AN ABSTRACT OF THE DISSERTATION OF

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Title: *Chlamydiae* Enhance Self-Antigen Presentation in a Mechanism that is Dependent on Biosynthesis of Lipooligosaccharide.

Abstract approved:

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Daniel D. Rockey

*Chlamydia* is a genus of obligate intracellular bacteria that undergo a unique, biphasic developmental cycle. The infectious and metabolically inert Elementary Bodies (EBs) begin the cycle of infection by attaching and invading a host cells. Once inside, the EBs differentiate into the metabolically active, dividing Reticulate Bodies (RBs) within their own cellular compartment.

A significant portion of the chlamydial developmental cycle takes place within the host cell, which presents a challenge for the host immune system to detect and clear the infection. MHC Class I antigen presentation is the adaptive immune system's answer to this problem by displaying both host and parasitic antigens, if present, to other cells such as $CD8^+$ cytotoxic T cells. Previous research has identified chlamydial interactions with host-cell metabolism such as alteration of host transcription and translation. Other previous work has identified chlamydial antigens displayed in MHC Class I

molecules. This body of work is the first to describe the impact of *Chlamydia spp.* infection on MHC Class I self-antigen presentation by enhancing presentation of peptides derived from defective ribosomal products (DRiPs). We hypothesize that enhancing self-antigen presentation is a novel immune evasion strategy by which *Chlamyidae* saturate MHC Class I molecules with self-antigen and therefore decrease the likelihood that chlamydial antigens are presented.

Mechanisms of pathogenesis have often been used to elucidate host molecular pathways such as herpesvirus and the MHC Class I and MHC Class II antigen presentation pathways. The DRiPs pathway is currently unknown, and future endeavors will aim to utilize chlamydial infection to discover the molecular mechanisms of the DRiPs pathway.

Additionally, we used a small molecule inhibitor of chlamydial lipooligosaccharide biosynthesis and identified the sensitivities of *C. trachomatis* L2, *C. trachomatis* J6276, *C. muridarum, C. caviae,* and *C. suis* to the drug. Treatment of infected cells with the inhibitor of LOS resulted in aberrant inclusions in some, but not all species as well as a differential effect on output of infectious EBs. Future experiments will seek to validate the use of the LOS inhibitor as an agent of selection in chlamydial recombination.

# *Chlamydiae* Enhance Self-Antigen Presentation in a Mechanism that is Dependent on Biosynthesis of Lipooligosaccharide

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Erik D. Cram

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APPROVED:

Major Professor, representing Molecular and Cellular Biology

Director of the Molecular and Cellular Biology Program

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Erik D. Cram, Author

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## Chapter 3

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# Pathogenic evasion strategies of MHC Class I and MHC Class II antigen presentation pathways

Erik D. Cram and Daniel D. Rockey

## Introduction

Antigen presentation is an essential component of the adaptive immune system that distinguishes and controls infection by displaying pathogen-derived molecules to cytotoxic $CD8^+$ T cells and $CD4^+$ T helper cells. MHC Class I antigen presentation results in the surface expression of peptide resulting from intracellular infection and is a reflection of the endogenous host proteome. MHC Class I molecules are found on the surface of almost all nucleated cell types. MHC Class II presentation of antigens originates from extracellular molecules endocytosed by the antigen-presenting cell. MHC Class II complexes are constitutively expressed in professional antigen presenting cells including B cells, dendritic cells, thymic epithelial cells and macrophages. The MHC Class I and MHC Class II antigen presentation pathways activate killer $CD8^+$ and helper $CD4^+$ T cells respectively. Despite the differences between the MHC Class I and MHC Class II pathways, the resulting immune response of either presentation pathway places selective pressures on pathogens to develop strategies of avoidance or evasion.

## MHC Class I presentation of endogenous peptides are altered as a consequence of infection

MHC Class I surface molecules display antigens from intracellular peptides that allows for the targeted destruction of infected or transformed cells by cytotoxic $CD8^+$ T cells. One major source of host peptide presented by

surface MHC Class I molecules are derived from defective ribosomal products, termed DRiPs, which include products from the pioneer round of translation, misfolded proteins, and errors in translation such as early termination [4]. DRiPs are targeted for degradation by the proteasome and are then shuttled into the MHC Class I pathway for surface expression. The other pool of host antigens come from the normal protein turnover of “retiree” proteins that reach the end of their functional lifespan and are degraded by the proteasome. While the relative contribution of retirees or DRiPs to antigens presented at the cell surface can vary, the resulting surface HLA peptidome is a reflection of the cell’s metabolic state [5]. Therefore, it is logical that infection leading to changes in host protein expression or manipulation of other cellular processes is consequently reflected in the surface HLA peptidome. Indeed, influenza virus both modifies host self-antigen repertoire and minimizes the surface HLA presentation of viral peptides (Fig 1-1) [6, 7]. Similarly, HIV infection modifies host gene regulation and expression resulting in both upregulating and alteration of the presentation of specific self-peptides in order to minimize viral antigen (Figure 1-1) [8]. It has been thought that these changes to the self-HLA repertoire may be partly responsible for the various autoimmune pathologies rampant in patients infected with HIV. While it remains unclear what advantages such changes to the HLA repertoire may provide for HIV or influenza virus during infection, future vaccination strategies include exploiting presentation of uniquely presented host antigens for CTL destruction [7, 8].

In addition to these viral studies, we have made the unique observation that the obligate intracellular bacteria, *Chlamydia trachomatis* and *Chlamydia caviae* increase self-antigen presentation of a model host protein by enhancing the DRiP pathway (Figure 1-2) [3]. Another study found infection of AE2N cervical cells with *C. trachomatis* serovar D resulted in the downregulation of surface MHC Class I molecules in both infected and neighboring AE2N cells [9]. While clinical studies support the idea of a faulty $CD8^+$ T responses *in vivo*, other *in vitro* studies were unable to detect changes in MHC Class I levels in other epithelial or fibroblast cell lines [10-12]. Our own data show that surface levels of MHC Class I molecules are unchanged in JY lymphoblastoid cells [3]. Taken together, the data suggest that downregulation of MHC Class I molecules may be cell line specific and conditional upon induction of IL10 production and stimulation of CXCL12/CXCR4 [11, 12].

Another contributing factor to skewed host antigen presentation are bacterial-mediated changes to the host transcriptome and peptidome. *C. trachomatis* alters both the host cell transcriptome as well as host protein stability [13, 14]. While researchers have yet to address if any host peptides are uniquely presented during chlamydial infection, this may be a viable scheme to the development of a chlamydial vaccine.

The obligate intracellular lifestyle that *C. trachomatis* shares in common with both HIV and influenza virus is one in which pathogen-derived antigens are readily accessible to MHC Class I machinery. The pathogenic shift in host peptide presentation suggests a common evolutionary pathway independently developed in three diverged lineages in which host peptides are exploited to mask the presence of each parasite. What these mechanisms are and how they affect the MHC Class I pathway remain a mystery. Currently, assays and technologies for detecting alterations of host antigen presentation require specialization and can be expensive to use. Emergence of model systems such as that of Dolan *et al.* will pave the way for future studies characterizing the interactions of infection on the MHC Class I pathway.

**Inhibition of proteasomal degradation and generation of antigenic peptides**

Peptide presentation from the direct MHC Class I pathway is dependent on host proteasomal degradation of DRiPs and retiree proteins in order to generate epitopes that are 8-10 amino acids in length [15, 16]. Pathogenic strategies to overcome peptide generation might involve 1) regulating transcription and translation of pathogenic peptides as to not express excess protein and therefore limit antigenic substrates, 2) producing stable proteins that are resistant to unfolding, degradation and post-translation modifications such as polyubiquitination and, 3) directly inhibiting proteasomal and/or nonproteasomal degradation machinery. Epstein-Barr virus (EBV) EBNA1 is

one such peptide that contains a Gly-Ala rich repeat, which directly inhibits translation initiation of EBNA1 mRNA [17]. While the Gly-Ala repeat does not affect protein stability of EBNA1 itself, fusion of the Gly-Ala repeat of an EBNA1 homolog in lymphocrypto-Papio virus to p53 mRNA increased stability of p53 transcripts and avoided MDM2 E3 ligase mediated destruction (Figure 1-1) [17]. Lastly, direct modification of the host proteasome to alter peptide presentation has not yet been observed by any known animal pathogens. However, Groll *et al.* identified GlbA, as novel proteasome inhibitor originating from an unknown member of the proteobacteria group, Burkholderiales which includes many mammal pathogens [18].

**Inhibition of TAP-mediated translocation of antigenic peptides into the ER**

Subsequent to protein degradation in the cytosol is the translocation of antigenic peptides into the ER mediated by the “transporter associated with antigen processing”, or TAP. The TAP complex is an ATP-dependent heterodimer consisting of TAP1 and TAP2 and is a popular target for bacterial and viral pathogens [19].

The most abundant mechanism to inhibit TAP-mediated antigen is through protein complexing with TAP such that the ATP binding site is blocked, preventing hydrolysis. This mechanism is targeted by ICP47 from Herpes simplex virus, BNLF2a from Epstein-Barr virus, CPXV012 from Cowpox virus, US6 from Human cytomegalovirus, and UL49.5 from Equine herpes virus

(Figure 1-4) [20-27]. Bovine herpes virus-1 UL49.5 affects this process in two ways. First, UL49.5 physically inhibits antigen translocation by binding to the TAP complex and second, the protein polyubiquitinates TAP for proteasomal degradation [25, 26, 28]. MK3 protein from Murine γ-herpesvirus-68 causes degradation of TAP1 and TAP2 that is dependent on its RING finger domain (Figure 1-5) [29-31]. Boname *et al.* (2004) reported that MK3 mitigates the effects of IFNγ-induced increased MHC Class I presentation by degrading TAP1, TAP2 and other members of the peptide loading complex (PLC), which will be discussed in the following sections [29]. MK3 is predicted to contain a RING finger domain which target proteins for degradation via a E3 ubiquitin ligase-like activity, though the ability of MK3 to ligate ubiquitin groups on its targets has never been directly tested [32]. Finally, the opportunistic bacterium *Pseudomonas aeruginosa* secretes the toxin CFTR inhibitory factor, or Cif, that inhibits the deubiquitinase USP10 in airway epithelial cells resulting in increased levels of polyubiquinated TAP1 targeted for proteasomal degradation (Figure 1-5) [33, 34].

**Targeting tapasin destabilizes bridging with TAP and empty MHC preventing antigenic peptide editing and loading**

Upon translocation of an antigen into the ER lumen, peptidyl loading onto the MHC complex is mediated through tapasin, which stabilizes empty MHC Class I molecules and docks them to TAP [35]. The stability of TAP is also dependent on binding affinity for tapasin [36]. Additionally, tapasin is

responsible for peptide editing and loading onto MHC Class I molecules, which selects for stable binding of high-affinity antigens [37]. The essential roles of tapasin in the PLC make tapasin a key target for virulence factors to interfere with MHC Class I antigen presentation. Adenovirus protein E19 uniquely binds either TAP1 or MHC Class I molecules, and thusly prevents the bridging of the PLC by tapasin (Figure 1-7) [38]. Along with causing degradation of TAP, MK3 from Murine γ-herpesvirus-68 triggers degradation of tapasin in murine antigen presenting (RMA) cells (Figure 1-7) [30]. Perhaps the most interesting instance of tapasin inhibition is that of Human cytomegalovirus which employs dual strategies to target tapasin. In addition to downregulating tapasin mRNA transcripts, Human cytomegalovirus also inhibits formation of the PLC, however, both mechanisms are still unknown (Fig, 1-7) [39].

**Arresting, degrading and counterfeiting polymorphic MHC Class I molecules**

MHC Class I heavy chains in association with the $\beta_2$-microglobulin subunit are the terminal carriers of antigenic peptides. After peptidyl loading within the ER lumen, MHC Class I molecules are trafficked through the Golgi and directed towards the plasma membrane. MHC Class I molecules are recognized and bound by $CD8^+$ Killer T lymphocytes which detect peptides indicative of infection or transformation and initiate killing. Classical heavy chain portions of MHC Class I molecules are polymorphic and are derived from three genes such as the HLA-A, HLA-B and HLA-C. Each gene encodes eight exons and

variation of peptide binding affinity between polymorphs is due to SNPs in exons 2 and 3 encoding for the extracellular-facing α1 and α2 domains which together, act to bind peptides [40]. Surface presentation efficiency of MHC Class I molecules varies between each polymorphism and is correlated to preferential peptide loading as well as stable binding with tapasin [41].

The Human cytomegalovirus genomic toolbox contains many mechanisms that directly interact with MHC Class I heavy chains. One strategy utilizes expression of two ER resident glycoproteins, US3 and US10, which delay trafficking of MHC Class I heavy chains to the cell surface by protein-protein interactions resulting in retention within the ER (Fig 1-7) [42-44]. In addition, Human cytomegalovirus expression of US11 and US2 result in the targeted degradation of ER resident heavy chain molecules by the endoplasmic reticulum-associate degradation (ERAD) and the proteasomal degradation pathways (Fig, 1-7) [45, 46]. Similarly, E19 from Adenovirus and gp40/m152 from Murine cytomegalovirus are other ER resident, glycoproteins that inhibit MHC Class I trafficking by associating with the heavy chains (Figure 1-7) [47-50]. MK3 protein from Murine γ-herpesvirus-68 triggers the degradation of MHC Class I molecules through the same predicted RING finger domain activity involved in the targeted destruction of TAP1, TAP2 and tapasin (Figure 1-7) [29-31].

In addition to $CD8^+$ T cell-mediated killing of infected the cells, NK cells also survey surface levels of MHC Class I molecules. While few surface receptors

of NK cells are dedicated to pathogenic ligands, the majority of NK receptors are dedicated to surveillance of endogenous self-peptides. This poses a problem to pathogens that decrease surface levels of MHC Class I molecules which can act as a signal to induce NK cell-mediated killing of the infected cell [51]. To address this issue the Human cytomegalovirus employs the “fake ID” strategy of protein UL18 (Figure 1-7). UL18 can form a trimeric mimic of MHC Class I heavy chain that is capable of not only interacting with β2 microglobulin (β2m), but can also bind endogenous peptide and interact with NK receptors [52]. Additionally, the MHC Class I related chain A protein (MICA) acts as a stress-induced signal for the NKG2D receptor of NK cells. Expression of the *cis*-Golgi Human cytomegalovirus resident glycoprotein, UL142 retains MICA in the *cis*-Golgi preventing surface presentation (Figure 1-7) [53].

**Sequestering β2-microglobulin from the PLC decreases PLC stability and inhibits peptide loading to the MHC Class I complex**

The β2m subunit of the MHC Class I molecule associates via non-covalent linkages with the α1 subunit and is parallel with the α3 subunit by the cell surface, but unlike the α3 subunit, β2m lacks a transmembrane domain. β2m is critical for stabilizing the MHC Class I complex, and promotes peptide loading [54, 55]. The stabilizing properties of β2m on the complex is also dependent on the binding affinity for the antigenic peptide within the complex

[56]. The resulting interactions of peptide with the MHC Class I complex lead to β2m associations ranging from hours to days [57, 58].

The early-secreted effector ESAT-6 protein from the facultative intracellular bacterium, *Mycobacterium tuberculosis*, escapes the phagosome into the host cytosol in dendritic and macrophage cells. ESAT-6 localizes to the ER lumen where it interacts directly with β2m resulting in a downregulation of surface MHC Class I molecules (Figure 1-7) [59]. Tanapox virus protein TPV-2L and MC80R from Molluscum contagiosum virus (MCV), share some sequence identity to MHC Class I heavy chains. The heavy chain mimics are able to bind free β2m and sequester within the ER to prevent MHC Class I molecules from forming (Figure 1-7) [60, 61].

**ER resident chaperones BiP, calnexin, calreticulin, and ERp57 stabilize and fold MHC Class I heavy chains and viral glycoproteins**

The immunoglobulin binding protein, BiP, and calnexin are both ER resident chaperones that promiscuously bind MHC Class I heavy chain molecules before association with the PLC. These chaperones serve many functions including involvement with the ER stress unfolded protein response (UPR) triggered by the accumulation of unfolded proteins. BiP and calnexin both bind MHC Class I heavy chains by interacting with hydrophobic residues. In addition to assuring proper protein folding, BiP and calnexin prevent aggregation of MHC Class I complexes [62]. Calnexin is a lectin that is a

chaperone to nascent glycoproteins and membrane proteins that are monoglycosolated [63].

Because many viruses induce the UPR response pathway as an advantage in establishing acute, chronic and latent infections, it is hypothesized that downregulating BiP or calnexin would result in a negative outcome for viral infection [64]. So far, the only known pathogenic strategy to subvert the MHC Class I pathway by manipulating BiP and calnexin belongs to the facultative intracellular bacterium, *Legionella pneumophila. L. pneumophila* bacteria replicate within a cellular compartment that contain ER protein markers. *L. pneumophila* infection of human monocyte cells resulted in the adsorption of BiP and calnexin to the parisitopherous vacuole and subsequent downregulation of surface MHC Class I that was correlated to microbial growth (Figure 1-6) [65].

The ER resident chaperone ERp57 is a thiol oxidoreductase involved in the formation of disulfide bonds within the α2 and α3 domains of MHC Class I heavy chain [66, 67]. Calreticulin, like calnexin, is a lectin chaperone that interacts with N-terminal glycosylated α1 domain of the MHC Class I heavy chain [67]. Turnquist *et al.* found that tapasin was unable to associate with MHC Class I heavy chains in the absence of calreticulin, suggesting that calreticulin plays a central role in forming the PLC [68]. Additionally, Wearsch *et al* found that disruption of either ERp57 or calreticulin completely prevented

the PLC from forming [69]. Because both ERp57 and calreticulin are vital to PLC formation, it would be reasonable to hypothesize these enzymes as lynchpin mechanisms for defeating MHC Class I presentation. However, the sole known instance of targeting either of these chaperones belongs to Nairobi sheep nairovirus which translocates ERp57 to the cell's surface where it is hypothesized that it interacts with integrins and tissue factors in a pro-inflammatory response (Figure 1-7)[70]. The absence of identified viral mechanisms that target ERp57 and calreticulin may be simply explained by the fact that many known viral glycoproteins rely on both enzymes as chaperones for viral protein folding and stability [71-75].

**Preventing vesicular trafficking of MHC Class I molecules to the cell surface**

After loading of the peptide on to MHC Class I – β2m heterodimer, the PLC disassembles and the MHC Class I molecule exits the ER via the vesicular trafficking pathway. COPII-coated vesicle bearing the MHC Class I molecules then fuse with the *cis*–Golgi where new buds form and fuse until reaching the *trans*-Golgi compartment and, finally, the plasma membrane. Poliovirus protein 3A disrupts vesicular trafficking by interfering with the ER to Golgi intermediate complex, ERGIC, resulting in the sequestering of MHC Class I molecules within the ERGIC (Figure 1-8) [76-78]. Similarly, the zoonotic Orf virus restricts MHC Class I molecules to the *trans*-Golgi by disrupting and fragmenting the Golgi structure through early expression of viral genes

(Figure 1-9) [79]. Cowpox virus protein CPXV203 is active in the Golgi and binds non-classical and classical MHC Class I molecules in the same highly conserved domain recognized by members of the PLC. CPXV203 then exploits the KDEL receptor ER retrieval pathway to restrict MHC Class I molecules to the ER (Figure 1-8) [80]. Equine herpesvirus expresses pUL43 and pUL56 that are localized to the Golgi. Together, they hijack vesicles containing MHC Class I molecules and reroute them to the lysosome for degradation (Figure 1-9) [81]. In a similar mechanism, HIV Nef protein acts together with binding partner, adapter protein 1 (AP-1) to misdirect *trans*-Golgi trafficking of MHC Class I molecules heading towards the plasma membrane into pre-lysosomal endosome compartments (Figure 1-9) [60].

**Targeting surface MHC Class I molecules for destruction within the lysosome**

Once at the surface of the cells, MHC Class I complexes are recognized by CTLs and NK cells. Following surface presentation, antigenic peptides disassociate from MHC Class I molecules that are then endocytosed and recycled for subsequent antigen presentation. Kaposi's sarcoma herpesvirus proteins K3 and K5 target fully mature, surface MHC Class I molecules for endocytosis and degradation within the lysosome (Figure 1-10). K3 and K5 are E3 ubiquitin ligases with predicted N-terminal RING domains similar to MK3 from Murine γ-herpesvirus-68 [82]. Oddly, microscopy has shown K3 and K5 to be ER resident proteins, however these results have been called

into question as a possible artifact of overexpression [82]. While K3 targets all classical and non-classical MHC Class I isotypes, K5 only targets HLA-A and HLA-B molecules [83].

**The MHC Class II pathway displays antigens derived from exogenous sources**

MHC Class II molecules present both self and non-self peptides from antigenic substrates that are first endocytosed and then fragmented within endosomes and lysosomes. The MHC Class II pathway is therefore not redundant to the MHC Class I antigen presentation because it is not limited to displaying antigens from intracellular pathogens only. MHC Class II molecules are only constitutively expressed in professional antigen presenting cells including macrophage, dendritic cells, thymic epithelial cells, and B cells [84]. Other cell lines express MHC Class II molecules when activated by cytokines such as IFN-Y[85]. MHC Class II molecules are recognized by helper T cells expressing CD4 protein. Unlike cytotoxic $CD8^+$ T cells, $CD4^+$ T cells do not initiate the killing of the antigen-presenting cell rather recruit other immune cells, including $CD8^+$ T cells by secreting cytokines [86, 87].

**Transcriptional regulation and post-translational modification of MHC Class II heterodimers are targets for pathogenic manipulation**

Classical MHC Class I molecules are heterodimers consisting of α and β chains that include HLA-DR, DP and DQ isoforms in humans. Gene loci for

each α and β gene are located adjacently in all three cases [88]. Regulation of classical MHC Class II HLA transcription is controlled through three promoters S, X and Y and are highly conserved for all three isoforms [89]. The class II transactivator, or CIITA, is both essential for and a master co-regulator of MHC Class II molecule transcription by recruiting and complexing with numerous other transcription factors. The essential role of CIITA in MHC Class II molecule transcription makes CIITA a prime target for pathogens to avoid the MHC Class II pathway.

Epstein-Barr virus protein Zta is a DNA binding protein with affinity for CIITA and HLA-DRα promoter sequences resulting in repressing transcription of either molecule and downregulation of MHC Class II HLA (Figure 2-1) [90]. Similarly, Kaposi's sarcoma herpesvirus LANA protein and latency associated protein, vIRF3, block CIITA transcription by binding the pIII and pIV promoter sites (Figure 2-1) [91-93]. The obligate intracellular protozoan pathogen *Toxoplasma gondii* indirectly inhibits CIITA expression in multiple cell lines by blocking IFNγ production and subsequently, inducible CIITA transcription (Figure 2-1) [94]. Human cytomegalovirus also downregulated IFNγ-induced CIITA activation in a model system of U373 MG astrocytoma cells resulting in reduced MHC Class II surface expression (Figure 2-1) [95]. *Bordetella pertussis* infection of human monocytes collected from primary PBMCs leads to a reduction in surface HLA-DR and blocked IFNγ-inducible expression (Figure 2-1). The data collected by Shumilla *et al.* suggest that pertussis

toxin is responsible for reduced HLA-DR expression while the mechanism responsible for blocked IFNγ induction remains unknown [96]. The herpesvirus family has evolved mechanisms to cease global host protein synthesis upon infection by altering mRNA stability. This strategy is an indirect mechanism that includes targeting CIITA and MHC Class II transcripts resulting in reduced antigen presentation in infected cells. The viral host shutoff genes include UL41 from Herpes simplex virus type 1, SOX from Kaposi's sarcoma herpesvirus BGLF5 from Epstein-Barr virus (Figure 2-1) [97]. Lastly, CIITA upstream regulatory activator USF-1, and MHC Class II HLA co-regulator RFX5, are degraded *in vitro* by the *C. trachomatis* protease-like activity factor, CPAF, however these findings have been challenged as authentic substrates (Figure 2-1) [98, 99].

Targeted destruction or vesicular re-routing of MHC Class II HLA molecules is another popular strategy for pathogenic evasion of $CD4^+$ T cell recognition. The facultative intracellular bacterium *Francisella tularensis* is known to survive and proliferate within macrophage cells. Wilson *et al.* observed that IFNγ-activated macrophage infected with *F. tularensis* resulted in ubiquitin-mediated destruction of MHC Class II molecules due to bacterial expression of prostaglandin E2 (PGE2) and another unidentified factor (Figure 2-2) [100]. The facultative intracellular pathogen *Salmonella typhimurium* interferes with MHC Class II HLA molecules in a variety of mechanisms. One study identified *S. typhimurium* pathogenicity islands, SPI-1, and SPI-2, were

partially responsible for downregulating MHC Class II molecules in porcine alveolar cells (Figure 2-2) [101]. Another study demonstrated that an unknown T3SS effector protein enhances ubiquitination of classical HLA-DR, DP and DQ molecules for protease-mediated degradation in DCs [102]. US2 and US3 proteins from Human cytomegalovirus are glycoproteins that respectively target HLA-DRα chains for proteasomal degradation and retain MHC Class II HLA within the ER (Figure 2-3) [103]. Additionally, Human cytomegalovirus pp65 targets HLA-DRα for destruction within the lysosome (Figure 2-3) [104].

**Blocking degradation of invariant chain (Ii) or competing with Ii chaperone complexes with MHC Class II molecules prevents antigenic loading**

The invariant chain, sometimes known as Ii or CD74, is a membrane-associated chaperone that interacts with MHC Class II molecules beginning in the ER. Ii contains the class II invariant chain-associated peptide, or CLIP, domain that binds the antigen-binding grove. This prevents the binding and presentation of self-peptides intended for MHC Class I presentation pathway. Ii gene expression is also under the control of CIITA [105]. MHC Class II-bound Ii complexes are presented on the plasma membrane and subsequently endocytosed and trafficked to the lysosome [84]. After delivery to the lysosome, proteases cleave Ii leaving only CLIP bound to the MHC Class II αβ dimer that must then be removed before peptide loading [84].

Hepatitis C virus has evolved a unique strategy for MHC Class II antigen presentation by inhibiting cathepsin S expression, which is responsible for Ii degradation within the lysosome. In a study by Kim *et al.* HCV core and NS5A proteins downregulated cathepsin S resulting in an increase expression of surface MHC Class II/Ii complexes in DCs and hepatocytes (Fig, 2-4) [106]. Another study found that Human papillomavirus type 16 protein E5 blocks degradation of Ii in the lysosome in human foreskin keratinocytes (Figure 2-4) [107]. While the exact mechanism remains unknown, VacA toxin produced by *Helicobacter pylori* blocked the degradation of Ii within the lysosome of DCs *in vitro* (Figure 2-4) [108]. Alternatively, Epstein-Barr viral protein BZLF1 downregulated surface Ii complexes through an unknown post-transcriptional mechanism in human melanoma cells [109]. Herpes simplex virus type 1 viral envelope glycoprotein, or gB, not only reduces expression of Ii, but is a competitive binding partner with HLA heavy chains preventing Ii binding, and HLA-DR trafficking (Figure 2-3) [110].

**Sequestering, retaining or degrading non-classical HLA-DM, DO chaperone prevents peptide editing and loading on to classical HLA molecules within the lysosome**

Classical HLA heavy chains bound to CLIP are ready for peptide loading, a process that is facilitated by the non-classical and functionally distinct HLA-DM (DM) and HLA-DO (DO). DO functions to negatively inhibit DM within the

lysosome by competitive binding [111]. DM functions as a peptide exchange factor loading self and foreign antigen onto classical complexes, though many intricacies of this process remain to be solved [112]. The essential nature of DM in MHC Class II antigen presentation makes it a key target of pathogenesis.

One study identified SPI-1 gene expression of *sifA* recruited non-classical MHC Class II molecules HLA-DM to the *S. typhimurium*-containing vacuoles to avoid surface presentation (Figure 2-5) [113]. Other studies focusing on the herpesvirus families reveal additional pathways to defeat MHC Class II presentation. In addition to binding HLA-DR, the Herpes simplex virus type 1 envelope glycoprotein gB can bind DMα and prevent the HLA-DR/DM complex from forming (Figure 2-5) [110]. Human cytomegalovirus US2 and US3 also target DMα for degradation or retention as mentioned in previous sections (Figure 2-5) [103].

**Endocytosis of extracellular molecules supplies MHC Class II antigenic pool**

Substrates of antigenic peptides gain entry into the cell via endocytosis, which is typically initiated by cellular recognition of pathogens through stimulation of receptors on the plasma membrane. Some cells such as macrophage use chemotaxis to target and engulf pathogens via phagocytosis [114]. Phagocytosis is mediated by cellular receptors that recognize pathogenic

structures (pattern recognition receptors, or PRRs), or molecules that have been opsonized (Fc receptor) or coated in the C3b complement (complement receptor 1) [115]. After receptor stimulation, the actin skeleton is rearranged such that the extracellular particle is engulfed. Similarly, macropinocytosis begins with the formation of large ruffles on the plasma membrane stimulated by receptor activation. The actin cytoskeleton is rearranged and external macromolecules are internalized [116]. Self MHC Class II antigens are generated through the process of autophagy and fusion with the endosome [117].

One of the main strategies used by pathogens to subvert phagocytosis is to use antigenic variation to avoid recognition by specific immune receptors. Antigenic variation refers to a clonal population of pathogens that differentially express external proteins in order to avoid recognition by the adaptive immune system. Mechanisms of antigenic variation are diverse and employed by viral, bacterial and eukaryotic animal pathogens and have been extensively reviewed [118-122].

A similar mechanism employed by many numerous bacteria is to produce external molecules to protect from opsonization. Bacterial capsules such as *Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus* prevent C3b from coating the bacteria and the subsequent Fc-mediated phagocytosis (Figure 2-6) [123-125]. Additionally, *S. aureus* expresses the immunoglobulin-

binding proteins SpA and Sbi, which attach to the Fc portion of IgG and form complexes effectively blocking access to Fc-receptors and subsequent phagocytosis (Figure 2-6) [126].

The *Yersinia* genus of bacterial pathogens has developed multiple approaches for avoiding phagocytosis by secreting the Yop family of TIII effector proteins (Figure 2-6). YopE acts as a GTPase-activating protein that interacts with Rho GTPase, while YopT modifies RhoA, Rac1 and Cdc42 to destabilize the actin cytoskeleton [127]. YopH dephosphorylates proteins at focal adhesion junctures [127]. Lastly, YopP is a MAPK inhibitor that block clathrin-mediated endocytosis in dendritic cells [128].

**Avoidance or remodeling of the lysosomal compartment impedes MHC Class II peptide generation and surface HLA expression**

After endocytosis, antigen-containing endosomes are fused with lysosomes. Antigenic peptides are then generated via degradation and subsequently loaded onto MHC Class II molecules. Because of the harsh environment of the phagolysosome, including low pH and the presence of destructive enzymes, intracellular, intravacular parasites such as *C. trachomatis* need a strategy to avoid phagolysomal maturation (Figure 2-7) [129]. Alternatively, the obligate intracellular bacterium *Coxiella burnetii* remodels the lysosome compartment to facilitate growth in the otherwise uninhabitable organelle (Figure 2-7) *[130]*. Intriguingly, the facultative intracellular genus of bacteria

*Mycobacterium* develop within a phagosome that does not fuse with the lysosome, however, *M. avium* phagosomes actively sequester HLA-DR from both intracellular vesicular transport and the plasma membrane in macrophage cells (Figure 2-7) [131, 132].

**Surface expression of MHC Class II molecules and Ii-independent recycling pathway**

After MHC Class II molecules are loaded with antigen, they are then trafficked from the lysosome to the plasma membrane where they can be recognized by $CD4^+$ T cells. While at the surface, MHC Class II molecules can be endocytosed via clathrin, dynamin and AP-2 pathways for antigen recycling within endolysosomal compartments [133]. Vaccinia virus disrupts MHC Class II antigen presentation, although the physiological significance is under debate as the phenotype is conditional. Recently, Rehm *et al.* demonstrated that the highly conserved poxvirus protein A35 from Vaccinia virus AR35 is responsible for downregulating MHC Class II surface expression by disrupting peptide loading of a model peptide in B cells (Figure 2-8). As well as blocking nascent peptide loading and increasing surface expression of CLIP, A35 inhibited the recycling of MHC Class II molecules in the endosomal compartment (Figure 2-8) [134]. Alternatively, HIV-1 Nef protein induces endocytosis of MHC Class II molecules by exploiting normal protein turnover mechanisms, redirecting the MHC Class II complexes to lysosomal compartments in monocyte cells (Figure 2-8) [135].

### *Chlamydia* persistence within the host cell

The *Chlamydia* genus is comprised of unique obligate-intracellular organisms that undergo a biphasic developmental cycle in a broad range of hosts. Infection begins when a metabolically inert, and spore-like Elementary Body (EB) attaches and invades a host cell. Once inside its own cellular compartment, termed the “inclusion”, the EB differentiates into the metabolically active, Reticulate Body (RB) form. The chlamydial developmental cycle proceeds and RBs continually divide and differentiate back into the infectious EB phase. Late in the cycle, EBs are released into the environment to restart the infection process on a different host cell (Figure 3).

Chlamydial persistence is defined as a reversible state of viable, but non-cultivable, enlarged, aberrant, non-dividing RBs within the inclusion [136]. Many factors are known to induce this stress response *in vitro* including nutrient/amino acid starvation, host IFN-γ, host cell differentiation state, concomitant infection with herpes virus, and treatment with antibiotics such as ampicillin [136-142]. *In vivo* evidence for persistence has been compelling, however, less direct. One 1962 study by Allison *et al.* described *C. muridarum* susceptibility to ampicillin in a mouse model of infection. The study found that although infected mice treated with ampicillin did not develop lung lesions, EBs were cultivable from processed lung tissue [143]. Another early human study by Oriel *et al* from 1976 showed human patients co-infected with

*Neisseria gonorrhea* and *C. trachomatis* and treated with a single dose of ampicillin/probenecid resulted in successful clearing of *N. gonorrhea*, but rarely eliminated *C. trachomatis* infections [144]. Finally, evidence in the form of direct imaging of EBs from infected tissues in animal models has been previously demonstrated [145]. These and other studies aim to address the issue of persistent infections contribution to the severe immunopathologies that are known outcomes of repeat infections.

The evidence for the numerous stimuli that induce the chlamydial persistency strongly suggests the necessity of this diversion in the developmental pathway in order for the bacteria to survive *in vivo*. *Chlamydia* spp. face an additional problem to the successful establishment of a persistent infection: detection by the immune system. Such a challenge to pathogen survival would likely come in the form of MHC Class I presentation of chlamydial antigens to the surface of the cell. This pathway would likely drive selection towards mechanisms to defeat the MHC Class I pathway. We propose that infection with *Chlamydia* spp. leads to saturation of MHC Class I complexes with host antigen by shifting the outcome of translation towards an increased pool of DRiPs and away from stable peptides [3]. Future work should address the effects of persistent chlamydial infections on self-antigen presentation.

## Conclusion

Elucidation of the molecular mechanisms associated with MHC Class I and MHC Class II pathways remains a developing field of study. Much of what we have learned regarding antigen presentation results from uncovering pathogenic mechanisms to manipulate and evade each pathway. Members of the herpesvirus family have been especially useful, as they have evolved a variety of mechanisms to defeat both antigen presentation pathways. There remains, however, much to learn about antigen presentation because mechanisms evolved to defeat antigen presentation are as diverse as the pathogens themselves. Additionally, the tools for studying the subject are becoming increasingly more reliable and widespread, allowing researchers of host cell-pathogen interactions to address related questions. The similarities in mechanisms for many steps of both pathways, is reflective of the strong selective forces derived from the efficiency and efficaciousness of antigen presentation in clearing infection. In addition to addressing pathogen interactions with the antigen presentation pathway, it would be of worth to investigate pathogenic interactions with host cell self-peptide presentation. To date, we are the only lab that has addressed the topic and have discovered that *C. trachomatis* increases antigen presentation of model self-peptide. We would hypothesize that other pathogens have evolved similar capabilities.

We hypothesize that the obligate intracellular developmental cycle of *Chlamydae* have presented selective forces that require subversion of the MHC Class I antigen presentation pathway. In this body of work we aim to examine the effects of chlamydial infection on host antigen presentation and define the mechanisms that may contribute to skewed self-antigen presentation. We further hypothesize that the conserved nature of the chlamydial lipooligosaccharide (LOS) molecule may play a role in both chlamydial development and interactions with host processes. We utilized a model of self-antigen presentation in conjunction with a TCRm monoclonal antibody to examine the effects of chlamydial infection on antigenic substrates from pools of defective ribosomal products (DRiPs) and pools of normal protein turnover. Finally we utilized a small molecule inhibitor of chlamydial LOS biosynthesis to characterize the role LOS in skewed antigen presentation and chlamydial development in several species of *Chlamydia.*

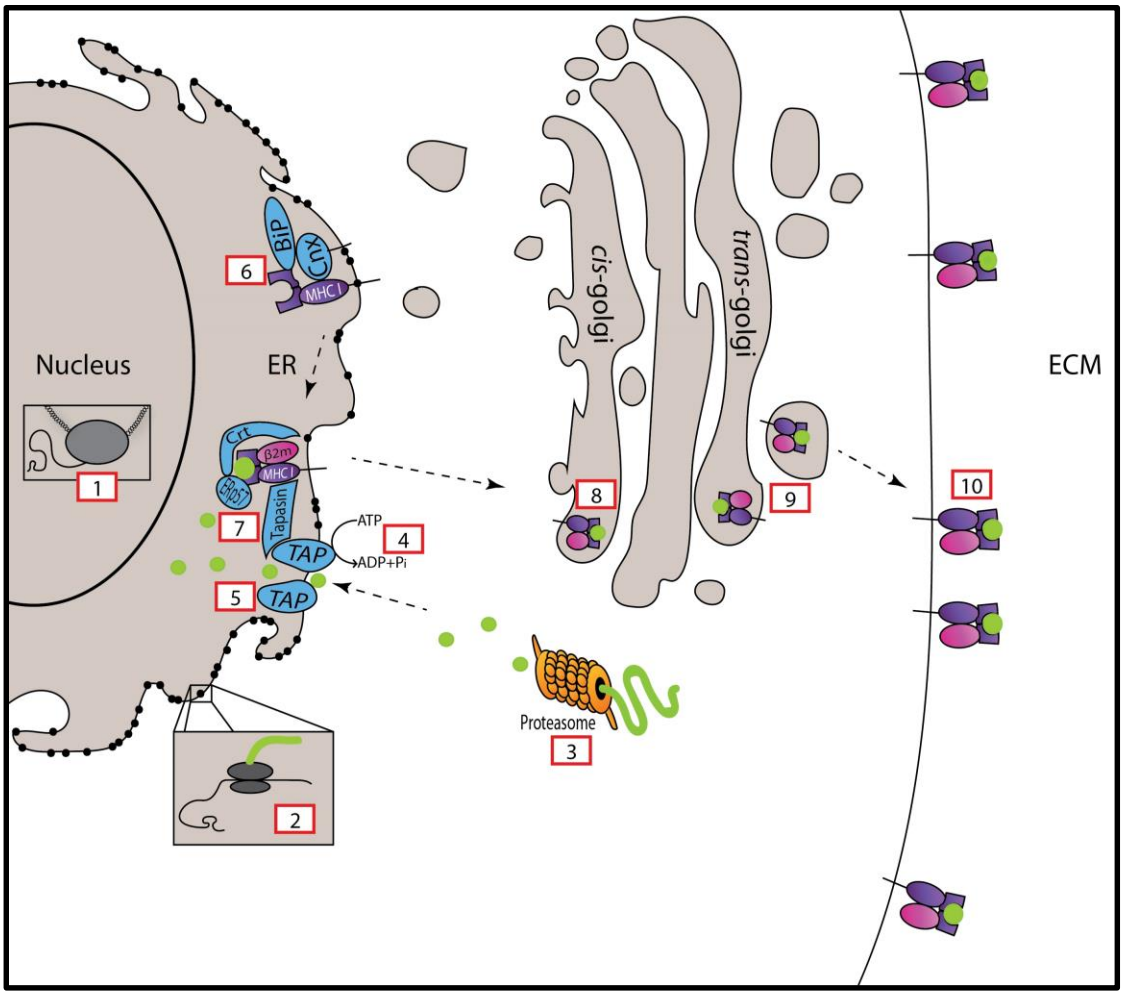


**Figure 1. Summary of MHC Class I pathway and mechanisms of subversion:** Red boxes indicate target(s) of pathogenic manipulation. If the mechanism is known, it is listed after the organism name. **1: Transcription** Influenza, HIV, Epstein-Barr virus EBNA1 **2: Translation** *Chlamydia trachomatis*, *Chlamydia caviae* Epstein-Barr virus EBNA1 **3: Proteasomal degradation** *Pseudomonas syringae* SylA **4: Hydrolysis of ATP by TAP** Herpes simplex virus ICP47, Epstein-Barr virus BNLF2a, Cowpox virus CPXV012, Human cytomegalovirus US6, Equine herpesvirus UL49.5 **5. Degradation of TAP** Bovine herpesvirus UL49.5, Murine γ-herpesvirus-68 MK3, *Pseudomona aeruginosa* Cif **6. Heavy chain chaperones** *Legionella pneumophilla* **7. Formation of the PLC** Adenovirus E19, Murine γ-herpesvirus-68 MK3, Human cytomegalovirus US3, US10, US11, US2, UL18, UL142, Murine cytomegalovirus gp40/m152, *Mycobacterium tuberculosis ESAT-6,* Tanapox virus TPV-2L, Molluscum contagiosum virus MC80R, Nairobi sheep virus **8. *cis*-Golgi (ERGIC) trafficking** Poliovirus 3A, Cowpox virus CPX203 **9: *trans*-Golgi trafficking** Orf virus, Equine herpesvirus pUL43, pUL56, HIV Nef **10. Surface presentation** Kaposi's sarcoma virus K3, K5

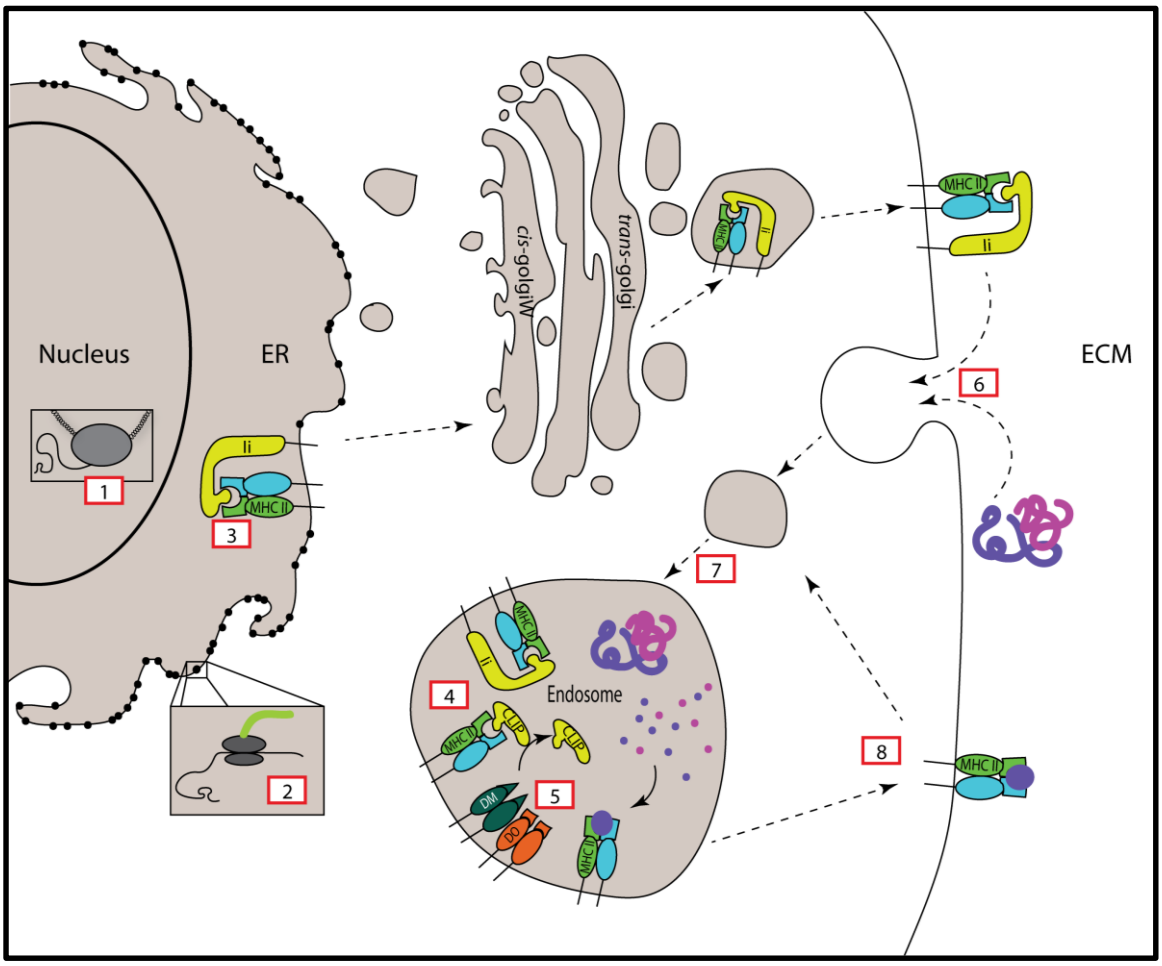


**Figure 2. Summary of MHC Class II pathway and mechanisms of subversion:**
Red boxes indicate target(s) of pathogenic manipulation. If the mechanism is known, it is listed after the organism name. **1: Transcription** Epstein-Barr virus Zta, BGLF5, Kaposi's sarcoma virus LANA, vIRF3, SOX, *T. gondii*, Human cytomegalovirus, *B. pertussis* pertussis toxin, Herpes simplex virus type 1 UL41, *C. trachomatis* CPAF **2: Post-translational modification** *F. tularensis* PGE2, *S.* typhimurium SPI-1, SPI-2 **3: HLA-Ii complexing within the ER** Human cytomegalovirus US2, US3, pp65, Epstein-Barr virus BZLF1, Herpes simplex virus type 1 gB **4: Trimming of Ii** Hepatitis C virus NS5A, Human papillomavirus type 16 E5, *H. pylori* VacA **5: CLIP exchange and peptide loading** *S. typhimurium* SifA, Herpes simplex virus type 1 gB, Human cytomegalovirus US2, US3 **6: Endocytosis** *E. coli*, *K. pneumoniae*, *S. aureus* SpA Sbi, *Yersinia* YopE, YopH, YopP, YopT **7. Fusion with the lysosome** *Chlamydia trachomatis, Coxiella burnetii, Mycobacterium avium* **8. Surface expression and recycling of MHC Class II molecules** Vaccinia virus AR35 A35, HIV-1 Nef

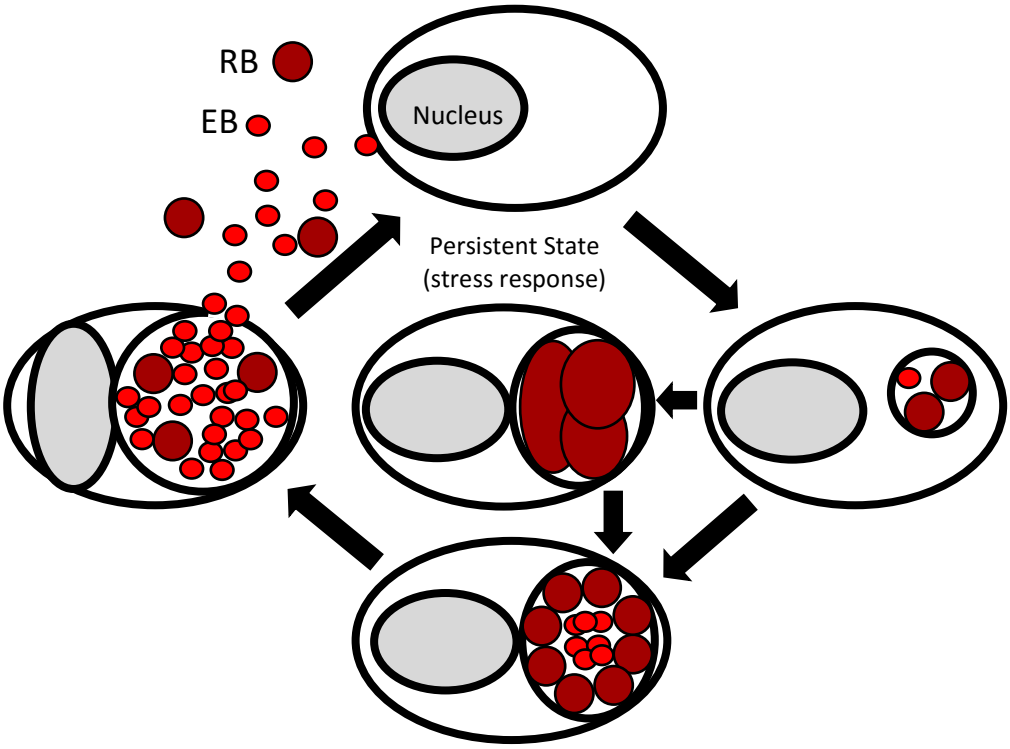


**Figure 3. Illustration of the chlamydial developmental cycle.** Infection begins with metabolically inert, infectious Elementary Bodies (EBs) attaching to and invading the host cell. Once inside, EBs differentiate in to Reticulate Bodies (RBs) within their own cellular compartment. RBs are metabolically active and undergo multiple rounds of binary fission which differentiate back in to EBs. If there are stressors present, RBs stop dividing, but continue growing into the enlarged, Aberrant Bodies (ABs) stage that is reversible upon removal of the stressor. After maturation of the inclusion, which is characterized by many EBs and few RBs, the bacteria are released into the environment by either destructive (lysis) or non-destructive (extrusion) means to begin the cycle of infection again.

# Enhanced Direct Major Histocompatibility Complex Class I Self-Antigen Presentation Induced by Chlamydia Infection

Erik D. Cram, Ryan S. Simmons, Daniel D. Rockey, Brian P. Dolan

**Abstract**

Infections caused by obligate intracellular *Chlamydia* bacteria can lead to serious diseases in both humans and animals. $CD8^+$ cytotoxic T cells are the adaptive immune system's primary resource in removing self-cells that have become infected with intracellular pathogens and should therefore play an important role in controlling chlamydial infections. It is also likely that *Chlamydia* have evolved mechanisms to avoid cytotoxic T cell responses, including interfering with direct MHC Class I antigen presentation. Here we take advantage of a model system of self-antigen presentation to examine how *Chlamydia* infection impacts antigen presentation, and find that infection with either *C. trachomatis* or *C. caviae* alters the presentation of self-peptides. Infection limited the accumulation of a model host-protein while simultaneously increasing the presentation of peptides derived from the model antigen. Skewed antigen presentation was dependent on a bacterial synthesized component as evidenced by reversal of the observed phenotype upon preventing bacterial transcription, translation and lipooligosaccharide biosynthesis. These data suggest that *Chlamydia* have evolved a mechanism to alter the host antigen presentation machinery to favor presentation of defective and rapidly degraded forms of self-antigen, possibly as a mechanism to prevent or diminish the presentation of peptides derived from bacterial proteins.

## Introduction

*Chlamydia trachomatis* is a bacterial pathogen which can cause serious diseases in humans. Clinical signs and long term consequences of *C. trachomatis* disease are particularly skewed towards women and include pelvic inflammatory disease (PID), ectopic pregnancy, premature birth, hydrosalpinx, and infertility [146]. Additionally, *C. trachomatis* is the leading cause of preventable blindness (trachoma) worldwide [147]. Other chlamydial pathogens such as *C. abortus* negatively impact livestock by causing abortion in sheep, cattle, goats, horses, pigs and less commonly, their human caretakers [148].

Bacteria of the genus *Chlamydia* are obligate intracellular organisms. As such they should be subject to control by $CD8^+$ T cells, the adaptive lymphocytes which are responsible for eliminating self-cells which have become infected or transformed. In order to spare healthy cells, $CD8^+$ T cells must first recognize a pathogen-specific peptide bound to a host cell-expressed MHC Class I molecule. The “peptidome” observed by T cells at the cell surface is composed of peptides from both self-proteins and infectious antigens that have been degraded by the proteasome and assorted proteases. Several chlamydial peptides have been identified (reviewed in [149]) that elicit $CD8^+$ T cell responses.

Often intracellular pathogens, such as viruses, have evolved a multitude of

ways to evade $CD8^+$ T cell responses [150, 151]. It is therefore likely that *Chlamydia* species manipulate the host cell antigen presentation machinery in order to circumvent the cytotoxic T cell responses. The chlamydial protease CPAF has been implicated in down-regulating MHC Class I molecules on infected cells [152], though this finding has recently been challenged [12]. In order to better understand the biology of chlamydial infections, it is imperative to determine what, if any, mechanisms are employed by *Chlamydia* species to alter antigen presentation.

Here we provide evidence that Chlamydial infection alters the host cells' ability to present peptides-derived from self-proteins. We observed that infection enhanced presentation of peptides from a particular class of substrates termed Defective Ribosomal Products or DRiPs [153]. DRiPs represent the outcome of protein translation when the polypeptide fails to gain its proper function and is rapidly degraded by the cell to prevent the build-up of potentially toxic, mis-folded proteins [154, 155]. Our data indicate chlamydial infections diminish synthesis of a model self-protein while simultaneously increasing the presentation of DRiPs derived from an antigenic peptide embedded in the fusion protein. These data suggest that *Chlamydia* species may evade CD8+ T cell responses by enhancing the presentation of self-peptides derived from DRiPs which may limit the ability of the host cell to present bacteria-derived peptides.

## Materials and Methods

### Chlamydia strains and cell lines.

McCoy cells were grown in minimal essential medium (Life Technologies) with 10% fetal bovine serum (LifeTechnologies). *Chlamydia trachomatis* serovar L2 transformed with mCherry plasmid, L2/pBRmChE (here L2-RFP, kindly provided by Robert J. Suchland, University of Washington), *C. trachomatis* D/UW-3, and *C. caviae* were propagated in McCoy cells. For all figures, *C. trachomatis* refers to the L2 serovar unless otherwise noted. JY/SCRAP cells were established as previously described by Cram *et al*. [3]. Titers of chlamydial stocks were determined by counting the number of inclusion-forming units (IFU) per ml in JY/SCRAP cells. Chlamydial growth kinetics were established by infecting JY/SCRAP cells, and at different time points, cells were removed into a new 1.5-ml tube, centrifuged, and resuspended in phosphate-buffered saline (PBS). Lysing matrix C beads (MP Biomedicals) were added to each tube, which were then vortexed vigorously for 30 s. Samples were then centrifuged at 20,000 relative centrifugal force (RCF) for 10 min, and the supernatant was transferred to a new 1.5-ml tube and stored at -80°C. McCoy cells were then grown to confluence in a 24-well tray. Three serial dilutions were made from each sample and used to infect each monolayer in triplicate. The numbers of inclusion-forming units per ml were determined on monolayers fixed with methanol at 48 h post infection (hpi).

**Antigen presentation assays**

JY/SCRAP cells were harvested and cultured at 5 x $10^5$ cells/ml for indicated times and with the indicated compounds before harvesting. Following cell harvesting, cells were washed in 0.1% BSA/HBSS and labeled with Alexa-Fluor 647-coupled RL15A mAb or the pan-HLA A,B,C antibody W6/32 at 4°C for 30 minutes. Cells were then washed, and W6/32-labeled cells were stained with DyLight 649 goat-anti-mouse IgG for 30 minutes at 4°C. After washing to remove excess Ab, cells were analyzed by flow cytometry using a BD Accuri C6 Flow cytometer. For kinetic analysis, single data points were analyzed. When single time points were analyzed, a minimum of three individual cell stains were included. Data analysis was completed with Accuri software. Single color controls were used to ensure proper color compensation. Each iteration of an antigen presentation experiment was repeated a minimum of three times.

**Electron Microscopy**

JY cells were infected at an MOI of 0.3 and at 24 hpi, cells were harvested and washed 2 times with DPBS and resuspended in 1 ml of EM Fixative A (2.5% gluteraldehyde, 1% paraformaldehyde, 0.1M sodium cacodylate). Cells were submitted to the Oregon State University Electron Microscopy facility for thin-sectioning and imaging with a FEI-Titan 80-200 in STEM mode. Approximately 100 JY cells were examined.

**Fluorescence Microscopy**

Fluorescence microscopy. JY cells infected with C. trachomatis L2-RFP or mock infected were harvested and washed with excess PBS and fixed in methanol. Cells were then rinsed in PBS and mixed with VectaShield (Vector Laboratories) containing DAPI (4',6'-diamidino-2-phenylindole; Sigma-Aldrich) to stain DNA and imaged with a Leica DML fluorescent scope with Chroma 49004 TRITC (tetramethyl rhodamine isothiocyanate) and 49021 DAPI filters. Ten fields were examined with a 100X oil immersion lens for RFP/DAPI, and images were taken with Retiga 2000R camera and QCapture Pro 6.0 software. A minimum of 10 fields (representing almost 50 cells) were examined.

**Infection and antigen presentation assays**

5 x $10^5$ JY/SCRAP-SVG cells were centrifuged at 200 RCF for 5 min. Medium was removed and cells were re-suspended in PBS pH 7.4. Cells were then aliquoted into individual wells of a 24-well tissue culture plate in 400 µl of PBS. *Chlamydia* spp. were added to the cells and the plate was centrifuged at 980 RCF for 1 hour. Following infection, 1 ml of RPMI media supplemented with 2% L-glutamine and 10% FBS was added to each well. At 12 hpi, Shield-1 was added to the appropriate wells at a final concentration of 1.0 µM. Cells were incubated at 37°C in 6% CO2 for 24 hours. Antigen presentation assay was then carried out as described above followed by 10 minutes of incubation in 2% paraformaldehyde at room temperature and in the dark.

Paraformaldehyde was then neutralized with 0.2% L-lysine (Sigma-Aldrich). Cells were then washed and resuspended in PBS. Unless otherwise noted, a multiplicity of infection (MOI) of 0.3 was used and was determined by measuring percentage of $RFP^+$ cells by FACS analysis (Figure 2). For kinetics assays, cells were resuspended in PBS, fixed, and Venus Fluorescent Protein (hereafter referred to as VFP) was determined by flow cytometry at each time point. In some experiments, chloramphenicol (CAM, Sigma, 1.5 µg/ml), rifampin (RIF, Sigma, 10 ng/ml), or LPC-011 (LPC, a kind gift from Dr. Pei Zhou and Dr. Raphael Valdivia, 1.92 µg/ml) was added to cells during and after the infection and maintained in culture throughout the remainder of the experiment. In other experiments epoxomicin (Enzo, 1 µg/ml), lactacystin (Enzo, 5 µg/ml) or Brefeldin A (BFA, MP Pharmaceuticals, 10 µM) were added to cells approximately 17 hpi. For infection of MCF7 cells, following a 2 hour incubation period post-transfection, cells were infected with C. trachomatis D/UW-3 as described above. Prior to staining with RL15A, at 30 hpi, all medium was removed and the monolayer was disrupted by treatment with 0.25% trypsin-EDTA (Life Technologies) and neutralized with RPMI. For titering of *C. caviae* infected JY/SCRAP cells, at 24 hpi, cells were washed with PBS and resuspended in C6-NBD-Ceramide (Invitrogen, 0.1 µM) for 1 hour at 37°C. Cells were then centrifuged at 200 RCF and the supernatant was aspirated and replaced with fresh RPMI. After a 3 h incubation period at 37°C, cells were fixed in 2% paraformaldehyde and analyzed by FACS. Each antigen presentation assay was repeated a minimum of three times.

**Western blot**

Cells were harvested and pellets were resuspended in SDS protein gel loading buffer (Amresco) with DTT at $10^7$ cells/mL. Cells were lysed at 95°C for 30 min with occasional vortexing. SDS-PAGE was performed with cell lysates using the Bolt electrophoresis system followed by blotting onto nitrocellulose membranes using the iBlot 2 (Invitrogen). Membranes were blocked in 5% non-fat dehydrated milk, probed with primary antibodies, and incubated with secondary antibodies (LI-COR, IR Dye donkey-anti-goat 680 or 800). Membranes were imaged with an Odyssey infrared imager (LI-COR).

**Quantification of SCRAP-SVG transcripts**

Three wells containing $5X10^5$ JY/SCRAP-SVG cells were either infected with L2-RFP or mock infected and then cultured for 24 h. RNA was extracted with the NucleoSpin RNA isolation kit (Clontech), and RNA was stored at -20°C. Synthesis of cDNA from RNA extracts was performed using the RNA to cDNA EcoDry Premix (Clontech). For quantitative PCR (qPCR), a Fast SYBR green master mix (Life Technologies) was used with FKBP12 cloning primers 1 and 2, as mentioned above, with an input of 1 µl of cDNA template for each reaction, repeated in triplicate. Transcript levels of SCRAP-SVG were normalized with ACTB amplicons generated from ACTB primers F 5'-ATCCTGCGTCTGGACCCTG-3'and R 5'-TAGCT CTTCTCCAGGGA-3'. Plasmids containing either SCRAP-SVG or ACTB were

made into 10-fold serial dilutions ranging from $10^3$ to $10^{10}$ to serve as standards. ABI StepOne real-time PCR machine and StepOne software determined the transcript level of ACTB and SCRAP-SVG transcripts based on their abundance interpolated from each respective standard curve. Both biological and technical replicates were done in triplicate.

**Quantification of chlamydial genome copies.**

In a 24-well tray, eight wells of McCoy cells were set to full confluence and infected with *C. trachomatis* L2-RFP as described above. Dulbecco's modified minimal essential medium (DMEM) containing either dimethyl sulfoxide (DMSO) or 1.92 _g/ml LPC-011 was added to each well. At each time point, medium was removed, and the cells were washed in PBS. After the removal of the PBS, 200 µl of sterile, nuclease-free water was added, and the cells were incubated for 5 min at 37°C. The cells were then lifted from the plate by gently pipetting and then stored at -80°C. Dithiothreitol (DTT) was added to a final concentration to 5 mM, and the samples were heated to 95°C for 1 h. DNA was then extracted by using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's recommendations.

Quantitative PCRs were set up using TaqMan Universal master mix (Life Technologies) and *C. trachomatis ompA* primers as previously described [156]. The experiment was performed in triplicate.

## Statistics

Linear regressions, ANOVA analysis, and t-tests were performed with Prism software (GraphPad)

## Results

### Quantification of surface antigen presentation from a model self-peptide

JY/SCRAP cell lines were constructed as previously described [3, 157-159]. Figure 1 is adapted from *Cram and Simmons* et al. 2016 [3] and describes the Shield-1 controlled recombinant antigenic protein (SCRAP) construct (Fig. 1A), FACS histogram of SCRAP peptide accumulation and HLA-A2-SVG presentation in the presence/absence of Shield-1 (Fig. 1B), dose response of JY/SCRAP cells with Shield-1 (Fig. 1C), HLA-A2-SVG presentation kinetics from nascent SCRAP peptides (Fig. 1D), and HLA-A2-SVG presentation of JY/SCRAP cells stained in triplicate at 8 hpi (Fig. 1E).

### *Chlamydia* infect and develop normally with human lymphoblastiod B cells

Before testing the ability of *C. trachomatis* to alter host protein synthesis and MHC Class I antigen presentation, we worked to demonstrate that our model cell line, the human lymphoblastoid line JY/SCRAP, was susceptible to *C. trachomatis* infection. JY cells were infected with *C. trachomatis* serovar L2 expressing mCherry red fluorescent protein (RFP) by centrifugation. Cells were then cultured for up to 24 hours before subsequent analysis. Electron

micrographs of cells 24 hours after infection clearly show reticulate bodies (RBs) and elementary bodies (EBs) sequestered to a large inclusion within the cell, typical of *C. trachomatis* infection (Figure 2A). Fluorescence microscopy confirmed infection by detecting RFP expression in the inclusion body (Figure 2A). We also measured the amount of RFP expression using flow cytometry and we can readily discriminate between infected and uninfected cells to determine the multiplicity of infection (Figures 2B and 2C). Next, we monitored RFP expression over time and found a steady increase in RFP beginning approximately 12 hours after infection and rising at a linear rate for the next 12 hours (Figure 2D). To ensure detected RFP derived from active *C. trachomatis* infection, we added chloramphenicol (CAM) to the cells immediately after infection and assayed for RFP expression 24 hours later. As shown in Figure 2E, blocking bacterial protein synthesis with the addition of CAM completely inhibited RFP expression. Finally, JY/SCRAP cells were infected with *C. trachomatis,* harvested and passaged over time in order to quantify infectious progeny production as a result of the chlamydial developmental cycle (Figure 2F). Together, these data demonstrate that *C. trachomatis* L2 can infect JY cells.

### ***C. trachomatis* L2 decrease SCRAP accumulation in JY SCRAP cells in a mechanism independent of the proteasome**

To determine if *C. trachomatis* infection altered SCRAP stability we infected JY/SCRAP cells and added Shield-1. At 24 hpi we examined VFP levels by

flow cytometry. As shown in Figure 3A, *C. trachomatis* infection diminished accumulation of VFP (Figure 3A). Kinetic analysis (Figure 3B) revealed that SCRAP accumulation loss in infected cells began at approximately 16 hpi and resulted in plateaued VFP levels. The diminished levels of host VFP may be due to enhanced proteolysis by the proteasome, and therefore, we treated both infected and non-infected JY/SCRAP-SVG cells with the proteasome inhibitors epoxomicin or lactacystin. Inhibiting proteasome function did not restore the levels of VFP (Figure 3C) demonstrating the *C. trachomatis* infection did not result in enhanced proteolysis of host proteins. Lactacystin is also known to inhibit the chlamydial protease CPAF [160]. Because VFP levels did not recover upon lactacystin treatment, we can also rule out degradation of SCRAP-SVG by CPAF.

Fluorescent proteins require proper folding in order for the fluorochrome to function. To test if loss of SCRAP-SVG fluorescence was due to improper protein folding, we analyzed total cell lysates of infected and mock infected cells treated with Shield-1 and epoxomicin by western blot analysis. Levels of SCRAP-SVG were diminished in infected cells and did not recover to uninfected levels upon proteasome inhibition (Figure 3D). Therefore, the lack of SCRAP-SVG accumulation, either in its functionally folded form or non-fluorescent state, in *Chlamydia*-infected cells is not due to proteasome-mediated degradation. Finally, in order to investigate whether the loss of SCRAP accumulation was due to decreased SCRAP transcripts, SCRAP

transcripts were quantified and normalized to actin. As Figure 3E shows, levels of SCRAP transcripts were similar in mock and *C. trachomatis* infected JY/SCRAP cells.

### *C. trachomatis* enhance self-antigen presentation from DRiP substrates

The SVG peptide embedded in SCRAP-SVG can be presented on the MHC Class I molecule, HLA-A2, following degradation of SCRAP-SVG. The levels of HLA-A2-SVG complexes were measured by flow cytometry using the monoclonal antibody RL15A. As shown in Figure 1D, the MFI of JY/SCRAP cells stained with RL15A shows some variability over the course of a 12 hour period, but remains relatively constant. If cells are treated with BFA to prevent trafficking of newly formed peptide-MHC complexes from the ER to the Golgi, the levels of HLA-A2-SVG gradually fall. Treating cells with Shield-1, which allows SCRAP-SVG to fold properly and gain fluorescence, partially diminishes levels of HLA-A2-SVG complexes (Figure 1D). For simplicity sake, we can examine cells at one time point (12 hours post addition of Shield-1 or BFA) to visualize the loss of HLA-A2-SVG induced by Shield-1 or BFA treatment (Figure 1E). In agreement with our previous work, stabilization of SCRAP-SVG did not completely abolish presentation of the SVG peptide suggesting that a residual pool of SCRAP-SVG molecules are still degraded and are likely some form of DRiP. This finding is consistent with other data showing both DRiP-derived and “retiree” peptides are presented by JY/SCRAP cells [157-159]. We can therefore measure the antigenic

contribution of both DRiPs and substrates undergoing normal degradation independently from one another.

When JY/SCRAP cells are infected with *C. trachomatis* and treated with Shield-1, levels of HLA-A2-SVG complexes increase (Figure 4A, $p<0.05$), suggesting that chlamydial infections increase the presentation of peptides derived from DRiP substrates. In the absence of Shield-1, levels of HLA-A2-SVG were equivalent to uninfected cells or decreased slightly (Figure 4A). Global levels of HLA-A, B, and C also do not change 24 hours post-*C. trachomatis* infection (Figure 4B). The increase in self-peptide presentation observed during infection is therefore not the result of a general increase in the antigen presentation capability of the cell, but rather some aspect specific to host DRiPs. To determine if this phenotype was restricted to our cell line or a general feature of chlamydial infection, we infected an epithelial cell line with a clinically prevalent strain of *C. trachomatis*. Infection of the human epithelial cell line MCF7 expressing SCRAP-SVG with C. trachomatis D/UW-3 resulted in loss of VFP signal ($p < 0.01$) (Figure 4C) and an increase in surface HLA-A2-SVG complexes ($p < 0.05$, student's t-test) (Figure 4D).

### *C. caviae* infection of JY SCRAP cells also results in increased surface HLA-A2-SVG and reduced SCRAP accumulation

The skewing of self-antigen presentation favoring peptides from DRiP substrates upon infection of *C. trachomatis* is novel and may play an

important role in the pathology of chlamydial infections. To determine if this phenomenon was unique to *C. trachomatis* or was a general feature of chlamydial infections, we repeated the experiment with the distantly related bacteria *C. caviae*. *C. caviae* infection of JY/SCRAP cells was visualized by incubating cells with C6-NBD-ceramide which stains the *C. caviae* inclusion membrane and can be visualized by flow cytometry (Figure 5A). Similar to *C. trachomatis*, infection with *C. caviae* resulted in enhanced DRiP presentation (Figure 5B) while simultaneously diminishing VFP accumulation (Figure 5C). Presentation of peptides derived from normal protein turnover (i.e., in the absence of Shield-1) is slightly diminished. Therefore, enhanced self-peptide presentation from DRiP substrates is applicable to multiple species of the genus *Chlamydia*.

**Enhanced antigen presentation is dependent on active bacterial metabolism and lipooligosaccharide biosynthesis**

*Chlamydia* infection may alter the host cell in many ways to facilitate enhanced DRiP-antigen presentation. We first tested to see if the mere presence of the *C. trachomatis* bacterium was sufficient to alter the host cell by treating infected cells with rifampin, to block bacterial transcription (Figure 6A), or CAM to block bacterial protein synthesis (Figure 6B), and measuring SVG peptide presentation in the presence of Shield-1. Treatment with either drug reversed the *Chlamydia*-induced presentation of DRiP-derived peptides (Figure 4A, B, $p < 0.05$). These data suggest that the growth of bacteria was

necessary to alter the host cell, and skewed peptide-presentation was not the result of materials present in the infectious inoculum.

One feature shared between *C. trachomatis* and *C. caviae* is the genus-common chlamydial lipooligosaccharide (LOS). To determine if LOS may be responsible for enhancing DRiP-presentation, we treated infected cells with *C. trachomatis* and treated with LPC-011 (LPC) immediately following infection to inhibit the synthesis of chlamydial LOS. LPC successfully prevented the accumulation of LOS in infected cells (Figure 6C) but did not prevent *C. trachomatis* genome replication as determined by qPCR (Figure 6D). LPC treatment partially reversed the enhanced DRiP-presentation induced by *C. trachomatis* infection (Figure 6E), returning levels of HLA-A2-SVG peptide complexes to levels similar to uninfected cells, indicating that LOS may be responsible for altering the cellular antigen presentation machinery.

**Discussion**

The exact role of DRiPs in direct MHC Class I antigen presentation and the molecular pathways which govern DRiP presentation are not fully understood. DRiPs have largely been studied in the context of viral infections and presentation of self-antigens but not during the study of intracellular bacterial infections. As DRiP generation is intimately tied to protein translation [161], it

would be unlikely for bacterial proteins to be impacted by DRiP presentation, as bacterial proteins are synthesized within the bacterial cell, sequestered from the host cell cytoplasm and antigen presentation machinery. Here we describe a surprising finding: the infection of human tumor cells with *C. trachomatis* enhanced the repertoire of self-peptides presented on MHC Class I molecules to favor the defective form of a model antigenic protein. Combined with our observation that stable protein accumulation diminished during infection through a process other than proteasomal mediated decay, these data suggest a chlamydia-specific factor alters the host translational machinery and converts ribosomal products that would normally be fully functional to a defective product that would be rapidly degraded, yielding peptides for antigen presentation. This hypothesis is conceptualized in Figure 7. We are unable to determine what fraction of SCRAP-SVG is defective and which is functional, owing to the difficulty in measuring DRiPs, which makes exact determination of the shift during chlamydia infection difficult. However the shift from functional protein to DRiP is rather significant as our kinetic data (Figure 2C) suggest that SCRAP-SVG accumulation plateau's during *C. trachomatis* infection, indicating that very few newly synthesized forms of SCRAP-SVG are functional.

Our data suggest that Chlamydia infections may skew the peptidome of host cells to reflect more self DRiP-derived peptides, limiting the ability of the infected cell to present peptides derived from chlamydial proteins. If true, this

mechanism may offer an advantage to the infecting bacterium by masking its presence from pathogen-specific $CD8^+$ T cells. If DRiPs are preferentially presented, as has been previously proposed, then fewer chlamydial peptides would bind to MHC Class I and thus diminish the likelihood for elimination by chlamydia-restricted CD8+ T cells. Because total MHC Class I levels remain unaltered during infection, infected cells are unlikely to be targeted for elimination by NK cells, providing an additional way for *C. trachomatis* to avoid detrimental immune responses. Indeed Ibana *et al*. report that modulation of the MHC Class I like molecule MICA can render *C. trachomatis*-infected epithelial cells targets of NK cell killing [162].

While our data suggest that *Chlamydia* infected cells can subvert antigen presentation, this cannot be a complete prevention of bacterial antigen presentation as previous work has identified $CD8^+$ T cells specific for chlamydial peptides [149, 163, 164] presented by human MHC Class I molecules. Importantly, peptide-reactive T cells levels in healthy patients were far lower than infected patients, demonstrating the *Chlamydia*-specific $CD8^+$ T cells expanded in response to infection [165, 166]. If cytotoxic T cells targeting *Chlamydia* species are generated during an infection, it would be advantageous to the bacteria to evolve mechanisms to escape detection and/or elimination. Enhancing DRiP presentation to ensure over representation of self-peptides may be such a mechanism.

There is recently published evidence for the role $CD8^+$ T cells play in the control of chlamydial infections. Olivares-Zavaleta et al discovered an attenuated vaccine leads to a anamnestic response by $CD8^+$ T cells and elimination of $CD8^+$ T cells reversed protection to ocular challenge in macaques [167] although other vaccine studies in mice have shown $CD8^+$ T cells to be dispensable [168]. We would therefore expect the $CD8^+$ T cell barrier to pathogenesis to be a strong selective pressure which chlamydia might develop diverse methods to escape or exploit. Indeed, a recent study by Frankhauser and Starnbach in mice showed enhanced upregulation of PD-1L following Chlamydia infection, which is suspected to lead to a faulty $CD8^+$ T cell response and increase host susceptibility to subsequent infection [10]. Additionally, memory $CD8^+$ T cells in endocervical tissues of infected women expressed low levels of perforin [169]. It is therefore plausible that *Chlamydia* species will actively try to avoid detection and elimination by CD8+ T cells.

Pathogen alteration of self-peptide presentation has been observed in other systems. Several studies have found alteration of the self-peptide repertoire following infection with viruses such as HIV [8], measles virus [170], respiratory syncytial virus [171], and influenza virus [172]. Changes in cellular metabolic activity can also skew the repertoire of self-peptides displayed at the cell surface [173]. While these studies did not examine directly if substrates were derived from the defective or functional form of a protein, they certainly lend credence to our hypothesis that significant changes to the

self-peptidome of cells occurs following infection. Indeed *Chlamydia* infections are known to alter both the transcriptome [174-176] and more importantly, the stability of host proteins [13], which almost certainly will have a quantitative effect on presentation of host-peptides.

DRiP presentation may be compartmentalized in some manner to ensure efficient antigen presentation [158]. Several different potential "compartmental" models have been proposed including specialized ribosomes [177] and translation of DRiPs in the nucleus of cells [178-180]. The compartment could also resemble a scaffold of particular proteins designed to complete a cellular process not necessarily related to antigen presentation, such as nonsense-mediated decay of mRNA [180]. Another possible way to compartmentalize DRiP presentation would be through a dedicated ribosome, termed the immunoribosome [181]. If DRiP presentation is indeed compartmentalized, our findings indicate that *Chlamydia* may have evolved to exploit this process to evade cytotoxic T cells. Future experiments should focus on how this occurs in order to better understand DRiP presentation.

**Acknowledgements**

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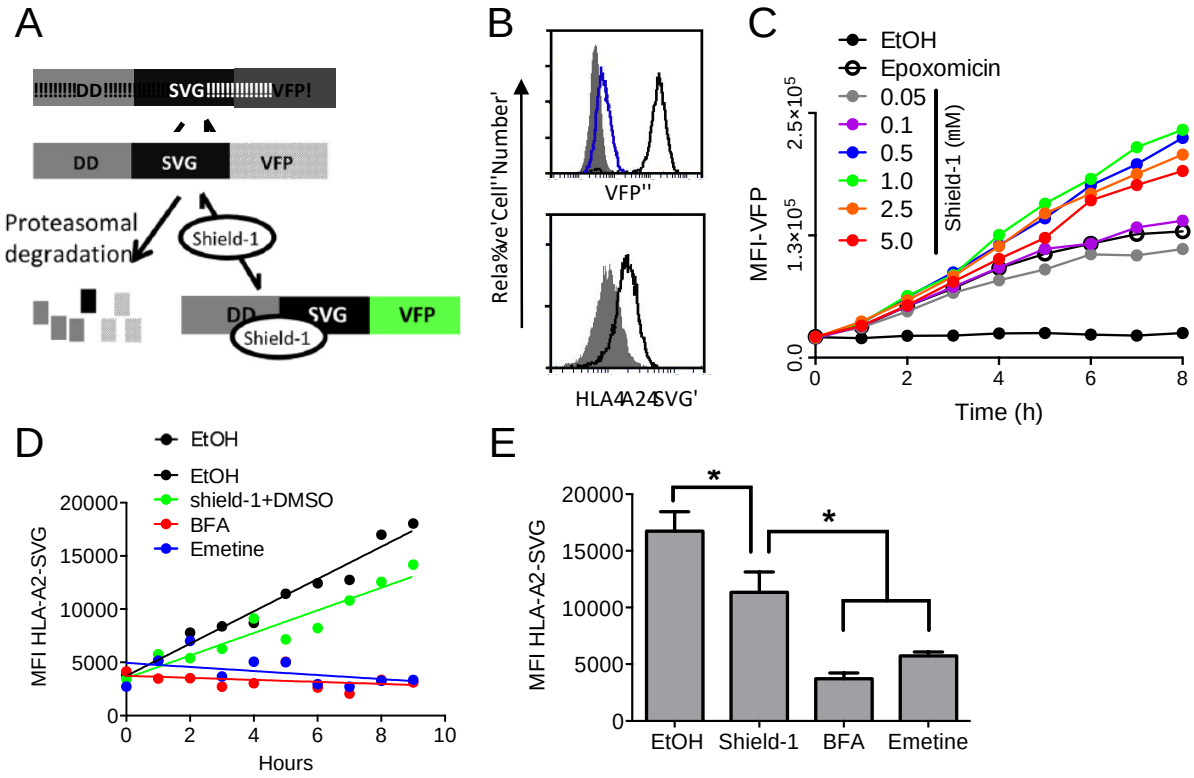


**Figure 1. Stabilization and presentation of SCRAP-SVG in JY cells.** (A) Depiction of SCRAP-SVG, including the destabilization domain (DD), the SVG peptide, and VFP. In the absence of Shield-1, SCRAP-SVG is degraded by the proteasome. The addition of Shield-1 allows SCRAP-SVG to fold and gain fluorescence. (B) Flow cytometry histograms depicting VFP fluorescence of parental JY cells (shaded), JY/SCRAP-SVG cells treated with Shield-1 (black histogram), and JY/SCRAP-SVG cells treated with ethanol alone (blue trace). RL15A-stained JY/SCRAP-SVG cells (bottom histogram, black trace) and parental JY cells (shaded histogram) are used to measure HLAA2-SVG complexes at the cell surface. (C) Dose response of JY/SCRAP-SVG cells with various concentrations of Shield-1 or epoxomicin, measuring the mean fluorescence intensity (MFI) of VFP fluorescence. (D)JY/SCRAP-SVG cells were washed in citric acid and cultured with ethanol (EtOH), 1MShield-1, BFA, or emetine and harvested at the indicated times for RL15A staining. (E) The same as in panel D except the cells were harvested at 8 h and stained in triplicate. Results for Shield-1-treated cells are statistically significantly different from results for ethanol-treated and either BFA- or emetine-treated cells (*, *P*0.05). Figure 1 was adapted from [3].

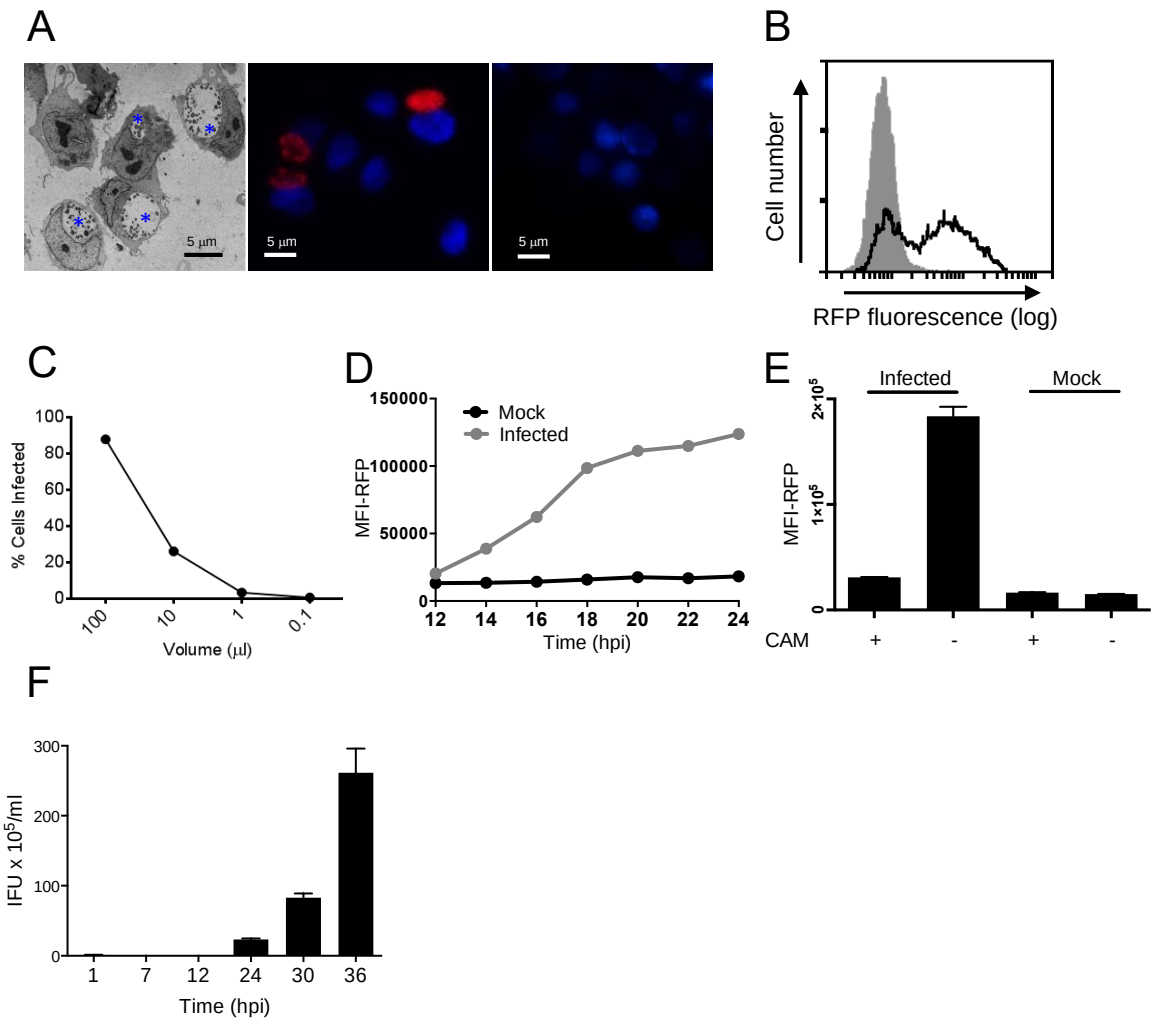


**Figure 2. *C. trachomatis* develop typically within JY/SCRAP cells** (A) Electron micrograph of JY cells 24 h after infection with *C. trachomatis* (Left). Blue asterisks show locations of *C. trachomatis* inclusions. Fluorescence microscopy image of JY cells infected (middle) or not infected (right) with RFP-expressing *C. trachomatis*. Cells were stained with DAPI to delineate nucleic acid. (B) RFP expression of *C. trachomatis*-infected JY cells (black trace) and uninfected cells (shaded histogram) analyzed by flow cytometry. (C) Determining titer of L2-RFP by infecting JY/SCRAP cells with increasing doses of bacteria (x axis) and measuring percent of RFP positive cells (y axis) by flow cytometry. (D) Measure of RFP fluorescence in *C. trachomatis* infected JY/SCRAP cells (grey) increase over time in correspondence with chlamydial growth compared to mock-infected (black). (E) CAM was added to either infected or mock-infected cells, and the MFI of RFP fluorescence was recorded at 24 hpi. (F) C. trachomatis-infected JY cells were harvested at the indicated times post-infection and disrupted, and the supernatants were used to infect McCoy cells. The infectious dose at each time point is plotted.

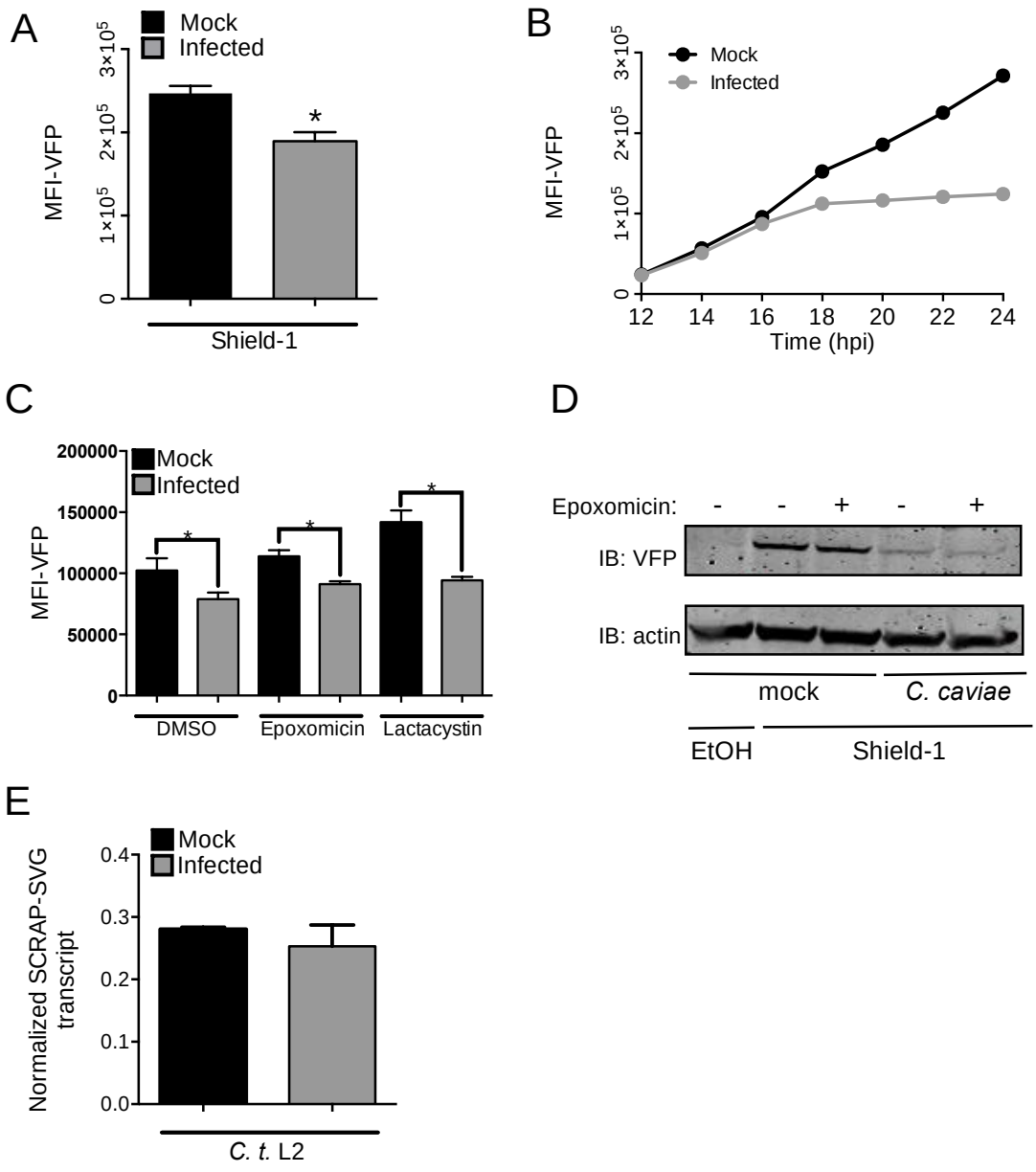


**Figure 3. *C. trachomatis* inhibits Shield-1 stabilized SCRAP accumulation in infected JY/SCRAP cells.** (A) SCRAP accumulation as measured by VFP expression and detection by flow cytometry is decreased in *C. trachomatis* infected JY/SCRAP cells. (B) A kinetic measurement of SCRAP by VFP fluorescence shows accumulation loss beginning at 16 hpi in *C. trachomatis* infected JY/SCRAP cells. (C) Treating infected cells with the proteasome inhibitor epoxomicin or the known CPAF and proteasome inhibitor lactacystin did not rescue SCRAP accumulation in infected cells indicating that loss of SCRAP is neither due to enhanced proteasomal decay nor to CPAF activity within the cytosol. (D) Western blot of VFP in infected and mock-infected cells with and without treatment of epoxomicin did not reveal an excess of VFP in epoxomicin treated, infected JY/SCRAP cells indicating that SCRAP loss is neither due to protein misfolding or VFP loss of function. (E) SCRAP transcripts normalized to actin do not differ between *C. trachomatis* infected and mock-infected JY/SCRAP cells.

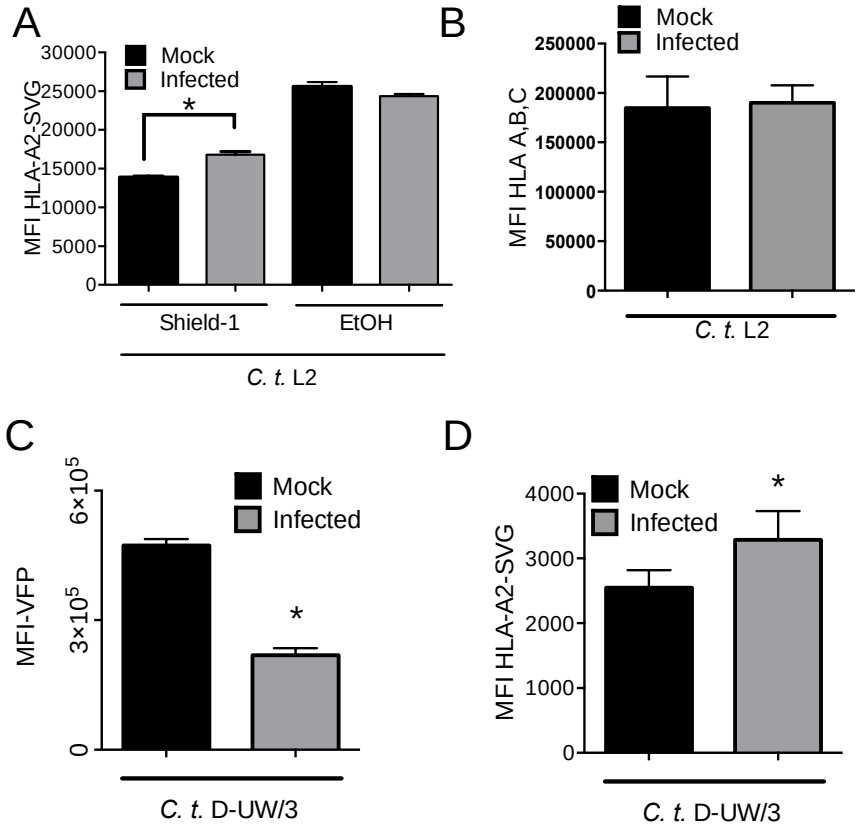


**Figure 4. *C. trachomatis* serovars L2 and D/UW-3 enhance HLA-A2-SVG self-antigen presentation in infected cell lines.** (A) JY/SCRAP-SVG cells were infected and treated with Shield-1 or ethanol and HLA-A2-SVG complexes detected by staining with the RL15A mAb. Shield-1 treated infected cells had significantly more peptide-MHC complexes ($p<0.05$) than mock-infected cells. (B) Total MHC Class I was quantified by flow cytometry in both mock and infected cells. (C) MCF-7 cells were transiently transfected with SCRAP-SVG and infected with *C. trachomatis* serovar D/UW-3 and treated with Shield-1 12 hours post infection. Cells were analyzed 15 hours later by gating on VFP+ cells and the average MFI of both the Venus fluorescence (*, $p <0.05$) and (D) HLA-A2 staining on VFP+ cells is depicted (*, $p <0.05$).

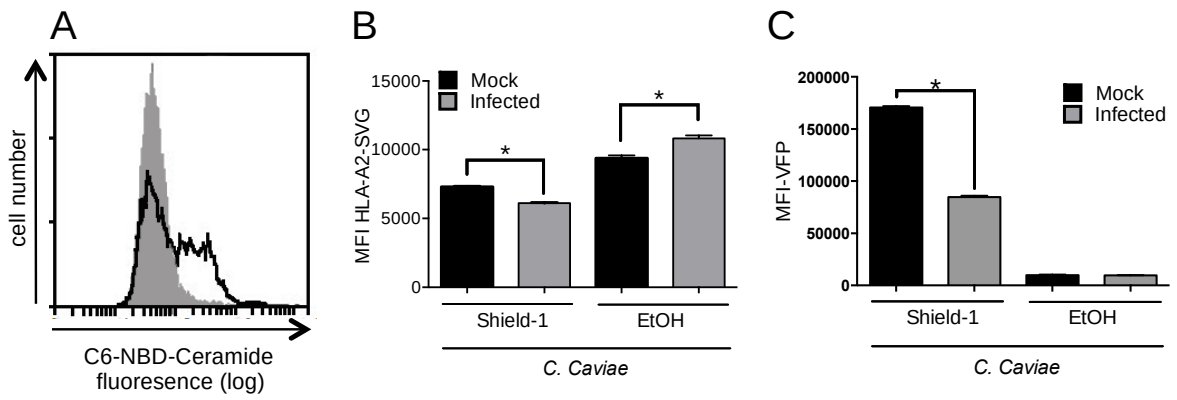


**Figure 5. *C. caviae* infection of JY/SCRAP cells show enhanced HLA-A2-SVG presentation with SCRAP accumulation loss phenotypes.** (A) JY/SCRAP cells were infected with *C. caviae* (black trace) or mock infected (shaded histogram) and identified by staining cells with C6-NBD-Ceramide. (B) JY/SCRAP cells were infected with *C. caviae* and treated with either Shield-1 or ethanol at 12 hpi, harvested at 24 hpi and stained for HLA-A2-SVG as in 4A. *C. caviae*-infected JY/SCRAP cells had a significant increase (*, $p<0.05$) in surface SVG presentation compared to mock-infected cells. Increase in SVG presentation from normal protein turnover (*, $p<0.05$) was not a consistent feature of *C. caviae* infection in JY/SCRAP cells and varied between experiments. (C) *C. caviae*-infected JY/SCRAP cells had a concomitant decrease (*, $p<0.05$) in Shield-1 stabilized SCRAP accumulation compared to mock infected cells.

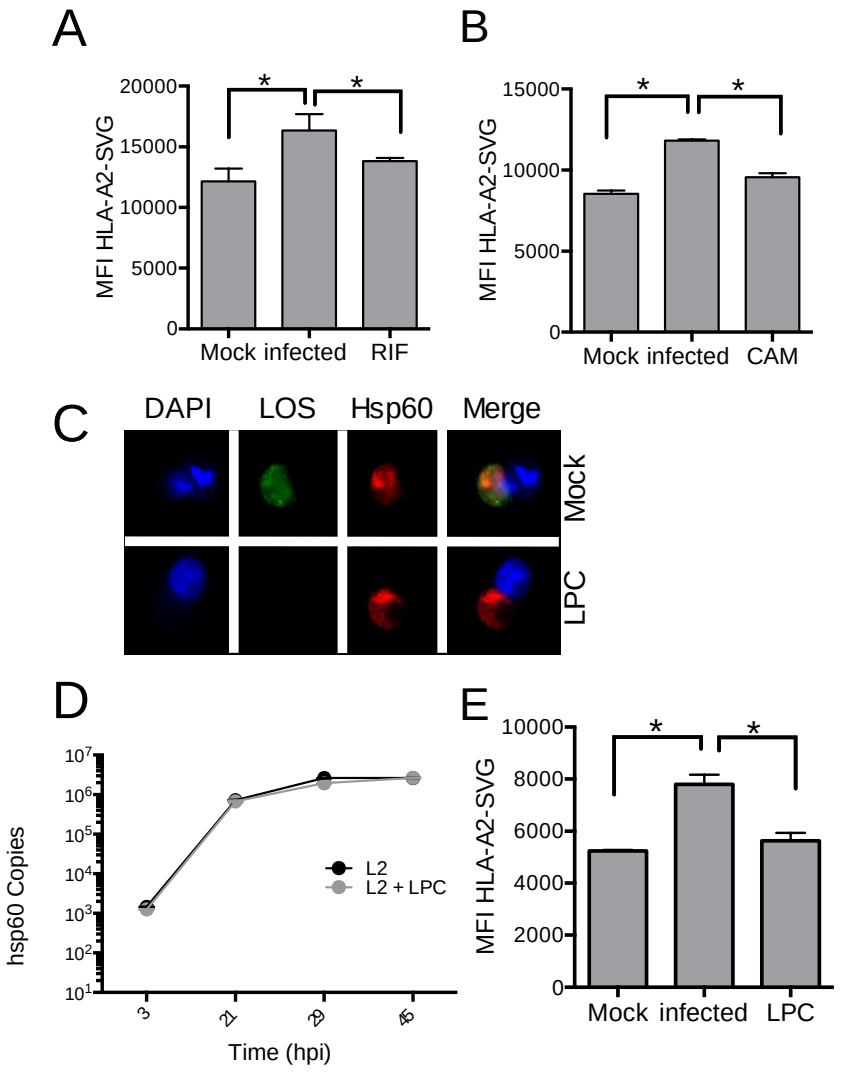


**Figure 6. *C. trachomatis* protein synthesis and LOS are necessary for skewing peptide presentation.** Infected and mock infected JY/SCRAP-SVG cells were treated with RIF (A) or CAM (*B*) immediately following infection and Shield-1 was added 12 hpi and HLA-A2-SVG levels were measured 24 hpi *C.* JY/SCRAP-SVG cells were infected with *C. trachomatis* and treated with LPC. After 24 hours, cells were visualized using fluorescence microscopy with antibodies to LOS and bacterial Hsp60. A representative cell from treated and non-treated cells is shown. *D.* Cells were treated as in (*C*) and DNA extracted from cells 24 hpi to determine the *C. trachomatis* genome copy number by qPCR. *E.* JY/SCRAP-SVG cells were infected and treated with LPC or left untreated, exposed to Shield-1 and HLA-A2-SVG complexes quantified by RL15A staining 24 hpi. LPC treated cells are statistically different from untreated, infected cells ($p < 0.05$).

# *Chlamydia* spp. development is differentially altered by treatment with the LpxC inhibitor, LPC-011, and reverts enhanced MHC Class I self-antigen presentation

Erik D. Cram, Daniel D. Rockey, Brian P. Dolan

**Abstract**

*Chlamydia* is a genus of obligate intracellular bacteria that infect a broad range of mammalian hosts. Although members of the genus infect a wide variety of hosts causing a similarly wide variety of diseases, many aspects of chlamydial biology are highly conserved. This conservation includes the outer membrane lipooligoasaccharide that decorates the surface of these Gram-negative bacteria. All chlamydial species contain outer membrane lipooligosaccharide that is comprised of a genus-conserved, and genus-defining, trisaccharide 3-deoxy-D-manno-octulosonic acid Kdo region. Recent studies with lipopolysaccharide inhibitors demonstrate that LOS is important for the *C. trachomatis* developmental cycle during RB->EB differentiation. Here, we examine the effects of LPC-011, an inhibitor of bacterial LOS synthesis on the developmental cycle of five chlamydial species. We observed that inhibition of LOS biosynthesis in some chlamydial species induced formation of aberrant RBs and *C. muridarum* was able to produce infectious EBs in the presence of high concentrations of LPC-011. In our previous studies we found that *C. trachomatis* and *C. caviae* enhance MHC Class I antigen presentation of a model self-peptide and here we observe these findings as a feature of *C. muridarum, C. abortus* and *C. suis* infection. Treatment of cells infected with *C. trachomatis* reversed the enhanced antigen presentation phenotype. Here we report that LOS inhibition and induction of aberrancy of chlamydial species abrogates enhanced self-antigen presentation.

## Introduction

Members of the genus *Chlamydia* are obligate intracellular, intravacular, bacteria that undergo a biphasic developmental cycle inside cells. Infectious, metabolically inert elementary bodies (EBs) attach and enter the host cell and differentiate, forming metabolically active reticulate bodies (RBs). Following several rounds of binary fission, RBs then differentiate a second time to initiate the infectious EB phase. At the conclusion of the developmental cycle, bacteria are released from the host cell by either lysis or extrusion [182]. While this process represents the typical, unobstructed chlamydial developmental cycle, stress factors such as nutrient starvation, host interferon-γ, coinfection with herpesvirus, and exposure to selected antibiotics causes RBs to become aberrant [136-141, 143]. Aberrancy and persistence *in vivo* is reversible resulting in continued production of infectious progeny.

Although the basic chlamydial developmental cycle is conserved among species, there is a wide variety of animal hosts susceptible to different chlamydiae. *Chlamydia trachomatis* is the most significant human pathogen, causing which causes a variety of serious conditions following infection of the urogenital tract, and blinding trachoma following infection of the eye [183, 184]. Veterinary species include *C. caviae, C. muridarum, C. abortus,* and *C. suis* which infect guinea pigs, mice, sheep, and pigs respectively [185-192].

All *Chlamydia* species have an outer membrane that is partially comprised of a lipooligosaccharide molecule with a highly conserved trisaccharide Kdo region (Fig. 1)[193]. The role of LOS in the *C. trachomatis* developmental cycle facilitates attachment and entry of EBs into the host cell by and is a necessary component for the generation of infectious EBs, but it is unclear if these findings are true for other species, or if there are novel roles for LOS yet to be discovered [194, 195]. Here we utilize LPC-011 (LPC), a potent inhibitor of the enzyme LpxC which functions in the chlamydial LOS biosynthesis pathway, to examine the sensitivity and growth phenotype on other species of *Chlamydia*. We observed that growth in the presence of LPC at the minimum effective concentration (MEC) produced either normal or aberrant RBs. This finding suggests a role of LOS in active RB metabolism as well as RB->EB differentiation.

We have previously shown that *C. trachomatis* and *C. caviae* enhance MHC Class I self-antigen presentation from DRiP substrates in a model of quantitative antigen presentation [3]. The diverse lineage of the two species suggests a genus-wide mechanism that alters host antigen presentation. Treatment of *C. trachomatis* L2 with LPC to block the biosynthesis of the conserved chlamydial LOS molecule abrogated the enhanced antigen presentation phenotype. Treatment with rifampicin or chloramphenicol inhibited the enhanced self-antigen presentation phenotype suggesting that a bacterial synthesized compound is important to preserve the phenotype.

However, it remained unknown whether the induction of aberrant inclusions had any effects on the previously observed antigen presentation phenotype. We further demonstrated that enhanced antigen presentation is the outcome of infection with all of the species of *Chlamydia* that we tested. Additionally, treatment of all species with LPC negated enhanced antigen presentation.

## Materials and Methods

### Cell lines and organisms

Cultured murine fibroblast cells (McCoy; ATCC® CRL-1696™) were grown in DMEM (Life Technologies) with 10% FBS (Life Technologies) at 37°C in 5% $CO_2$. The human B lymphoblastiod cell line JY expressing Shield-1 Controlled Recombinant Antigenic Protein (SCRAP) were grown in RPMI (Life Technologies) supplemented with 10% FBS (Life Technologies), GlutaMAX (Gibco, 20mM), and HEPES (Gibco, 10 mM). Cells were incubated at 37°C in 6% $CO_2$.

Infections with *Chlamydia trachomatis* L2/pBRmChE (hereafter referred to as L2, a generous gift from Robert J. Suchland, University of Washington), *Chlamydia caviae* GPIC*, Chlamydia trachomatis* J6276, a clinical isolate of *Chlamydia abortus* from sheep placenta, *Chlamydia suis* R19, and *Chlamydia muridarum* were all carried out in McCoy cells as previously described [196].

**Antibody labeling and fluorescence microscopy**

McCoy cells were grown to 20% confluency on glass coverslips within individual wells of a 24-well tissue culture treated plate and infected. After 48 hpi (unless stated otherwise) all media were removed and cells were fixed with 100% methanol for ten minutes at room temperature. Cells were then washed 3X with Dulbecco's phosphate-buffered saline (DPBS: Life Technologies). LOS was labeled with mAb EVI-HI (a gift from Harlan Caldwell), Hsp60 was labeled with mAb B9, *C. trachomatis* IncA with mAb 12 E7, and *C. caviae* IncA with mAb 17 [197-200]. After an hour of incubation and 3X wash with DPBS, secondary labeling was performed with the appropriate secondary antibody conjugated to either fluorescein (FITC) or tetramethylrhodamine (TRITC; (Southern Biotech). VectaShield (Vector Laboratories) containing 4',6-diamidino-2-phenylindole DAPI (Sigma-Aldrich) was used to stain DNA. Images were collected with a Leica DML scope fitted with a Retiga 2000R and QCapture Pro 6.0 software (Q Imaging).

**Chlamydial growth kinetics and quantification**

McCoy cell monolayers in 24 well trays were infected with an MOI of 0.5 either in the presence of LPC 1.92 µg/mL or mock-treated with DMSO diluent. At each indicated time point, all media was removed and cells were rinsed with DPBS. EBs were released from cells by incubation in water for 10 min, disrupted with serial pipetting, and stored at -80°C. After thawing, samples were processed using the DNeasy Blood and Tissue kit (Qiagen) with the

single modification of adding dithiothreitol (DTT) to a final concentration of 5mM before lysing of EBs with proteinase K. Genome copy number was determined by TaqMan q-PCR using probes to *ompA* (*C. caviae)* or *hsp60* (L2) with plasmids containing *ompA* or *hsp60* for standards as previously described [156, 201].

**Growth recovery assay**

McCoy cells were grown to a fully confluent monolayer in a 24-well tissue culture tray and then infected with either *C. caviae* or L2 at an MOI of 0.5. In order to better facilitate chlamydial growth, cells were cultured in DMEM supplemented with 10% FBS, 1 µg/mL cycloheximide (Sigma-Aldrich) plus or minus added LPC in triplicate. The concentration of LPC is indicated for each experiment in the results. In order to remove LPC at 24 hpi, media was removed and cells were washed 3X with DPBS and incubated in fresh DMEM + 10% FBS and 1 µg/mL cycloheximide. At 48 hpi, cells were lysed in $H_2O$ and an equal volume of 2X PBS was then added back to cells which were then lifted by pipetting and pooled in a single 2 mL tube and stored at -80°C. To titer the EBs, a new monolayer of McCoy cells in wells of a 24-well tissue culture tray were infected in triplicate with EB-containing lysed cell inoculum in a dilution series. At 48 hpi, cells were methanol-fixed and stained with monoclonal antibody, directed at chlamydial Hsp60 [197-199]. The number of inclusions in each of ten 40X fields were counted and a mean value calculated. The averages were multiplied by the number of total possible

fields in a well (1019) and then divided by the dilution factor, to determine IFU/mL.

**Phylogenetic analysis of LpxC**

LpxC amino acid sequences from *C. caviae* (GenBank: AAP04840.1), *C. abortus* (GenBank: CAH63545.1), *C. muridarum* (UniProtKB/Swiss-Prot: Q9PJK9.2), *C. trachomatis* J/6276 (GenBank: AGS02347.1), *C. trachomatis* L2 434/Bu (GenBank: CAP04233.1), and *Escherichia coli* K12 (GenBank: BAB96664.1) were aligned by ClustalW and a neighbor-joining approach was used to generate a tree representing phylogenetic relationships (MacVector 11.1.2).

**Infection and antigen presentation assay**

Infection of JY SCRAP cells with *Chlamydia*, and a quantitative measure of antigen presentation, were carried out as previously described with the single exception of cells infected with *C. muridarum* were harvested at 20 hpi [3]. Experiments were repeated a minimum of three times and the Student's t-tests were performed using GraphPad Prism 6 software (GraphPad Software, Inc.).

## Results

### *Chlamydia trachomatis L2* are less sensitive to the LOS biosynthesis inhibitor LPC than *C. caviae*

We first determined the sensitivity of L2 to the LpxC inhibitor drug, LPC, to completely inhibit LOS biosynthesis. LPC was added to L2-infected McCoy cells in serial dilutions. At 48 hpi, cells were fixed with methanol, stained for LOS and Hsp60 and visualized with fluorescent microscopy. The MEC was determined to be 1.92 µg/mL as LOS was visualized with treatment of 1.5 µg/mL LPC (Fig. 2-A). It has previously been reported by [194] that inhibiting LOS biosynthesis in L2 resulted in blocking RB to EB differentiation and resulted in the production of non-infectious intermediate bodies (IBs). We confirmed this finding by passaging chlamydia grown in the presence of 1.92 µg/mL, which resulted in no subsequently infected McCoy cells. However, formation of aberrant RBs (ABs) was induced with treatment of LPC 19.2 µg/mL, a concentration 10-fold greater than the MEC (Fig. 2-A).

LpxC in *C. caviae* and L2 434/Bu share a 74% identity between amino acid sequences (accession AAP04840.1 and YP_001654866 respectively). In order to determine if *C. caviae* are sensitive to LPC, *C. caviae* was grown in McCoy cells, in the presence of LPC in serial dilutions, methanol fixed at 48 hpi, stained for Hsp60 and LOS and visualized by fluorescent microscopy. *C. caviae* exhibited a greater sensitivity compared to L2 as the MEC of LPC was 0.25 µg/mL with ABs prevalent at concentrations as low as 0.004 µg/mL (Fig.

2B). To measure growth of *C. caviae* and L2 in the presence of LPC, McCoy cells were infected in triplicate and grown in the presence of LPC 1.92 µg/mL. At each indicated time point cells were harvested, and genome copy was determined (Fig. 2C). While treatment of LPC on *C. caviae* resulted in nearly a 2-log genome copy reduction by 36 hpi (two-sided t test, P>0.05), by 45 hpi L2 growth was not significantly impacted (two-sided t test, P=0.76) (Fig. 1-C).

### *C. caviae* multi-lobed inclusion phenotype is altered by treatment with LPC

*C. caviae,* in contrast to *C. trachomatis,* produce multi-lobed inclusions within the host. Rockey *et al.* have previously proposed that formation of the multi-lobed inclusions is a byproduct of RB replication and division [2]. Fusion of *C. trachomatis* inclusion compartments is mediated by inclusion membrane protein A (IncA) that contains a putative SNARE-like domain. Clinical isolates of *C. trachomatis* bearing mutations in IncA develop within non-fusogenic inclusions [202]. While *C. caviae* contains a homologue of IncA, it remains unknown whether the multi-lobed inclusions are a product of IncA loss-of function, kinetics of fusion vs. division, or host-cell factors. In order to determine if the inhibition of LOS biosynthesis affects the multi-lobed inclusion phenotype, *C. caviae* infected McCoy cells were treated with increasing doses of LPC. Following methanol fixation, visualization of IncA via fluorescent microscopy, we observed a decreased number of inclusion

compartments with few, large ABs (Fig. 3A) suggesting LOS as an essential component of *C. caviae* development. Coinfection of L2 and *C. caviae* in the presence of LPC did not enable the two species to fuse, however, fewer, larger *C. caviae* inclusion compartments were observed compared to treatment with DMSO (Fig 3B).

### ***C. trachomatis* L2 and *C. caviae* are differentially sensitive to ampicillin and LPC**

The persistent state of chlamydial infections are characterized by smaller inclusions containing few, large, aberrant RBs. The persistent state of chlamydial infection *in vivo* and *in vitro* are induced by treatment with antibiotics such as penicillin, IFNγ production by the host cell, and nutrient abundance [203]. In evaluation of LPC as an antichlamydial compound, we determined to compare the ability of *C. caviae* and *C. trachomatis* RBs and IBs to differentiate into infectious EBs following exposure to LPC and a recovery time. In order to determine the kinetics of LOS production following exposure to LPC, *C. caviae* was exposed to LPC at 5, 24 and 45 hpi, washed three times with PBS and replaced with fresh media for the remainder of time until 48 hpi. It was determined by fluorescent antibody microscopy that 24 hours of recovery was enough time to see a modest production of LOS in infected cells (Fig. 4A). Next, McCoy cells were infected with either L2 or *C. caviae* and following infection, grown in DMEM + 10% FBS, 1 µg/mL cycloheximide (10 µg/mL) and with or without LPC (1.92 µg/mL) for 24 hours.

At 24 hpi, all media was removed and cells were washed three times with PBS, and replaced with fresh DMEM + 10% FBS and 1 µg/mL cyclohexamide to grow for an additional 24 hours. At 48 hpi, cells were harvested in PBS and stored at -80°C. A fresh monolayer of McCoy cells were infected in triplicate with serial dilutions of inoculum and allowed to culture for an additional 48 hours which all wells were methanol-fixed and stained with anti-Hsp60. IFU/mL was determined and revealed a 100-fold reduction in *C. caviae* IFU compared to mock-treated, infected cells while L2 exhibited a 10-fold decrease in IFU/mL (Fig. 4B). FA microscopy of *C. caviae* and inclusions allowed to recover after treatment of LPC revealed few, large ABs with LOS present while L2 inclusions appeared to produced normal-looking inclusions (Fig. 4C, 2A). In contrast, 24 hour exposure to ampicillin (10 µg/mL) with a subsequent 24 hour recovery yielded a ½-log reduction in *C. caviae* IFU/mL while L2 IFU/mL was once again reduced 10-fold (Fig. 4D).

**Sensitivity of other Chlamydial species to LPC**

*C. caviae* and *C. trachomatis* are both members of the *Chlamydiaceae* phylum but are both distantly related and grouped in two different phylogenetic clades formerly known respectively as chlamydophila and chlamydia [204, 205]. In order to determine if aberrant outcome of LPC treatment is distinguishable by clades, *Chlamydia abortus* from chlamydophila, and *C. trachomatis* J6276, *C. suis* and *C. muridarum* were tested from the chlamydia clade. *C. abortus* exhibited greater tolerance to

LPC than *C. caviae* with an MEC of 0.7 µg/ml (Fig. 5A), however, *C. abortus* inclusion phenotype contained few, large ABs even at LPC levels permissive of LOS biosynthesis (Fig 5A). Similar to L2, the MEC for LPC in *C. suis* was 1.92 µg/ml with LOS detectable at 1.5 µg/mL, however, like the chlamydophila, few, large ABs were present at both concentrations (Fig. 5B). *C. trachomatis* J6276 had identical inclusion phenotypes to L2 treated with 1.92 µg/ml which produce inclusions similar to their vehicle-treated controls (Fig. 2A, 5C). *C. muridarum* develop full-sized inclusions, typical-looking inclusions in the presence of LPC. However, unlike the other chlamydia species, *C. muridarum* was far more sensitive to LPC with an MEC of 0.4 µg/ml (Fig. 5D). A summary of MEC results is provided in Table 1. LpxC amino acid sequences from *C. muridarum, C. trachomatis* L2 434/Bu, *C. trachomatis* J6276, *C. abortus*, *C. caviae,* and *E. coli* K12 show a phylogenetic relationship similar to previously published 16S phylogenetic analysis (Fig. 6A) [205].

Additionally, *C. muridarum, C. caviae* and L2 were treated with increasing doses of LPC and passaged in order to determine output of infectious progeny. Surprisingly, in contrast to L2 and *C. caviae, C. muriduram* was able to produce infectious progeny even when treated with concentrations of LPC well above the MEC (Figure 7A). *C. caviae* exhibited the most sensitivity with a full log reduction in infectious progeny when treated at the lowest concentration of LPC. The experiment was repeated with *C. muridarum* and

L2 to include a higher concentration of LPC at 5 µg/mL *C. muridarum* was able to still produce infectious progeny while L2 remained unable to (Figure 7B). Treatment of *C. caviae* and L2 with a range of concentrations of ampicillin revealed equal drug sensitivity at higher concentrations, but *C. caviae* produced more infectious EBs than L2 at lower concentrations (Figure 7C).

**Treatment of LPC on *Chlamydia* spp. Infection abrogates enhanced MHC Class I self-antigen presentation phenotype**

Our previous data has shown that inhibition of LOS biosynthesis during the course of L2 infection reverts the enhanced self-antigen presentation phenotype [3]. In order to further show that LOS is important to the enhanced self-antigen presentation phenotype, L2 infected cells were treated with a concentration of LPC that is permissive to LOS biosynthesis resulting in no significant decrease in HLA-A2-SVG antigen presentation compared to mock-treated, infected cells (Figure 8A). We then sought to establish which, if any, other *Chlamydia* species increase self-antigen presentation upon infection and the necessity of LOS biosynthesis for that phenotype. *C. caviae*-infected JY SCRAP cells showed an increase in surface HLA-A2-SVG that was abrogated by treatment with LPC (Figure 8B). Similarly, *C. muridarum* (Figure 8C), *C. abortus* (Figure 8D), and *C. suis* (Figure 8E) all were able to enhance presentation of surface HLA-A2-SVG molecules, which phenotype was abrogated by treatment of LPC (2 µg/mL).

## Discussion

The reason for the highly conserved nature of the chlamydial LOS molecule between chlamydial species remains one of the greatest mysteries in chlamydial biology [206]. Various selective forces within a range of host organisms have not yielded much change in the structure within the genus. Known interactions of LOS with host biology include cell attachment and entry, as well as a interacting as a hemagglutinin with erythrocytes [195, 207, 208]. Little is known about the role of LOS in the chlamydial developmental cycle and uncovering this information may be the key to unlocking the anomaly of conserved LOS structure. We were able to induce formation of L2 ABs with a dose of LPC much greater than the MEC, although it is unclear whether this was due to host toxicity, or secondary interactions with host factors. It is also possible that LOS is still being synthesized by L2 when treated with the MEC, though levels are below our ability to detect using FA microscopy. It is clear that as *C. caviae* LOS is allowed to be synthesized in increasingly permissive amounts, there is an increased accumulation of RBs and a decrease of ABs. Division and septation are linked in the development of multi-lobed *C. caviae* inclusions, and the lack of active growth is evident in few, large inclusion compartments when treated with LPC which is supported by the concomitant reduction in *C. caviae* genome copies. Formation of ABs when treated at the MEC with LPC seemed to be a conserved trait with members of the chlamydophila clade, however we were surprised to also observe this phenotype with the chlamydia clade member, *C. suis.* Members

of the chlamydia clade all shared the same MEC of LPC with the lone exception of *C. muridarum*, which had a MEC at 20% of the other chlamydia. Despite having a lower MEC, *C. muridarum,* treated with LPC exhibited an inclusion phenotype similar to other chlamydia, which was marked with many RBs and no ABs. The exact mechanisms of replication and differentiation are all still unknown in chlamydial biology. It is therefore plausible to consider these genus-common factors that must rely on the presence of LOS are evolutionary selective forces that maintain the stringent nature of the chlamydial LOS molecule.

Many factors are known to induce the persistent state of chlamydia *in vitro* including amino acid starvation, IFN-γ, NO, β-lactam antibiotics, and now, inhibition of LOS biosynthesis [203, 209, 210]. When challenged with LPC or ampicillin followed by a brief recovery in the absence of drug, L2 was able to recover from each treatment with only a ten-fold reduction in infectious progeny, while *C. caviae* exhibited a greater resistance than L2 to ampicillin and a greater susceptibility to LPC. The dichotomy may be due to the ability of drug to bind LpxC or the other penicillin-binding proteins and the slight differences in protein structure between the two species. Alternatively, downstream regulators or other factors dependent on membrane integrity or may be differentially impacted by treatment with ampicillin compared to LPC and thusly affect reversion of persistence. The ability of *C. trachomatis* to

recover after treatment with LPC might suggest LPC as a poor clinical choice as a novel antichlamydial compound.

Chlamydial recombination has been an increasing topic of interest in chlamydial biology and remains an important tool for genetic manipulation of the bacteria [148, 201, 211-214]. Our data has identified differential MECs and phenotypic outcomes of treatment with LPC including aberrancy for *C. caviae, C. abortus* and *C. suis*, ability of *C. muridarum* to produce infectious progeny, in addition to the previous findings that *C. trachomatis* do not produce infectious progeny [194]. Taken together, these data suggest that LOS biosynthesis aid to progress the chlamydial developmental cycle at different stages for different species. The differential phenotypic outcomes of different species as a result of LPC treatment make LPC a good candidate as a selecting force for chlamydial recombination.

Additionally, we have identified that the ability of *Chlamydia* to enhance self-antigen presentation during the course of infection is present in three more species, further indicating a genus-conserved mechanism. Treatment of all species of *Chlamydia* we tested with LPC, regardless of the developmental outcome, disabled the ability to increase self-antigen presentation during infection.

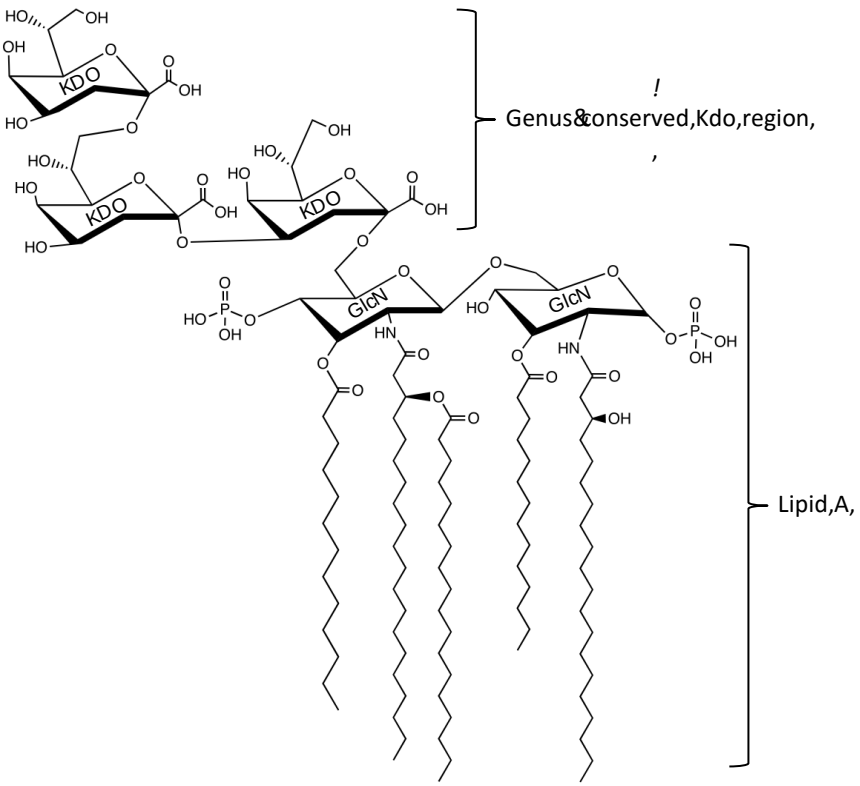


**Figure 1. L2 lipooligosaccharide consisting of the genus conserved trisaccharide Kdo (α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo) region and lipid A.**

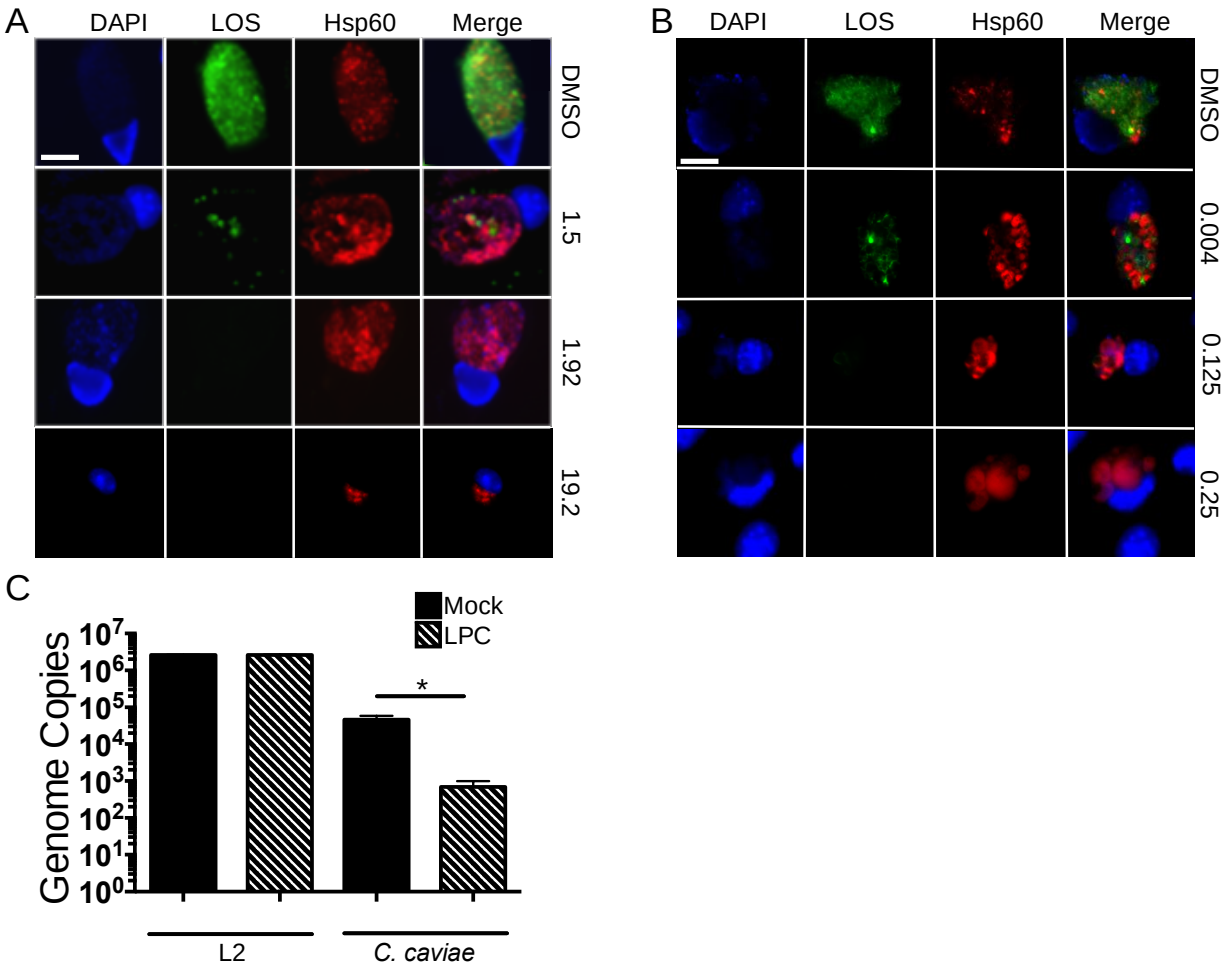


**Figure 2. Treatment of *C. trachomatis* and *C. caviae*-infected cells with LPC reveals an aberrant phenotype and differential sensitivity of each species to LPC:** (A) MEC for LPC treatment of C. trachomatis L2 is 1.92 µg/mL which produces inclusions comparable in size and frequency to DMSO-treated inclusions. Treatment of L2 with 10x the MEC resulted in small inclusions containing aberrant RBs. (B) Treatment of LPC on C. caviae infected cells resulted in the formation of aberrant RBs both at, and well below the MEC of drug. (C) Treatment of L2 with 1.92 LPC µg/mL (MEC) did not result in a reduction of genome copy number compared to mock-treated L2, while treatment of C. caviae with 0.25 µg/mL resulted in 2-log decrease of genome copy number (*, $P<0.05$).

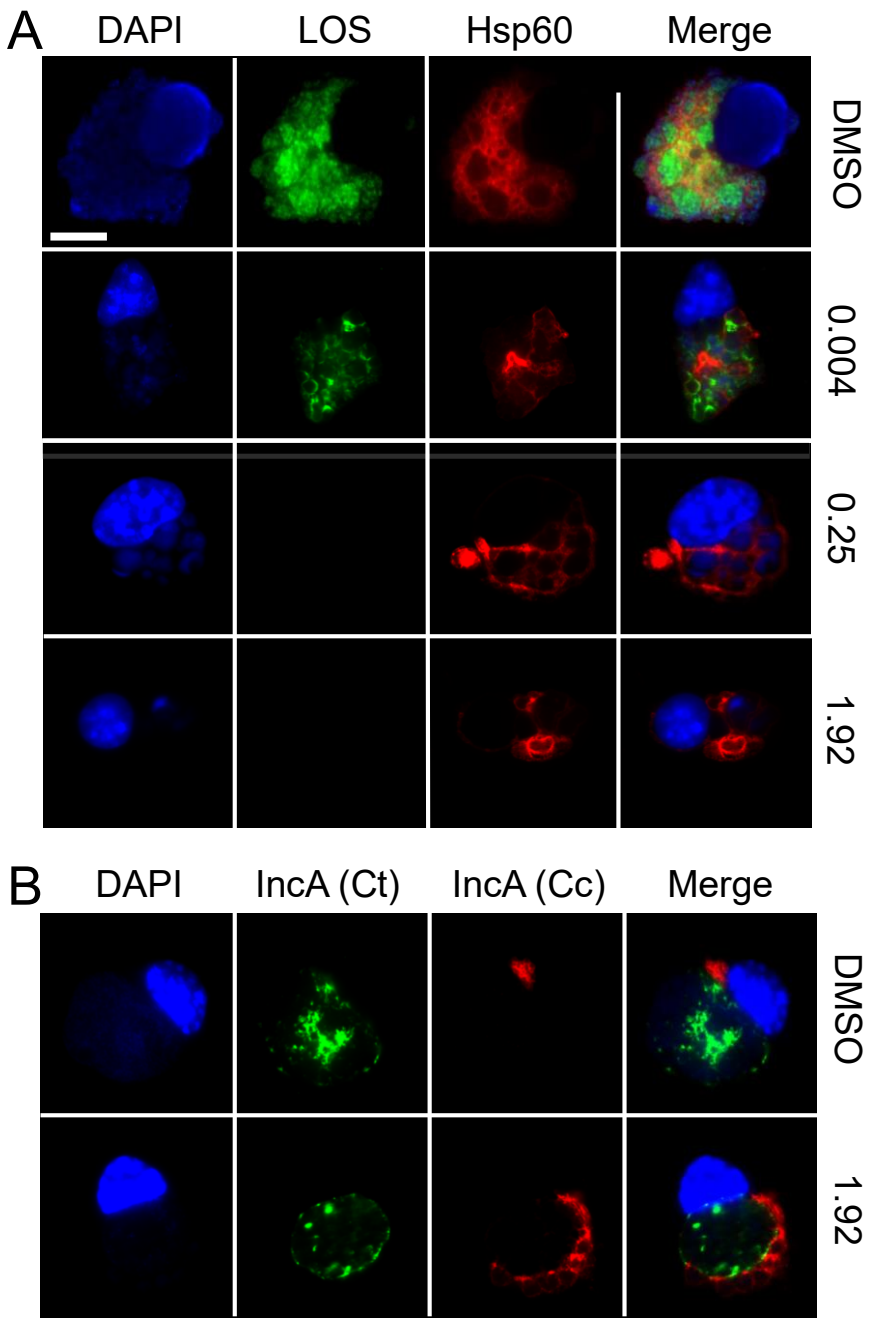


**Figure 3. Treatment with LPC does not alter the multi-lobed phenotype of *C. caviae* infection:** Unlike *C. trachomatis, C. caviae* produce multi-lobed inclusions as visualized by the inclusion membrane protein IncA. Division and septation are intimate biological process during the *C. caviae* developmental cycle [2]. (A) Increasing concentrations of LPC results in diminished *C. caviae* inclusion compartments and few, large ABs as visualized by DAPI. Treating *C. caviae* with a concentration of LPC that is permissive of LOS production results in increased inclusion septation. (B) Treatment of LPC on cells dually infected with *C. trachomatis* L2 (Ct) and *C. caviae* (Cc) did not allow for the formation of mixed-species inclusions.

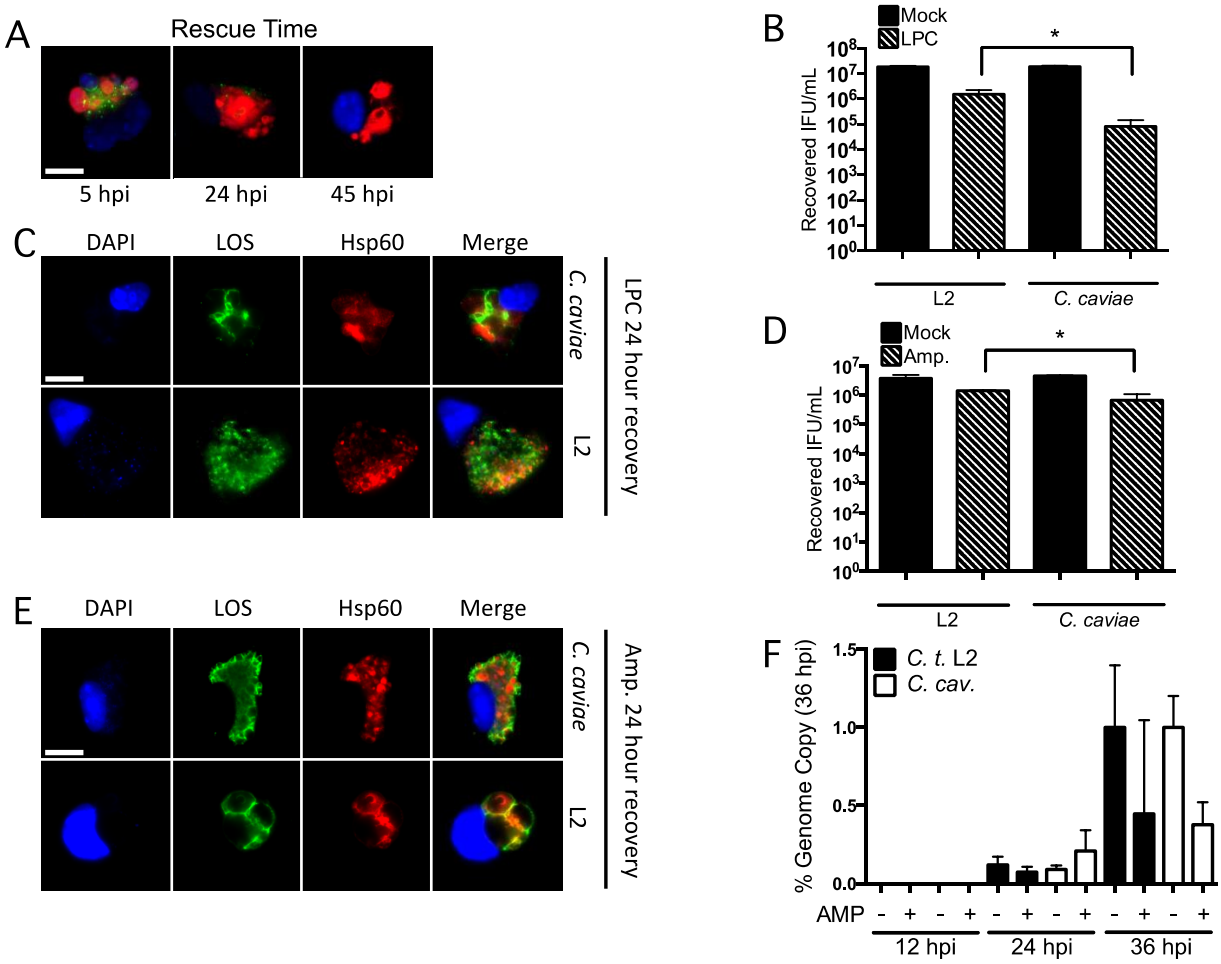


**Figure 4. *C. caviae* and *C. trachomatis* are differentially sensitive to ampicillin and LPC: (A)** *C. caviae* infected cells were treated with 1.92 µg/mL LPC at 0 hpi and at 5, 24, and 45 hpi cells were washed and drug was removed. At 48 hpi, infected cells were methanol fixed, and labeled with the anti-LOS mAb (green) and anti-Hsp60 mAb and total DNA with DAPI (blue). At 24 hpi, a modest production of LOS was observed within *C. caviae* inclusions (B) *C. caviae* and L2 were treated with LPC for 24 hpi, and allowed to recover for an additional 24 h in the absence of drug. Infectious progeny were recovered an enumerated, revealing a significant decrease in *C. caviae* compared to *C. trachomatis* L2 (*, $p < 0.05$). (C) Fluorescent microscopy of *C. caviae* and L2 after a 24 h exposure to LPC and an additional 24 recovery period in the absence of drug. LOS is clearly visible in both inclusions, however, large ABs are visible in the *C. caviae* inclusion. (D) The experiment described in B was repeated with ampicillin revealing a significant decrease in L2 infectious progeny compared to *C. caviae* (*, $p < 0.05$) (E) Fluorescent microscopy of *C. caviae* and L2 after a 24 h treatment with ampicillin followed by a 24 recovery period in the absence of drug. The L2 inclusion showed several, large ABs compared to the more typical-looking *C. caviae* inclusion. (F) Genome copy abundance relative to the final harvest was quantified by qPCR showing similar genome replication kinetics of *C. trachomatis* L2 and *C. caviae* following treatment with 10 µg/mL ampicillin.

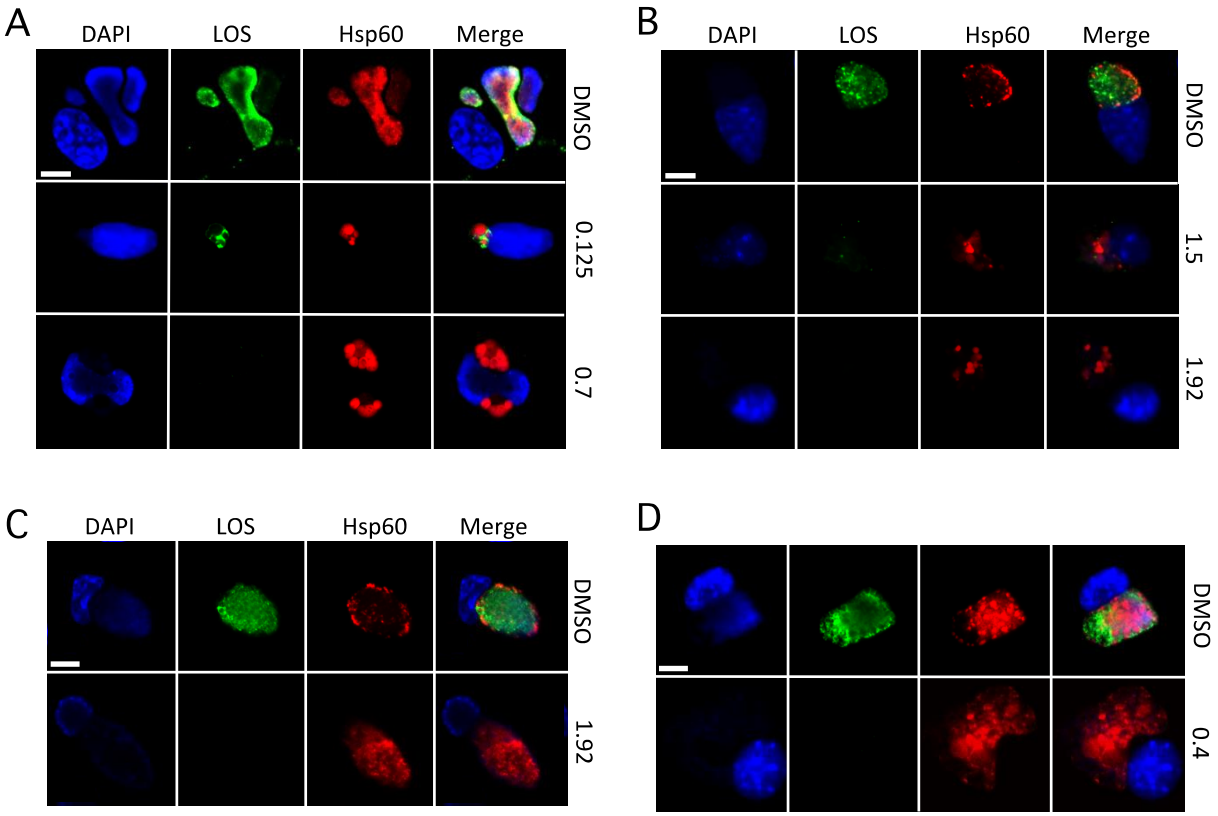


**Figure 5. *Chlamydia* spp. inclusion phenotype and sensitivity to LPC:** (A) *C. abortus* treated with 0.7 µg/mL (MEC) of LPC show an aberrant phenotype. LPC concentrations permissive of LOS production also contain ABs. (B) The MEC of LPC for *C. suis* is 1.92 µg/mL, however, unlike L2, treatment with the MEC concentration results in the formation of ABs within the inclusion, as well as at concentrations permissive of LOS biosynthesis. (C) The MEC of LPC for the non-LGV species, *C. trachomatis* J6276, was 1.92 µg/mL, with inclusions similar of frequency and size compared to mock-treatment. (D) *C. muridarum* was more sensitive than L2 to treatment of LPC with an MEC of 0.4 µg/mL, however inclusion frequency and size was similar to mock treatment.

| *Chlamydia* species | MEC of LPC (µg/mL) | Aberrant bodies visible at MEC? | Infectious progeny? |
|---|---|---|---|
| *C. muridarum* | 0.4 | No | Yes |
| *C. trachomatis* L2 | 1.92 | No | No |
| *C. trachomatis J6276* | 1.92 | No | No |
| *C. abortus* | 0.7 | Yes | No |
| *C. caviae* | 0.25 | Yes | No |
| *C. suis* | 1.92 | Yes | No |

**Table 1: A summary of MEC, inclusion phenotype, and infectious progeny output of different chlamydial species treated with LPC.**

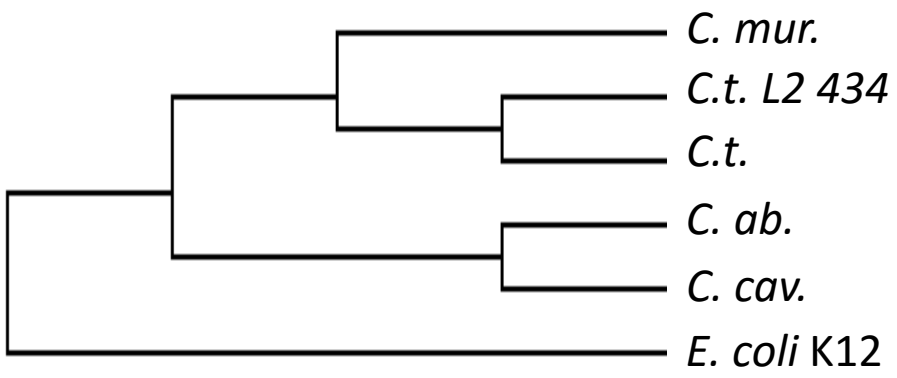


**Figure 6. Phylogenetic analysis of LpxC amino acid sequence from *Chlamydia* species listed in Table 1 and *E. coli* K12.**

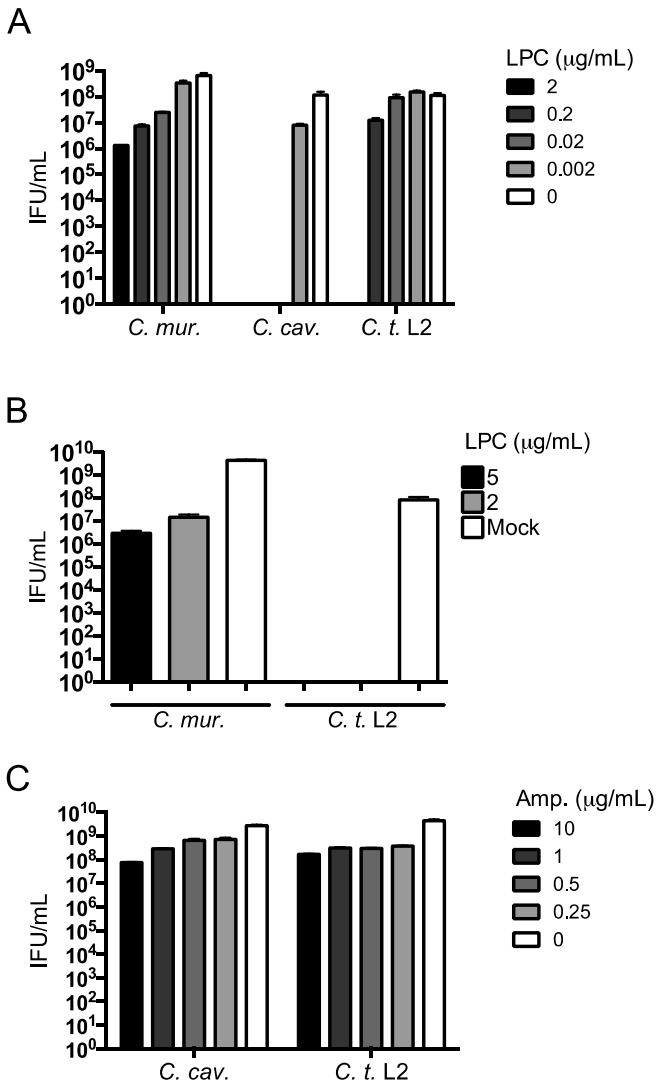


**Figure 7. Infectious progeny production of *Chlamydia* spp. in response to treatment with LPC or ampicillin:** (A) Infectious progeny production of *C. muridarum*, *C. caviae* and *C. trachomatis* L2 when treated with increasing doses of LPC. *C. muridarum* was the only species able to produce infectious progeny when treated with LPC concentrations above the MEC. *C. caviae* exhibited the greatest sensitivity to LPC as the only species not able to produce infectious progeny when treated with a concentration of LPC that was 10-fold less than the MEC. (B) Experiment described in A was repeated to include LPC at a concentration of 5 μg/mL, which resulted in the production of *C. muridarum* but not *C. trachomatis* L2 offspring. (C) Infectious progeny output of *C. caviae* and *C. trachomatis* L2 infected cells treated with increasing doses of ampicillin revealed a greater sensitivity of L2 to lower doses of ampicillin compared to *C. caviae*.

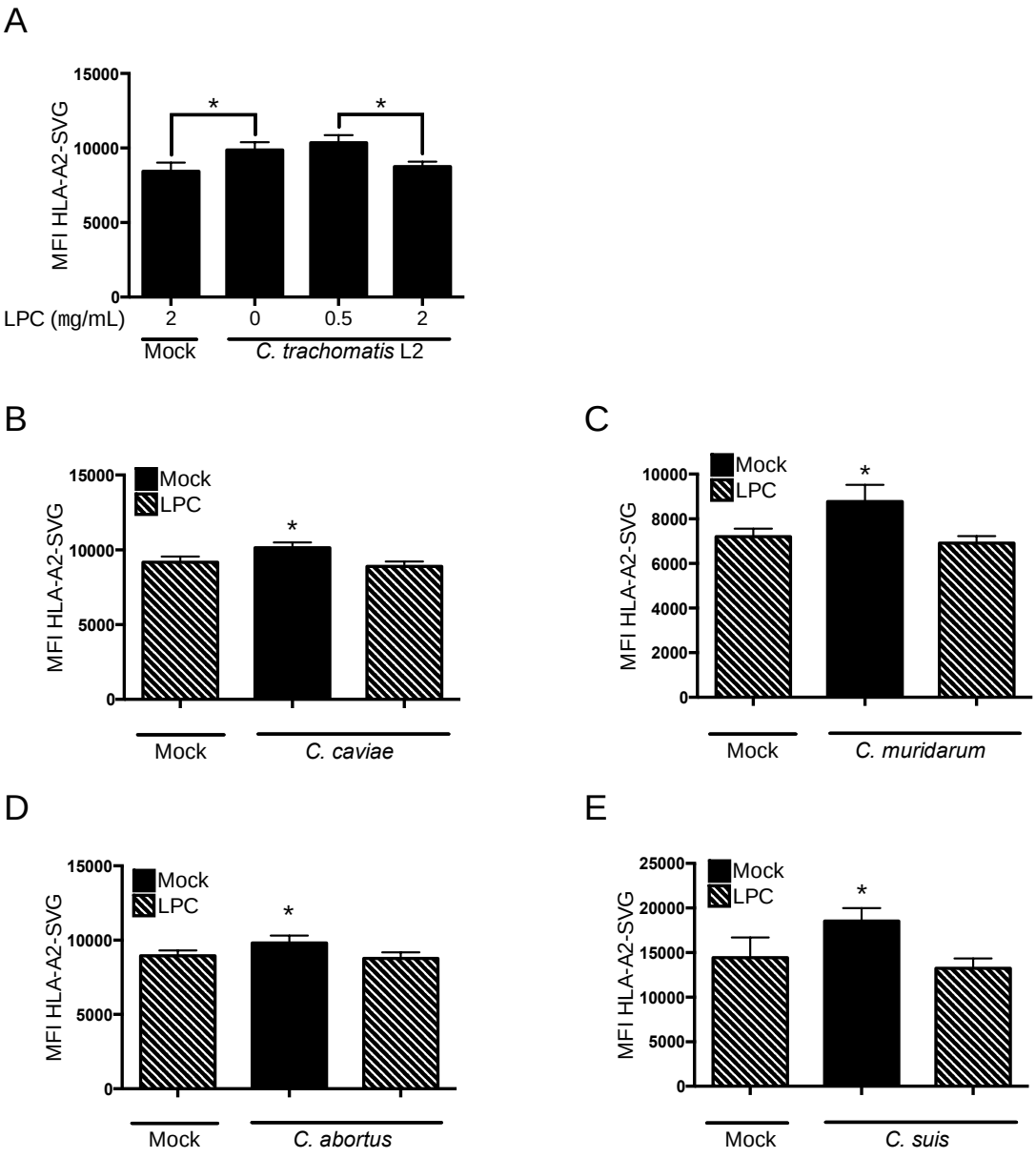


**Figure 8. *Chlamydia* enhance self-peptide presentation in a LOS-dependent mechanism:** (A) L2 infected cells were treated with mock, sub-MEC and MEC concentrations of LPC. At the MEC of LPC (2 µg/mL) surface HLA-A2-SVG were reduced to mock-infected levels. 0.5 µg/mL of LPC did not rescue increased surface HLA-A2-SVG phenotype. (B) Surface levels of HLA-A2-SVG were increased during *C. caviae* infection which was abrogated by treatment with LPC (2 µg/mL). The same results were true for (C) *C. muridarum*, (D) *C. abortus*, and (E) *C. suis*.

# GENERAL CONCLUSION

*Chlamydia trachomatis* is currently the leading cause of bacterial sexually transmitted disease in the United States, and has more recently become the most common notifiable disease [215]. Despite increased surveillance and treatment programs initiated by the CDC and other governmental institutions, the record for *C. trachomatis* infections was broken in 2014 with more than 1.4 million cases reported [215]. These statistics, however, are estimated to only reflect half of the total infections within the population because around half of *C. trachomatis* infections are asymptomatic and therefore go undiagnosed [216].

To date, *Chlamydia* infections and interactions with the host immune system remain shrouded in mystery. This is due, in part, to the difficulty and only recent advances of routine genetic tools, including the development of a transformation system with *C. trachomatis* that is ethically limited to use in clinically infrequent serovars [217]. Another difficulty in understanding interactions of chlamydial infection with the host immune system is a product of the limitations of human studies in order to maintain the integrity and ethics involved in clinical research. Instead, decades of *in vivo* studies have utilized animal models including guinea pig, mouse, and macaque models of infection, which do not completely reflect human physiology (reviewed in [218]). Finally, our understanding of chlamydial biology is basic in nature, and is only beginning to account for co-infections of other pathogens and

microbiomes in urethral, vaginal, rectal, ocular, and pharyngeal infections which likely result in combinatorial effects on the adaptive immune system.

Current unresolved aspects involving chlamydial interactions with the host immune system pose some very important questions to be answered by basic and clinical researchers. Firstly, it is well known that chlamydial infections are often asymptomatic, but the molecular pathways of asymptomatic infections in both sexes and the elevated prevalence of asymptomatic infections in women compared to men are poorly understood [216]. Secondly, sequelae associated with *C. trachomatis* infections are generally immunopathological, and are outcomes of repeat infections. While molecular mimicry and PD1-L downregulation play a role, it is likely that other chlamydial mechanisms exist which result in an immunopathological outcome [10, 219]. Thirdly, both *in vivo* and *in vitro* data suggest that the chlamydial persistent state may play a significant role in the development of chlamydial sequelae and immunopathologies. These analyses began in the 1970's when it was discovered that men and women who were co-infected with *Neisseria gonorrhea* and *C. trachomatis* and treated with ampicillin plus probenecid, cleared *N. gonorrhea* infection, but were unable to clear *C. trachomatis,* which lead to postgonoccocal urethritis in men [144]. While many factors that induce persistence are known, the *in vitro* frequency of persistence, the chlamydial mechanisms leading to persistence, and the mechanisms of persistence that lead to immunopathologies are all unknown. Finally, mechanisms of the

adaptive immune system that are responsible for the spontaneous clearance of *C. trachomatis* infections remain unsolved. When understood, this data is anticipated to lead to the development of the world's first *C. trachomatis* vaccine.

The results of the work described in this dissertation may help address several of the aforementioned questions. In explanation of the occurrence and sex-skewed ratios of asymptomatic infections, we propose that *Chlamydia spp.* are able to saturate MHC Class I molecules with host antigens in order to avoid detection by members of the adaptive immune system such as $CD8^+$ cytotoxic T cells and NK cells. While this immune evasion strategy only lowers probability of chlamydial antigens being presented, as backed by clinical evidence of $CD8^+$ T cell response, it may allow the bacteria, or a portion of bacteria, to subvert the immune system and therefore not manifest obvious pathologies to patients and clinicians, some of the time. Our data is preliminary and further experiments might determine that this enhanced self-antigen presentation phenotype may be cell-line dependent and may be sex-dependent. Additionally, our data only support the hypothesis that self-antigen presentation is being enhanced, and therefore that relationship to presentation of chlamydial antigens is only hypothetical. Future work in the Dolan and Rockey laboratories will be aimed to define the relationship of host antigen presentation to chlamydial antigen presentation. Fortunately, some of our findings may make our hypothesis easier to test.

The discovery that treatment with the LOS inhibitor reverts the enhanced MHC Class I self-antigen presentation phenotype during chlamydial infection would allow a means to modulate the phenotype in an “on and off” state. Treatment of infected cells with LOS inhibitor coupled with the more difficult and expensive goal of developing a TCRm monoclonal antibody against a chlamydial antigen, may allow for a model system to manipulate and quantitate *Chlamydia*-to-host antigen presentation by multiparameter flow cytometry. Other experiments, such as exposing *C. trachomatis*-infected cells grown in the presence and absence of LOS inhibitor to PBMCs from *C. trachomatis*-infected patients in an *ex vivo* killing assay may show a differential rate in cytotoxicity to infected cells.

The contribution of increased self-antigen presentation during infection with *Chlamydia spp.,* as identified in this dissertation, may have a direct correlation with the development of some immunopathological outcomes such as molecular mimicry. The development of autoimmune disorders such as atherosclerosis and reactive arthritis are well-documented outcomes of chlamydial infections [174, 175]. Previous studies identifying chlamydial modifications of the host peptide repertoire taken together with our data of increased host-peptide presentation may have implications in the outcome of chlamydial-induced autoimmune syndromes [13]. In order to better address this hypothesis, future experiments would be aimed to examine the dynamics of the HLA peptidome of infected cells, including DRiP contributions, using

the large-scale dynamic stable isotope labeling by amino acids in cell culture method as pioneered by Arie Admon's group at the Israel Institute of Technology [220].

Furthermore, the discovery of differential phenotypes, including the development of chlamydial aberrant bodies in some species may prove to be useful in understanding the regulation of persistence and the chlamydial developmental cycle. While many stressors have been identified as causing aberrancy and persistence in chlamydial-infected cells, our work is the first, to our knowledge, that identifies the differential effects of a stressor towards the developmental cycle in different species of *Chlamydia*. This includes the aberrant forms of *C. caviae, C. abortus* and *C. suis*, and the new finding that *C. muridarum* are able to produce infectious progeny. Genetically tractable tools, while useful, have only been developed in *C. trachomatis.* Other classical means of studying the biology of *Chlamydia,* such as recombination techniques between species and comparative genomics, remain vital to basic research. Future experiments that couple these classical tools with transcriptomics and proteomics analyses may add more insight towards mechanisms of chlamydial persistence and regulation of the developmental cycle.

Finally, the pathways and mechanisms that lead to antigen presentation from DRiPs remain unknown. All other previous and contemporary research by the

Dolan lab has identified genetic and chemical factors that influence the downregulation of surface antigen from DRiP substrates. This collaborative effort between the Rockey and Dolan labs is the first to identify an agent capable of increasing antigen presentation from DRiP substrates. It is possible that through the aid of mutant library screens and other molecular biological techniques, that the elements of the DRiPs pathway that are modulated by chlamydial infection may be discovered similar to other work using herpesvirus to discover the mechanisms of MHC Class I and MHC Class II antigen presentation.

In conclusion, there remains a lot of information left to uncover about the nature of chlamydial interactions with the host immune system. The development of the SCRAP antigen presentation assay by the Dolan lab has been essential in its application towards further understanding *Chlamydia* biology as described in this dissertation. Undoubtedly, other novel tools are currently being developed for use in other disciplines of biology. Interdisciplinary collaboration has been a large part of the work in this dissertation and future collaborations will be essential to build upon this body of work.

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# APPENDIX

# Identification of a unique, 125 kDa neddylated protein in *C. caviae* infected cell lysates

Erik D. Cram, Daniel D. Rockey and Brian P. Dolan

**Abstract**

Our previous work has shown that the obligate intracellular *Chlamydia* enhance MHC Class I self-antigen presentation in a model system of quantitative antigen presentation. Concomitant to enhanced antigen presentation from a model peptide is the loss of accumulation of the antigenic peptide substrate during infection, by an unknown mechanism. While our data shows that this loss is not due to proteasomal degradation, protein misfolding, other non-proteasomal, pathways have not been explored. *Chlamydia* genomes contain deubiquitnating enzymes that cleave ubiquitin-like protein modifications such as SUMO or NEDD8 from protein substrates. While neddylation activate E3 ligases, neddylation of other substrates results in a myriad of consequences. Here we aim to investigate the role of NEDD8 modifications to the host proteasome during infection with *Chlamydia* and identify a unique, 125 kDa, neddylated protein band by western blot that is present in multiple cell lines infected with *C. caviae,* but not with any other chlamydial species.

**Introduction**

*Chlamydiae* are obligate intracellular parasites that undergo a unique, biphasic developmental cycle. The generation of the infective elementary body (EB) form from the metabolically active, dividing reticulate body (RB) form take place within the host cell. *Chlamydia* are dependent on host cell resources for growth [137] and extensively remodel the host transcriptome

and proteome in order to optimize growth conditions within the host cell [13, 152, 174-176]. Modification of these processes often involves regulation of protein activity through covalent post-translational modifications that alter half-life and stability of the substrate with groups such as ubiquitin, SUMO and NEDD8 [221-223]. While much of the *Chlamydia* genome remains cryptic, several enzymes have been discovered that regulate host processes through these pathways such as the chlamydial protease-like activity factor (CPAF), and deubiquitinase enzymes Dub1 and Dub2 [152, 224].

NEDD8 is a 9 kDa, ubiquitin-like protein that is used to post-translationally modify and activate mainly the E3 ligase family of cullin-RING ligases [225, 226]. Identifying new substrates of neddylation is challenging as overexpression of NEDD8 results spurious neddylation of ubiquitin substrates [227]. However, neddylation has been implicated in affecting cellular processes such as receptor tyrosine kinase signaling, apoptosis, DNA damage repair, and nucleolar stress signaling (reviewed in [221]).

All chlamydial species have proteins with Ovarian Tumor-like (Ota) or ubiquitin-like protease (Ulp) domains [1, 228]. ChlaDub1 and ChlaDub2 have been identified in *C. trachomatis* L2 and D/UW3 genomes and have demonstrated both deubiquitinating and deneddylating activities *in vitro* via their Ulp domains [1]. That same study identified a homologue of ChlaDub2 in the *C. caviae* genome designated CCA00718 [1]. A similar study identified a

gene encoding a conserved hypothetical protein with a predicted Ota domain, CCA00261, which when overexpressed, rapidly deubiquitinated proteins found at the chlamydial entry site [228]. However, it is unknown if the protein encoded by CCA00261 has any deneddylation activity. The conserved presence of these large deubuiquitinating/deneddylating protein-coding genes within the minimal chlamydial genome suggests the necessity of these functions in optimizing the host for chlamydial growth. In an effort to identify differences in neddylation profiles between infected and uninfected cells, we observed a unique, neddylated, 125 kDa protein band unique to *C. caviae* infected cells by western blot.

## Materials and Methods

### Cell lines and Organisms

The human lymphoblastoid B cell line, JY, expressing Shield-1 controlled recombinant antigenic protein (SCRAP), were cultured in RPMI (Gibco) supplemented with GlutaMAX (Gibco, 20mM), 10% fetal bovine serum (Invitrogen), and HEPES (Gibco, 10mM) and 6% $CO_2$ [3]. Vero cells (ATCC CCL-81) were cultured in DMEM (Gibco) supplemented with 10% FBS (Invitrogen), L-Glutamine (Gibco, 2mM), and 5% $CO_2$. Infection with *C. trachomatis* L2 expressing mCherry (L2/pBRmChE, hereafter referred to as L2, a generous gift from Robert J. Suchland, University of Washington) [3], *Chlamydia caviae* GPIC, *Chlamydia muridarum* str. Nigg were carried out as

previously described [3, 196]. In some instances infection was carried out in the presence of ampicillin (100 µg/mL, Sigma).

**Western Blot**

Cells were harvested at 24 hours post infection and resuspended in 4X Bolt LDS protein loading buffer (Thermo Fisher) with DTT and NEM added. Cells were lysed by incubation at 95°C with occasional vortexing for 30 min. An equal volume of water was added bring the final concentration of cells to $10^7$ cells/mL and incubated for an additional 30 min. SDS-PAGE was performed with the cell lysates using the Bolt electrophoresis system, followed by protein transfer to a nitrocellulose membrane with an iBlot 2 (Invitrogen). Membranes were then blocked in 5% nonfat, dehydrated milk for 30 min, and probed with rabbit anti-Nedd8 (Cell Signaling) overnight. After a brief wash with TBS-T, membranes were incubated with secondary (LI-COR, IRDye donkey-anti-rabbit 800) and imaged with an Odyssey infrared imager (LI-COR). Membranes were then rinsed a second time and incubated with the monoclonal mouse-anti-p97 ATPase antibody (Fitzgerald) for 1 hour. Following a brief rinse with TBS-T, cells were then incubated with goat-anti-mouse secondary antibody (LI-COR, IRDye 680) for 1 hour, rinsed a final time and imaged.

**Bioinformatic analysis**

*C. trachomatis* D/UW-3 ChlaDub1 (UniProtKB/Swiss-Prot: O84876.1) and ChlaDub2 (UniProtKB/Swiss-Prot: O84875.1) amino acid sequences were obtained by accessing the NCBI public database. The *C. caviae* genome (ID: 1052) was queried against each ClaDub sequence using basic local alignment search tool (BLASTp). Alignments were made with ChlaDub2 using MacVector 11.2.1 ClustalW. *C. caviae* genes predicted to produce protein products between 100 kDa and 135 kDa were identified by using Geneioous 8.1.5 software (Biomatters LTD).

**Results**

JY SCRAP cells were infected with *C. trachomatis* L2 and *C. caviae*. Cells were lysed at 24 hpi and western blot probed with anti-NEDD8 antibody revealed a neddylated, ~125 kDa protein band in *C. caviae* infected cells only (Figure 1A). This band was not present in *C. caviae*-infected JY cells that were treated with ampicillin suggesting that neddylation of this protein was dependent on chlamydial growth (Figure 1A). The same experiment was then repeated to include *C. muridarum,* which only resulted in the 125 kDa band in the *C. caviae*-infected lysate lane only (Figure 1B). In order to examine if these results were cell line dependent, the same experiment was carried out in Vero cells, with the addition adding three concentrations of *C. caviae* EBs. Once, again, a unique 125 kDa protein band was visible only in the *C. caviae*-infected JY cell lysates, while a band of the same size was observed in the

lane of 100 µL of purified EBs (Figure 1C). Alignment of the amino acid sequences for the *C. trachomatis* D/UW-3 ChlaDub1 and ChlaDub2 were aligned to the *C. caviae* CCA 00718, *C. muridarum* TC 0258, and *C. abortus* WP 006344298 revealed that all contain the conserved, predicted catalytic residues typical of ubiquitin-like proteases as reported previously by Mishagi *et al.* [1] (Figure 2). Finally, all genes predicted to produce a product between 100-135 kDa were identified from the *C. caviae* genome and listed in Table 1.

**Discussion**

*Chlamydia* bacteria are obligate intracellular bacteria that proliferate within the nutrient-rich environment of the host cell. Members of the *Chlamydia* genus possess a genome ranging between 1-1.3 million base pairs, which are considered to be reduced genomes [229]. Reduced genomes are a typical characteristic of obligate intracellular microbes [230]. While we may not understand all of the characteristics of the chlamydial proteins containing Ulp and Out domains, it is clear that despite their size, their conserved prevalence in all members of the genus suggests the importance of the proteins in the chlamydial developmental cycle.

Previous data has identified that *Chlamydia* are able to enhance host antigen presentation from a model self-peptide in a quantitative model of antigen presentation [3]. We observe a sharp decrease in the quantity of stabilized antigenic protein substrate during infection with *C. trachomatis*. This

phenotype was further exaggerated during infection with *C. caviae.* These findings suggest differential mechanisms between the two species that may serve to enhance the phenotypes in *C. caviae* infected cells. Further experimentation revealed that loss of stable peptide was not due to inhibition of the proteasome.

Our data presented here, demonstrate that neddylation profiles of infected cells differ between species of *Chlamydia* as evident by the unique 125 kDa band seen in all *C. caviae* infected cell lysates. Furthermore, the presence of this band was dependent on active bacterial growth, as treatment with ampicillin did not produce a band visible by western blot. Additionally, the band was observed in lysates made from high concentrations of *C. caviae* EBs, however it is possible that the EB purification process did not purify all host proteins out of the inoculum. Therefore, it remains unclear whether or not this neddylated protein is of *C. caviae* or of host origin. We found that nearly half of all of the chlamydial protein candidates that are between 100-135 kDa are annotated as membrane proteins. It also remains unknown whether there is a correlation of neddylation activity in *C. caviae* infected cells with the loss of antigenic peptide substrate from our earlier study. Future experiments will aim to address the identification of the unique 125 kDa protein from *C. caviae* infected cells and determine the role of neddylation on the enhances antigen presentation phenotype from our previous efforts.

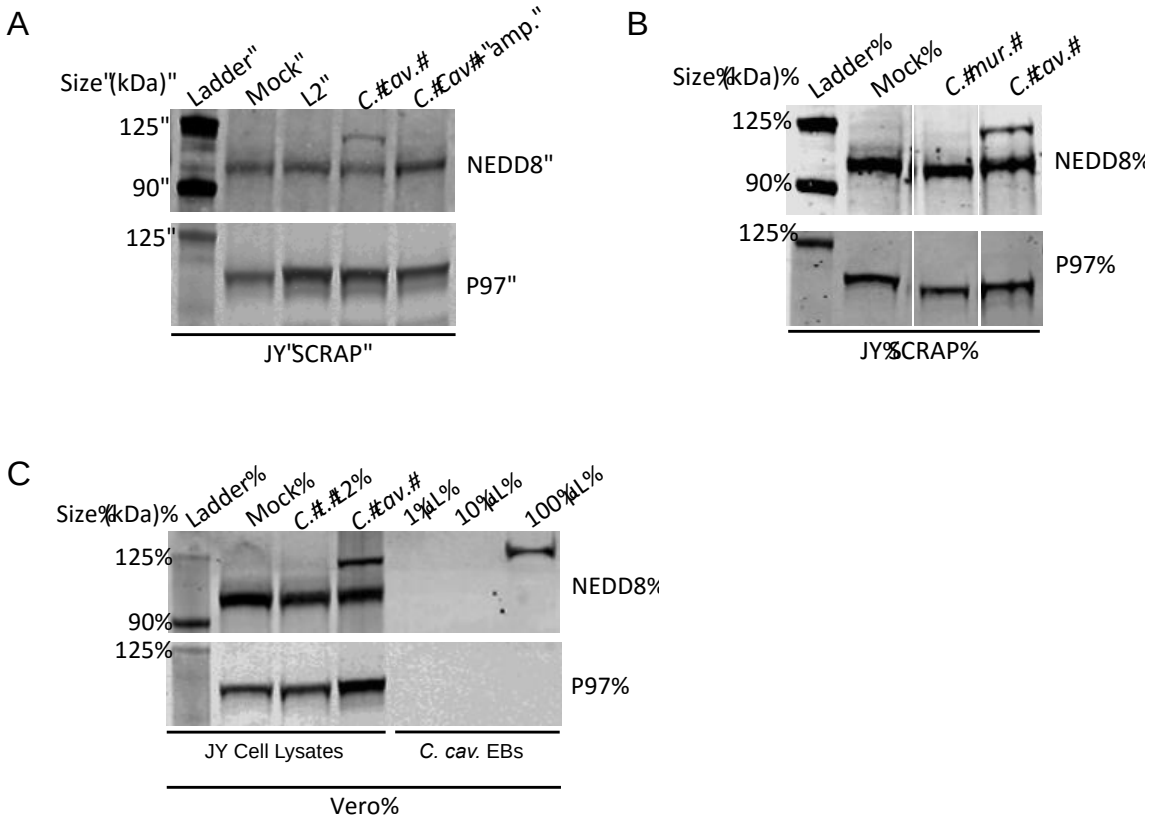


**Figure 1: Identification of a unique neddylated 125 kDa protein in cells infected with *C. caviae*:** (A) Western blot probed with anti-NEDD8 antibody revealed a 125 kDa protein band unique to lysates from JY SCRAP cells infected with *C. caviae*. This band was not observed when *C. caviae* was treated with ampicillin. Loading control was probed with anti-P97. (B) Same as in A, however with the addition of laysate from cells infected with *C. muridarum* str. Nigg, which revealed the neddylated125 kDa protein only in lanes containing *C. caviae*. (C) Same as in A, but in Vero cells which revealed a neddylated, 125 kDa protein in the *C. caviae* lane. Additionally, purified EBs were probed for anti-NEDD8 in increasing concentrations, which revealed a second, neddylated 125 kDa protein in the 100 µL lane.

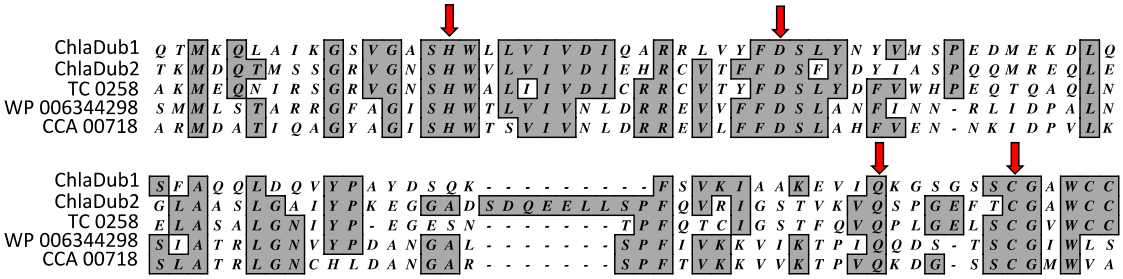


**Figure 2:Alignment of proteins containing Ulp domains from different species of *Chlamydia*:** Amino acid sequences of ChlaDub1 and Chladub2 from *C. trachomatis* D/UW-3, TC 0258 from *Chlamydia muridarum* str. Nigg, WP 006344298 from *C. abortus* S26/3, and CCA 00718 from *C. caviae* were aligned using ClustalW. Red arrows indicate the conserved amino acid residues responsible for the catalytic activity of the Ulp domain as reported by Misaghi *et al. [1]*

| CDS | Size (bp) | Function |
|---|---|---|
| 2-oxoglutarate dehydrogenase subunit E1 | 2,727 | 2-oxoglutarate dehydrogenase subunit E1 |
| secretin | 2,730 | secretin |
| cell division protein FtsH | 2,742 | cell division protein FtsH |
| membrane protein | 2,766 | membrane protein |
| membrane protein | 2,778 | membrane protein |
| membrane protein | 2,781 | membrane protein |
| hypothetical protein | 2,784 | hypothetical protein |
| hypothetical protein | 2,793 | hypothetical protein |
| pknD | 2,793 | serine/threonine protein kinase |
| membrane protein | 2,805 | membrane protein |
| membrane protein | 2,811 | membrane protein |
| peptidase | 2,814 | peptidase |
| valine--tRNA ligase | 2,823 | valine--tRNA ligase |
| membrane protein | 2,826 | membrane protein |
| membrane protein | 2,829 | membrane protein |
| membrane protein | 2,847 | membrane protein |
| hypothetical protein | 2,892 | hypothetical protein |
| secA | 2,913 | protein translocase subunit SecA |
| peptidase M16 | 2,925 | peptidase M16 |
| membrane protein | 2,946 | membrane protein |
| 23S rRNA | 2,946 | 23S ribosomal RNA |
| membrane protein | 3,021 | membrane protein |
| glycine--tRNA ligase subunit alpha/beta | 3,021 | glycine--tRNA ligase subunit alpha/beta |
| membrane protein | 3,036 | membrane protein |
| exodeoxyribonuclease V subunit gamma | 3,066 | exodeoxyribonuclease V subunit gamma |
| isoleucine--tRNA ligase | 3,132 | isoleucine--tRNA ligase |
| membrane protein | 3,135 | membrane protein |
| ribonucleotide-diphosphate reductase subunit alpha | 3,135 | ribonucleotide-diphosphate reductase subunit alpha |
| exodeoxyribonuclease V subunit beta | 3,138 | exodeoxyribonuclease V subunit beta |
| transcription-repair coupling factor | 3,258 | transcription-repair coupling factor |
| peptidase | 3,276 | peptidase |
| membrane protein | 3,450 | membrane protein |
| helicase | 3,501 | helicase |
| helicase | 3,618 | helicase |

**Table 1: *C. caviae* genes that are predicted to express proteins between 100 and 135 kDa are candidates as the unique neddylated 125 kDa protein band**