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Subunit vaccines for the prevention of mucosal infection with *Chlamydia trachomatis*

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Abstract

Chlamydia trachomatis is the most common preventable cause of tubal infertility in women. In high-income countries, despite public health control efforts, *C. trachomatis* case rates continue to rise. Most medium and low-income countries lack any *Chlamydia* control program; therefore, a vaccine is essential for the control of *Chlamydia* infections. A rationally designed *Chlamydia* vaccine requires understanding of the immunological correlates of protective immunity, pathological responses to this mucosal pathogen, identification of optimal vaccine antigens and selection of suitable adjuvant delivery systems that engender protective immunity. Fortunately, *Chlamydia* vaccinology is facilitated by genomic knowledge and by murine models that reproduce many of the features of human *C. trachomatis* infection. This article reviews recent progress in these areas with a focus on subunit vaccine development.

Keywords

Chlamydia; vaccine; antigen; adjuvant; tissue-resident memory T cells; immunoproteomics

Chlamydia trachomatis genital tract infection is the most prevalent bacterial sexually transmitted infection in the United States and likely globally. The World Health Organization (WHO) estimates that over 130 million new cases of *C. trachomatis* infection occur each year including 68 million new infections in women, of whom up to one million become infertile following the development of pelvic inflammatory disease (PID) [1]. In 2013, there were 1.4 million cases of *Chlamydia* infection reported in the United States to the Centers for Disease Control and Prevention (CDC) [2]. Reported cases are thought to represent only 50% of the actual number of cases. The highest age-specific rate was found among persons aged 14–24 years where the prevalence was nearly three times that found in persons aged 25–39 years. A recent study estimated that the direct lifetime medical cost in

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Declaration of interest

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United States from the estimated annual 2.8 million cases of *C. trachomatis* infections was greater than 500 million US dollars [3].

C. trachomatis is a Gram-negative obligate intracellular bacterium. This bacterium most commonly infects columnar epithelial cells of the endocervix of women and urethra of men to initiate local inflammation that causes mucopurulent cervicitis in women and nongonococal urethritis in men, respectively. Between 70–90% of women and 30–50% of men with infection are asymptomatic and consequently most people with *C. trachomatis* infection do not seek testing and treatment. Untreated infection is the source of onward transmission and in women can spread from the cervix to the upper reproductive tract (i.e., uterus, fallopian tubes) and cause PID, tubal factor infertility and ectopic pregnancy [4] (Fig. 1). It is estimated that about 15% of untreated *Chlamydia*l infections lead to PID and 10 to 15% of PID results in infertility. A *C. trachomatis* vaccine that prevents cervical infection, PID and its sequelae has tremendous potential to improve women's health worldwide.

Public health measures, including national screening recommendations, partner identification, and treatment to control *C. trachomatis* infection, have been ongoing for over two decades in many developed countries. However, despite these public health efforts, case rates of *C. trachomatis* infection have been rising over the past two decades. The failure of public health control program may be due to early antibiotic treatment blunting the development of individual protective immunity resulting in reduction of herd immunity to *C. trachomatis* [5]. This phenomenon has been termed as the arrested immunity hypothesis. Experimental studies that used the mouse model of *Chlamydia* genital tract infection have demonstrated that early antibiotic treatment interrupts the establishment of protective immunity [6]. As antibiotics are not the ultimate solution, development of an effective *Chlamydia* vaccine is the essential next step to control this persistent health problem [7].

Chlamydia vaccine research started in 1959 when *C. trachomatis* was first isolated in egg yolk in China [8]. Shortly after human *Chlamydia* vaccine trials were conducted using chemically inactivated whole organism formulated with a variety of oil in water adjuvants. The best results of these trials showed that up to 70% of vaccinees were protected against the ocular disease, trachoma. However, immunity quickly waned over time and was no longer detectable three years after vaccination [9]. Vaccine studies in non-human primates demonstrated that the best protection required the highest amount of organism indicating that the inactivated *C. trachomatis* vaccine was of limited immunogenicity. Furthermore the best protection was seen when the same *C. trachomatis* strain was used for vaccine and challenge infection. Of concern, *C. trachomatis* infection in some primates resulted in more severe disease with worse inflammation post-vaccination demonstrating a role for incomplete immunity in enhanced inflammatory pathology [9,10]. However a critical analysis of human trachoma vaccine trials failed to demonstrate similar vaccine immunopathology in humans [11]. Nonetheless concern about exacerbated inflammatory pathology in previously vaccinated non-human primates has continued to be a barrier that impedes human *Chlamydia* vaccine trial research.

Contemporary *C. trachomatis* vaccine research has focused on the development of subunit vaccines, live attenuated *C. trachomatis* vaccines or whole inactivated organism vaccines.

Among these three options, the whole inactivated organism vaccines are least attractive as they were the vaccine vehicles that elicited inflammatory pathology in earlier primate models of trachoma. Many promising studies have evaluated the use of plasmid-deficient *C. muridarum* as live attenuated vaccine in mouse models and recent studies have shown the efficacy of a plasmid-deficient *C. trachomatis* serovar A vaccine in the macaque trachoma model. Surprisingly, the rhesus macaque genital tract model revealed that plasmid-deficient *C. trachomatis* serovar D vaccine induced inflammation equivalent to wild-type and failed to prevent challenge infection. This observation raises concerns that plasmid-deficient genital strains may not be sufficiently attenuated to use as a live attenuated vaccine. This review therefore focuses on subunit vaccine research. Subunit vaccines hold the promise of a chemically defined product capable of eliciting a predefined protective immune response. Furthermore subunit vaccine development is based on the solid foundation of *Chlamydia* genomics [12,13]. A rational *Chlamydia* subunit vaccine design requires: 1) an understanding of the immunological correlates of protective immunity and pathological responses to this mucosal pathogen, 2) identification of effective vaccine antigens, 3) selection of a suitable adjuvant and delivery system to induce long lasting immunity, and, 4) appropriate animal models to test vaccine efficacy. This review summarizes recent progress in these four areas with a focus on concepts and approaches undertaken in our laboratory.

Immune correlates of protective immunity against *C. trachomatis*

Complete characterization of immunity to *C. trachomatis* genital tract infection and identification of immune correlates of protective immunity is important for the development of an effective subunit *Chlamydia* vaccine. However, since it is not ethically acceptable to withhold treatment for known infections in humans, it is impossible to directly define the natural course of *C. trachomatis* infection in humans and the risk of reinfection after primary infection that has naturally resolved. Experimental challenge with *C. trachomatis* in humans is fraught with ethical concerns regarding immunopathology. Therefore available human data of protective immunity to *C. trachomatis* is mostly indirect and inferred from epidemiological studies. Overall, the data from human studies reveal that a degree of partial protective immunity against reinfection is established after human genital infection of *C. trachomatis* [14]. Th1-type immune mechanism including CD4 lymphocytes and IFN-γ are correlates of acquired immunity in human *C. trachomatis* infections [15]. In 2005 we published a prospective study involving sex workers at high risk of *C. trachomatis* infection which showed that IFN-γ and IL-13 production by peripheral blood mononuclear cells (PBMCs) in response to *C. trachomatis* antigens was associated with reduced risk of incident infection, and thus could represent a phenotypic marker of human protective immunity to *C. trachomatis* [16]. Studies on women with *C. trachomatis* infection showed that *Chlamydia*-specific IgA in cervical secretions reduced the intensity of shedding [17]. However, anti-*Chlamydia* IgA in cervical mucus did not correlate with the risk of reinfection [16]. Direct evidence for protective mechanisms to *Chlamydia* genital infection has come from animal models including mouse and non-human primate models. The role of different immune components and their interactions in protective immunity to *Chlamydia* are described below.

CD4 T cells

Results from the mouse demonstrate that CD4 T cells play a dominant protective role in *Chlamydia* genital infection, which support results from human studies. The species-matched *Chlamydia muridarum* mouse model has identified two CD4 T cell-mediated mechanisms that are sufficient for clearing *Chlamydia* from the genital tract.

The first CD4 mechanism, discovered in the mid-1990s, is dependent on IFN-γ and inducible nitric oxide synthetase (iNOS) [18,19]. In this mechanism, *Chlamydia* specific CD4 T cells recognize infected epithelial cells and produce IFN-γ. IFN-γ and T cell-epithelial cell contact via ICAM-1 then induces high expression of epithelial iNOS that generates chlamydiacidal levels of nitric oxide [20]. This mechanism has been shown to be functional in both mouse and human epithelial cells. In recent murine vaccine studies our lab demonstrated that multi-functional CD4 T cells that co-secrete IFN-γ and TNF-α was a better correlate of immunity against *C. muridarum* infection than CD4 T cells that secreted IFN-γ alone [21]. Although the requirement for multifunctional Th1 is not well understood, it is possible that IFN-γ and TNF-α have a synergistic effect in the induction of iNOS and/or that multifunctional Th1 T cells possess a more robust degranulation phenotype compared to Th1 T cells producing only IFN-γ. Consistent with the importance of multiple cytokines, Johnson *et al.* [22] showed that treatment of murine epithelial cells with IFN-γ alone was not sufficient to terminate *C. muridarum* replication, while treatment of murine epithelial cells with activated T cell supernatants was a potent terminator of *C. muridarum* replication via an iNOS dependent mechanism

The second CD4-mediated *Chlamydia* replication termination mechanism was identified by Johnson's group in 2010 [23] and is dependent on a subset of CD4 T cells that express a granule associated protein called Plac8. This mechanism like iNOS dependent immunity relied on T cell-epithelial cell contact followed by T cell degranulation and was only seen in *Chlamydia*-specific CD4 T cells that expressed Plac8 [22]. The discovery of the Plac8-dependent mechanism resolved a longstanding mystery in the *Chlamydia* immunobiology that iNOS knockout mice were not significantly compromised in their ability to clear *C. muridarum* genital tract infections [24]. This may be because iNOS knockout mice still had the Plac8 clearance mechanism in place to clear *C. muridarum* genital tract infections. This was supported by the observation that Plac8 knockout mice were modestly compromised in their ability to clear *C. muridarum* genital tract infection at late points (> 3 weeks) after infection [22]. When Plac8 knockout mice were treated with an iNOS inhibitor (functionally a dual Plac8 and iNOS knockout) they were profoundly unable to clear *C. muridarum* genital infections over an 8 week period. All *Chlamydia* specific Th1 cells seem to possess iNOS-dependent clearance mechanism. However, only a subset of *Chlamydia* Th1 cells express Plac8 ($CD4_{Plac8}$) capable of utilizing both degranulation-dependent and iNOS-dependent clearance mechanisms. Based on these data, $CD4_{Plac8}$ cells that secrete IFN-γ and TNF-α may be the long sought biomarker for protective immunity. Further investigation on the role of this novel specific CD4 T cell subpopulation in natural and vaccine-generated immunity needs to be undertaken. Importantly the Plac8 mediated *Chlamydia* inhibition has been demonstrated only in the mouse model and needs to be identified in humans as well.

CD8 T cells

Although *Chlamydia* infections induce both CD4 and CD8 immune responses in humans and mice, the role of CD8 T cells in protective immunity is not clear. In the murine model, it is generally observed that CD8 T cells are not necessary for clearing genital tract infection or protecting against reinfection [25,26]. In contrast, they seem to contribute significantly to upper genital tract pathology [27,28] and infertility [29] at least in the murine model. There is a possibility that the protective role mediated by a subset CD8 T cells is redundant in the presence of CD4 T cell protective immunity. An early study reported that adoptive transfer of a multifunctional *Chlamydia*-specific CD8 T cell clone into chronically infected nude mice cleared *C. muridarum* infection from the genital tract [30]. A recent non-human primate study reported that CD8 T cells play an important role in live-attenuated trachoma vaccine-mediated protective immunity [31]. This study showed that CD8 T cells from protected macaques exhibited proliferation against *Chlamydia* antigen and that depletion of CD8 T cells completely abrogated protective immunity. Polyfunctional CD8 T cells have also been shown to better predict protection against HIV infection [32] and the HIV-specific responses are associated with certain HLA class I [33].

Tissue-resident memory T cells (Trm)

Protective T cells need to be at the site of infectious challenge in order to provide immunity. Given that *Chlamydia* is a mucosal pathogen, this implies that such cells need to be resident in tissue beneath the mucosa. During infection or immunization, a subpopulation of effector T cells seed both lymphoid tissue and non-lymphoid tissue where these cells differentiate and develop into tissue-resident memory T cells with distinct phenotype and function for long-term residency and survival. Such tissue-resident memory T cells are on standby and capable of immediately recognizing pathogens that enter through the local tissues and mount robust local immune responses to limit the spread of infection. An understanding of the biology of Trms should provide important insights into the protective immune mechanisms at the site of pathogen entry, which will be a basis for designing better vaccines against many mucosal pathogens including *Chlamydia*.

Trm was first described in the *Chlamydia* field in 1990 when Kiviat *et al.* [34] observed lymphoid follicles with T cells and plasma cells in endometrial biopsies of women with *C. trachomatis* infections. Morrison *et al.* [35] conducted an in situ immunohistochemistry study in the *C. muridarum* model and saw predominantly clustered CD4 T cells with few CD8 T cells and B cells present in the genital tract long after infection had resolved (day 70). These observations provide the best histopathological correlate of protective immunity. Recently, Stary *et al.* [36,37] demonstrated that two waves of protective CD4 Th1 cells develop after mucosal immunization (either intrauterine or intranasal application) with UV-inactivated *C. trachomatis* organism formulated with a cationic nanocarrier adjuvant incorporating the TLR-7/8 agonist, resiquimod. Mucosal vaccination induced a wave of effector T cells that seeded uterine mucosa and established resident memory T cells (first wave) in addition to circulating memory T cells. Upon genital *C. trachomatis* infection, local reactivation of uterine Trm cells efficiently triggered the recruitment of circulating memory T cells (second wave). Optimal pathogen clearance was shown to be dependent on both waves of memory cells. Importantly, systemic immunization did not induce Trm cells and

only induced the second wave, and mice were suboptimally protected, even when circulating memory cells were abundant (Fig. 2). Thus, the protective effect of this mucosal vaccine depended on the synergistic action of two memory T cell subsets: tissue resident memory T cells and circulating memory T cells. Early mucosal seeding with Trm CD4 T cells was the key to a successful *C. trachomatis* vaccine in this model system. This finding constitutes a major step forward in understanding *C. trachomatis* immunology and provides a mechanistic basis for mucosal immunization against *C. trachomatis*.

B cells and antibody

B cell and antibody-mediated immunity against *Chlamydia* infection is not completely understood [38]. In 1997, Su *et al.* demonstrated that B cell-deficient mice (μMT) cleared *C. muridarium* primary infections with normal kinetics of bacterial shedding from the genital tract but knockout mice were more susceptible to reinfection compared to wild type control mice [39]. Further studies conducted by Morrison and colleagues showed that immune wild type mice that were depleted of either circulating CD4 and/or CD8 T cells by parenteral antibody treatment were able to clear the secondary *Chlamydia* infection [26]. Strikingly immune B cell-deficient mice were unable to resolve secondary infection in the absence of T cells [40]. However adoptive transfer of immune serum into immune B cell deficient mice in the absence of T cells reconstituted their ability to clear secondary infection [41]. Surprisingly, passive transfer of immune serum into naïve wild type mice did not provide protection from primary infection. While these data show that B cells and antibody have a role in clearing secondary infection, direct antibody-dependent neutralization or complement-mediated killing is unlikely to account for antibody-mediated protection during secondary infection since passive transfer of immune serum only protected antigen-experienced hosts rather than naïve mice. It may be that parenternal anti CD4 antibody treatment to deplete CD4 T cells failed to completely deplete tissue resident CD4 T cells. An alternative protection mechanism mediated by antibody may be enhanced antigen presentation mediated by Fc receptors at the mucosal tissue level that expanded mucosal Trm cells. It is known that professional antigen presenting cells bear Fc receptors and are 1,000 times more efficient at presenting antibody coated antigen [42]. In support of this conjecture Moore *et al.* [43] reported that Fc receptor-mediated antibody effector mechanisms are indeed important in anti-*Chlamydia* secondary immune responses. These results reflect infection-induced immunity and parallel studies by Farris et al [44] used recombinant MOMP as vaccine. They observed that MOMP immunity is more strongly antibody dependent than is infection immunity, although both also required CD4 T cells.

Recently, Li and McSorley demonstrated that local *Chlamydia*-specific CD4 T cell priming was also significantly reduced in B cell-deficient mice. This reduced local response was accompanied by spread of *Chlamydia* throughout the peritoneal cavity, resembling the pathology of women with the Fitz-Hugh Curtis syndrome associated with *C. trachomatis* PID. These findings reveal a role for B cells in limiting the intra peritoneal spread of *Chlamydia* during primary infection [45].

Animal models for vaccine research

Various animal models have been developed to evaluate *Chlamydia* vaccine efficacy which include mouse, guinea pig and nonhuman primate models [46].

C. muridarum mouse model

Mice are the most widely used animals for the study of genital *Chlamydia* infection and evaluation of candidate vaccines. The mouse models have advantages because of their small size, ease of handling, a wealth of immunologic reagents and well-characterized inbred and knockout strains. *C. muridarum* genital infection model is established by intravaginal infection. Of note *C. muridarum* is a natural mouse pathogen that causes pneumonitis, was originally isolated from the lung of mice and may spontaneously spread via the respiratory route. Even though the genital tract model is somewhat artificial in its route of infection, it does recapitulate many significant features of human *C. trachomatis* pelvic infections including ascending infection that results in salpingitis, hydrosalpinx and infertility [47]. Macrophages and lymphocytes including B cells, CD4 T cells and CD8 T cells infiltrate the genital tract tissues and CD4 T cells predominate throughout the course of infection and are essential for protective immunity. Mice that clear genital infection with *C. muridarum* are significantly resistant to reinfection manifested as low shedding of short duration. Immunity to *Chlamydia* genital infection and vaccine development using the *C. muridarum* murine model was comprehensively and elegantly summarized by Farris and Morrison [48].

There are also some notable differences in immunity and pathogenesis between the mouse model and human infection, which need to be considered before translating mouse data into human pathobiology. *C. trachomatis* possess different *ompA* genotypes that are specifically associated with conjunctival, genital and LGV infections. However, *C. muridarum* has a nonvariant *ompA* genotype that does not reflect the antigenic diversity of human *C. trachomatis* infection. Protective immunity in the *C. muridarum* genital infection mouse model develops very quickly and infection is resolved within 4 weeks while it can take several months for humans to develop immunity and clear infection. The failure to develop immunity can result in long-term chronic infection after *C. trachomatis* primary infection in humans, which does not seem to occur with *C. muridarum* infection [49]. In addition, primary *C. muridarum* genital infection is enough to cause most mice to develop tubal dilatation followed by hydrosalpinx that leads to infertility, while the risk of fallopian tube pathology in women with *C. trachomatis* PID occurs in only 10 to 15% of women whose increases with repeated infection [50].

C. trachomatis transcervical mouse model

Mice can also be genitally infected with human *C. trachomatis* serovars. However intravaginal inoculation with *C. trachomatis* generally produces mild genital infection that is unable to ascend to the upper genital tract and is cleared mainly by innate immune responses [51]. Gondek *et al.* established the *C. trachomatis* serovar L2 transcervical infection model where the organism is directly inoculated into the uterine cavity to establish productive infection. Immune CD4 T cells were demonstrated to be essential for clearance in this model [52]. Transcervical inoculation of *C. trachomatis* is performed using a Non-Surgical Embryo

Transfer (NSET) device for mice. Mice previously transcervically infected with *C. trachomatis* serovar L2 were shown to be resistant to challenge infection, confirming that this model can be used to evaluate *C. trachomatis* vaccine candidates. Recently transcervical *C. trachomatis* serovar D infection was also shown to be an efficient and reliable model to evaluate *C. trachomatis* vaccine candidates [53]. An important caveat is the *C. trachomatis* transcervical model requires a challenge inoculum several orders of magnitude greater than the *C. muridarum* model or natural human *C. trachomatis* infection.

C. caviae guinea pig model

Another rodent model for *Chlamydia* genital infection is the guinea pig infected with *C. caviae*. Although the *C. caviae* guinea pig model is constrained by availability of reagent and lack of knockout animals, an important advantage of this model is that the pathology following *Chlamydia* infection, effects of reproductive hormones on infection, and sexual transmission of infection more closely resemble human *Chlamydia* pathobiology than does the mouse model. The model remains under utilized in *Chlamydia* vaccine research and may be especially useful for trachoma vaccine research.

C. trachomatis non-human primate models

Because of the closer evolutionary relatedness between human and non-human primates (NHP), primates have been valuable in the study of pelvic inflammatory disease caused by *C. trachomatis* [54]. However, the use of non-human primates to evaluate vaccine efficacy against *C. trachomatis* genital tract infection has had limited success. Although several studies have shown that the NHP model is a promising platform to evaluate trachoma vaccine, there is only one vaccine study that has targeted genital tract infection [55]. In this study, a rhesus monkey model of *Chlamydial* genital tract infection was used to investigate the efficacy of a plasmid-deficient *C. trachomatis* strain (CTD153) as a live attenuated vaccine. The CTD153 strain has an attachment/uptake defect and induces lower levels of cytokine production in vitro and in the murine genital tract [56]. Unfortunately, resistance to reinfection developed in the macaque genital tract only after multiple challenge infections irrespective of the presence or absence of the plasmid. Since there is no known natural NHP strain of *Chlamydia*, high doses of *C. trachomatis* inoculation were also required. In addition, differences in immune responses and disease states were found when using different *C. trachomatis* serovars and among outbred NHPs, which make the NHP model much more complicated to standardize for vaccinology studies. Therefore, the general consensus is that NHP model is not feasible nor necessary for use as a model to evaluate vaccines against *C. trachomatis* genital infection before proceeding to human trials.

Discovery of novel *Chlamydia* antigens

Much contemporary *C. trachomatis* vaccine research has focused on the production of subunit vaccines based on individual protective *C. trachomatis* proteins. To date, the *Chlamydia* major outer membrane protein (MOMP) has been the most widely investigated subunit vaccine candidate in multiple animal models. However studies indicate that vaccines based on MOMP alone afford only incomplete protection and efficacy is highly dependent on conformational structure. Other candidate antigens including outer membrane protein 2

(OMP2), heat shock protein 60 (HSP60), polymorphic membrane protein D (PmpD), cysteine rich protein A (CrpA), homolog of Yersinia pseudotuberculosis YopD (YopD), enolase and *Chlamydia* protease-like activity factor (CPAF) have been identified as vaccine antigens [48,57]), but none of these candidates is demonstrably better than MOMP. With the *Chlamydia* genome having been entirely deciphered [12,13], theoretically all potential vaccine antigens are now known. Recently novel strategies based on genomics to discover T cell antigens and antibody-inducing antigens for the development of *Chlamydia* vaccines have been reported resulting in a significant expansion in the number of antigens evaluated.

Reverse vaccinology approach

Reverse vaccinology is a term coined by Rappuoli *et al* [58]. It is based on unbiased bioinformatics analysis of the whole genome to predict and select novel antigens for vaccine development. Finco *et al.* [59] reported a strategy for discovery of *Chlamydia* vaccine candidates involving four major steps. (1) the *C. trachomatis* serovar D genome was bioinformatically analysed to identify proteins associated with the outer and inner membrane, secreted proteins and proteins predicted to be involved in virulence or pathogenicity; (2) 120 *Chlamydia* proteins were selected for expression and purification in an *Escherichia coli* expression system; (3) recombinant proteins were used to generate protein arrays for recognition by sera from *C. trachomatis*-infected humans and for their ability to stimulate IFN-γ producing CD4 T cells from *C. trachomatis*-infected mice; and (4) for the protective activity of combinations of selected antigens (*C. muridarum* homologs) as evaluated using the *C. muridarum* lung infection model. The study led to the discovery of 21 pure B cell antigens (antibody-inducing antigens), 16 pure T cell antigens (CD4/IFN-γ-inducing antigens) and 5 antigens inducing both T cell responses and antibody. Seven proteins were finally identified as protective antigens in the animal model and a combination of 4 antigens provided robust additive protection. The protection was mainly due to cellular immunity mediated by CD4 T cells. A second study [60] used a whole genome scale proteome array consisting of 908 *C. trachomatis* proteins to profile human antibody responses in 99 women with *C. trachomatis* infection. This study showed that 719 *Chlamydia* proteins were recognized by sera from at least one subject; only 27 proteins were recognized by 50% or more of the subject sera and were suggested as potential vaccine candidates. However the study by Finco *et al.* [59] showed that proteins with B cell epitopes do not substantially overlap with proteins containing T cell epitopes. Since only a minority of proteins have both T and B cell epitopes, this approach may not efficiently identify T cell antigens which are central for *Chlamydia* immunity.

Immunoproteomics approach

The immunoproteomic approach uses genomic information to guide the delineation of the T cell immunoproteome of a pathogen based on peptide binding by MHC molecules [61,62]. This approach became feasible due to advancements in tandem mass spectrometry MS/MS technology that provide improved sensitivity limits at, or below, one femtomole. The immunoproteomic approach identifies MHC class I and II bound pathogen-derived peptides that are directly eluted from infected primary dendritic cells (DCs). This approach has a false discovery rate of < 2% and creates a vast improvement for the positive validation rate compared to reverse vaccinology, presumably because its findings are empiric and result

from physiological antigen processing and presentation. Since the identified peptides depend on both the affinity for the MHC molecule as well as the frequency of their presentation, such peptides may only represent the most “fit” peptides rather than the entire antigenic repertoire. This approach involves the following steps; (i) generation of DCs from mouse bone marrow, (ii) infection of bone marrow derived DCs (BM-DCs) with the pathogen for12 or more hours, (iii) lysis and isolation of MHC class I and II molecules from pulsed BM-DCs using allele-specific anti-MHC monoclonal antibody affinity columns, (iv) elution of peptides from purified MHC molecules, (v) analysis of purified MHC-bound peptides by MS/MS and (vi) validation of the identified peptides, and their cloned parent proteins *in vitro* and *in vivo* to identify potential vaccine candidates.

The immunoproteomics approach is capable of identifying antigens able to stimulate CD4 T cells via MHC class II. With this technology 27 *C. muridarum* CD4 T cell antigens were identified in C57BL/6 mice, which represents about 3% of the *Chlamydia* proteome [53,61]. Excluding those *Chlamydia* proteins with significant sequence homology to human and other bacterial proteins,13 proteins were selected for evaluation as vaccine antigens. Eleven of these engendered significant protection against *C. muridarum* infection in mice and seven proteins (PmpG, PmpE, PmpF, TC0420, Aasf, TC0825, and RplF) induced protection better than or equal to the MOMP [63]. Further studies of DCs from C57BL/6 mice infected with the human strain, *C. trachomatis* serovar D revealed nine antigenic proteins that were orthologs between the *C. muridarum* and *C. trachomatis* proteome [53]. Of particular interest, from both *Chlamydia* species the polymorphic membrane family of proteins generated MHC class II binding epitopes at multiple sites within the protein sequence and on different MHC class II molecules. The *C. trachomatis* and *C. muridarum* genomes encode nine different Pmps (PmpA to PmpI) [12,13]. All nine *C. trachomatis* Pmps have been reported to mediate adhesion to human epithelial cells [64]. In aggregate five outer membrane proteins [four Pmps (PmpE, -F, -G, and -H) and MOMP] were identified as T cell antigens via the immunoproteomic approach suggesting that outer membrane proteins may have advantages over other groups of proteins in presenting to the immune system. It was previously reported that the three Pmps (PmpE, PmpG and PmpH) constitute 61% of the total Pmp protein abundance [65] and MOMP is already known to constitute over 60% of the total outer membrane protein mass [66]. Therefore high abundance proteins as well as outer membrane localization may play a role in favoring antigen presentation.

PmpG is the most protective *C. muridarum* T cell antigen yet discovered via the immunoproteomic approach. Vaccination with PmpG resulted in 20 times and 1,000 times reduction in day 6 and day 13 shedding post *C. muridarum* challenge, respectively compared to that of mice with no vaccination [67]. Two subsequent studies validated the immunodominance of PmpG in the murine model [34,68].

A single-component subunit vaccine however may not provide optimal protection against infection due to MHC variation in the human population, or because the antigen may be antigenically or phase variable. A successful *Chlamydia* vaccine will likely need to be composed of multiple *Chlamydia* recombinant proteins in order to provide a broad coverage in an outbred population and cross-protect against multiple variants of *C. trachomatis*. A shortcoming of MOMP is allelic variation. A shortcoming of Pmps is phase variation. Both

forms of antigenic variation are likely the evolutionary result of immune selection because these proteins are the targets of immunity. However combination of both antigens may be an ideal *Chlamydia* vaccine. Our studies demonstrate that a recombinant protein vaccine consisting of four Pmps (PmpEFGH) with MOMP formulated with a Th1 polarizing adjuvant significantly accelerated clearance in the C57BL/6 mouse *C. trachomatis* transcervical infection model [53] and in the *C. muridarum* genital infection mouse model using mice of different MHC backgrounds [69]. Overall we suggest that *Chlamydia* outer membrane proteins are likely to be the most important T cell antigens useful in the development of a *C. trachomatis* subunit vaccine.

Recombinant MOMP protein with VD4 multimers to engender protective B cell responses

MOMP has been studied as a leading vaccine candidate for 3 decades in multiple animal model systems and is capable of inducing both strong T and B cell immune responses. MOMP is an integral membrane protein that is very difficult to prepare in its native conformation. Recombinant MOMP immunization has provided only partial and variable protection most likely due to lack of the native structure of the protein [70]. Given that B cells and antibody play a role in T cell priming and limiting spread during initial infection as well as accelerating clearance during reinfection, a universally appealing approach in *Chlamydia* vaccine development is to select antigens such as the MOMP that are able to elicit antibody capable of binding to the surface of the native organism. However, the expense and technical difficulty related to the production of MOMP in its native conformation make it difficult to scale for vaccine development. Recently Olsen *et al.* [71] developed a novel recombinant MOMP vaccine construct based on engineering variable domain 4 (VD4) multimers within the MOMP sequence. The VD4 region from MOMP protein contains the highly conserved species-specific neutralizing B cell epitopes (LNPTIAG) that appears relevant to antibody-mediated protection against genital infection. Interestingly, the LNPTIAG epitope was also previously identified as a MOMP T cell epitope via the immunoproteomic approach [53]. They engineered MOMP with multimers of VD4 and this novel recombinant MOMP vaccine raised high titered antibody responses that neutralized most *C. trachomatis* serovars *in vitro*. Besides broadly neutralizing antibody responses, the construct also induced robust T cell responses, which conferred protection against vaginal infection and upper genital tract pathological changes in the mouse intravaginal *C. trachomatis* model. The inclusion of this newly developed recombinant MOMP with VD4 multimers may represent a novel solution for *Chlamydia* MOMP-based vaccine formulations. Determining the mechanism by which MOMP-VD4 multimers elicit functional antibodies is now an important research goal. Perhaps multimerization of the B-cell epitope allows this novel recombinant protein to directly activate antigen specific B cells.

Adjuvants and delivery systems for efficient vaccines against *Chlamydia*

Since recombinant proteins generally demonstrate poor immunogenicity, a significant challenge in developing an effective *Chlamydia* subunit vaccine is to discover an optimal adjuvant that delivers antigens in an appropriate way to elicit potent protective immunity *in vivo*. Immunological advances have revealed a much higher degree of complexity and

exquisite specificity of the innate immune system than previous thought, which has inspired a new generation of novel adjuvant formulations that tailor the induction of defined immune responses against specific pathogens [72]. Pattern recognition receptors (PRRs) are expressed in DCs either on cell surfaces or intracellular compartments. The binding of pathogen-derived molecules to the PRR receptors to detect invading pathogens is crucial for initiating innate immunity and consequently activating adaptive immune responses. To date, various families of PRRs have been identified including Toll Like Receptors, C-type lectin receptors (CLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR). The discovery of the prominent roles played by these receptors has clarified the mechanisms behind novel adjuvant technologies. Characteristics of innate immune receptors and their ligands were comprehensively reviewed [73]. In the past ten years, the range of adjuvants and delivery system used in experimental and clinical vaccines against infectious diseases, including *C. trachomatis*, has rapidly increased [48,74,75]. Below we highlight several studies that applied novel adjuvants and delivery systems for *Chlamydia* vaccines that have shown promise.

Liposome DDA formulated with TDB or MPL (DDA/TDB or DDA/MPL)

In the past several years, our laboratory screened a series of adjuvants using PmpG as a model antigen to evaluate vaccine protection in the *C. muridarum* genital tract infection model [63,67]. These adjuvants include three cationic liposome formulations (dimethyldioctadecylammonium bromide -trehalose 6,6=-dibehenate [DDA-TDB], DDA-monophosphoryl lipid A [DDA-MPL], and DDA-monomycolylglycerol [DDA-MMG]), ISCOM (AbISCO-100), CpG-ODN1826 and Montanide ISA720–CpG-ODN1826. We found that vaccination with DDA-MPL or DDA-TDB (also named CAF01) formulation conferred the greatest level of protection that generated the highest frequency of T cells coexpressing IFN-γ and TNF-α. The frequency of multifunctional CD4 T cells induced by different adjuvant formulations accurately tracked the corresponding pattern of protection against *C. muridarum* genital tract infection, supporting the hypothesis that IFN-γ and TNF-α-secreting CD4 T cells are a correlate of protective immunity [21].

The small cationic molecule DDA forms liposomes that act as antigen “depot” to ensure the long-term release of antigen. DDA does not have direct effects on the maturation of DCs, and the combination of DDA with TDB or MPL delivers the PAMPs to DCs via the liposomal surface charge, thereby potentiating immunostimulation. TDB selectively activates the FcR-Syk-Card9 pathway in antigen-presenting cells to induce a unique innate immune activation program that directs protective Th1 and Th17 immunity [76]. Ishikawa *et al.* [77] reported that the monocyte-inducible C-type lectin (Mincle) is the essential cell receptor for TDB. DDA-TDB (CAF01) promotes both strong humoral and cell mediated immune responses and has been found to be a very useful adjuvant for a number of different vaccines [78,79]. DDA/TDB is currently undergoing clinical trials in humans for both a tuberculosis [80] and HIV subunit vaccine [81]. MPL is a derivative of LPS but more than 100 times less toxic. MPL activates via TLR4 and triggers the Trif-dependent pathway [82]. MPL is licensed for vaccines against human papillomavirus types 16 and 18 (Cervarix GSK) and hepatitis B virus (Fendrix GSK) and thus may be a feasible adjuvant component for a human *C. trachomatis* vaccine.

Mucosal adjuvants

Mucosa are the major entry ports for most human pathogens including *Chlamydia*. Therefore mucosal adjuvants should be ideal to generate local immunity in mucosal compartments to prevent infections. However, most licensed vaccines are adminisitered parenterally and most are ineffective in generating immune responses that localize to the mucosa. Currently the most commonly used experimental mucosal adjuvants can be divided into toxin-based adjuvants (e.g. LT, CT), immunostimulatory adjuvants (e.g. MPL, CpG, QS21) and particulate adjuvants serving as delivery vehicles (e.g. virus-like particles, liposomes and emulsions). Newsted *et al.* [83] recently reviewed advances and challenges in mucosal adjuvant technology and the mucosal administration routes, targets, and human clinical testing.

LT and CT are potent, but also toxic, mucosal adjuvants. New generation of LT and CT adjuvants using site-directed mutagenesis can mitigate toxicity. LTK63 is one mutant that preserves adjuvant activity in the absence of enzymatic activity and toxicity. LTK63 combined with CpG was shown to be effective in both mucosal and systemic immunization [84]. The study by Finco *et al.* [59] used LTK63/CpG adjuvant co-administrated systematically with newly discovered T and B cell antigens to evaluate vaccine efficacy against *Chlamydia*. That study demonstrated that LTK/CpG vaccine formulations generated strong Th1 immune responses characterized by induction of antigen-specific CD4/IFN-γ co-expressing by TNF-α or IL-2. The impact on tissue resident T cells was not studied.

Charge switching synthetic adjuvant particles (cSAPs) recently reported by Stary *et al.* [36] is an exciting advancement in *Chlamydia* vaccine development. A triblock copolymer, poly(D,L-lactic-co-glycolic acid)-b-poly(L-histidine)-b-poly(ethylene glycol) (PLGA-PLH-PEG) is a biodegrdable nanocarrier that was developed recently to target encapsulated antibiotics to the bacterial surface [85]. The adjuvant cSAPs was created by incorporating a second polymer PLA that was covalently coupled to R848 (resiquimod), a potent TLR 7/8 intracellualr agonist (PLA-R484) into PLGA-PLH-PEG polymer. The cSAPs form a hydrophobic core PLGA with R484 and a hydrophilic surface consisting of PLH and PEG. At physiologic pH 7.4, cSAPs carry a slight negative surface charge but acidification to below pH 6.5 makes cSAPs cationic and thus able to conjugate with negatively charged bacteria such as *C. trachomatis*. The study demonstrated that mucosal immunization (either via an intrauterine or intranasal route) with UV inactivated *C. trachomatis* (UV-Ct) organism complexed with the adjuvant cSAPs elicited an excellent long-lived protection comparable to that conferred by live *C. trachomatis* immunization, whereas immunization with UV-Ct alone induced tolerogenic effect that rendered mice hypersusceptible to subsequent *C. trachomatis* challenge. This study showed that activation of naïve CD4 cells in the mucosal environment, in the presence or absence of the adjuvant cSAPs, differentially recruited CD103 negative or positive DCs that induced Th1 or Treg cells respectively. The mucosal immunization of UV-Ct with cSAP generates Trm that seeded the uterine mucosa and strongly correlated with protective immunity. Systemic immunization with cSAP and UV-Ct failed to elicit Trm and protection.

Expert commentary and five-year view

Although there is no licensed vaccine for human *C. trachomatis* infection, recent research is getting us closer to this goal. The findings on tissue-resident memory T cells have provided insight into protective immunity mechanisms to *C. trachomatis* infection [86]. A vaccine to prevent sexually transmitted *C. trachomatis* needs to develop tissue-resident memory T cells that are capable of detecting incoming *C. trachomatis* and mount a robust local immune response to limit the spread of infection. Since the frequency of circulating multifunctional CD4 T cells coexpressing IFN-γ and TNF-α most accurately correlates with the pattern of protection against *C. muridarum* genital tract infection, IFN-γ producing CD4 T cells that highly coexpress TNF-α could be used as a systemic marker for the evaluation of candidate vaccines. Immunoproteomics and reverse vaccinology approaches have identified several novel protective antigens and recombinant outer membrane proteins, such as Pmps and MOMP are particularly promising subunit vaccine candidates. Adjuvants and delivery systems are critical for subunit vaccine formulations. For example, the protective antigen PmpG formulated with various adjuvants showed different levels of protective immunity depending on the adjuvant chosen. PmpG formulated with the adjuvant DDA/TDB induced potent IFN-γ/TNFα/IL-17 CD4 immune responses and conferred substantial protection against *Chlamydia* challenge. However when PmpG was formulated with adjuvant CpG or alum, low immune responses were detected and no protection was observed. The discovery of pattern recognition receptors as part of the innate immune system is spurring many novel adjuvant strategies for vaccine delivery. The optimal *Chlamydia* vaccine adjuvant needs to induce multifunctional Trm in genital tissues and maintain them for long duration. The mucosal adjuvant cSAP formulated with a TLR 7/8 ligand and UV inactivated *C. trachomatis* generated Trm that seeded the genital tissue and provided excellent protection against infection and pathology. Since whole organism vaccines previously induced inflammatory pathology, a subunit *Chlamydia* recombinant protein vaccine formulated with cSAP should be attempted. While the mucosal adjuvant (charge switching synthetic adjuvant particles, cSAPs) is an exciting breakthrough for *Chlamydia* vaccine development its manufacture remains complex and its delivery to the human genital mucosa problematic.

Developing a *Chlamydia* vaccine is scientifically tractable due to progress in understanding protective immune mechanisms, genome based antigen identification, adjuvant and vaccine delivery technologies and animal models. Research efforts in the next five years will build on these four areas to optimize vaccine formulations and delivery systems in suitable animal models and generate a suitable *Chlamydia* vaccine(s) to be tested in human clinical trial. The era of *Chlamydia* vaccine discovery is nearing an end. It is imperative that the best vaccine candidate now be taken in human trials. Computer modeling suggests that even a partially protective vaccine would rapidly reduce the prevalence of genital infection [87]. Health economic analysis suggests that a *C. trachomatis* vaccine is cost-effective [88]. The current major obstacle for *Chlamydia* vaccine trials is the failure of public health leaders to explicitly identify *C. trachomatis* vaccines as a high priority. Collaborations among academic, public health and the private sector are necessary to overcome this tragic inertia given that up to 1 million women per year become infertile from this preventable infection.

References

Reference annotations

* Of interest

** Of considerable interest

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Key issues

- Optimal protection conferred by mucosal vaccine requires the synergistic action of two memory T cell subsets: tissue resident memory T cells and circulating memory T cells, and early mucosal seeding with Trm CD4 T cells is the key to a successful *C. trachomatis* vaccine.
- Multifunctional CD4 T cells secreting IFN-γ/TNFα are the most efficient cells to terminate *Chlamydia* replication and will likely lead to a potential biomarker for protective immunity.
- Immunoproteomic approaches resulted in a significant expansion in the number of T cell antigens for the development of a *Chlamydia* vaccine.
- *Chlamydia*l outer membrane proteins (Pmps and MOMP) are among the major T cell antigens useful in the development of a *C. trachomatis* subunit vaccine and should be used in combination to enhance coverage. MOMP is the most important antigen for the development of functional antibodies and a novel VD4 multimeric MOMP offers a practical solution to using recombinant MOMP able to stimulate functional antibodies as a vaccine antigen.
- Adjuvants play important roles in modern vaccine formulations by acting as bridge between innate and adaptive immune responses.
- Transcervical *C. trachomatis* infection mouse model is useful for the evaluation of *C. trachomatis* vaccine candidates. Non-human primate models need to be standardized and are not currently ready for evaluation of vaccine against *Chlamydia* genital infection. Robust vaccine protection in the *C. muridarum* and transcervical *C. trachomatis* murine models is sufficient to advance a *Chlamydia* vaccine into human trial.
- Recent remarkable progress renders *Chlamydia* vaccine scientifically tractable, and effective collaborations of academic, public health and private sectors are required for successful development of a *C. trachomatis* vaccine, a major infertility prevention strategy.

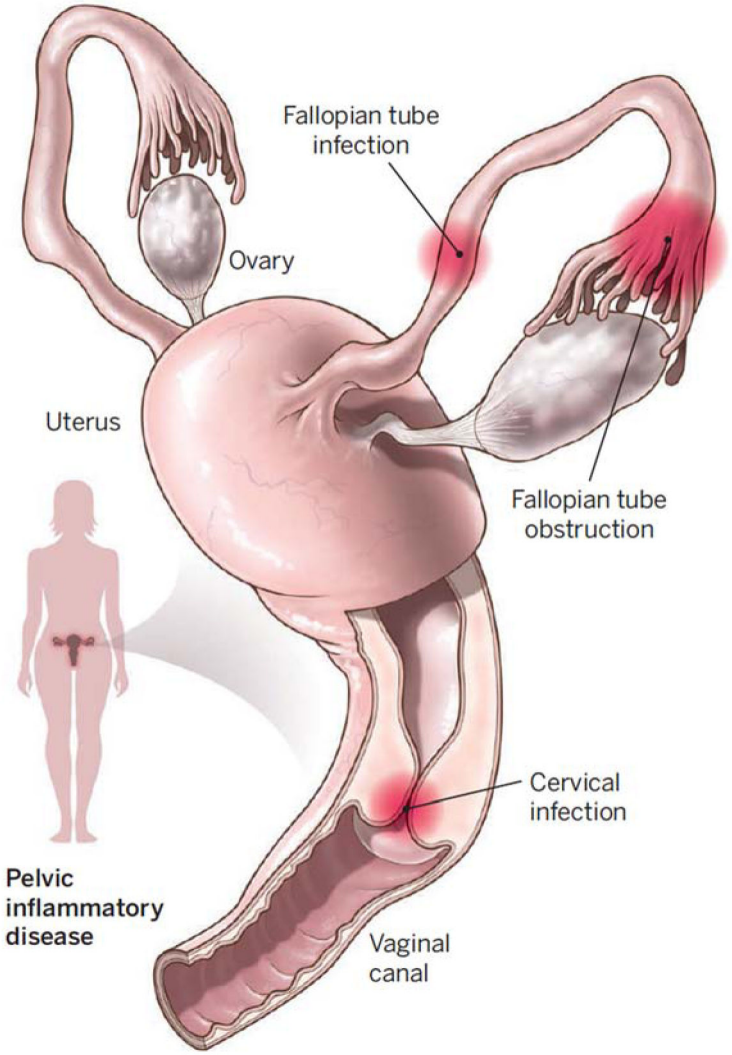

Figure 1. Reproductive damage

Pelvic inflammatory disease in women caused by *C. trachomatis* (sites of infection shown) can result in tubal factor infertility, ectopic pregnancy, and chronic pelvic pain. Reproduced with permission from [37].

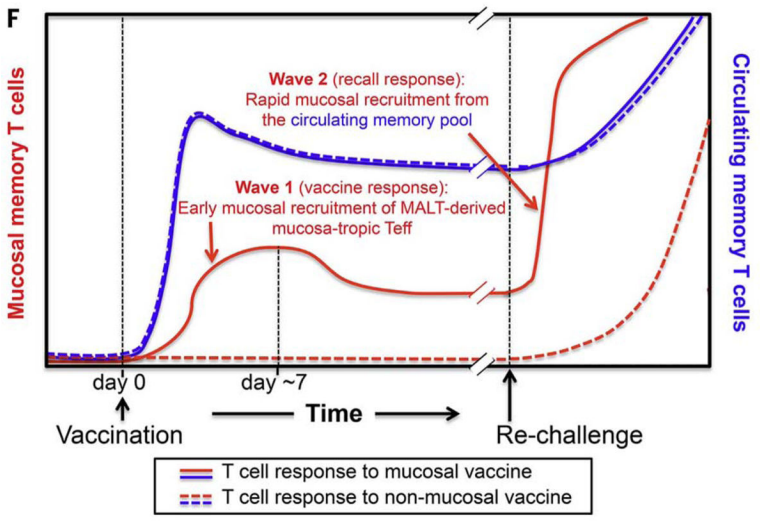

Figure 2. Two waves of vaccine-induced *C. trachomatis*-specific memory T cells

The first wave generated mucosal T cells which provided early protection and a second wave generated systemic T cells which augmented early and late protection. Mucosal immunization with adjuvant induced both the first and second waves of T cells. Systemic immunization induced only the second wave and generated incomplete protection. Reproduced with permission from [36].