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Role of CD8+ T cells in the host response to *Chlamydia*

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Abstract

Chlamydia infections constitute a major public health problem. Although multiple arms of the immune system participate in the control of *Chlamydia* in infected hosts, T lymphocytes are essential. This review focuses on the roles that $CD8^+$ T cells may play in immunoprotection and immunopathology following recognition of *Chlamydia*-infected cells.

Keywords

Chlamydia; $CD8^+$ T cells

2. Introduction

Members of the *Chlamydiaceae* family are obligate intracellular gram-negative bacteria that include the human pathogens *Chlamydia trachomatis* (*Ct*) and *Chlamydia pneumoniae* (*Cpn*). While *Ct* is responsible for ocular and sexually transmitted diseases that can result in blindness and infertility, *Cpn* is a common cause of upper respiratory infections and pneumonia and has been associated with several chronic inflammatory conditions such as atherosclerosis and chronic obstructive pulmonary disease (COPD) [1–3]. When diagnosed early, *Chlamydia* infections can be treated with antibiotics. However, the high costs required to identify and treat individuals with mild or no symptoms limits the feasibility of this control strategy. Moreover, hosts can remain chronically infected despite chemotherapy, and some antibiotics may induce chlamydial persistence [4]. Thus, development of safe and effective vaccines represents a cost-effective approach that would have a greater impact on the high prevalence of *Chlamydia* infections and the prevention of severe long-term sequelae.

Like all chlamydiae, *Ct* and *Cpn* have a unique biphasic developmental cycle alternating between an infectious metabolically inert elementary body (EB) and a replicating metabolically active reticulate body (RB). After entry into susceptible cells such as epithelial cells, macrophages, endothelial and smooth muscle cells, the EB remains within a nonacidified vacuole known as an inclusion, where it differentiates into a RB, which replicates by binary fission. The generated progeny differentiate back into EBs that are then released upon host cell lysis to infect other cells. Under certain conditions, however, *Chlamydia* enters a persistent non-replicating stage but remains capable of resuming a productive cycle when the adverse

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conditions are no longer present. During *Chlamydia* infections, the immune system of the infected host encounters antigens expressed at various stages of the chlamydial developmental cycle and during persistence.

Although our knowledge of bacterial antigens and defense mechanisms that lead to protective immunity against *Chlamydia* has increased substantially in recent years, developing vaccines or immunotherapies against *Ct* and *Cpn* will require an improved and comprehensive understanding of all the elements of the immune system that act in concert to control chlamydial growth and facilitate pathogen clearance without causing immunopathology. Because type 1 T cells play a central role in anti-*Chlamydia* immunity, immune-based control strategies against *Ct* and *Cpn* will need to stimulate this group of lymphocytes. However, to develop T cell-stimulating *Chlamydia* vaccines it will be important to dissect the antigen-specific T cell responses that correlate with protective effector mechanisms from those that associate with the promotion of chlamydial persistence and tissue damage.

Numerous studies have shown that type 1 cytokine-secreting $CD4^+$ T (Th1) cells inhibit *Chlamydia* replication mostly via the secretion of IFNγ and by stimulating the protective function of other immune and inflammatory cells [5]. However, given the obligate intracellular nature of *Chlamydia*, there is an increased interest to determine the contribution of $CD8^+$ T cells in controlling replication of these pathogens. This review describes the evidence supporting a role for $CD8^+$ T cells in the response to *Chlamydia* infection and the consequences of $CD8^+$ T cell-mediated recognition of *Chlamydia*-infected cells as it relates to immunoprotection and immunopathology.

3. Evidence of a role for $CD8^+$ T cells in the immune control of *Chlamydia*

An intact T cell compartment is required for resistance against *Chlamydia* infection. T cell-depletion and -adoptive transfer experiments have, respectively, ablated and reconstituted protection in naïve mice challenged with *Chlamydia* [6,7]. Moreover, in *Chlamydia*-infected experimental animals, both $CD4^+$ and $CD8^+$ T cell subsets are detected at the site of infection [8–11]. Using mice made deficient of $CD4^+$ or $CD8^+$ T cells by antibody treatment or as a result of mutations in the CD4, CD8, major histocompatibility complex (MHC) class II, or β_2-microglobulin genes, the relative contribution that each of these two T cell subsets play in protective immunity against *Chlamydia* has been investigated. Although both $CD4^+$ and $CD8^+$ T cells contribute to protection, differences exist depending on the model of chlamydial infection studied. For instance, depletion of $CD8^+$ but not $CD4^+$ T cells in immune mice abrogates protection upon challenge with *C. psittaci* [12]. Similarly, in the absence of $CD8^+$ T cells, increased bacterial burdens and disease severity are observed during both a primary and secondary infection with *Cpn* [13,14]. By contrast, in *C. trachomatis*-infected and reinfected mice, depletion of $CD4^+$ T cells abrogates protection more significantly compared to the depletion of $CD8^+$ T cells [15,16]. Nevertheless, protective $CD8^+$ T cells are elicited following *Ct* infection [15,17]. It should be noted that $CD4^+$ T cells are often needed for the induction and preservation of a functional $CD8^+$ T cell response and in their absence, both $CD4^+$ and $CD8^+$ T cell effector functions are impaired. Thus, the minor role that some studies have reported for $CD8^+$ T cells in the immune control of *Chlamydia* may be underestimated.

Most information on the immune response to *Chlamydia* has been obtained from work with mice. In general, mouse models have proven to be excellent systems to study the immune mechanisms that are thought to control *Chlamydia* in humans. However, the successful design of a vaccine for *Chlamydia* will require validation of mouse data in humans and an increased understanding of the correlates of protective immunity in infected humans. Thus far, however, relatively few studies have evaluated human T cell immune responses to *Chlamydia*. Yet, like in mice, both T cell subsets are detected at the site of infection, and available data strongly

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suggest that T cells play an important role in protective immunity [18–20, unpublished]. However, the contribution of $CD4^+$ and $CD8^+$ T cells to the human anti-*Chlamydia* immure response remains unknown.

4. Pathogen-specific $CD8^+$ T cells are elicited during *Chlamydia* infection

An increasing body of evidence indicates that *Chlamydia* infection primes a pathogen-specific $CD8^+$ T cell response in mice and humans. In pioneering studies using *Ct* murine infection models, it was shown that splenic $CD8^+$ T cells could specifically lyse *Chlamydia*-infected fibroblasts, and that *Ct*-specific type 1 cytokine-producing $CD8^+$ cytotoxic T (Tc1) cells were partially protective when adoptively transferred into infected mice [17,21,22]. Nearly five years later, human leukocyte antigen (HLA) class I-restricted *Ct*-specific cytolytic $CD8^+$ T cells were detected in the peripheral blood mononuclear cells (PBMC) from individuals with history of previous *Ct* infections of the genital tract [23].

More recently, the lungs of *Cpn*-infected mice were shown to include pathogen-specific $CD8^+$ T cells with an ex vivo capacity to produce IFNγ and exert cytolytic effector function upon recognition of *Cpn*-infected macrophages [24,25]. *Cpn*-reactive $CD8^+$ T cells have also been detected in PBMC from *Cpn*-exposed individuals, in sputum from patients with COPD that are infected with this pathogen, and in *Cpn*-positive plaque from atherosclerotic persons [26,27].

Most studies supporting the priming of $CD8^+$ T cells during *Chlamydia* infection have searched for T cells that are restricted by classical MHC class Ia molecules. However, a *Chlamydia*-specific nonclassical MHC class Ib-restricted $CD8^+$ T cell response is also stimulated in *Ct*- or *Cpn*-infected hosts. Studies with *Cpn*-infected mice showed that primed pathogen-specific $CD8^+$ T cells include a subpopulation of Tc1 effectors that exerts nonclassical MHC class Ib-(H2-M3)-restricted lysis of *Cpn*-infected macrophages and that upon adoptive transfer into naïve mice, reduce lung *Cpn* loads following infectious challenge [25]. Using PBMC-derived $CD8^+$ T cells from *Ct*- or *Cpn*-exposed humans, the majority of *Chlamydia*-reactive $CD8^+$ T cells recognize infected cells in a nonclassically restricted manner [28, unpublished].

5. Access of *Chlamydia* antigens to the MHC class I processing and presentation pathway

$CD8^+$ T cells keep a constant vigil for signs of infection by surveying a vast array of peptides presented in complex with MHC class I molecules on the surface of all nucleated cells. These MHC class I-bound peptides are generated through a process known as antigen processing. In the classical pathway of MHC class I antigen processing, proteins located in the cytosol are ubiquinated and then cleaved by the proteasome. The resulting peptide fragments are then translocated into the lumen of the endoplasmic reticulum (ER) via the transporters associated with antigen processing (TAP) where a subset of these peptides bind MHC class I molecules. After peptide loading, stable peptide-MHC class I complexes move through the Golgi apparatus and become displayed at the cell surface [29]. Because MHC class I-presented peptides are commonly derived from cytosolic proteins, a cell infected with microorganisms that replicate in the cytosol can readily process secreted pathogen-derived antigens and present MHC class I-bound antigenic peptides on its surface for subsequent recognition by $CD8^+$ T cells.

However, MHC class I molecules can also present peptides from exogenous soluble or cell-associated proteins, including nonsecreted antigens from intravacuolar and cytosolic pathogens. This process, known as cross-presentation, is performed most efficiently by dendritic cells and macrophages. While these professional antigen-presenting cells (pAPC)

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prime naïve $CD8^+$ T cells via the endogenous MHC class I presentation pathway when they themselves become infected, priming may also occur through one or more of several intricate and not fully elucidated cross-presentation routes after the uptake of exogenous antigens like dead infected cells or debris thereof [30]. One potential route involves the translocation of endocytosed or phagocytosed antigens from the phagosome into the cytosol where these then follow the same fate as endogenous proteins. Another mechanism entails fusion of the phagosome with the ER. In this case, internalized antigens are exported to the cytosol for proteasomal processing via the Sec61 channel, and the resulting peptides are then re-imported into phagosomes by TAP where they bind MHC class I molecules. Evidence for another cross-presentation route implicates the endosomal compartment. In this pathway, exogenous soluble antigens are cross-presented from stable early endosomes to which TAP is recruited in an endotoxin-induced, Toll-like receptor 4–MyD88–dependent manner [31]. Regardless of the route, for cross-primed pathogen-specific $CD8^+$ T cells to recognize infected cells that are unable to cross-present, the priming antigens need to access the cytosol of the infected cell.

In the context of *Chlamydia* infection, with the exception of those antigens that translocate through or associate with the inclusion membrane, most chlamydial proteins remain compartmentalized within the inclusion. To date, it is uncertain if infection-primed $CD8^+$ T cells target any of the secreted chlamydial antigens reported to gain entry into the cytosol of infected cells. By contrast, several proteins intimately associated with the developing organisms or that localize to the inclusion membrane are known to prime antigen-specific $CD8^+$ T cells in *Chlamydia*-infected hosts [23–25,32–39, unpublished]. Importantly, effector T cells to these antigens recognize *Chlamydia*-infected cells regardless of the cross-presentation competence of host cells. It is difficult to explain how these nonsecreted chlamydial antigens become substrates for MHC class I processing and presentation, especially since *Ct* and *Cpn* infect and replicate predominantly within epithelial cells and other nonprofessional APCs that are unable to cross-present. Nevertheless, a number of potential mechanisms can be postulated from the existing data.

With regards to the chlamydial products that insert into the inclusion membrane, multiple members of the large family of inclusion membrane proteins known as the Incs elicit MHC class I-restricted $CD8^+$ T cell responses in infected hosts [24,34,39, unpublished]. Incs share minimal primary sequence identity with each other, but bear a conserved secondary structure consisting of a unique bilobed hydrophobic region of 50–80 amino acids and domains that are exposed at the cytosolic face of the inclusion [40]. These antigens are thought to be inserted in the inclusion membrane via the chlamydial type III secretion apparatus. Because most defined Inc-derived $CD8^+$ T cell epitopes map to the predicted cytosolic domains [24, unpublished], it was proposed that these exposed regions may be cleaved by cytosolic proteases, and after proteasomal processing of the released fragments, the resulting determinants may become surface-displayed as MHC class I-bound peptides [24]. Evidence supporting the entry of Incs into the endogenous MHC class I processing pathway include the elution of an Inc-derived $CD8^+$ T cell epitope from MHC class I molecules purified from *Cpn*-infected epithelial cells, the ability of $CD8^+$ T cells specific for a *Ct* Inc to secrete IFNγ upon interaction with *Ct*-infected fibroblasts, and the failure of *Cpn* Inc antigen-specific $CD8^+$ T cells to recognize brefeldin A-treated infected macrophages [24,39, unpublished]. Moreover, the lysis of *Ct*-infected fibroblasts by $CD8^+$ T cells specific for a *Ct* inclusion membrane protein that lacks the typical bilobed hydrophobic motif of Incs [34] further suggests that chlamydial proteins at the interface of the inclusion and the host cell cytosol enter the classical pathway of MHC class I antigen processing.

Of the *Chlamydia* antigens that remain confined within the inclusion and are recognized by infection-primed $CD8^+$ T cells, many are in the outer membrane of the developing organisms. Cross-presentation has been reported as a mechanism to prime *Chlamydia*-specific $CD8^+$ T

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cells [41] and could thus contribute in the priming of $CD8^+$ T cell responses to these chlamydial-associated antigens. However, murine *Ct*-specific $CD8^+$ T cells target *Ct*-infected fibroblasts for lysis via the endogenous antigen processing pathway [21]. Also, human and murine $CD8^+$ T cell effectors to defined *Cpn* and *Ct* outer membrane proteins recognize infected epithelial cells, fibroblasts, or pAPC in a brefeldin A-sensitive manner, suggesting that these antigens can be processed via the conventional MHC class I processing pathway in both humans and mice [23,24,35, unpublished]. As no report has localized *Chlamydia* envelope antigens in the cytosol of infected cells, some of these proteins may reach the cytosol in a pre-processed form through unidentified mechanisms. It was proposed that *Chlamydia*-derived proteases in the lumen of the inclusion may partially cleave envelope antigens from intact organisms during the extensive membrane remodeling that occurs during chlamydial replication and differentiation, from the membranous material present within a typical inclusion, or from a small fraction of developing chlamydiae undergoing autolysis [24].

6. Potential contribution of other immune components to the induction of anti-*Chlamydia* $CD8^+$ T cells

In addition to pAPCs that prime $CD8^+$ T cells, numerous studies support the participation of other immune and inflammatory cells in the induction, polarization, and maintenance of $CD8^+$ T cell responses via the production of cytokines and through recruitment of $CD8^+$ T cells to the site of infection. For instance, although the role of NK cells in anti-*Chlamydia* immunity is still unclear, these cells are a well known early source of IFNγ, including *Chlamydia* infections, and as such they likely contribute to the induction of $CD8^+$ T cell responses by ensuring that an optimal level of this cytokine is available to decrease bacterial loads and to drive the polarization of $CD4^+$ and $CD8^+$ T cells to become Th1 and Tc1 cells [42,43]. NKT cells, on the other hand, play divergent roles following infection with the mouse biovar of *Chlamydia* (*C. muridarum*) and *Cpn*; secreting IL-4 and exacerbating disease in the former, but producing IFNγ and decreasing bacterial loads in the latter [44]. Thus, during infection with *Cpn*, the IFNγ produced by NKT could result in the preferential activation and differentiation of *Cpn*-specific Tc1 cells. Another early source of IFNγ are MHC class Ib-restricted $CD8^+$ T cells, which besides their direct participation in the control of *Chlamydia* growth [25], they could also do so indirectly by their demonstrated ability to increase $CD4^+$ and class Ia-restricted $CD8^+$ T cell responses and a likely role in the polarization of Th1 $CD4^+$ T cells [45].

The induction of strong and durable MHC class Ia-restricted T cell responses often requires $CD4^+$ T cell help, which acts by promoting the development and preservation of a functional memory $CD8^+$ T cell pool. $CD4^+$ Th1 cells are undoubtedly critical for the generation of an optimal *Chlamydia*-specific $CD8^+$ T cell response. Nevertheless, $CD4^+$ T cells may be expendable for the induction and maintenance of MHC class Ib-restricted $CD8^+$ T cell responses, as *Chlamydia*-specific, MHC class Ib (H2-M3) restricted $CD8^+$ T cells are primed and functional memory cells are recalled in the absence of $CD4^+$ T cell help [25].

Another mechanism that presumably contributes to the induction of *Chlamydia*-specific $CD8^+$ T cells is the migration of this lymphocyte subset to the site of infection. Polymorphonuclear neutrophils have been shown to be necessary for the recruitment of $CD8^+$ T cells in a mouse model of *C. psittaci* infection [46]. Moreover, EBs and RBs can be found inside infiltrating neutrophils [47]. Because apoptotic neutrophils containing bacterial antigens are a known substrate for DC cross-presentation [48], their presence during *Chlamydia* infection may enhance the $CD8^+$ T cell response to secreted and non-secreted antigens.

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B cells may also establish interactions with $CD8^+$ T cells that help induce an optimal response by this T cell subset. Interactions between the two cell types is strongly suggested in a study showing that chlamydial burdens are significantly higher in mice that lack both $CD8^+$ T cells and B cells than those lacking only $CD8^+$ T cells [49]. Indeed, B cells may enhance $CD8^+$ T cell activity through the increased activation of Th1 cells that occurs after cross-presentation by DCs of antigens acquired through IgG2a and IgA Fc receptor-mediated uptake [50].

7. Effector mechanisms of $CD8^+$ T cells associated with anti-*Chlamydia* activity

$CD8^+$ T cells control infection by intracellular pathogens via a number of effector mechanisms. These mechanisms include cytotoxicity via the granule exocytosis (perforin/granzymes) or Fas/FasL (CD95/C95L) pathways, production of antimicrobial peptides, and production of cytokines and chemokines. With this arsenal, $CD8^+$ T cells may contribute to anti-*Chlamydia* immunity presumably by lysing infected cells and depriving the pathogen of its intracellular niche, and through the release of inflammatory mediators that render developing bacteria noninfectious or that recruit and activate other cells to limit intracellular survival of the pathogen.

IFNγ has been extensively reported as a critical mediator in immunity to *Chlamydia*. In mice lacking IFNγ or signaling by this type 1 cytokine, chlamydial loads are higher and clearance of organisms is greatly hampered [51]. Multiple cells of the immune system can produce IFNγ, and during *Chlamydia* infection they are all likely needed to ensure that this type 1 cytokine is present at optimal levels to stimulate and effect protective innate and adaptive immune responses. Although both $CD4^+$ and $CD8^+$ T cells produce IFNγ in response to infection, several mouse studies have shown that the release of IFNγ by $CD8^+$ T cells is associated with protective anti-*Chlamydia* activity. In adoptive transfer experiments, a protective *Ct*-specific $CD8^+$ T cell line failed to confer protection in infected recipient mice that were previously treated with a neutralizing anti-IFNγ antibody, and the transfer of *Chlamydia*-specific $CD8^+$ T cells derived from wild type but not IFNγ-deficient mice protected naïve mice against *Ct* challenge [22,52]. Indirect evidence showing that $CD8^+$ T cell-derived IFNγ contributes to the control of *Cpn* growth in infected mice was obtained using animals lacking $CD4^+$ and $CD8^+$ T cells. The higher susceptibility of CD8-deficient mice correlated with an immune deviation from a type 1 to a type 2 cytokine pattern and $CD8^+$ T cells were found to modify the cytokine secretion of $CD4^+$ T cells from a *Cpn* growth promoting type 2 to a protective type 1 phenotype [14]. Further proof was recently obtained by the partial yet five-fold higher levels of protection against *Cpn* infection observed in untreated mice compared to IFNγ-neutralized animals infused with an IFNγ-producing, perforin-deficient *Cpn* Inc antigen-specific $CD8^+$ T cell line (unpublished). That $CD8^+$ T cells can suppress *Cpn* growth by production of IFNγ was also shown in vitro where neutralization of IFNγ partially reversed the inhibition of *Cpn* inclusion formation in infected cells treated with the supernatants from IFNγ-producing *Cpn*-specific $CD8^+$ T cell lines [24]. These studies also suggested a contribution of other $CD8^+$ T cell-secreted factors in the control of chlamydial growth. Such factors could include TNFα, a cytokine known to synergize with IFNγ in inhibiting *Cpn* replication [53], and MIP-1α, a chemokine with anti-*Cpn* growth activity (unpublished).

$CD8^+$ T cell-derived IFNγ is likely to contribute to the inhibition of chlamydial replication by at least four mechanisms [51]. First, IFNγ mediates the activation of the inducible nitric oxide synthase, which catalyzes production of NO and inhibits chlamydial growth. Second, it enhances the function of pAPCs, which may increase the MHC class I- and II-dependent presentation of *Chlamydia* antigens to T cells. Third, IFNγ activates indoleamine 2,3-dioxygenase, which catalyzes decyclization of L-tryptophan, depriving *Chlamydia* of this amino acid in the host cell. Finally, the IFNγ-mediated intracellular deficiency of iron may

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also limit the replication capacity of *Chlamydia*. Intriguingly, reduced intracellular levels of tryptophan and iron are also amongst the known inducers of chlamydial persistence [4]. Thus, IFNγ may have a dual role in controlling the outcome of chlamydial infections in vivo. While production of IFNγ by *Chlamydia*-specific $CD8^+$ T cells may be critical to achieve the concentration required to inhibit chlamydial growth and prevent persistence, a suboptimal or waning $CD8^+$ T cell response during *Chlamydia* infection may also induce the formation of persistent organisms. Because IFNγ levels rise and decline repeatedly due to the consecutive cycles of chlamydial reactivation and persistence, the resulting bouts of inflammation can lead to tissue damage.

$CD8^+$ T cells from *Chlamydia*-infected mice and humans have in vitro lytic activity for infected and peptide epitope-sensitized cells [21–25,28,32–34,38,39]. Although a key effector function of $CD8^+$ T cells is to trigger apoptotic-mediated lysis of target cells through the release of perforin and granzymes and by engaging Fas, three studies seem to suggest that these cytolytic mechanisms are not required for resistance against *Chlamydia* infection. First, as noted above, the adoptive transfer of *Ct*-specific cytotoxic $CD8^+$ T cell lines derived from wild type but not from IFNγ-deficient mice protected naïve mice against challenge [52]. Second, the clearance of genital infections by *C. muridarum* was shown to be normal in mice lacking perforin, Fas, or Fas ligand [54]. Finally, the kinetics of pulmonary *Cpn* infection in perforin-deficient mice was found to be similar to that of wild-type mice [14]. However, it is important to emphasize that the interpretation of these studies is complicated by at least two factors: the variability of effector mechanisms for different $CD8^+$ T cell lines and the wider effects of gene perforin knockouts on the abnormal expansion of IFNγ-producing pathogen-specific $CD8^+$ T cells. In this context, recent data indicate that lung-infiltrating $CD8^+$ T cells from *Cpn*-infected mice express upregulated levels of perforin mRNA compared to lung $CD8^+$ T cells from naïve mice, and that freshly isolated pulmonary $CD8^+$ T cells from infected mice include pathogen-specific effectors that express perforin protein, and display perforin-dependent killing of *Cpn*-infected but not uninfected macrophages (unpublished). Moreover, cells coated with *Cpn* $CD8^+$ T cell epitopes are killed in the lungs of infected but not in the lungs of naïve mice [25, unpublished]. Altogether, these data suggest that in addition to the secretion of IFNγ, the granule exocytosis pathway may also represent a mechanism by which $CD8^+$ T cells may control chlamydial growth in vivo.

8. Chlamydial antigens recognized by infection-primed $CD8^+$ T cells

Despite their intravacuolar location, chlamydiae interact with multiple host cell processes to ensure that the inclusion is a safe niche for their survival and replication. These interactions are needed to acquire nutrients, avoid fusion with lysosomes, obtain membrane components from Golgi-derived exocytic vesicles, and alter host cell functions. The most likely products that control these processes are chlamydial proteins that gain access to the host cell cytosol. Because $CD8^+$ T cells usually recognize antigens processed from cytosolic proteins [29], and $CD8^+$ T cell recognition of *Chlamydia*-infected cells can inhibit bacterial growth [24], various groups have used available *Cpn* and *Ct* genome sequence information and a number of different technologies to identify antigens that become degraded by the MHC class I processing machinery, and subsequently induce $CD8^+$ T cell responses in infected hosts (Table 1).

Using MHC class I motif-based epitope prediction strategies, 14 *Cpn* antigens have been identified as targets of infection-primed murine $CD8^+$ T cells. These T cell effectors recognize epitopes in a MHC class Ia-(H-2^b or H-2^d)- and class Ib-(H2-M3)-restricted fashion in 12 and 2 of the target molecules, respectively [24,25,38]. All identified $CD8^+$ T cell antigens are endogenously processed, and nearly all defined determinants are presented to $CD8^+$ T cells on *Cpn*-infected cells (Table 1). An in-depth characterization of multiple *Cpn* epitope-specific $CD8^+$ T cells revealed that these effectors displayed a Tc1 phenotype, secreting IFNγ, TNFα,

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and MIP-1α. Moreover, they suppressed chlamydial growth in vitro by direct lysis, exhibited in vivo pulmonary killing of peptide epitope-coated splenocytes, and conferred different levels of anti-*Cpn* immunity upon adoptive transfer [24,25, unpublished]. Importantly, immunization of mice with a $CD8^+$ T cell epitope-based DNA minigene vaccine encoding seven determinants from different *Cpn* antigens elicited a potent and durable multifunctional $CD8^+$ Tc1 response that provided immunized animals with an unprecedented level of protection against infectious *Cpn* challenge [55].

Recently, the mass spectrometric analysis of H-$2K^b$-eluted peptides from *Cpn*-infected cells led to the identification of a $CD8^+$ Tc1 epitope from Cpn0369 (unpublished). This antigen is a predicted member of the Inc family of inclusion membrane proteins. Incs from the same or different chlamydial species share minimal primary sequence identity with each other or with proteins in public databases, but they bear a conserved secondary structure consisting of a unique bilobed hydrophobic region of 50 to 80 amino acids and domains that are exposed at the cytosolic face of the inclusion [40]. That Cpn0369 is localized to the *Cpn* inclusion membrane was confirmed by immunofluorescence using specific antisera. Cpn0369 epitope-specific $CD8^+$ T cells generated from *Cpn*-infected mice displayed similar phenotypic and functional characteristics as the T cells specific for most of the defined MHC class I motif-predicted epitopes. However, the in-vivo anti-*Cpn* activity of these effectors was only comparable to the protection conferred by Tc1 cells specific for three other epitopes, including one in Cpn0585. Because Cpn0585 is an Inc, most predicted members from this family of proteins were also tested as potential targets of *Cpn*-specific $CD8^+$ T cells using a MHC class I motif-based approach. Interestingly, 13 putative Incs emerged as antigens recognized by *Cpn* infection-primed IFNγ-producing $CD8^+$ T cells and all identified epitopes were found to map to regions of these Incs that are presumably exposed to the host cell cytosol (unpublished). Thus, proteins that associate with the *Cpn* inclusion membrane represent a significant source of chlamydial peptides loaded onto MHC class I molecules.

In *Ct* murine infection models, three $CD8^+$ T cell antigens have been reported to date. The first two target molecules, Cap1 and CrpA, were identified using *Ct*-specific T cell lines as probes to screen *Ct* DNA expression libraries [34,39]. Interestingly, both antigens also localize to the inclusion membrane. Although Cap1 lacks the typical bilobed hydrophobic region of Incs, it includes a transmembrane domain and thus, likely a protein integral to the inclusion membrane. Immunization of mice with recombinant vaccinia virus expressing Cap1 or CrpA was found to elicit partial protective immunity. The third $CD8^+$ T cell antigen, PmpI, was recently identified using a MHC I tetramer array prepared with H-2^b motif-predicted *Ct* peptide epitopes [36]. Like most identified *Chlamydia* $CD8^+$ T cell antigens, PmpI is also a membrane protein.

Information on human $CD8^+$ T cell responses to *Chlamydia* is sparse and the antigens eliciting such responses have only begun to be identified. Thus far, the only *Cpn* antigen reported as recognized by $CD8^+$ T cells from *Cpn*-exposed HLA-A*0201$^+$ individuals is DnaK, a heat shock protein first identified as a target of murine anti-*Cpn* Tc1 cells [24,33]. Recently, human *Cpn*-specific $CD8^+$ Tc1 cells were also found to include specificities for HLA-A*0201-, A*0301-, B*0801-, and B*3501-restricted epitopes in 13 of 18 chlamydial antigens targeted by Tc1 cells from *Cpn*-infected mice (unpublished). These targets include inclusion membrane proteins such as Cpn0585 and Cpn0369, and *Cpn* outer membrane proteins like MOMP and Omp2. It is noteworthy that the *Ct* MOMP and Omp2 orthologues are also recognized by $CD8^+$ T cells from *Ct*-infected individuals [23,35]. Thus, these studies demonstrate the validity of mouse *Chlamydia* infection models to identify target antigens of human anti-chlamydial $CD8^+$ T cells. Moreover, the study of *Chlamydia* antigen-specific $CD8^+$ T cells in mice can provide an insight into the pathogen components and aspects of the $CD8^+$ T cell response that contribute to protection and those that mediate tissue damage. Pathogen-derived proteins that

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stimulate protective murine $CD8^+$ T cells, among other effective immune responses, represent potential candidates to develop vaccines against *Chlamydia*.

9. Induction of *Chlamydia*-specific $CD8^+$ T cell responses through vaccination

Chlamydia infection generates short-lived partial immunity against reinfection. Early attempts at vaccinating humans with whole inactivated chlamydial organisms led to short-term protection. However, whole organism-based vaccines also induced responses that exacerbated disease upon reinfection [56]. Although attenuated live *Chlamydia* vaccines lacking immunopathogenic components could circumvent the safety concerns of whole organism-based immunization and elicit a multispecific protective $CD8^+$ T cell response that would otherwise be inefficiently induced using inactivated chlamydiae, attenuated organisms cannot yet be used because the methods to manipulate chlamydial genes have not been developed. Thus, current *Chlamydia* vaccine efforts are focused on developing subunit vaccines and vaccine delivery vehicles that improve the suboptimal immunity conferred by previous exposure to chlamydial agents.

Ideally, such vaccines should include chlamydial components that when properly delivered rapidly induce a comprehensive immune response, including strong, broad, and sustained antibody, $CD4^+$ and $CD8^+$ T cell responses that prevent bacterial entry into cells, microbial growth, spread, and persistence in the infected host. Because suboptimal resistance induced following *Chlamydia* infection is likely due to the poor priming, expansion, and maintenance of type 1 T cells to multiple antigens, immunization strategies that enhance the magnitude and quality of Th1 and Tc1 responses against several antigens could potentially be superior to protection induced by whole organisms.

In fact, the antigenic complexity of *Chlamydia* suggests, and experimental vaccine studies confirm, that effective and protective immune responses to *Ct* and *Cpn* are distributed among many antigens. Thus, of the available vaccine technologies, the multicomponent-based subunit approach is perhaps the best suited to mimic or augment whole organism-induced immunity and prevent potential immunopathogenic or suppressive responses, given that each component is tested first for their pathologic and immune-dampening potential. Multicomponent vaccines circumvent a number of the potential short-comings of single-component subunit vaccines, in particular the genetic restriction of responses to individual antigens and epitopes. However, the rational design of multicomponent *Chlamydia* vaccines that induce responses to a cadre of $CD8^+$ T cell and other protective T and B cell specificities will require studies that address a number of issues. What are the best criteria for selection of *Chlamydia* vaccine candidates? Is it abundance? Timing and length of expression in the bacterium? subcellular location? Conservation across *Chlamydia* species and serovars? Level of protection observed in validated animal models? What is the minimal or optimal number of subunits in a multicomponent vaccine? How many components are too many? Should the vaccine include antigens or epitopes? What is the best way to deliver such a vaccine? Clearly there are many questions to deal with in this area.

Chlamydia $CD8^+$ T cell target antigens identified from sequence analysis, expression data, and bioinformatic approaches that help predict the function, cellular compartment and the developmental stage of expression of proteins can be used both for vaccine discovery and development as well as for studies aimed at unraveling the interactions that *Chlamydia* needs to establish its intracellular niche.

For a multicomponent vaccine to confer universal protection against *Chlamydia*, it is important that it primes a Tc1 response against multiple conserved *Ct* and *Cpn* target molecules that are differentially expressed at different developmental stages so that induced $CD8^+$ T cells could potentially impair bacterial viability at any stage of the pathogen's intracellular existence.

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Epitope-based multicomponent vaccines can induce protective immunity against *Chlamydia*. This was first demonstrated using a DNA minigene vaccine based solely on multiple *Cpn* $CD8^+$ Tc1 epitopes. Minigene-immunized mice were shown to be highly protected against *Cpn* challenge [55]. Multiepitope-based constructs offer several advantages over the use of whole antigens. For instance, extensive antigenic complexity can be packed into a single immunogen, and constructs can be designed to include a mix of protective T and B cell epitopes and exclude potentially immunosuppressive and immunopathogenic determinants. On the other hand, one major disadvantage is the high degree of HLA polymorphism in humans. However, this limitation can be addressed by the inclusion of multiple supertype-restricted epitopes that can be recognized in the context of several related HLA alleles, and which would allow for coverage in the majority of all racial and ethnic populations.

An alternative to the epitope-based approach as a vaccination strategy against *Chlamydia* is to develop a subunit construct that incorporates multiple antigens. A recent study providing proof-of-principle for this approach showed that a subunit vaccine including two *Ct* antigens conferred a greater level of protective immunity than the single subunit constructs [57]. To date, the protective immunity induced by single antigen-based *Chlamydia* vaccines delivered as DNA, protein, viral vector, or via heterologous prime-boost immunization strategies has generally been lower or comparable to the suboptimal resistance generated by previous infection. Additional studies will be needed to determine if the levels of protection induced with multicomponent vaccines are further enhanced when the construct includes three or more antigens and to ensure that proteins targeted by protective $CD8^+$ T cells effectively prime these lymphocytes.

Finding conserved and immunogenic antigens is not enough for development of a successful *Chlamydia* vaccine. It is well known that the choice of delivery vehicles and adjuvants is also important. Indeed, there are apparent differences between DNA- and protein-based immunizations because some *Chlamydia* antigens are protective when delivered as protein but not as DNA [49,58,59]. The route of administration is also crucial to ensure that robust immune responses are induced and mobilized where they are needed [49]. Furthermore, when *Chlamydia* antigens are evaluated for their protective capacity, they should be tested with adjuvants that favour the induction of Th1 and Tc1 cells as there is evidence that protection against infection can be abrogated when antigens are formulated in adjuvants that tilt responses towards those mediated by type 2 cytokine-producing T cells [60].

Finally, since *Chlamydia* infections are largely confined to mucosal surfaces, a vaccine will need to induce vigorous Tc1 and Th1 responses at mucosal sites. Intranasal immunization elicits protective immune responses in both the airways and in the genital tract [61]. Thus, a universal *Chlamydia* vaccine that primes mucosal T cell immunity could potentially provide protection to both respiratory *Cpn* and genital *Ct* infections.

10. Potential role for $CD8^+$ T cells in *Chlamydia* infection-associated immunopathology

As stated previously, the host immune response often fails to completely clear an initial infection with *Chlamydia* and the pathogen persists in the host. While some chlamydial antigens can directly contribute to tissue damage, the repeated cycles of chlamydial reactivation and the nonsterilizing rounds of immune reactivity are largely the cause of immunopathology. It is still unclear which cells are responsible for the harmful bouts of inflammation that ensue after *Chlamydia* infection and which lead to tissue fibrosis and scarring. However, it is likely that the same cell types that contribute to protective anti-*Chlamydia* responses may also be to blame for triggering the pathologic changes associated with chlamydial infection. Because IFNγ is a major player in promoting *Chlamydia* persistence and immunopathology [4,53] and

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infection-primed $CD8^+$ T cells are a significant source of this type 1 cytokine, Tc1 cells may represent a double-edge sword in the immune response to *Chlamydia*.

Following ocular and genital tract infection with different *Ct* serovars, the chronic inflammation that develops at these sites can lead to blindness, tubal scarring, ectopic pregnancy, and infertility [1]. Available data demonstrate that IFNγ is amongst the cytokines that are present at higher levels in the conjunctiva and cervical secretions of *Ct*-infected individuals [20,62]. *Ct* infection can also cause reactive arthritis (ReA), as it can be found in the joints of arthritis patients [63]. However, a *Ct* infection is not enough for development of ReA. Individuals with a history of *Ct* infection and who also express the MHC class I allele HLA-B27 have an increased risk of developing this joint disease [64]. Because MHC class I is involved, this could point to an immunopathological function of $CD8^+$ T cells. *Ct* infection-primed $CD8^+$ T cells could cause joint damage by inducing inflammation following the recognition of HLA-B27-presented chlamydial peptides on the surface of *Ct*-infected synovial cells. Alternatively, HLA-B27 could act as an autoantigen, as there is evidence that a peptide derived from this MHC class I allele has homology with a chlamydial peptide, and this molecular mimicry could lead to an autoimmune response in the joint [65].

Cpn infection has been associated with a spectrum of chronic inflammatory conditions including COPD and atherosclerosis [2,3]. *Cpn*-reactive $CD8^+$ T cells have been detected in the sputum from patients with COPD that are infected with this pathogen, and in *Cpn*-positive plaque from atherosclerotic persons [19, unpublished]. However, the contribution of *Cpn*-specific $CD8^+$ T cells to airway and coronary artery damage is unknown. Because *Cpn* can infect and grow within vascular endothelial cells, macrophages and smooth muscle cells, the inability of the immune response to clear *Cpn* infection from the vessel wall may set the stage for chronic inflammation, exacerbation of atheroma formation, and subsequent cardiac events. The increase of $CD8^+$ T cells in *Cpn*-positive symptomatic carotid plaque [19] suggests that an enhanced proinflammatory Tc1 response in atherosclerotic lesions may contribute to plaque destabilization.

Because of the serious sequelae caused by *Chlamydia* infections, it is imperative that vaccines are evaluated for their pathological potential. Indeed, vaccines that stimulate the immune system, yet fail to effectively control pathogen growth may only exacerbate tissue damage.

11. Immune evasion strategies of *Chlamydia*

Despite the evidence for an induction of protective *Chlamydia*-specific $CD8^+$ T cell responses, infected hosts frequently fail to completely clear the organism. Because a chlamydial infection only generates short-lived partial immunity, this may explain why reinfections are common and the establishment of bacterial persistence is favored. A suboptimal $CD8^+$ T cell response during *Chlamydia* infection may result from host genetic factors that influence the breadth and magnitude of the response and from strategies co-opted by the pathogen to evade immune recognition. Several studies support the existence of different immune evasion mechanisms that may allow *Chlamydia* to persist within the host. However, thus far there is no data proving that these strategies actually operate in a *Chlamydia*-infected host.

One of these strategies is based on the demonstrated ability of *Ct* and *Cpn* to inhibit apoptosis of host cells [66,67]. By exhibiting antiapoptotic activity, these two pathogens ensure that host cell lysis does not occur prior to the completion of the developmental cycle. Inhibition of apoptosis could also potentially limit the number of apoptotic infected cells available to pAPCs for cross-priming of $CD8^+$ T cells, and allow infected cells to resist killing by effector $CD8^+$ T cells.

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A second strategy that *Chlamydia* may employ to avoid T cell-mediated immune recognition is by downregulating MHC class I and II expression on infected cells. Accordingly, a chlamydial protease-like activity factor (CPAF) secreted into the cytosol of *Ct*- or *Cpn*-infected cells degrades the host transcription factors RFX5 and USF1, which are needed for the constitutive and IFNγ-inducible expression of MHC class I and II molecules, respectively [68–71]. Through this immune evasion strategy, *Chlamydia* could hamper both, T cell priming and $CD8^+$ T cell-mediated recognition of infected cells.

Another potential mechanism by which *Chlamydia* could reduce the pathogen-specific $CD8^+$ T cell response is via altered peptide ligand-(APL)-mediated antagonism (unpublished). MHC class I-binding chlamydial peptides representing antagonistic variants of defined *Chlamydia* $CD8^+$ T cell epitopes may upon interaction with such epitope-specific $CD8^+$ T cells transduce qualitatively different TCR signals that could result in the partial elimination of effector functions or in T cell anergy. For instance, two *Cpn* Inc-derived peptides may participate in this phenomenon during *Cpn* infection as the Cpn0126 peptide LQQCFSDL acts as an in vitro antagonistic APL for protective $CD8^+$ Tc1 cells to the Cpn0585 epitope LQQRYSRL (unpublished). $CD8^+$ T cell antagonism could also occur in *Cpn*-infected hosts that are also acutely or persistently infected with *Ct* or vice versa. This antagonism across related chlamydial pathogens is suggested by the existence of *Ct* peptides with sequences that only differ from known *Cpn* $CD8^+$ T cell epitopes at potential TCR contact positions, such as the *Ct* Omp85 peptide GTYQFTKL, which antagonizes the functional activity of $CD8^+$ Tc1 cells to the *Cpn* Omp85 epitope GTYHFTKL (unpublished). Thus, APL-mediated antagonism could hamper the ability of effector $CD8^+$ T cells to detect and destroy *Chlamydia*-infected cells and allow the pathogen to persist in the host.

12. Concluding remarks

$CD8^+$ T cells are primed during *Chlamydia* infection. Depletion, adoptive transfer, and vaccination studies indicate that $CD8^+$ T cells contribute to protective immunity against *Ct* and *Cpn*. Of the chlamydial antigens targeted by infection-primed murine and human $CD8^+$ T cells, most are intimately associated with the developing organisms or localize to the inclusion membrane. Nevertheless, an endogenous processing pathway appears to be mostly responsible for the MHC class Ia- or class Ib-restricted recognition of *Chlamydia*-infected cells by pathogen-specific $CD8^+$ T cells. Although IFNγ secretion is a key mechanism by which $CD8^+$ T cells control *Chlamydia* replication, it is unlikely that monofunctional IFNγ-producing T cells are as protective and long-lived as those that are multifunctional.

Future studies on $CD8^+$ T cell priming, trafficking, antigen-specificity, surface phenotype, and multifunctionality of *Chlamydia*-specific $CD8^+$ T cells will provide insight into the aspects of the response that are most likely to contribute to protection and those that mediate immunopathology. Moreover, this information will prove essential to determine the feasibility of generating *Chlamydia* vaccines capable of reducing the pathogen's ability to grow and spread in the infected host and thereby prevent the development of chronic or persistent infections. Such vaccines will most likely incorporate multiple antigens or epitopes and a potent and safe adjuvant that stimulate multiple arms of the immune system, including multifunctional $CD8^+$ T cells capable of eliminating infected cells without causing serious tissue damage.

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Table 1

Chlamydia antigens recognized by infection-primed $CD8^+$ T cells

Protein	Chlamydia species	Predicted Localization	Number of epitopes (restricting MHC I allele)	Antigen Identification Method	$CD8^+$ T cell recognition of infected cells	Reference
Omp2 (Cpn0557)	*C. pneumoniae*	Outer membrane	4 (H-2 K^b, H-2 D^b, H-2 K^d, H-2 D^d)	MHC I motif prediction	Yes, No	[24,38]
DnaK (Cpn0503)	*C. pneumoniae*	Cytosolic, surface-exposed	2 (H-2 D^b, HLA-A*0201)	MHC I motif prediction	Yes (H-2); N.T. (HLA-A*0201)	[24,33]
76 kDa protein (Cpn0728)	*C. pneumoniae*	Surface-exposed	1 (H-2 D^b)	MHC I motif prediction	Yes	[24]
43 kDa homolog protein 1	*C. pneumoniae*	N.D.	3 (H-2 K^b, H-2 D^b)	MHC I motif prediction	Yes	[24]
43 kDa homolog protein 2	*C. pneumoniae*	N.D.	2 (H-2 K^b)	MHC I motif prediction	Yes	[24]
43 kDa homolog protein 3	*C. pneumoniae*	N.D.	1 (H-2 K^b)	MHC I motif prediction	Yes	[24]
43 kDa homolog protein 4	*C. pneumoniae*	N.D.	2 (H-2 K^b, H-2 D^b)	MHC I motif prediction	Yes	[24]
MOMP (Cpn0695)	*C. pneumoniae*	Outer membrane	3 (H-2 K^b, H-2 D^b, HHD Transgenic HLA-A2)	MHC I motif prediction	Yes, No (H-2); N.T. (HHD)	[24,32,38]
Pmp10 (Cpn0449)	*C. pneumoniae*	Outer membrane	2 (H-2K^b)	MHC I motif prediction	Yes	[24]
YaeT (Cpn0300)	*C. pneumoniae*	Outer membrane	1 (H-2K^b)	MHC I motif prediction	Yes	[24]
OmpB (Cpn0854)	*C. pneumoniae*	Outer membrane	1 (H-2K^b)	MHC I motif prediction	Yes	[24]
IncA homolog (Cpn0585)	*C. pneumoniae*	Inclusion membrane	2 (H-2 K^b, H-2 D^b)	MHC I motif prediction	Yes	[24]
Conserved hypothetica l protein (CP0021)	*C. pneumoniae*	Putative secreted (heterologous T3S)	1 (H2-M3)	MHC I motif prediction	Yes	[25]
SmpB (CP0421)	*C. pneumoniae*	Cytosolic	1 (H2-M3)	MHC I motif prediction	Yes	[25]
Cap1 (CT529)	*C. trachomatis*	Inclusion membrane	1 (H-2 K^d)	Expression cloning system	Yes	[34]
CrpA (CT442)	*C. trachomatis*	Inclusion membrane	1 (H-2 D^b)	Expression cloning system	Yes	[39]
PmpI (CT874; CTL0254)	*C. trachomatis*	Outer membrane	2 (H-2 D^b)	Caged MHC I tetramers	N.T.	[36]
Omp2 (CT443)	*C. trachomatis*	Outer membrane	1 (HLA-A*0101)	Expression cloning system	Yes	[35]
MOMP (CT681)	*C. trachomatis*	Outer membrane	5 (HLA-A2, HLA-B51)	MHC I motif prediction	Yes (HLA-A2); N.T. (HLA-B51)	[23]
PmpC (CT414)	*C. trachomatis*	Outer membrane	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
ClpC (CT286)	*C. trachomatis*	N.D.	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
Hypothetic al protein (CT339)	*C. trachomatis*	Predicted membrane	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]

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Protein	Chlamydia species	Predicted Localization	Number of epitopes (restricting MHC I allele)	Antigen Identification Method	$CD8^+$ T cell recognition of infected cells	Reference
PapQ (CT601)	*C. trachomatis*	N.D.	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
Hypothetic al protein (CT610)	*C. trachomatis*	N.D.	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
NADH-ubiquinone oxidoreduct ase (CT634)	*C. trachomatis*	Predicted membrane	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
RecC (CT640)	*C. trachomatis*	Predicted cytosolic	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
YgeD (CT641)	*C. trachomatis*	Predicted membrane	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
FtsH (CT841)	*C. trachomatis*	Inner membrane	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
Hypothetic al protein (CT847)	*C. trachomatis*	Predicted secreted	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]
Hypothetic al protein (CT850)	*C. trachomatis*	Predicted inclusion membrane	1 (HLA-B*2705)	MHC I/proteasome cleavage motif prediction	N.T.	[37]