Published in final edited form as:

Trends Microbiol. 2018 July ; 26(7): 611–623. doi:10.1016/j.tim.2017.12.002.

***Chlamydia* Spreading from the Genital to Gastrointestinal Tracts —A Two-Hit Hypothesis**

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Abstract

Chlamydia trachomatis, a leading bacterial cause of sexually transmitted infection-induced infertility, is frequently detected in the gastrointestinal tract. *Chlamydia muridarum*, a model pathogen for investigating *C. trachomatis* pathogenesis, readily spreads from the mouse genital tract to the gastrointestinal tract, establishing long-lasting colonization. *C. muridarum* mutants, despite their ability to activate acute oviduct inflammation, are attenuated in inducing tubal fibrosis and are no longer able to colonize the gastrointestinal tract, suggesting that *C. muridarum* spreading to the gastrointestinal tract may contribute to its pathogenicity in the upper genital tract. However, gastrointestinal *C. muridarum* cannot directly auto-inoculate the genital tract. Both antigen-specific CD8⁺ T cells and profibrotic cytokines such as TNF α and IL-13 are essential for *C. muridarum* to induce tubal fibrosis, which may be induced by the gastrointestinal *C. muridarum* as a second hit to transmucosally convert tubal repairing initiated by *C. muridarum* infection of tubal epithelial cells (serving as the first hit) into pathogenic fibrosis. Testing the two-hit mouse model should both add new knowledge to the growing list of mechanisms by which gastrointestinal microbes contribute to pathologies in extra-gastrointestinal tissues and provide information for investigating the potential role of gastrointestinal *C. trachomatis* in human chlamydial pathogenesis.

I. *Chlamydia* infection in the genital tract

Sexually transmitted infection with *Chlamydia trachomatis* (CT) in humans can lead to tubal adhesion/fibrosis/hydrosalpinx (<http://www.cdc.gov/std/tg2015/chlamydia.htm>). However, the pathogenic mechanisms remain unknown. *Chlamydia muridarum* (CM) has been used for investigating CT pathogenesis due to its ability to induce long-lasting tubal fibrosis/hydrosalpinx in mice following an intravaginal inoculation [1–5]. The vaginal CM must overcome both the cervical barrier [6–11] and uterotubal junction [12] in order to reach the oviduct. After arriving in the oviduct, CM infects tubal epithelial cells and triggers

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pyosalpinx [13]. The acute tubal inflammatory responses may cause collateral damages both injuring tubal epithelia and clearing chlamydial infection, which is followed by repairing the damaged epithelial tissue with matrix components such as collagens secreted by fibrocytes and replacing the acellular fiber structure with new epithelial cells through re-epithelialization. An optimistic outcome is to restore the reproductive function of the oviduct. However, in some cases, tubal fibrosis continues and becomes pathogenic by occluding the oviduct lumen, leading to long-lasting hydrosalpinx and tubal infertility. The question is how the pathogenic tubal fibrosis is maintained after chlamydial infection is completely cleared from the genital tract.

Over the years, extensive efforts have been made to understand the chlamydial pathogenic mechanisms in the female upper genital tract, which has revealed that both chlamydial and host factors can impact chlamydial ascension and induction of tubal fibrosis/hydrosalpinx. The chlamydial plasmid is a key pathogenic determinant for CM to induce hydrosalpinx [9,10] and deficiency in the plasmid-encoded pGP3 phenocopied plasmid-deficiency [14, 15], indicating a major role of pGP3 in plasmid-dependent pathogenicity [16]. The chlamydial chromosome also encodes many putative virulence factors [9–11, 14, 17–28]. Loss of function mutations in CM chromosomal proteins TC0237/TC0668 [29, 30] consistently reduces hydrosalpinx induction. Many host pathways have been found to impact chlamydial induction of upper genital tract pathology [8, 31–34]. For example, TNF α and IL-13 signaling pathways are required for promoting CM induction of hydrosalpinx [35–37]. In terms of adaptive immune responses, CD8 $^{+}$ T cells play a critical role in CM induction of hydrosalpinx [36, 38] while CD4 $^{+}$ T cells are required for protective immunity [39]. However, the accumulated knowledge is still insufficient for addressing how the long-lasting pathogenic tubal fibrosis is maintained after oviduct infection with *Chlamydia* is completely cleared.

II. CT is frequently detected in the gastrointestinal tract

Despite the fact that CT is a pathogen of the genital tract, it is also routinely detected in human gastrointestinal (GI) tracts [40–44], which is consistent with the observation that CT can infect human enteroendocrine cells [45]. Women practicing oral/anal sex could introduce CT into their GI tracts [43, 44] while those without practicing these behaviors were also found positive for CT in their rectal swabs [41, 42], suggesting that CT may spread from women's genital tracts to the GI tracts via sexual behavior-independent pathways. Interestingly, CT has been detected in human blood samples [46, 47] and shown to infect human peripheral blood cells [48], suggesting that CT may enter and survive in the circulation. This assumption may partially explain the observations that sexually transmitted CT has been detected in synovial tissues [49] and high titers of anti-CT IgG antibodies are detected in women with tubal infertility [50–53]. Regardless how CT gets into women's GI tract, a burning question is whether the GI tract CT can affect CT pathogenicity in the genital tract [54]. Addressing this question is imperative because guidance is urgently needed for dealing with the frequent detection of CT in the human GI tract. However, addressing this question in humans may require large-scale clinical investigations likely involving therapeutic intervention. An alternative approach is to evaluate the contribution of

the GI tract *Chlamydia* to the genital tract pathogenicity using the CM induction of hydrosaplinx murine model.

III. CM spreads from the mouse genital to GI tracts

1. Real time imaging of genetically engineered CM infection in mice

The successful transformation of CT L2 serovar organisms [55–57] made it possible to genetically modify CM [58]. When a CM clone engineered to express luciferase was characterized in HeLa cells, both the luciferase gene expression and enzymatic activity (measured as bioluminescence intensity) correlated well with the level of CM reticulate body (RB) proliferation [59]. The bioluminescence signals from replicating RBs had a short half-life and the mature elementary bodies (EBs) displayed minimal level of bioluminescence, suggesting that the bioluminescence signal can be used for monitoring active chlamydial replication in mice. Following an intravaginal inoculation with luciferase-expressing CM organisms, bioluminescence signal was first detected at the lower genital tract area within a week, but the signal migrated to the lower abdominal area where the upper genital tract is located, suggesting that CM underwent rapid ascension in the female mouse genital tract.

2. Detection of CM in the GI tract tissues following genital inoculation

CM is known to undergo a self-limited infection course in the genital tract of female mice following an intravaginal inoculation with most mice clearing CM infection in 4–5 weeks [8]. However, when a luciferase-CM clone was monitored in mice using *in vivo* imaging for >4 weeks after the initial inoculation, significant bioluminescent signal was still detectable in the mouse abdominal area [60]. This bioluminescent signal lasted for > 100 days. *Ex vivo* imaging of the mouse organs revealed that these long-lasting bioluminescent signals were from the GI tract tissues including the stomach, small intestine, cecum, colon and rectum but not the genital tract or other tissues. These GI tract signals represented replicating CM organisms as revealed by direct detection of live chlamydial organisms and genomes in the same organ tissue homogenates. Thus, the genital tract chlamydial organisms can spread to and establish a long-lasting infection in the GI tract.

3. The genital chlamydial spreading into the GI tract is independent of oral/anorectal routes

The next question is how the genital CM spreads to the GI tract. After an intravaginal inoculation, CM organisms were recovered from many tissues including extra-GI organs during the first two weeks while the genomes persisted in these organs for ~4 weeks depending on the mouse strain [60], indicating that the genital inoculation caused systemic spreading. However, it remains unknown whether systemic spreading is responsible for CM to traffick to the GI tract. Following an intravaginal inoculation, CM organisms are shed to the vagina, which may be excreted for mice to take up orally or via anorectal contact. To exclude these possibilities, mice wearing Elizabethan collars and singly housed in netted cages were monitored for genital and GI tract infections following an intravaginal inoculation. Despite the physical restraining of mice from coprophagy, all mice still developed long-lasting infection in the GI tract [60], suggesting that oral uptake is not

required for genital chlamydial organisms to spread to the GI tract. However, the above experiment cannot exclude the possibility of vagina-anorectal cross-contamination, which might contribute to the observed GI tract spreading. This possibility was tested by comparing CM shedding in the vaginal and rectal swabs between mice intravaginally versus intrabursally inoculated with CM [60]. Although mice with intravaginal inoculation developed positive shedding in the vaginal swab on day 3 and in the rectal swab on day 7, the intrabursally inoculated mice failed to shed organisms in the vaginal swabs on either day 3 or 7 but developed positive shedding in the rectal swabs on day 7. Since the intrabursally inoculated CM organisms successfully spread to the GI tract by day 7 when no live organisms were present in the vaginal excretion, the intrabursally-inoculated CM organisms must have spread to the GI tract via a route independent of either oral uptake or anorectal contact. Thus, it can be concluded that genital chlamydial organisms must spread to the GI tract by tissue penetration or blood/lymph circulation.

4. Intravenous CM establishes long-lasting colonization restricted to the GI tract

In support of the circulatory route spreading is the observation that CM organisms survived in the blood stream for more than two weeks following an intravenous injection [61]. Furthermore, when an intravenously inoculated luciferase-expressing CM was monitored using *in vivo* imaging, all luciferase-generated bioluminescence signals were detected in the GI tract tissues shortly after the inoculation. It is clear that hematogenous CM can successfully establish a long-lasting colonization in the GI tract. The question is how the genital tract chlamydial organisms enter and survive in the blood. Naked chlamydial organisms are rapidly inactivated by serum complement [21], suggesting that the organisms must hide inside cells during the blood stage. Monocytes/macrophages (M ϕ s) and dendritic cells (DCs) may serve as the vehicle since these cells have been shown to disseminate intracellular bacteria [62–65] and to also support chlamydial replication/persistence [27, 66–70]. Neutrophils generally kill *Chlamydia* [71–73]. Mice expressing diphtheria toxin (DT) receptor under the control of CD11b [CD11b-DTR; ref:[74]] or CD11c [CD11c-DTR; ref:[75, 76]] promoters can be used to evaluate the roles of M ϕ s or DCs in chlamydial spreading.

IV. Lack of autoinoculation into the genital tract by GI tract CM

Since *Chlamydia* was detected in the GI tracts of animals [77] and humans [41–44], some have proposed that the GI tract *Chlamydia* may serve as a reservoir for auto-inoculating into the genital tract to promote chlamydial pathogenicity in the upper genital tract [78, 79]. However, this hypothesis has not been tested and there is no direct evidence from either animal model studies or human investigations for supporting this hypothesis. On the contrary, it was recently reported that the GI tract CM organisms failed to autoinoculate the genital tract of the same mice after colonization in the GI tracts for >70 days [80]. Despite the continuously shedding of infectious organisms from the rectal swabs of mice inoculated either intragastrically or intrarectally, no significant chlamydial organisms were detected in the vaginal swabs. More importantly, these mice did not develop any significant inflammatory pathology in the genital tract or any other organs [81]. It is worth pointing out that lack of autoinoculation by GI CM [60, 61, 80] does not necessarily suggest that the

autoinoculation theory is invalid in humans. For example, a person may use fingers or practice oral and/or anal sex to spread CT from his/her GI to genital tracts. To determine whether and how the GI tract CT serve as a reservoir for auto-inoculating the genital tract in humans, well-controlled large-scale clinical studies are required.

V. Chlamydial spreading from the genital to GI tracts correlates with chlamydial pathogenicity in the upper genital tract

1. Temporal correlation of CM colonization in the GI tract with CM induction of hydrosalpinx

Although chlamydial persistence in the genital tract has been proposed, there is no direct evidence and it is difficult to differentiate persistent infection from reinfection [82]. On the contrary, CM infection in the female mouse genital tract is hardly persistent, only lasting about four to six weeks depending on the specific mouse strains [8], after which chlamydial organisms or genomes were no longer detectable in either vaginal swabs or the genital tissue, including the oviduct/ovary tissues [10, 60, 83]. It is clear that CM infection in the mouse genital tract is self-limited. However, CM-induced tubal hydrosalpinx persisted for much longer [8, 13], creating a temporal gap between the genital CM and the upper genital tract pathology. Interestingly, once genital CM spreads to the GI tract, CM can persist in the GI tract for long periods of time [60], temporally correlating with the CM-induced long-lasting hydrosalpinx.

2. Attenuated CM with chromosomal gene mutations are defective in spreading to the GI tract

CM clones carrying mutations in chromosomal genes *tc0237* and/or *tc0668* were attenuated in inducing hydrosalpinx in the mouse upper genital tract [29, 30]. The mechanisms of the attenuation remained unknown since these CM mutants still maintained robust live organism shedding courses in the genital tract. Interestingly, when both vaginal and rectal swabs were simultaneously monitored, the attenuated CM mutants consistently decreased their spreading to the GI tract [84]. The decreased spreading is likely due to their reduced colonization in the GI tract since a direct intragastric inoculation failed to rescue their colonization. The defective colonization in the GI tract may represent a primary phenotype of these CM mutants since the functions of TC0668 and/or TC0237 appear to be more important for CM to colonize the GI tract than to infect the genital tract. It remains unknown how TC0237 or TC0668 promotes CM colonization in the GI tract since both are conserved hypothetical proteins of *Chlamydia*.

TC0237, found on the complementary strand of CM genome, codes for a 159 amino acid (AA) protein with no known function [85]. TC0237 contains a domain of unknown function 720 (DUF720) motif, which is also found in its neighboring ORFs TC0236 (coding for a 172AA hypothetical protein) and TC0235 (170AA hypothetical protein). These three sequential proteins are paralogous to each other and predicted to be encoded in an operon [85]. TC0237, TC0236, and TC0235 are all highly conserved within the *Chlamydiae* and *Chlamydophila* genera, an example of such being ~90% AA sequence identity with their *C. trachomatis* serovar D homologs CT849, CT848, and CT847. Interestingly, a Q119K

substitution mutation in TC0236 was also reported although its role in CM pathogenicity remains unknown [86]. Obviously, more studies are required to reveal the mechanisms by which TC0237-TC0236-TC0235 may promote CM fitness in the GI tract.

TC0668 is a 408 amino acid hypothetical protein consisting of a series of transmembrane domains in the N-terminal 70 amino acids and the domain of unknown function 1207 (DUF1207) motif in the remaining region. TC0668 is predicted to associate with chlamydial outer membrane complex [87]. Phylogenetic analysis of TC0668 indicates that *C. muridarum* inherited the DUF1207 motif from a distant chlamydial progenitor [29]. Tertiary protein structure prediction and a homology search of TC0668 with the I-TASSER suite revealed that TC0668 shares structural homology with various eukaryotic integrins including alpha V beta 1 ($\alpha 5\beta 1$, fibronectin receptor), alpha IIb beta 3 ($\alpha IIb\beta 3$, fibrinogen receptor), and alpha V beta 3 ($\alpha 5\beta 3$, vitronectin receptor) [29]. These integrins engage in diverse cellular activities, which makes difficult to predict the function of TC0668. A hypothesis is that TC0668 may be an essential outer membrane component required for CM to colonize the GI tract. Interestingly, TC0668 shares 97% protein identity with its CT serovar D counterpart, CT389, which is consistent with the observations that both CM and CT have been frequently detected in the GI tracts of mice and humans [88]. Furthermore, like CM [78, 89], CT has not been associated with any significant GI tract pathologies [90]. Further investigation of DUF720- & 1207-containing homologs may provide useful information for understanding how TC0237/0668 promote both chlamydial colonization in the GI tract and pathogenicity in the upper genital tract.

3. Plasmid-free CM is deficient in both inducing hydrosalpinx and spreading to the GI tract

The chlamydial plasmid is a key pathogenic determinant in the mouse upper genital tract [9, 10]. As with the chromosomal gene mutants described above, plasmid-deficient CM significantly delayed/reduced spreading from the mouse genital to the GI tracts [91]. However, CM with or without plasmid maintained similar levels of survival in the mouse circulatory system following an intravenous inoculation, suggesting that CM plasmid is not required for CM survival in the blood [61]. Intragastric inoculation failed to restore the plasmid-deficient CM to normal colonization in the GI tract, suggesting that the cryptic plasmid is more important for chlamydial organisms to colonize the GI tract than to infect the genital tract [91]. To map the plasmid genes responsible for promoting CM colonization in the GI tract, CM deficient in plasmid-encoded pGP3, 4, 5, 7 or 8 were compared for colonizing the GI tract following an intragastric inoculation [84]. The pGP3- or pGP4-deficient strains failed to colonize the GI tract while other plasmid gene mutants maintained robust colonization. Since pGP4 regulates pGP3, while pGP3 does not significantly affect pGP4 expression [56–58], it is reasonable to conclude that pGP3 is critical for CM to colonize the GI tract. This conclusion is supported by the fact that CM mutants deficient in GlgA, a chromosomal gene-encoded protein that is up-regulated by pGP4 [56, 57], were able to colonize the mouse GI tract [92]. The latter also suggests that chlamydial glycogen synthesis is not essential for CM colonization in the GI tract.

The next question is how pGP3 promotes CM colonization in the GI tract. Since CM deficient in pGP3 was able to infect the genital tract but completely lacked the ability to

colonize the GI tract, GI colonization assay may provide a more sensitive platform for defining the mechanisms by which pGP3 promotes CM pathogenicity. The CM long-term colonization in the GI tract has been localized to the cecum/colon [60, 80, 93]. Thus, the intragastrically inoculated CM has to overcome the gastric acid barrier, the small intestinal defense mechanisms and gut microbiota competition in order to reach its final destination. The question is how pGP3 helps CM to overcome these GI tract barriers. pGP3 is a stable trimer [94–96] and is both associated with the chlamydial organism outer membrane and secreted into the host cytosol [97]. The outer membrane-associated pGP3 may promote CM resistance to gastric acid while the secreted pGP3 may neutralize the antimicrobial factors in the small intestine. The latter is supported by an observation that pGP3 bound to host antimicrobial cathelicidin peptide LL-37 (human) or CRAMP (mouse) and neutralized its anti-chlamydial activity [98]. Furthermore, the pGP3 C-terminal trimerization domain is similar to the trimer structure of the TNF α family members [94], suggesting that the secreted pGP3 may also be able to modulate host inflammatory pathways mediated by TNF to promote CM colonization in the GI tract. The secreted pGP3 may also target other bacteria or bacterial products to create a niche for CM to maintain long-term colonization in the colon. *Chlamydia* is known to take up bacterial metabolites for its own biosynthesis [99–103]. As the cecum/colon is full of bacterial metabolites, the question is whether the outer membrane-associated pGP3 can promote CM biosynthesis by taking up bacterial metabolites.

4. Correlation of chlamydial pathogenicity in the upper genital tract with chlamydial loads from the GI tract but not the genital tract

As described above, CM mutants deficient in either chromosomal or plasmid gene-encoded proteins are attenuated in both inducing hydrosalpinx and colonizing the GI tract [84, 91] but maintaining robust infectivity in the lower genital tract [10, 14, 29, 30, 104], suggesting a correlation of chlamydial colonization in the GI tract with chlamydial pathogenicity in the upper genital tract. Indeed, the hydrosalpinx scores correlate with chlamydial loads in the vaginal but not rectal swabs [84, 91]. Furthermore, direct inoculation of the CM mutants into the oviduct failed to enhance the CM mutants' pathogenicity in the upper genital tract or their spreading to the GI tract. These observations suggest CM-induced tubal inflammation alone is not sufficient for inducing long-term hydrosalpinx and CM colonization in the GI tract may be required for the chlamydial pathogenicity. It appears that both the CM ascension along the genital tract to the oviduct and spreading via the circulatory system to the GI tract, which may depend on two independent mechanisms [59–61], are required for CM induction of long-lasting hydrosalpinx. The next question is how CM colonization in the GI tract may promote CM induction of pathology in the upper genital tract.

VI. A two-hit model

The *in vivo* imaging-based discovery of CM spreading from the mouse genital to GI tracts [60, 61] suggested a temporal correlation between the CM spreading to and colonizing in the GI tract and CM pathogenicity in the upper genital tract. This correlation was strengthened by the observation that a series of CM mutants are attenuated in both inducing hydrosalpinx [14, 29, 30, 104] and spreading to the GI tract but maintaining robust infection courses in the

genital tract [84, 91]. The correlation remained true even when the mutants were directly delivered into the upper genital tracts [84, 91]. Since the GI CM is restricted to the GI tract without auto-inoculating the genital tract [80], the GI tract CM must promote hydrosalpinx indirectly. CD8⁺ T cell depletion significantly reduced hydrosalpinx in mice infected intravaginally with a wildtype CM that also spread to the GI tract [36, 38, 105], suggesting that the GI tract CM may promote hydrosalpinx by inducing these “pathogenic” CD8⁺ T cells. Thus, a 2-hit model for chlamydial pathogenicity in the upper genital tract has emerged (Fig. 1): To produce the 1st hit, CM ascends from the lower to upper genital tract to infect the oviduct, causing epithelial damage, triggering transient fibrosis to repair injured tissue, and generating MHC:CM peptide complexes. The 1st hit can be induced by tubal infection with a mutant CM, which alone is not sufficient for driving long-term tubal fibrosis/hydrosalpinx in the absence of a 2nd hit. To produce the 2nd hit, a wildtype CM spreads from the genital to the GI tract to induce CD8⁺ T cells that are then recruited to the genital tract for converting the 1st hit into long-lasting tubal fibrosis/hydrosalpinx. The “pathogenic” CD8⁺ T cells induced in the GI tract may possess a profibrotic phenotype for example by secreting IL-13/TNF α since IL-13^{-/-} or TNFR1^{-/-} mice were resistant to hydrosalpinx induction [35, 37]. Indeed, GI tract infections are known to induce profibrotic lymphocytes [106–108]. IL-13⁺CD8⁺ T cells were isolated from CM-infected mice [109] and associated with fibrotic pathology [110–112]. The 2-hit model is also consistent with the concept that gut bacteria-induced responses can impact extra-GI tissues [113–116].

1. The 1st hit is necessary but not sufficient for CM to induce long-lasting fibrosis/hydrosalpinx

CM ascends to the oviduct to induce pyosalpinx followed by clearance of CM and a reparative response [13, 117]. CM antigen processing & presentation must also occur in the infected oviducts since CM-specific CD4⁺ Th1 cells are recruited to clear the tubal infection [39, 118], suggesting that MHC class I-restricted epitopes may also be produced in the oviduct. Although CM mutants can induce both early-induced responses and adaptive immune responses, the mutants fail to induce long-lasting hydrosalpinx. The mouse long-lasting hydrosalpinx mimicks the tubal adhesion/hydrosalpinx observed in women under laparoscopy [50–52]. It is important to understand how pyosalpinx is driven into long-lasting hydrosalpinx. Our hypothesis is that “pathogenic” CD8⁺ T cells with a profibrotic phenotype induced by the GI tract CM may be recruited to the oviduct to drive the conversion.

2. The 2nd hit may be mediated by CD8⁺ T cells

When CD4⁺ and CD8⁺ T cells are compared for their roles in controlling CM infection and contributing to CM induction of pathology, it is clear that CD8⁺ T cells are required for hydrosalpinx development in mice infected in the genital tract with a wildtype CM that is able to spread to the GI tract [36, 38, 105]. First, antibody depletion of CD8⁺ but not CD4⁺ T cells significantly reduced CM induction of hydrosalpinx without alteration in chlamydial infection courses [36]. Second, OT1 T cell receptor transgenic mice failed to develop hydrosalpinx in response to CM infection in the genital tract [105], indicating that antigen-specific CD8⁺ T cells are required for CM induction of hydrosalpinx. The T cell receptors of CD8⁺ T cells from OT1 mice are engineered to only recognize the ovalbumin (OVA) 257SIINFEKL₂₆₄ or OT1 peptide in the context of H-2K^b while the CD4⁺ T cells are wild

type. Finally, supplementing OT1 mice with wild type CD8⁺ T cells rescued the mice to develop hydrosalpinx following CM infection [38], suggesting that the donor wild type CD8⁺ T cells were induced to promote hydrosalpinx. The pathogenic CD8⁺ T cells must be specific to CM. The CM-specific CD8⁺ T cells must promote hydrosalpinx in the oviduct by detecting CM epitopes presented by antigen presenting cells remaining in the oviduct tissues even after the infection is cleared. However, direct evidence for demonstrating a role of CM-specific CD8⁺ T cells in CM induction of hydrosalpinx remains lacking, which makes it difficult to phenotypically characterize these pathogenic CD8⁺ T cells and to investigate how the pathogenic CD8⁺ T cells are induced during CM infection. Strategies to move the field forward may include identifying CM epitopes recognized by the pathogenic CD8⁺ T cells during CM infection followed by isolating these T cells or using a surrogate system with CM expressing a model CD8⁺ T cell epitope. A convenient surrogate is the OT1 transgenic mouse system. Since OT1 mice resisted hydrosalpinx induction by wildtype CM [105], if CM engineered to express the OT1 epitope OVA₂₅₇SIINFEKL₂₆₄ rescue the OT1 mice to develop hydrosalpinx, it could be concluded that the CM-associated CD8⁺ T cell epitope is required for CM to induce pathogenicity. The OT1 mouse surrogate system would allow both the phenotypic characterization of the pathogenic CD8⁺ T cells and the investigation of how these pathogenic CD8⁺ T cells are induced during CM infection.

3. Profibrotic cytokines may be secreted by CD8⁺ T cells to promote CM induction of hydrosalpinx

Although CD8⁺ T cells are required for CM to induce hydrosalpinx, it remains unknown whether these pathogenic CD8⁺ T cells secrete cytokines IL-13, TNF α or TGF α . Mice deficient in TNF α or TNFR1 became resistant to CM induction of hydrosalpinx [35, 36], suggesting that the pathogenic CD8⁺ T cells may produce TNF α [36]. However, the TNF α -responding cells may not be CD8⁺ T cells since TNFR1-expressing CD8⁺ T cells are not required for CM induction of hydrosalpinx [119]. Mice deficient in the profibrotic cytokine IL-13 [37] had significantly reduced hydrosalpinx after genital infection with wildtype CM that is able to spread to the GI tract. However, these mice also significantly shortened CM infection in the genital tract [37]. Thus, it is not clear whether the reduced pathology was due to the decreased genital CM infection or reduced profibrotic response. A soluble IL-13 receptor alpha 2-human Ig Fc fusion protein (IL-13Ra2Fc) that is able to neutralize the profibrotic role of IL-13 may be used to differentiate these two possibilities. The effect of IL-13Ra2Fc on the infection courses can be minimized by adjusting the timing of the infection and fusion protein application.

4. Pathogenic CD8⁺ T cells that secrete both TNF α and IL-13 may be preferentially induced by Chlamydia in the GI tract

C. muridarum is known to spread systemically during the first two weeks after inoculation regardless of the routes of inoculation [60, 120]. However, it only establishes long-lasting colonization in the GI tract [61] without causing any pathology [80, 81]. This distribution pattern suggests that *C. muridarum* has well adapted to the GI tract but not other tissues. In non-GI tract tissues, *C. muridarum* is known to induce Th1-dominant responses [121–123] while gut microbial species are known to induce immune responses with suppressing/ tolerogenic phenotypes such as Treg [124–126] and Th2-type cytokines [127]. Many Th2-

cytokines such as IL-13 are potent in promoting tissue repair and fibrosis [128–131]. Thus, it is likely that GI tract *Chlamydia* may induce CD8⁺ T cells that secrete profibrotic cytokines. Once induced in the GI tract, the pathogenic CD8⁺ T cells may be recruited to the oviduct tissues where chlamydial epitopes may be preserved by antigen presenting cells even after the tubal infection is cleared. The GI tract-derived CD8⁺ T cells can be activated to release profibrotic cytokines for converting the transient tubal repairing/fibrosis into long-lasting fibrosis/hydrosalpinx. Testing whether GI tract *Chlamydia* can indeed induce *Chlamydia*-specific pathogenic CD8⁺ T cells and whether the pathogenic CD8⁺ T cells induced by the GI tract *Chlamydia* are both necessary and sufficient for promoting chlamydial induction of hydrosalpinx in the upper genital tract will provide crucial evidence for supporting the 2-hit model.

VII. Concluding remarks and Caveats

The temporal gap between the self-limited genital tract infection with CM and its induction of long-lasting hydrosalpinx in mice has puzzled many chlamydiologists for years. Testing the proposed 2-hit model that emphasizes the roles of both the 1st hit caused by the CM organisms that ascend along the genital tract to the oviduct and the 2nd hit produced by the CM organisms that spread to the GI tract may provide the knowledge for filling in the gap. The 1st hit consists of the initial epithelial infection/damage, antigen processing and presentation and wound-healing response while the 2nd hit consists of recruitment of CD8⁺ T cells and release of profibrotic cytokines in the oviduct by CD8⁺ T cells induced by CM in the GI tract. Although there is still lack of direct evidence for supporting that the 2nd hit is mediated by the GI tract CM-induced CD8⁺ T cells with a profibrotic phenotype (see Outstanding Questions), the hypothesis is consistent with the following facts: CM-specific CD8⁺ T cells are required for CM induction of hydrosalpinx [36, 38, 105]. Mice deficient in either TNFR1 [35] or IL-13 [37] significantly reduced hydrosalpinx upon CM infection. IL-13⁺CD8⁺ T cells have been isolated from CM-infected mice [109]. IL-13⁺CD8⁺ T cells have been shown to promote pathogenic fibrosis [110–112]. GI tract infections are known to induce fibrosis-promoting lymphocytes [106–108].

Although the 2-hit model is well supported by observations described in the literature from mouse model studies, caution should be taken when applying the 2-hit mouse model to human chlamydial pathogenesis. It is true that CM infection in the mouse genital tract can induce hydrosalpinx similar to that observed in CT-infected women [50–52]. However, CM may be transmitted naturally among mice via oral-fecal route instead of sexually. This hypothesis is supported by the finding that CM plasmid or plasmid-encoded proteins or chromosomal proteins are more important for CM to colonize the GI tract than to infect the genital tract [84, 91]. However, CT has been transmitted sexually among humans, forcing CT to adapt to the human genital mucosa. Nevertheless, CT has been frequently detected in the GI tracts of humans but without any significant association with the GI tract pathologies [40–44], suggesting that CT may have experienced selection pressures from both the genital and GI tracts, which is testable by comparing the roles of the virulence factors in CT infection/colonization in the genital versus GI tracts.

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Highlights

- That chlamydial factors identified as virulence determinants in the genital tract are more important for CM colonization in the GI tract suggests that CM has acquired its virulence factors for primarily adapting to the GI tract.
- CM interactions with the mouse GI tract may represent a more productive platform for dissecting chlamydial pathogenic mechanisms in the genital tract.
- Investigating how the mouse GI tract CM affects CM pathogenicity in the upper genital tract should provide another example for the growing list of cases in which the GI microbes impact physiology and pathology in extra-GI tissues.
- Understanding the molecular basis of CM interactions with the mouse GI tract may uncover the rules governing gut microbe-microbe and microbe-host interactions.

Outstanding Questions

Is GI tract CM both necessary and sufficient to promote CM pathogenicity in the mouse upper genital tract?

Are GI CM-induced profibrotic CD8⁺ T cells both necessary and sufficient for CM induction of hydrosalpinx?

How do the GI tract-derived profibrotic CD8⁺ T cells promote CM induction of tubal fibrosis? Is CT able to spread from the genital to GI tracts?

Can GI tract CT induce profibrotic CD8⁺ T cells that promote fallopian epithelial mesenchyme transition?

Are women positive for CT in rectal swabs more vulnerable to tubal infertility?

Figure 1. A 2-hit Hypothesis

Sexually transmitted *Chlamydia* ascends to the oviduct by overcoming the genital tract mucosal defenses at both the cervix and uterotubal junction to induce the 1st hit. As the tubal infection is resolved, tubal function should be restored. However, the transient tubal reparative response is converted into a long-lasting fibrotic blockage in many mice, which may be catalyzed by fibrosis-promoting CD8⁺ T cells (defined as the 2nd hit) induced by *Chlamydia* in the GI tract. This 2-hit model, supported by many studies [8, 13, 29, 30, 36–38, 41–44, 50, 60, 61, 77–80, 87, 89, 105–112, 117, 120, 132, 133], has both explained how a self-limited infection in the genital tract can induce long-lasting hydrosalpinx and stimulated our imagination to ask more questions.