]. Proteomic analyses have revealed distinct differences in the EB and RB proteomes [63,149], and transcriptional profiling indicates that differentiation depends on the expression of early genes [100,150]. RNA profiles from EBs at 1 hour post-infection reveal that transcription of a large proportion of the *C. trachomatis* genome begins by this timepoint, marking the beginning of primary differentiation early after invasion and highlighting potential pathways important for this process [151]. However, the mechanism(s) underlying primary differentiation from EB to RB remains an interesting area for future study.

During primary differentiation, *C. trachomatis* chromosomal DNA undergoes decondensation, mediated by the inhibition of histone-like proteins Hc1 and Hc2 binding to chromatin (Figure 1). This inhibition is mediated by a small molecule, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEC) [152,153]. Additionally, disulfide crosslinking of the *C. trachomatis* membrane decreases during the EB-RB transition, potentially enabling a more flexible membrane and differential secretion of T3SS effectors at various development stages [154,155]. Repressors of *C. trachomatis* late genes, such as early upstream ORF (Euo), are expressed by 1 hour post-infection in *C. psittaci* and *C. trachomatis* to prevent premature conversion back into EBs [100,156]. These repressors specifically bind to and repress the promoters of late cycle genes, including the *omcAB* operon [157], which encodes proteins involved in disulfide crosslinking with MOMP [158]. Repressing these genes may help reduce crosslinking in RB membranes, facilitating their distinct physiology.

IncS has also been implicated in primary differentiation in that an IncS mutant largely fails to differentiate into RBs [126]. However, some RBs form in non-complemented inclusions, potentially due to promoter leakiness or additional factors that allow for very low levels of conversion in the absence of IncS [126]. While it is clear IncS promotes primary differentiation, its precise mechanism has yet to be determined.

Additionally, glutamine (Gln) has recently been shown to play a key role in the transition from EB to an intermediate body (IB), a stage between EB and RB, with reduced infectivity but some replicative activity [146]. *C. trachomatis* infection induces expression of c-Myc, increasing Gln transporter expression and glutaminolysis by host cells, which in turn promotes *C. trachomatis* replication [146]. However, Gln supplementation alone is not sufficient to promote the production of infectious progeny [146]. Further studies are required to explore the host and bacterial factors that regulate primary differentiation.

Replication

C. trachomatis divides in phases, with slower duplication in the first 6 hours post-infection followed by exponential growth from 8 to 18 hours, achieving 7–8 cycles of replication before replication progressively slows down as RBs asynchronously transition to non-replicative EBs beginning at 24 hours post-infection [159–161]. The average size of RBs decreases over rounds of replication, limiting the number of duplications an RB can undergo [160]. Two models for division have been proposed. The first model is binary fission, in which an RB divides into two daughter cells by elongating and forming a septum at the center [162,163]. The second is budding, where one daughter cell emerges from the pole of the mother cell [164].

Both models of *C. trachomatis* cell division converge on certain identified replication-mediating – or divisome – proteins. During cell division, peptidoglycan accumulates at the septum of the dividing RB to form a ring structure (Figure 1) [165–167]. The addition of β-lactam antibiotics blocks division, leading to the

formation of large, aberrant RBs with multiple copies of chromosomal and plasmid DNA [159,162–164,168,169]. Removal of β-lactams leads to further replication via budding of RBs from the aberrant RB, but the aberrant RBs persist until host cell death rather than resuming normal division [159,168]. This shows that peptidoglycan synthesis plays an essential role in RB division, but inhibition of division does not prevent DNA replication.

C. trachomatis divisome proteins mediate the normal division process. The *C. trachomatis* MreB homologue, a cytoskeletal protein used by rod-shaped bacteria to elongate their cell wall [170], forms a ring at the septum and recruits additional divisome proteins [166], in a manner analogous to the mechanism of FtsZ used in the division of other prokaryotes [171]. In *C. trachomatis*, MreB recruits RodZ [166,172], FtsK [166], and FtsQ [173], and acts upstream of penicillin binding proteins (PBPs) PBP2 and PBP3 [169,174]. Though involved in the division process, they are not directly recruited by MreB [169,174]. Inhibition of MreB disrupts peptidoglycan synthesis, while blocking PBP2 prevents the initiation of division, and inhibition of PBP3 leads to the formation of elongated RBs that lack a septum [167,169].

Historically, *C. trachomatis* cell division was thought to occur exclusively by binary fission [162,163], with MreB substituting for FtsZ to form a ring at the center of the elongated RB, followed by septum formation. Evidence supporting this model includes electron microscopy observations of dividing RBs, which show parent and daughter cells of similar size [160,162,163]. Additionally, inhibition of peptidoglycan synthesis results in the formation of elongated RBs [169] consistent with the expected phenotype of incomplete binary fission [175]. Recent studies utilizing fluorescent imaging to examine localization of divisome proteins [164,174,176], as well as live cell microscopy to visualize dividing RBs [174,176], have brought into question whether this is the only mechanism of *C. trachomatis* RB duplication and proposed that *C. trachomatis* replicates via budding-like polarized cell division [164].

According to the budding-like polarized cell division model [164], *C. trachomatis* division occurs in distinct stages. During primary differentiation, RBs exhibit polar expression of the T3SS and Hsp60, which localize on the opposite pole from the bacterial membrane protein MOMP and sphingomyelin. In the polarized cell division stage, the daughter cell begins to grow from the MOMP-rich pole. Finally, during the two-cell stage, the daughter cell reaches a comparable size to the mother cell before undergoing FtsZ-independent separation [164]. This model suggests that MreB is recruited to the budding daughter cell by polar synthesis of cardiolipin [176]. Further evidence supporting this model includes the observation that inhibition of PBP2 prevents the formation of the peptidoglycan ring and the initial growth of the daughter cell, despite continued peptidoglycan accumulation at the MOMP-rich pole [174]. In contrast, PBP3 is not required for formation of the peptidoglycan ring, or for the initial growth of the daughter cell, but is important for the transition into the two-cell stage of division [174]. While the role of PBP3 in septum maturation aligns with both the polar budding and binary fission models, the phenotype observed with PBP2 inhibition is harder to reconcile. However, recent 3D electron microscopy analysis reveals that parent/daughter cell volume ratio and symmetrical division point more towards a binary fission model over the polarized budding model [160].

These two models converge on MreB as a substitute for FtsZ during RB division, facilitating peptidoglycan ring formation at the septum of the dividing cell as well as the recruitment of the divisome proteins listed above. However, the mechanisms underlying *Chlamydia trachomatis* replication, including its peptidoglycan synthesis and unique method of cell division, remain highly debated and unresolved within the field. Further characterization of the mechanisms of action for several of the divisome proteins and whether their functions differ significantly from their homologues, the mechanism of recruitment of the PBPs, and, most critically what role, if any, both models play in RB division.

Persistence

C. trachomatis persistence has long been observed *in vitro* and during infections, suggesting it is an important survival strategy in response to various stressors (reviewed in [177]). During replication, *C. trachomatis* RBs can transition into an aberrant body (AB) form in response to various stresses, including iron starvation, tryptophan starvation, IFNγ exposure, beta lactams, and co-infections with other pathogens (Figure 1) [159,168,178–182]. ABs are capable of surviving longer within an inclusion and can produce infectious progeny if they are formed during mid-stages of infection [159,168,183]. In penicillin-induced ABs, DNA replication still occurs, and upon removal of the stressor, infectious progeny begins to bud from the AB [159,168]. Notably, inhibition of peptidoglycan synthesis in ABs appears to mask them from host immune recognition [183]. ABs have been isolated from patients and are implicated in infection persistence following antibiotic treatment [184,185].

The ability of *C. trachomatis* to form ABs in response to stress represents a key mechanism by which it evades host immune defences and therapeutic interventions, enabling the establishment of long-term infections.

Blocking premature apoptosis

During normal replication, *C. trachomatis* protects its niche by blocking apoptosis, exerting a strong and broad anti-apoptotic effect on host cells [186]. The mechanisms by which *C. trachomatis* prevents and promotes cell death are thoroughly reviewed in [187]. The anti-apoptotic host Bcl-2 family protein Mcl-1 is upregulated via MAPK signaling pathways and stabilized by the PI3K/AKT pathway during infection and is generally considered a key factor in *C. trachomatis*'s anti-apoptotic effects (Figure 3a) [188]. Early in infection, Mcl-1 transcription is promoted by HIF-1α; however, at later stages of infection, HIF-1α levels decrease greatly while Mcl-1 expression remains high [189], indicating that an alternate mechanism upregulates Mcl-1 during later infection stages. Results from studies conducted with both serovars L2 and D suggest that host EphA2 activates the PI3K/AKT pathway during mid-cycle infection, which is important for *C. trachomatis* anti-apoptotic effects (Figure 3a) [61]. However, the mechanism by which *C. trachomatis* hijacks EphA2 and whether this interaction directly impacts Mcl-1 remains experimentally unconfirmed. Chlamydia de-ubiquitinase 1 (ChlaDUB1) (Cdu1) (CT868) has been shown to de-ubiqitinate Mcl-1 at the inclusion membrane (Figure 3a), though a Cdu1 mutant did not exhibit a significant decrease in Mcl-1 levels during infection [190]. The additional *C. trachomatis* factors that contribute to Mcl-1 expression and stabilization remain undetermined.

C. trachomatis additionally also inhibits pro-apoptotic factors, including Bcl-2 family proteins Bax and Bak, which induce mitochondrial cytochrome c release, to promote host cell survival [191]. A recent study demonstrated that ectopically expressed major outer membrane porin (MOMP) localizes to the mitochondrial fraction of HeLa cells and inhibits Bcl-2 protein mediated apoptosis (Figure 3a) [192]. However, whether this localization occurs during *C. trachomatis* infection remains unknown. *C. trachomatis* additionally inhibits apoptosis upstream of Bax/Bak by mediating the degradation of host BH3-only proteins [193–195]. Degradation of BH3-only proteins is mediated by the *C. trachomatis* type II secreted cysteine protease, chlamydial protease- or proteasome-like activity factor (CPAF) [196]. Many other factors previously attributed to CPAF cleavage have been found to be experimental artifacts caused by degradation in host cell lysates post-lysis [197], and CPAF-null *C. trachomatis* mutants have not yet been tested to show whether the degradation of BH3-only proteins is CPAF-dependent [198]. Thus, the precise mechanism by which *C. trachomatis* mediates BH3-only degradation to block apoptosis remains undetermined.

Premature lysis of the *C. trachomatis* inclusion and exposure of its contents to the host cell cytosol triggers host cell upregulation of host cell autophagic pathways and ultimately apoptosis [136]. To maintain inclusion membrane integrity, *C. trachomatis* stabilizes its membrane with Incs [136], subverts host cell actin scaffolding and microtubules to support the growing inclusion, and prevents recognition by cell autonomous immune factors [199]. CT383, IncC, CpoS, and IncS have been identified as Inc proteins whose absence leads to premature lysis of the inclusion membrane (Figure 3a) [125,136]. While the mechanisms by which these proteins stabilize the inclusion membrane remain unknown, in the case of CpoS, its interactions with other Incs and its role in stabilizing microdomains could partially explain its function.

Modulation of host cell cytokinesis

C. trachomatis targets specific stages of cell division to promote its intracellular survival and replication. Cytokinesis is a complex and tightly regulated process that concludes mitosis, ensuring proper segregation of cytoplasmic and nuclear contents between daughter cells. *Chlamydia trachomatis* infection has been shown to impair host cell cytokinesis while leaving mitosis unaffected, leading to increased multinucleation in infected cells [200]. By interfering with this process, *C. trachomatis* may create a more favorable intracellular environment, potentially increasing host cell size or altering organelle distribution to benefit bacterial growth. The precise mechanisms by which *C. trachomatis* disrupts cytokinesis remain unclear, but several bacterial effectors have been identified that target key regulatory proteins or structural components of the cytokinetic machinery [118,119,201–204].

In addition to IPAM's interactions with microtubules, ectopic expression of IPAM disrupts cytokinesis and induces supernumerary centrosomes (Figure 3b) [201] through mechanisms that remain undefined. Dre1 interacts with dynactin and plays a role in positioning centrosomes and mitotic spindles around the inclusion during infection, potentially contributing to blocked cytokinesis (Figure 3b) [118]. IncM also contributes to multinucleation [119] and centrosome relocalization to the inclusion through interactions with

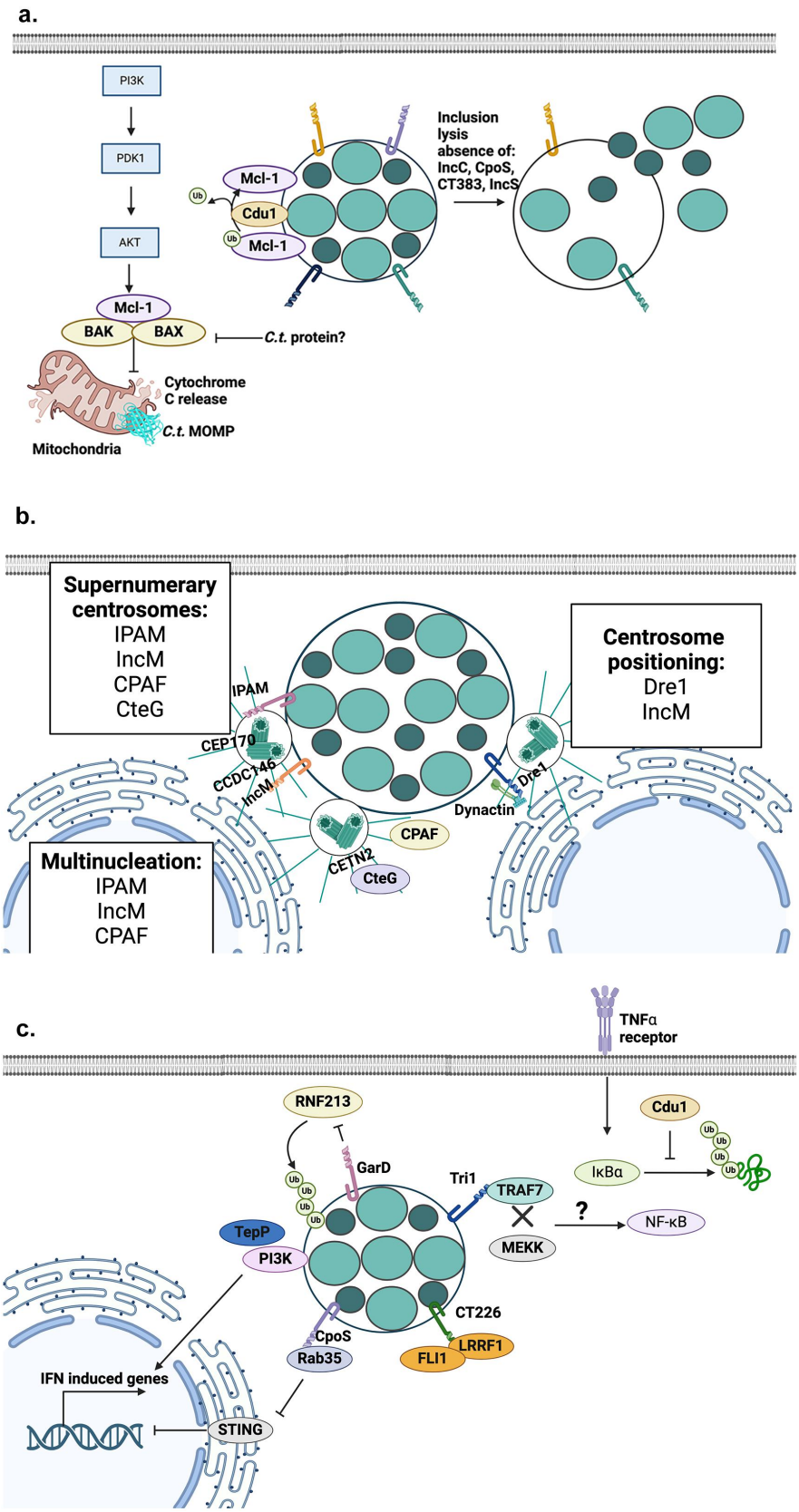

Figure 3. *C. trachomatis* secreted proteins manipulate host cell viability, centrosome dynamics and the immune response to promote infection. (a) The anti-apoptotic protein mcl-1 is upregulated via MAPK signaling, stabilized by PI3K/AKT pathways, and de-ubiquitinated by Cdu1. Ectopically expressed MOMP localizes to the mitochondria and inhibits apoptosis. *C. trachomatis* inclusion membrane stability is maintained by inc proteins, including CT383, IncC, CpoS, and IncS, which prevent premature lysis and promote

centrosome protein CCDC146 (Figure 3b) [202]. While the phenotypes of a IncM and Dre1 mutant appear strikingly similar, their potential overlap and cooperative roles in centrosome relocalization and multinucleation remain unexplored.

In addition to the Incs, secreted effector proteins have also been implicated in manipulating the centrosome and cell cycle. CPAF also contributes to multinucleation and centrosome amplification (Figure 3b) [203]. A CPAF-null mutant lacked multinucleation and multipolar spindles but does not appear to play a role in centrosome repositioning [203]. Despite its clear role in inducing centrosome overamplification and disrupting cell cycle progression, the host targets of CPAF remain unidentified, and how CPAF crosses the inclusion membrane to reach the host cytosol is still unknown. Further research is needed to elucidate how CPAF contributes to multinucleation and multipolar spindle formation.

Finally, the T3SS effector *Chlamydia trachomatis* effector associated with the Golgi (CteG)(CT105) associates with host centrin-2 (CETN2), a key regulator of centriole duplication, and contributes to the supernumerary centrosome phenotype seen in host cells during *C. trachomatis* infection (Figure 3b) [204]. The absence of CETN2 or CteG significantly impairs *Chlamydia's* ability to induce centrosome amplification, suggesting CteG subverts centriole duplication machinery to promote centrosome overamplification [204]. Notably, CteG appears to be cleaved during infection [204–206]. It has been proposed that CPAF cleaves and activates CteG [206,207], highlighting the need to examine potential interplay between these effectors in modulating host centrosome dynamics during infection.

As these *C. trachomatis* proteins induce cellular abnormalities similar to those observed in precancerous cells, it is imperative to examine how these proteins work in concert to perturb cytokinesis. It is possible that these proteins contribute to the increased risks of cervical [7–9] and ovarian [10–12] cancers seen in individuals with a current or previous *C.t.* infection.

Secondary differentiation

Secondary differentiation from RB to EB is critical for producing infectious progeny, a process that begins at 24 hpi, but reaches its peak between 28 and 40 hpi [160]. During this process, RBs become progressively smaller, an observation that gave rise to a model in which *C. trachomatis* uses size to regulate replication and trigger expression of genes required for differentiation [160]. This model also explains the asynchronous nature of secondary differentiation, yet the mechanism by which *C. trachomatis* detects and responds to this size threshold remains unknown.

Another model proposes that during the late stage of replication, after an initial period of RB proliferation, each division produces one RB capable of continued replication and one IB, which transitions into an EB without further replication (Figure 1) [175]. This is supported by the upregulation of *hctA* expression even when replication is inhibited [175]. In this model, EB production relies on the conversion of hypothetical replicating RBs (RB_Rs) into mother/stem cells (RB_Es) that generate EB progenitor IB [175]. However, markers for these proposed RB subtypes have yet to be identified, and the stimuli and mechanisms driving differential gene expression in the RB_E and the IB following division remains unknown. It is intriguing to speculate that the two models of replication could help determine the fate of the daughter cells, with binary fission contributing to RB expansion and budding accounting for RB_E and IB differentiation.

The transcriptional regulator general regulator of genes A (GrgA) (CT504) is essential for secondary differentiation in *C. trachomatis* [208,209]. GrgA upregulates the expression of several key genes involved in this process, including *tsp*, *hctA, hctB, omcA*, and multiple genes encoding components of the T3SS and its late-expressed effectors [209]. GrgA functions by binding to *C. trachomatis* sigma factors, thereby modulating gene expression throughout the intracellular developmental cycle [208–211]. Notably, in an inducible GrgA-deficient mutant, loss of GrgA function prevents the formation of infectious EBs, underscoring its critical

host cell survival. (b) The inc protein IPAM induces cytokinesis defects and promotes supernumerary centrosome formation, while Dre1 interacts with dynactin to position centrosomes around the inclusion. IncM contributes to multinucleation and centrosome localization by interacting with CCDC146. CPAF and CteG also induce centrosome amplification, with CteG targeting host centrin-2, a regulator of centriole duplication, to promote supernumerary centrosome formation and intracellular survival. (c) TepP recruits scaffolding proteins and PI3K to the inclusion, modulating host chemokine expression to diminish neutrophil recruitment. The effector Cdu1 inhibits NF-κB signaling by stabilizing IκBα, while Tri1 displaces TRAF7-associated kinases to potentially suppress NF-κB signaling. Other effectors, such as CpoS and GarD, suppress STING and RNF213 pathways to evade interferon responses. CT226 recruits LRRF1 to the inclusion membrane, potentially regulating innate immune responses.

role as a transcriptional activator during secondary differentiation [209].

During secondary differentiation, *C. trachomatis* employs proteases to degrade RB-specific proteins, enabling the transition to the EB form. This turnover depends on cytosolic $ClpP_2$ and ClpX [212,213], as well as the periplasmic Tail-specific protease (Tsp)(CT441) (Figure 1) [214]. In the *C. trachomatis* Clp protease system, the ATPase/unfoldase ClpC interacts with the $ClpP_2$ subunit of the proteolytic $ClpP_1P_2$ proteolytic complex during replication [215]. During secondary differentiation, the ATPase ClpX also interacts with the ClpP1P2 complex via the $ClpP_2$ subunit, and dominant negative overexpression of ClpX results in a failure to complete secondary differentiation [212].

ClpX and ClpC ATPases likely target distinct substrate pools, with ClpX interacting with SsrA-tagged proteins [212], ClpC does not (unpublished data [215], though this requires further confirmation. Moreover, the differences in ClpX and ClpC binding to the $ClpP_1P_2$ complex, the specific substrates of these interactions, and the regulatory mechanisms that upregulate or activate ClpX to drive secondary differentiation remain critical areas for future investigation.

C. trachomatis Tsp is a late stage expressed protein that is important for the production of infectious EBs [214]. It is an ortholog of Gram-negative Tsps, which are involved in periplasmic maintenance and the regulation of peptidoglycan-associated proteins [216]. *C. trachomatis* Tsp is necessary for proper EB morphology, as CRISPR interference (CRISPRi) knockdown results in a large "void" space between either the inner and outer membranes or between the nucleoid and the inner membrane during secondary differentiation. Tsp disruption also leads to a loss of EB viability and infectivity [214]. The specific substrates of Tsp in the periplasm and how their lack of degradation contributes to these morphological and functional changes has yet to be determined. Interestingly, while Tsp overexpression impairs replication, knockdown only impacts secondary differentiation [214]. This raises the intriguing possibility that *C. trachomatis* Tsp degrades peptidoglycan synthesis proteins, a process that could terminate replication and facilitate secondary differentiation, as proposed in the direct RB-to-EB transition model. Further investigation is needed to elucidate these mechanisms and their role in *C. trachomatis* pathogenesis.

During the transition from RB to EB, at the IB stage, Euo expression ceases, allowing for expression of late genes, including the *omcAB* operon, to drive progression to the EB state [157,217,218]. Euo expression is self-regulated, as it represses its repressor, though the identity of the repressor and the mechanism that decrease Euo levels remain unknown [217,218]. The upregulation of OmcA and OmcB expression likely allows for increased disulfide bonds formation between components of the COMC, such as MOMP, OmcA, OmcB, LPS, and the *C. trachomatis* T3SS proteins CdsF, CdsD, and CdsC (Figure 1) [154,155,158,219]. The unique Kdo-trisaccharide is important for this cross-linking, as its absence significantly reduces EB progeny [219]. Similarly, cysteine restriction markedly decreases EB formation, likely due to the inability to the impaired ability to form disulfide crosslinks [220].

During secondary differentiation, the *C. trachomatis* chromosome condenses into an electron-dense nucleoid (Figure 1). Prior to condensation, chromosomal replication is blocked, and active replication forks are completed, though the specific *C. trachomatis* factors responsible for this are yet to be fully elucidated [221]. During late infection, *hctA* and *hctB* are expressed [222–224]. These genes encode the *C. trachomatis* DNA-binding histone homologues Hc1 and Hc2, respectively [222–224]. Hc1 is conserved across serovars, while Hc2 varies in size [225], but both histones bind DNA and RNA with varying affinities – Hc1 for supercoiled DNA and Hc2 for RNA and linearized DNA [226]. This binding activity inhibits transcription and translation, as demonstrated both in *E. coli* and *in vitro* [226,227]. Additionally, both condense DNA with distinct phenotypes: Hc1 produces spherical nucleoids, while Hc2 forms coil-like structures of condensed DNA [224,228]. An additional protein that has been shown to play a role in DNA remodeling during RB to EB conversion is developmental DNA binding protein (DdbA) [221]. DdbA is thought to functions as an endonuclease, as a temperature sensitive DdbA mutant fails to produce infectious progeny and is arrested at the RB to EB transition, though its role in secondary differentiation requires further investigation [221,229]. Notably, both *ddbA* and *hctA* —but not *hctB* —are regulated by the sRNA inhibitor IhtA, which is expressed during early-mid to mid *C. trachomatis* growth [153,230,231]. This shared regulation supports the notion that repression of *ddbA* and *hctA* is critical for primary differentiation, while their expression is necessary for secondary differentiation.

Exit from the host cell

After secondary differentiation, infectious EBs exit the host cell through two distinct strategies: lysis and extrusion (Figure 2d) [232]. Extrusion involves the release of intact inclusions, referred to as extrusions, exiting the

host cell without inducing host cell lysis in a host N-WASP, actin, and myosin II pathway-dependent manner [232–234]. Extrusion has been proposed to facilitate immune evasion, promote dissemination, and enhance the extracellular survival of EBs [235], making it a compelling aspect of *C. trachomatis* pathogenesis.

Prior to extrusion, components of the myosin II pathway are recruited to microdomains on the inclusion membrane [234]. CT228, an inclusion membrane protein, has been shown to localize to these microdomains and recruits' myosin phosphatase target subunit 1 (MYPT1) [234]. However, CT228 is not conserved across all *Chlamydia species*. Its absence in certain serovars [234] does not abolish extrusion [236,237] but rather increases it in mutant strains compared to the parent strain [237]. This suggests that CT228 plays a regulatory role in extrusion rather than being essential for its induction.

C. trachomatis Inc protein Myosin Regulatory Complex subunit A (MrcA) (CT101) interacts with the host calcium channel type 3 inositol-1,4,5-trisphosphate receptor (ITPR3) within inclusion microdomains (Figure 2d) [238]. A MrcA mutant exhibited reduced extrusion, which correlated with reduced myosin light chain 2 (MLC2) phosphorylation, and consequently impaired myosin motor activity [238]. This phenotype was also observed in ITPR3 or STIM1 knockdown cells, highlighting the critical role of calcium signaling in regulating myosin II pathways during MrcA-mediated extrusion [238].

Several *C. trachomatis* inclusion-localized proteins have been shown to play a role in extrusion. Cdu1 protects itself and the *C. trachomatis* Inc proteins CT228, InaC, and IPAM from host cell ubiquitination through lysine acetylation during the late stages of infection (Figure 2d) [239]. Mutants lacking Cdu1, InaC, or IPAM exhibit reduced extrusion formation [239], highlighting the critical role of Cdu1 in regulating this process. While InaC and IPAM are known to influence actin and microtubule dynamics, the precise mechanisms by which they contribute to extrusion remain unknown.

The final step of inclusion extrusion, during which the extrusion is pinched off from the host cell, relies on host Rho GTPases, in particular RhoA, and endosomal sorting complex required for transport (ESCRT) family abscission proteins (Figure 2d) [232,240]. Due to InaC's established interactions with RhoA [114] and its importance for extrusion [239], it is interesting to speculate that InaC contributes to this critical step of the process.

CT619 has been identified as an interactor of the ESCRT family protein Tsg101 through a domain of unknown function (DUF), DUF582, which it shares with four other hypothetical *C. trachomatis* effectors [241]. It has been suggested that these conserved proteins play a role in extrusion [241], though this hypothesis remains untested for both the DUF582-containing proteins and Tsg101.

Extrusion has been shown to occur *in vivo* in both serovars D and L2 as well as *C. muridarum* [242], while extrusions have been observed *in vitro* in serovars B, D, and L2 and multiple *C. spp*. [232,236], indicating that it is a highly conserved *Chlamydia* exit strategy. The benefit of extrusion appears to be in both increasing survival time of EBs in the extracellular environment [235] and avoidance of the host immune response [235,243]. EBs within extrusions are capable of surviving within macrophages and eventually escape, although these EBs are incapable of primary differentiation into RBs while inside macrophages [235]. This macrophage infiltration strategy may facilitate *C. trachomatis* dissemination. Similarly, extrusions engulfed by dendritic cells promote EB survival, while upregulating production of immunosuppressive cytokines followed by apoptosis of the dendritic cells [243]. *In vivo* studies further support the functional benefits of extrusion, where despite similar disease burdens, mice infected with a CT228 mutant that displays increased extrusion, exhibited delayed clearance and had a reduced immune response to infection [237]. Additionally, because extrusion leaves the host cell intact – albeit highly transformed – surviving cells may retain infection-induced cellular abnormalities such as aneuploidy, supernumerary centrosomes, and multinucleation, which highlight its potential role in promotion of oncogenesis. Collectively, these data present a unique model of immune evasion and pathogenesis that appears to be broadly conserved across the *Chlamydia* genus.

In contrast, host cell lysis allows for release of infectious EBs that can infect nearby cells immediately, rather than after breakdown of an extrusion. The lysis process involves the disassociation of the inclusion from stabilizing actin scaffolds followed by lysis of both the inclusion and the host cell, releasing individual EBs [232,244]. Lysis can be blocked by protease inhibitors before inclusion lysis, and the lysis of the host cell's plasma membrane is calcium-dependent [232].

CPAF has been implicated in host cell lysis, as evidenced by the inability of a serovar L2 CPAF null mutant to induce host cell lysis in cycloheximide-free media [244]. Live cell microscopy has revealed CPAF-mediated cleavage of host targets such as the nuclear envelope protein lamin-associated protein-1 (LAP1) and vimentin filaments [198]. While vimentin cleavage

has been ruled out as essential for *C. trachomatis* mediated host cell lysis [244], the potential role of LAP1 cleavage remains unexplored. Identifying additional targets of CPAF and their relevance to inclusion and host cell lysis will help clarify its function in lytic egress.

Host cell lysis is also partially mediated by the *C. trachomatis* plasmid-encoded transcriptional regulator Pgp4 [244]. Pgp4 regulates the transcription of virulence-related genes on the *C. trachomatis* chromosome [245] and specifically induces expression of at least one unidentified T3SS effector protein that is necessary for actin depolymerization prior to lysis [244]. Further studies are needed to identify what protein or proteins, regulated by Pgp4, mediate this actin depolymerization.

The T3SS effector CteG has been proposed to play a role in the Pgp4-mediated pathway of host cell lytic egress, though its expression is not regulated by Pgp4 [206]. Overexpression of CteG rescues host cell lysis when cells are infected with CteG or Pgp4 deficient mutants [206]. However, what role CteG plays in Pgp4-mediated host cell lysis remains unclear. Since Pgp4-regulated targets are well-documented [245], future characterization of these targets will likely shed light on the mechanisms of *C. trachomatis* lytic egress.

Virulence plasmid

The highly conserved 7.5kb *C. trachomatis* plasmid has homologues in all *C. trachomatis* serovars [246] and has been identified in almost all clinical isolates to date [247], indicating that it plays an important role in *C. trachomatis* virulence. Although the plasmid is not essential, plasmid-free strains exhibit attenuated pathogenicity *in vivo* [248,249] and women infected with a serovar E strain containing a deletion in the plasmid displayed fewer clinical symptoms [250]. Studies using *C. muridarum*, which harbors a homologous plasmid, suggest that the plasmid is important for ascension into the upper genital tract [251], a major contributor to *C. trachomatis* pathology. Both *C. trachomatis* and *C. muridarum* plasmids are necessary for TLR2 signaling in infected mice, where TLR2 activation is associated with increased pathogenicity [252].

The *C. trachomatis* plasmid encodes eight ORFs that produce plasmid gene proteins 1–8 (Pgp1–8) and small anti-sense RNAs (sRNAs) [246,253]. The *pgps* are transcribed across serovars, though ocular serovars have lower levels of transcription compared to urogenital or LGV serovars [254]. Pgp1, Pgp2, Pgp6, and Pgp8 play a role in plasmid maintenance [245,255]. Pgp7, despite similarity to Pgp8, is not essential for plasmid maintenance [245,255]. Similarly, Pgp5 is not required for plasmid maintenance but serves as a negative regulator of chromosomal genes that are upregulated by Pgp4 [256]. This negative regulatory role is more pronounced in *C. muridarum* than in *C. trachomatis* [256], suggesting that *C. trachomatis* has additional, unidentified regulatory mechanisms for these genes or that differences in Pgp5 between the two species result in distinct regulatory outcomes.

Pgp4 upregulates the expression of Pgp3 and chromosomal genes in *C. trachomatis* [245]. It is important for host cell lysis at the end of the infectious cycle as well as secretion of Pgp3 into the host cell cytosol [244,245,257,258]. Pgp3, the only secreted Pgp, promotes inflammatory cytokine production during infection [259]. Recent findings also implicate Pgp3 in vaginal and intestinal colonization [260]. In *C. trachomatis*, Pgp3 expression confers resistance to lactic acid, while in *C. muridarum*, Pgp3 is important for colonization of both the vaginal and intestinal tracts in mice [260]. Additionally, Pgp3 increases *C. trachomatis* infectivity of human donor-derived primary gastric cells [261], a proposed niche for *C. trachomatis* survival during azithromycin treatment (reviewed in [262]. Taken together, the *C. trachomatis* plasmid contributes to *C. trachomatis* virulence in various ways, although further studies are needed to fully elucidate the roles of Pgp3, Pgp4, and potentially Pgp5 in pathogenesis.

Immune interacting effectors

During its intracellular developmental cycle, *C. trachomatis* employs a variety of effectors to subvert host cell immune responses. Translocated early phosphoprotein (TepP; CT875) recruits scaffolding proteins CrkI, CrkII, and CrkL as well as the lipid kinase PI3K, to the nascent inclusion (Figure 2a) [66,263]. TepP expression induces upregulation of interferon-induced proteins with tetratricopeptide repeats (IFITs), specifically IFIT1 and IFIT2, although its interaction with PI3K appears to dampen the IFIT upregulation [66,263]. Additionally, TepP reduces the expression of chemokines IL-6 and CXCL3 [66], which has been shown in organoid models to diminish neutrophil recruitment to *C. trachomatis* infected epithelial cells [264]. In murine models, serovar L2 and *C. muridarum* TepP mutants are cleared more rapidly than wild-type bacteria and exhibit impaired ascension to the upper genital tract [67]. However, it remains unclear how much of this clearance is directly due to TepP-mediated immune modulation versus other defects resulting from TepP deletion.

NF-κB signaling is another pathway that is potentially targeted by multiple *C. trachomatis* effectors. Cdu1 has been shown to downregulate NF-κB signaling by binding IκBα and preventing its TNFα-mediated ubiquitination and degradation (Figure 3c) [265]. A serovar L2 Cdu1 mutant displays increased sensitivity to IFNγ treatment [190], likely due in part to its altered interactions with the NF-κB signaling pathway. TRAF7 interactor (Tri1; CT224), interacts with host tumor necrosis receptor-associated factor 7 (TRAF7) at the inclusion membrane early in infection (Figure 3c) [266]. This interaction displaces endogenous interactors, including mitogen-activated protein kinase kinase (MEKK) MEKK2 and MEKK3 [266]. Since MEKK3-TRAF7 interactions lead to upregulation of NF-κB signaling [267], it has been hypothesized that Tri1-mediated displacement of TRAF7 interactors could suppress host cell NF-κB signaling [266], though this has not yet been experimentally demonstrated.

Other Incs have recently been shown to contribute to immune evasion. CpoS utilizes its coiled-coil domain to interact with Rab35 and suppress STING signaling, thus dampening IFN-I responses in infected cells (Figure 3c) [134]. Gamma resistance determinant (GarD) (CT135) acts as an antagonist of RNF213 in IFNγ treated cells, protecting the inclusion membrane from ubiquitylation (Figure 3c) [199]. CT226 interacts with leucine-rich repeat flightless-1 interacting protein 1 (LRRF1) and recruits it to the inclusion membrane [132,139]. Recent work has shown that FLI1 interacts with CT226 through its association with LRRF1, with the interaction between FLI1 and CT226 relying on LRRF1 [268]. However, both FLI1 and LRRF1 can independently localize to the inclusion suggesting that FLI1 utilizes additional pathways for localization beyond its interactions with CT226 and LRRF1 (Fig. C) [268]. Both LRRF1 and FLI1 have been shown to regulate the host innate immune response yet their role in chlamydial pathogenesis remains largely unknown.

Collectively, these findings highlight the sophisticated strategies employed by *C. trachomatis* to manipulate host immune signaling pathways, including interferon responses, NF-κB signaling, and chemokine production. Understanding the interplay between bacterial effectors and host immune components not only sheds light on *C. trachomatis* pathogenesis but also provides potential avenues for therapeutic interventions targeting these immune-modulating mechanisms.

Vaccine

Failure to adequately clear *C. trachomatis* from the reproductive tract is complex and multifactorial. Treatment failure in an individual can occur for several reasons, including patient noncompliance to the doxycycline antibiotic regimen, individual differences in mucosal medication absorption, or heterotypic antibiotic-resistant *C. trachomatis* in select local populations [269,270]. Moreover, a majority of *C. trachomatis* infection-associated pathologies are often quiescent, leading to gross underdiagnosis, clinically [6,271–273]. Thus, effective prevention strategies, such as vaccination, is of utmost importance in order to limit the downstream consequences of *C. trachomatis* infection [274].

Natural immunity to *C. trachomatis* following primary infection has been documented in humans. In a prospective cohort study of women who had not received antibiotics within 60 days of a positive *C. trachomatis* screening test, approximately ~ 18% of women returning to clinic for treatment presented with both culture- and PCR-negative results, suggesting spontaneous resolution of *C. trachomatis* infection [275]. However, this protection is not long-lived nor protective and the natural resolution of *C. trachomatis* infection without antibiotics can take as long as 16 months [275,276]. One of the most potent host immune defence against recurrent *C. trachomatis* infection is T-cell immunity, particularly IFN-γ secreting $CD4^+$ and $CD8^+$ T cells, however specific anti-chlamydial antibodies also play a role in humoral protection against *C. trachomatis* [277–279]. Early *C. trachomatis* vaccine efforts focused primarily on MOMP [280]. In a murine model of *C. trachomatis* infection, immunization with native MOMP purified from live *C. trachomatis* produced robust protection upon secondary challenge when compared to recombinantly purified MOMP [280]. However, optimization and production of an effective human MOMP vaccine is impeded by several challenges. Oculogenital *C. trachomatis* express differential serovar-specific MOMPs, and effective immunologic memory requires conformationally folded MOMP isolated from live bacteria rather than recombinant protein, thereby complicating the commercial production and public access to the human vaccine [280,281].

In the modern era of genomic sequencing and advanced chlamydial genetic tools, the repertoire of chlamydial vaccine targets continues to expand [282–287]. Many vaccine efforts focus on surface-exposed membrane proteins such as PmpD and PmpG that may be more immunologic and amenable to large-scale production [288]. The chlamydial vaccine repertoire has additionally expanded beyond surface-expressed bacterial proteins. Emerging vaccination studies utilizing recombinant T3SS secreted effectors, such as TarP, CPAF, and CopB, are showing promising

outlooks in murine studies [289–291]. A major aspect that has hindered chlamydial vaccine development is the lack of a genetic transformation system to generate gene mutants. However, with the rapid expansion of chlamydial genetic tools [282–287], the development of an effective human vaccine is greatly expedited and optimized, allowing for not only the identification of new immunologic targets but also the testing of key chlamydial virulence factors as potential vaccine candidates [292–294].

Conclusions/future directions

C. trachomatis poses a significant global health challenge due to its diverse mechanisms of direct and indirect pathogenesis, the prevalence of asymptomatic infections, and the lack of vaccine. In this review, we have highlighted several stages in the pathogen's developmental cycle that could be targeted for therapeutic intervention. Inhibiting the intracellular developmental cycle at any stage reduces the production of infectious progeny; however, focusing on the early stages of adhesion and invasion offers the greatest potential to mitigate pathogenesis as these stages precede the extensive host cell modifications induced by *C. trachomatis*.

Throughout its developmental cycle, *C. trachomatis* numerous strategies to ensure the production of infectious progeny. Inc proteins and conventional T3SS effectors constitute a critical component of its pathogenic arsenal [19,20,62,295–298]. Despite their importance, many of these effectors remain poorly characterized, presenting a valuable opportunity for further research to uncover additional mechanisms of pathogenesis and virulence. Historically, limitations in genetic tools have constrained much of the research to serovar L2, which does not fully represent the diversity across biovars. Variations in adhesion, as well as the expression of T3SS effectors and Pmps, differ between biovars and influence tissue tropism and molecular mechanisms of pathogenesis. This area warrants further investigation to achieve a comprehensive understanding of *C. trachomatis* pathogenesis across diverse strains.

Extrusions may facilitate the dissemination of *C. trachomatis* to distal locations via macrophages, effectively hindering localized immune responses. This process could enable ascension to the upper female reproductive tract, leading to severe complications, or potentially to the intestinal lining, where *C. trachomatis* could establish a more treatment-resistant niche. The *C. trachomatis* plasmid plays a significant role in driving more severe disease manifestations and enhancing colonization efficiency. Additionally, aberrant body formation during infection likely contributes to evasion of both natural host immunity and by therapeutic interventions, complicating clearance efforts.

Together, *C. trachomatis* pathogenesis and virulence requires completion of its intracellular developmental cycle, hinging on delivery of T3SS substrates to establish its niche, maintenance of its plasmid, subversion of host immunity, and use of extrusion and persistence mechanisms to avoid clearance and infect its next host.

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Author contributions

AM, SH, and MMW contributed to the conception, design, and drafting of the paper. All authors approved the final submitted version and agree to be accountable for the accuracy and integrity of the work.

Data availability statement

Data sharing is not applicable to this article as no new data were generated.

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