Defining the role of host cell chromatin traps in *Chlamydia trachomatis* pathogenesis.

by

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Date: 3/18/2016
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Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Molecular Genetics and Microbiology in the Graduate School of Duke University

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ABSTRACT

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Abstract

*Chlamydia trachomatis* (CT) is the most common bacterial agent of sexually transmitted infection and can cause damaging inflammation of the female reproductive tract. As an obligate intracellular pathogen, CT must exit exhausted host cells in a manner that favors successful dissemination. Epithelial cells infected with CT expel decondensed nuclear chromatin at the conclusion of an infectious cycle, and these ensnare CT particles. Whether these chromatin traps benefit the host or the pathogen is not obvious. The overall goal of this work is to begin discerning between these possibilities by determining how chromatin traps impact CT survival following exit and how traps contribute to CT-induced inflammatory processes.
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1. Specific Aims

*Chlamydia trachomatis* (CT) is the most common bacterial agent of sexually transmitted infection. CT can cause serious reproductive pathology in women, including pelvic inflammatory disease, ectopic pregnancy, and tubal factor infertility. These sequelae depend on CT ascending beyond the primary site of infection, the endocervical epithelia, and driving deleterious inflammatory processes in upper reproductive tract tissues.

CT is an obligate intracellular bacterium with a biphasic life cycle. Reticulate bodies (RBs) replicate inside host cells where they occupy a pathogenic vacuole called an inclusion. These non-infectious RBs give rise to elementary bodies (EBs): spore-like forms that must endure the extracellular environment and reach new hosts to continue propagation. EBs can’t replicate, have minimal metabolic activity, and are immotile; they must exit exhausted host cells in a state conducive to their survival and dispersal. The exit process is a critical last chance for CT to leverage host cell features to favor its successful dissemination, or for doomed host cells to execute last-ditch efforts to limit chlamydial spread.

I have observed that HeLa cells infected with CT expel decondensed nuclear chromatin into the cytosol just prior to rupture of the plasma membrane at the conclusion of an infectious cycle. Furthermore, I find large fibrous chromatin lattices covered with EBs in the supernatant of CT-infected cell cultures following host lysis.
Both the expulsion of chromatin preceding cell lysis, and the presence of fibrous chromatin structures in post-lysis cultures depends on the chlamydial protease, CPAF, as evidenced by studies with a CPAF null mutant.

That a chamydial protein is required for the production of these chromatin traps suggests that their formation is a programmatic step in chlamydial exit that likely has important consequences for CT pathogenesis. Whether these chromatin traps benefit the host or the pathogen is not obvious. The overall goal of this work is to begin discerning between these possibilities by determining how chromatin traps impact EB survival following exit and how traps contribute to CT-induced inflammatory processes.

1.1 *Determine how chromatin traps impact EB survival*

1.1.1 *Determine the direct effects of chromatin traps on EBs*

I hypothesize that EBs resist killing by chromatin traps. I will determine whether the expulsion of chromatin traps accelerates the inactivation of EBs upon exit from host cells by quantifying the kinetics of IFU loss from WT and *cpaf(-)* post-lysis supernatants normalized to that of purified EBs preps. If EBs do resist killing by traps, it would be of interest to determine whether the CT-induced traps have any antibacterial activity, and, if so, how this is mitigated by CT. In the case that traps reduce CT viability, exploring the mechanism of this activity will constitute an alternative research aim.
1.1.2 Determine how chromatin traps influence phagocyte interactions with EBs

Active influx of phagocytes is observed in CT-infected tissues. It is conceivable that traps could either facilitate or antagonize the clearance of EBs by phagocytes responding to lysed cells. I will take advantage of the “trapless” phenotype of the CPAF null mutant to study chlamydial uptake, clearance, and phagocyte survival upon introduction of neutrophils and macrophages to CT-infected cell cultures. After host cells lyse, many EBs appear to remain in close association with the debris. I hypothesize that chromatin traps could act as an escape route for EBs to evade clearance during efferocytosis of host cell debris, and thus expect more efficient clearance of the CPAF mutant.

1.2 Determine how chromatin traps influence CT-induced inflammatory pathology

Extracellular chromatin drives inflammatory pathology in numerous disease contexts. I hypothesize that CT-induced chromatin traps contribute to inflammatory signaling and destruction of genital tract tissue. The differential trap phenotypes of WT and CPAF null CT will be used to study the contribution of traps to the modulation of cytokine responses in A2EN cells, a human endocervical epithelial cell model. The impact of chromatin traps on upper genital tract tissue will be evaluated by comparing WT and CPAF null infections of a human fallopian tube organ culture model for differences in cell destruction and spatial expression of immunomodulatory proteins. To
control for phenotypic differences that may manifest through mechanisms extraneous to trap induction, infections will also be performed with DNA trap-degrading drugs.

Chromatin traps could be practical therapeutic targets for limiting CT sequelae because they can be dismantled by nucleases in extracellular environments. This strategy is already applied in the treatment of Cystic Fibrosis lung infections, where antibiotics are supplemented with DNase to augment clearance of DNA-ensnared bacteria. The proposed work will answer critical preliminary questions about the role of DNA traps in CT pathogenesis that will determine if a similar strategy has any traction in the management of CT infections.
2. Background and Significance

2.1 *Chlamydia trachomatis* sexually transmitted infection

Nearly 100 million new CT genital tract infections are estimated to occur throughout the world each year, more than for any other sexually transmitted bacterium. [1] Most CT infections are asymptomatic, complicating the identification of infected individuals and facilitating CT transmission. Untreated infections of the female reproductive tract can progress to uterine and fallopian tube scarring, resulting in pelvic inflammatory disease (PID), tubal factor infertility (TFI), and ectopic pregnancy. [2] In the US alone, the estimated cost of diagnosis, treatment, and management of sequelae for CT STI is approaching 4 billion dollars annually. [3] Despite the continued susceptibility of CT to conventional antibiotics, the incidence of infection is rising. Public health goals are focused on reducing CT transmission and interfering with the development of inflammatory sequelae in the female upper reproductive tract. [4]

2.2 The chlamydial life cycle

CT is an obligate intracellular bacterium with a biphasic life cycle. Reticulate bodies (RBs) replicate inside host cells where they occupy a pathogenic vacuole called an inclusion. These non-infectious RBs give rise to elementary bodies (EBs): spore-like forms that must endure the extracellular environment and reach new hosts to continue propagation. EBs can’t replicate, have minimal metabolic activity, and are immotile; they must exit exhausted host cells in a state conducive to their survival and dispersal.
exit process is a critical last chance for CT to leverage host cell features to favor its successful dissemination, or for doomed host cells to execute last-ditch efforts to limit chlamydial spread. Two distinct modes of exit have been described for CT: lysis and extrusion. [5] CT-induced lysis proceeds by the sequential loss of inclusion integrity, followed by loss of nuclear membrane integrity, and ending with disintegration of the plasma membrane. Extrusion describes the movement of intact inclusions into the extracellular environment, the significance of which is not yet understood.

2.3 CT induces chromatin traps from lysing host cells

I have preliminary microscopic evidence that HeLa cells infected with CT expel decondensed nuclear chromatin at the conclusion of an infectious cycle. The supernatant of CT-infected cell cultures contains fibrous chromatin lattices covered with EBs. (fig. 1)

Figure 1: Chromatin traps in the SN of post-lysis cultures are covered with EBs
Both the expulsion of chromatin from lysing cells, and the presence of fibrous chromatin structures in the supernatant depends on the chlamydial protease, CPAF, as evidenced by studies with a cpafl(-) mutant. (fig. 2, p.4) The chlamydial proteasome-like activity factor (CPAF) is conserved among Chlamydiaceae but is not essential for propagation of CT in cell culture. The work proposed here will take advantage of the “trapless” phenotype of the cpafl(-) mutant as a tool for investigating the consequences of chromatin traps for EB viability and host cell inflammatory signalling.

Figure 2: CT induces the release of chromatin traps from infected cells through a CPAF-dependent mechanism.
2.4 Relationship of the observed chromatin traps to neutrophil extracellular traps (NETs)

The association of bacteria with large, fibrous chromatin structures is reminiscent of the extracellular traps produced by neutrophils (i.e. NETs), which are thought to be an innate defense mechanism whereby ensnared bacteria are either killed directly by antibacterial properties of the traps themselves, or physically sequestered to enhance clearance by other means. [6, 7] While some bacterial pathogens are killed by extracellular traps, others are not, and there is evidence that some may even co-opt traps to promote their survival in the local tissue environment. [8-10] Beyond having proposed roles in antibacterial defense, extracellular chromatin has potent inflammatory and cytotoxic effects in a wide range of disease processes. [6, 11-15]

2.5 Summary of Research Aims

Whether the observed chromatin traps benefit the host or chlamydia is not obvious. The overarching goal of this project is to begin discerning between these possibilities by determining the consequences of chromatin traps for EB survival and epithelial inflammation. My overall hypothesis is that CT induces chromatin traps to its own advantage. Consistent with this, I predict that EBs are not susceptible to direct killing by the traps, and that trap-bound EBs evade clearance by phagocytes. Additionally, I hypothesize that traps have proinflammatory properties that contribute to the tissue pathology attributed to CT infection.
2.6 Innovation

While the EB-laden chromatin structures present in the supernatant of CT-infected cultures are superficially reminiscent of neutrophil extracellular traps (NETs), it should be emphasized that these are derived from epithelial cells following extensive manipulation by an obligate intracellular parasite. The current extracellular trap (ET) research field has thus far been limited to studies of granulocytic immune cells and their interactions with pathogens encountered in the extracellular space. [6] To my knowledge, the proposed research is the first investigation of chromatin-traps elicited by a host-pathogen interaction with a non-immune cell type.

The interaction being studied suggests a very approachable clinical application. Chromatin traps could be practical therapeutic targets for limiting CT sequelae because
they can be dismantled by nucleases in extracellular environments. This strategy is already applied in the treatment of Cystic Fibrosis lung infections, where DNase supplementation reduces mucus viscosity, aiding clearance of DNA-ensnared bacteria. [16] The same approach has been proposed as an adjunct for management of otitis media, where biofilms enriched with neutrophil derived DNA often resist current antimicrobial therapies. [17] The proposed work will answer critical preliminary questions about the role of DNA traps in CT pathogenesis that will determine if such a strategy has any future traction in the management of CT infections. This strategy has the advantages of a high safety profile, easy administration, and manageable cost that would make it practical to introduce in a clinical STD care setting.
3. Approach

3.1 Determine how chromatin traps impact EB survival

3.1.1 Determine the direct effects of DNA traps on EBs

3.1.1.1 Rationale

While seminal reports on neutrophil extracellular traps (NET) advanced the notion that chromatin traps kill enmeshed pathogens, it is now clear that this cannot be generalized across bacterial species, and that some bacteria may not only survive traps, but even co-opt them to their own advantage. [7-10, 18] All published data on extracellular traps reflect those derived from granulocytic immune cells. These cells synthesize large quantities of antimicrobial compounds, which are loaded onto chromatin traps. [7] The epithelial-derived traps in question here may lack many such antimicrobial factors. Nevertheless, histones have been shown to have potent antibacterial activity and could be toxic to EBs. [19] Aim 1A will test whether chromatin traps accelerate the inactivation of EBs upon exit from host cells. Because it appears that the chlamydial effector protein CPAF drives chromatin traps, it is reasonable to think that this obligate intracellular parasite is evolved to tolerate the consequence of its exit program. I hypothesize that epithelial-derived chromatin traps are not directly toxic to EBs.
3.1.1.2 Analysis of chromatin trap anti-chlamydial activity

I will take advantage of the trapless phenotype of the *cpaf(-)* mutant to determine whether the expulsion of chromatin traps accelerates the inactivation of EBs upon exit from host cells. I will measure the kinetics of IFU loss from SN samples from WT and *cpaf(-)* infected cell cultures that have lysed as a result of infection. These data will be normalized to the rate of IFU loss in purified EB preparations of WT and *cpaf(-)* strains incubated in cell-free media. It is not expected that purified EBs of these genotypes will differ significantly with respect to the kinetics of IFU loss, as EBs do not express CPAF, and it is not likely to have a direct role in the resilience of EBs. [20]

Quantification of total IFU in the above experiments will be done by treating all samples identically with DNase and sonication to liberate EBs from any cell debris structures. IFU/ul will be determined by infecting Vero cells with serial dilutions and counting inclusions using the Cellomics automated microscopy platform.
3.1.1.3 Expected outcomes and alternatives

I predict that EBs resist direct killing by chromatin traps. If there is no between genotype difference in the rate of IFU loss in the SN samples (normalized to purified EBs), I will conclude that chromatin trap expulsion does not have direct anti-chlamydial activity. (fig. 4A)

If a difference is observed (fig. 4B), it will remain unclear what factors in the trap-positive SN affect the loss of IFU. This will be addressed by testing whether IFU
inactivation depends on key features of chromatin traps: intact DNA and histones. The kinetic measurements described above will be performed in the presence of DNase to assess whether intact DNA is required for IFU inactivation. They will also be done in the presence of activated protein C, which cleaves and inactivates histones, to determine whether histone activity is required for IFU inactivation. [15]

3.1.2 Determine how traps influence phagocytosis of EBs released from lysed host cells.

3.1.2.1 Rationale

Neutrophils and macrophages infiltrate CT-infected tissues within 1-2 days of infection. [2] Thus, phagocytes are expected to be present when the first round of chlamydial replication is completing and EBs are being released from their primary host cells. Neutrophils can quickly inactivate purified EBs. [21] Macrophages ingest both bacteria and debris from lysed cells, which appears to contain many EBs. It is conceivable that chromatin traps could either facilitate or antagonize the clearance of EBs by phagocytes responding to lysed cells. [8] Using the trapless phenotype of the $cpaf(-)$ mutant I will determine the role of chromatin traps in chlamydial clearance and phagocyte survival upon introduction of neutrophils and macrophages to the SN of CT-infected cell cultures.

It has been proposed that NETs may facilitate the indirect clearance of pathogens by phagocytes via physical sequestration of microbes, though direct evidence for this is lacking. Aim 1B will test whether EBs associated with chromatin traps are cleared by
phagocytes faster than free EBs, or EBs associated with trapless cell debris. I hypothesize that chromatin traps impede the clearance of EBs by phagocytes.

3.1.2.2 Analysis of neutrophil anti-chlamydial activity

The rate at which infectious EBs are cleared from post-lysis supernatant (SN) samples by neutrophils will be measured by standard in vitro assays for neutrophil bactericidal activity. [22-25] My own implementation of these techniques will be optimized first using broth cultures of S. aureus, a standard organism used for measuring neutrophil bactericidal activity.

To measure neutrophil activity against chlamydial EBs, A2EN cells will be infected with WT and cpaf(-) CT and incubated for 72 – 96 hours, when the majority of infected cells will have lysed. The initial total IFU/ul in each supernatant (SN) will be counted by treating samples with DNase and sonication, then titering in Vero cell monolayers. Initial total IFU will be normalized between genotypes by gently diluting SN samples as needed. Neutrophils and serum will be collected from healthy human donors by standard methods. Serum will be added for 30 minutes at 37C to opsonize SN samples prior to addition of neutrophils. Autologous neutrophils will then be added to the samples at an EB:neutrophil ratio of 1:10 and incubated at 37C with gentle mixing for 15, 30, and 60 minutes. [21] Neutrophils will be lysed by raising solution pH to 10 to stop the assay. [23] The total remaining IFU will be counted by treating samples with DNase and sonication, then titering in Vero cell monolayers. A difference in the rate of IFU loss
between the WT and \textit{cpaf(-)} SN will suggest that the differing post-lysis conditions (\textit{i.e.}
with chromatin traps, or without) created by each genotype may influence the outcome
of interactions with neutrophils. To control for any differences in neutrophil killing that
may derive from direct interactions with the EBs themselves, neutrophil killing assays
will also be done with purified EBs of each genotype. As for the kinetic analysis
described in aim 1A, any difference appreciated for trap-positive condition will be
followed up by experiments including DNase and activated protein C, to assess the
requirement for intact DNA or histones in the observed effects.

It is possible that differences in the SN of WT and \textit{cpaf(-)} infected cultures
influence neutrophil clearance of EBs by effecting neutrophil survival. To assess this,
viable neutrophil counts will be taken at the start and stop points of these assays. To
determine how much of the neutrophil bactericidal activity depends on phagocytosis, I
will also perform the described experiments with neutrophils treated with cytocholasin-
D, which prevents phagocytosis.

\textbf{3.1.2.3 Analysis of macrophage anti-chlamydial activity}

In addition to phagocytosing microbes, macrophages clear debris generated by
dying cells. I have observed that when CT-infected cells lyse, many EBs appear to
remain in close association with cell debris and could be taken up along with debris
being ingested by macrophages. The workflow described for testing neutrophil anti-
chlamydial activity will also be employed using peripheral blood mononuclear cells
(PBMC) obtained from healthy donors, with modifications to accommodate macrophage biology. PBMCs will be activated by pre-treating cells with IFNγ prior to incubation with SN from chlamydia-infected cultures.

3.1.2.4 Expected outcomes and alternatives

Membranous cell debris contains “find-me” and “eat-me” signals that target the debris for clearance by phagocytes. Bacteria associated with cell debris are likely to be cleared along with the debris. Chromatin lattices are large structures that likely lack many of the “eat-me” signals found in membranous cell debris. I hypothesize that EBs ensnared in traps are less susceptible to clearance by phagocytes responding to zones of host cell lysis. However, it is possible that EBs ensnared in chromatin traps are equally, or even more susceptible to clearance by phagocytes. Figure 5 diagrams hypothetical outcomes of this aim.

![Figure 5](image-url)

**Figure 5:** Expected outcomes of assays for neutrophil anti-chlamydial activity.
3.2 Determine how DNA traps influence CT-induced inflammatory signaling

3.2.1 Rationale

The current synthesis of data from human CT infections and research in experimental systems supports the cellular paradigm of chlamydia pathogenesis; a framework emphasizing the central role of chlamydia-infected epithelial cells in the initiation and maintenance of immune signalling that culminates in reproductive tract scarring – the proximal cause of PID and TFI. [26] How the observed chromatin traps could influence inflammatory processes in infected epithelial cells is not obvious. It is conceivable that trap ensarement of EBs limits the number available to infect new cells, and thus opposes the inflammatory consequences that would ensue from an increased frequency of host cell infection at a tissue locale. If traps have bactericidal activity against EBs, they could reduce the number of infectious particles, but might also release chlamydial molecules that drive local inflammation. The trap structures themselves are also likely to contribute directly to inflammation, as numerous studies highlight the proinflammatory and cytotoxic action of extracellular histones deriving from ETosis and necrosis. [15] I hypothesize that the overall contribution of traps is proinflammatory. I will make use of complementary model systems for the investigation of chlamydia pathogenesis to evaluate the role of DNA traps in CT-induced inflammation at the cellular and tissue levels.
It should be noted that a recent study measured the cytokine output of CT-infected A2EN cells and reported a CT-induced release of IL1α at late stages of infection, with little appreciable secretion of other pro-inflammatory cytokines from CT-infected cells. In that study, infections were done with purified EBs, so cells would not have experienced chromatin traps except, perhaps, some that may have released at the 72hpi time point. That study also tested the potential of supernatants and lysates from infected cultures to drive cytokine secretion from naïve cells, and reported that neither induced a robust pro-inflammatory response. The supernatant and lysate tested was filtered through 0.1µm filters to exclude CT EBs. This filtration would also be expected to exclude large chromatin traps. [27] Thus, the modest pro-inflammatory responses recently reported for the A2EN model are in the absence of chromatin traps. It is reasonable to think that chromatin traps could drive inflammatory signaling beyond that observed with purified EBs or filtered supernatant and lysate from infected cells.

3.2.2 Analysis of cytokine secretion from CT-infected epithelial cells

Human endocervical epithelial cells (A2EN) will be infected with supernatants harvested from cell cultures that have lysed following infection with either WT or cpaf(-) CT. Infections will also be done with purified EBs of each genotype to control for differences between the WT and cpaf(-) genotypes that aren’t attributable to chromatin trap expulsion. Cell cultures will be washed and supplied with fresh media 2 hours after infection to synchronize the period of infection. Supernatant will then be analyzed at 24,
48, 72, and 96 hpi for secreted cytokines and chemokines using a multiplex cytometric bead assay for pro-inflammatory cytokines including IL1α, IL1β, IL6, TNFα, IL8, and GM-CSF. (table 1) Titering of the SN samples will also be carried out under the same infection conditions to ensure that infections being analyzed received comparable IFU for each genotype.

Table 1: Summary of precedents for cytokinds ot be assayed by cytometric bead approach

<table>
<thead>
<tr>
<th>cytokine</th>
<th>prior reports</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1α</td>
<td>up in A2EN and HFTOC + EBs</td>
<td>[27-29]</td>
</tr>
<tr>
<td>IL1β</td>
<td>up with extracellular histones</td>
<td>[11]</td>
</tr>
<tr>
<td>IL6</td>
<td>up in HeLa cells + CT seed</td>
<td>[27-29]</td>
</tr>
<tr>
<td>TNFα</td>
<td>up in HFTOC + EBs; up with extracellular histones</td>
<td>[30, 31]</td>
</tr>
<tr>
<td>IL8</td>
<td>up in primary endocervical cells + EBs; no change in A2EN + EBs</td>
<td>[27-29]</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>no change in A2EN + EBs</td>
<td>[27]</td>
</tr>
</tbody>
</table>

Key: HFTOC = human fallopian tube organ culture; EB = purified EBs, CT seed = EB samples that also contain cell debris.

3.2.3 Expected outcomes and alternatives

I expect that secretion of some pro-inflammatory cytokines will be differentially regulated by infection with WT and cpafl(-) SN inocula, but not by EBs of these genotypes alone. (fig, 6). For each cytokine assayed using the cytometric bead approach, 2-way ANOVA will be performed to determine whether between genotype differences are greater in the SN group than in the EB group.

This would show that factors in the post-lysis SN of WT infections drive increased cytokine production, but does not specifically implicate chromatin traps. Any
cytokines that have a profile like that diagrammed in figure 6 will be further analyzed. The infections will be repeated with SN inocula pre-treated with DNase or activated protein C to determine whether intact DNA or histones, respectively, are required for the observed effect. [32] These follow up experiments will be done by ELISA only for those cytokines that were differentially regulated in the multiplex assay. I anticipate that measuring cytokine secretion in SN should be straightforward. If no appreciable differences in cytokines are appreciable in the SN, it could be that cytokine responses are in fact being driven at the transcriptional level, but that CT interferes with cytokine protein expression or secretion. A microarray approach would be undertaken as an alternative to determine whether traps do contribute to induction of transcriptional responses for pro-inflammatory cytokines. [11-14]
# 3.2.4 Analysis of cytokine secretion and tissue destruction in human fallopian tube organ cultures

I will test whether chromatin traps enhance inflammatory cytokine responses and tissue destruction using the human fallopian tube organ culture (HFTOC) model for chlamydial infections of the oviduct. This model allows for the study of human responses in the tissue where ascending chlamydial infections drive scarring, the proximal cause of PID and TFI.

Human fallopian tube tissue will be obtained from post-hysterectomy material from individuals with no history of chlamydial infection and prepared by well-described methods. [29, 33, 34] Infections will be performed first with purified WT and *cpaf*(-) EBs to determine if chromatin trap expulsion can be induced in this system. For this experiment, I will use GFP expressing versions of the WT and *cpaf*(-) strains to allow easy visualization of EB-laden cell debris structures in the post-lysis SN. SN will be harvested from infections at 72-96 hpi, stained with DAPI, and checked for fibrous DAPI+ structures associated with EBs by fluorescence microscopy.

If trap-positive and trap-negative SN can be recovered from WT and *cpaf*(-) infections respectively, SN samples taken from HFTOC infections will be titered to enumerate IFU, and used to inoculate fresh HFTOC preparations. IL1α, IL1β, TNFα, IL8, and any other cytokines found to be differentially regulated in the A2EN model will be tested in the HFTOC model by immunohistochemistry (IHC). Infections of HFTOC preparations will be done with both purified EBs and SN samples of each genotype.
Cytokines with IHC signals showing a between genotype difference for the SN infections, but not for the purified EB infections, will be interpreted as depending on factors that differ between the SN produced upon lysis. Any cytokines with this profile will be further evaluated by performing infections with SN inocula pre-treated with DNase or activated protein C, to determine whether intact DNA, or histones, are required for the observed effect.

The HFTOC model is very amenable to scanning electron microscopy (SEM) of epithelial surface structure. [29] SEM imaging can reveal the extent, character, and spatial arrangement of cell damage. Additionally, chromatin traps with ensnared bacteria can be visualized by SEM, and key molecules, such as histones and chlamydial surface markers, can be immunostained to provide corroboration that trap-appearing structures contain chromatin and CT. [7]. I will visualize HFTOC epithelial surfaces following infection with WT and cpaf(-) SN inocula to determine if there are differences in the degree or character of surface architecture damage driven by chlamydial infection. [29]

3.2.5 Expected outcomes and alternatives

My expectation is that I will be able to identify inflammatory cytokines that are upregulated when organ cultures are infected with trap-positive SN inocula. IL1β has been shown to be secreted by epithelial cells exposed to extracellular histones. [11] TNFα secretion can be driven by extracellular histones, and has also been shown to be
upregulated in HFTOC during chlamydia infection, though only by ~1.5 fold. [30] I expect that TNFα secretion may be more substantially upregulated in the presence of chromatin traps. I also anticipate more profound damage to epithelial surfaces to be appreciable by SEM studies of infections with trap-positive inocula.

It is possible that infection with WT and cpaf(-) EBs does not result in differential trap expulsion phenotypes in this system. If this is found to be the case, the experiments could still be conducted with SN inocula derived from infected A2EN cells.
4. Summary and Future Directions

The proposed investigation will begin answering the question of whether chromatin traps benefit chlamydia or their host by determining whether they promote clearance of EBs, either directly or via phagocytes, and whether they contribute to inflammation and tissue damage in epithelial cells. If chromatin traps benefit the host by promoting chlamydial clearance, then they can likely be dismissed as a potential therapeutic target. If they help CT evade clearance, or if they drive inflammation, then consideration of traps as a therapeutic target warrants further study.

The answers provided by this study will prompt new avenues for investigation. If CT does resist killing by epithelial-derived chromatin traps, how is this accomplished? Are these traps bactericidal to other species? Are chlamydial EBs inherently resistant, or do they actively modify trap biochemistry to evade killing? If, on the other hand, these traps are toxic to EBs, what factors explain this? How does the composition of these traps compare to that of NETs? Proteomic analysis of epithelial-derived chromatin traps could provide insights that would be useful in dissecting the interactions between chromatin traps and EBs in greater detail.

This study will use the CPAF mutant as a tool to investigate downstream consequences of chromatin traps following their formation. The mechanism underlying the expulsion of chromatin, and the role of CPAF in this process, warrants a study of its own.
Testing the organismal consequences of chromatin traps is another obvious next step. If inflammatory signaling is enhanced by traps in cellular and tissue models, does this translate to increased upper genital tract pathology in a mouse model of CT STI? Could traps facilitate the ascension of EBs to the upper genital tract? Could they facilitate transmission between sexual partners? Establishing whether chromatin traps have a role in human STI would be supported by the identification of these structures in clinical samples.

Lastly, exploring whether this phenomenon is conserved across Chlamydiaceae could uncover possible significance for chromatin traps in other important diseases, such as C. trachomatis ocular infections, C. pneumoniae infections of the lung.
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