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Genital Chlamydia trachomatis: understanding the roles of innate and adaptive immunity in vaccine research.

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Running Head: Immunity and *Chlamydia* Vaccine

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SUMMARY

Despite significant advances in the understanding of the host response to chlamydial infection and over 30 years of vaccine research, *Chlamydia trachomatis* remains the leading cause of bacterial sexually transmitted disease worldwide. This gram-negative obligate intracellular bacterium, that often remains asymptomatic, may cause pelvic inflammatory disease (PID), ectopic pregnancies, scarring of the fallopian tubes, miscarriage and infertility when left untreated. In the genital tract, *Chlamydia trachomatis* primarily infects epithelium cells and requires Th1 immunity for optimal clearance. This review first focuses on the immune cells important in a chlamydial infection. Secondly, we will summarize the research and challenges associated with developing a chlamydial vaccine that elicits a protective Th1-mediated immune response without inducing adverse immunopathologies.

INTRODUCTION

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted diseases in humans. According to the WHO in 2008, there was 105 million new cases of STDs each year due to *C. trachomatis* worldwide and the infection rate has been steadily increasing (9, 103). When symptomatic, *C. trachomatis* can lead to mucopurulent endocervical discharge, hypertrophic cervix, and post coital bleeding. In 20-40% of untreated women *C. trachomatis* may reach the fallopian tubes via the endometrial epithelium and cause pelvic inflammatory disease (PID). However, *C. trachomatis* genital tract infections are often asymptomatic (75-90%) and therefore remain undiagnosed and untreated. This can lead to tubal factor infertility or ectopic pregnancies (68, 69), which is a life threatening condition. *C. trachomatis* can be easily treated with antibiotics such as erythromycin, azithromycin or doxycycline. However,

several studies have documented that within a year after treatment for a *C. trachomatis* infection, 13-26% of individuals showed evidence of persistent or recurrent infections (38, 51). Therefore, due to the high rate of asymptomatic infections, recurrent infections and the severity of pathologies induced by *Chlamydia,* the development of a vaccine is paramount. This review focuses on *C. trachomatis* and *C. muridarum* (a model organism that naturally infects rodents and largely used for animal experiments) immunity and the challenges associated with generating a vaccine against this bacterium. Table 1 summarizes recent developments in chlamydial research including *Chlamydia* strain or antigen used, cell type affected and immune response elicited.

CHLAMYDIA BIOLOGY

The genus *Chlamydia* includes species that infect humans (*C. trachomatis*, *C. pneumoniae*), and animals (*C. muridarum, C. suis*, *C. abortus*) (21). Presently, there have been 18 identified serovars of *C. trachomatis* based on the reactivity of patient sera to the major outer membrane protein (MOMP) (135). Some serovars are associated with ocular tissue infections (A-C) while others primarily infect genital tissues (D-K) (7). *C. trachomatis* is a gram-negative obligate intracellular bacterium that in the genital tissues normally infects the epithelium layer of the cervix of women and the urethra of men (12).

Chlamydia exists in two developmental forms, the infectious extracellular non-replicating elementary body (EB) and the non-infectious intracellular replicating reticulate body (RB). The EB displays no metabolic activity, is resistant to both chemical and physical factors, and is adapted for prolonged extracellular survival. Infection begins when the small (~0.2-0.3μm) EB attaches to the host cell and is internalized inside an entry vacuole which avoids fusion with the lysosome. After 8-10 hours the vesicle bound EB (termed an inclusion)

replicates by binary fission into the larger (~0.8 μm) RB (138). Following several rounds of division, the RB`s reorganize and revert back to the EB (131). Inside host cells, *C. trachomatis* circumvents endogenous stress mechanisms, prevents lysosomal fusion and escapes intracellular destruction by replicating in an inclusion outside of the endocytic pathway (138). *C. trachomatis*-infected cells have increased inducible oxide synthase (iNOS) and increased pro-inflammatory molecules such as activins, which may be involved in scarring (1, 115).

IMMUNITY TO *CHLAMYDIA*

T cells

A critical role for T cells in immunity to *Chlamydia* was demonstrated almost 30 years ago when Rank *et al*. observed that athymic nude mice established chronic infection with *C. muridarum* after intravaginal inoculation whereas wild-type (wt) mice resolved the infection in 20 days (114). In human and mouse models, both CD4+ and CD8+ T cells can be detected at the site of *C. trachomatis* infection (65, 71, 101, 129). T cells are unable to recognize pathogens or antigens without the help of antigen presenting cells (APC) such as dendritic cells (DC), macrophages, or B cells. APC are able to phagocytose chlamydial EBs in the extracellular space or engulf infected cells harboring RBs. After phagocytosis, APC degrade chlamydial components and present the peptides via MHC class II-antigen complex to CD4 + T cells or MHC class I-antigen complex to CD8 + T cells. In fact, numerous *C. trachomatis* antigens have been identified which can be recognized by human CD4+ and CD8+ T cells including the cysteine-rich outer membrane protein 2 (Omp2) (40), polymorphic outer membrane protein D (POMP-D) (41), MOMP (50, 72, 104), heat shock protein 60 (hsp 60)

(25, 50), chlamydial protease activating factor (CPAF) (75), PmpG, PmpF, and RpIF (66, 101). High-throughput proteomic screening has identified even more potential immunodominant *C. trachomatis* antigens including 36 that have been shown to react with sera from three strains of mice immunized with live *Chlamydia* and two protein antigens that were able to induce a polyfunctional Th1 CD4+ T cell response and high Th1 antibody titers (112, 126). Although *Chlamydia* is able to induce a Th2 response characterized by IL-4 and Th2-associated antibodies such as IgG1, a Th1 response predominates characterized by the production of IL-12 by APC (17) and the subsequent activation of IFN-γ producing T cells and plasma B cells that secrete Th1-associated antibodies such as IgG2a and IgG3 (97, 100). However, a recent study demonstrated that CD4+ T cells from women with genital tract *C. trachomatis* infection that were restimulated *ex vivo* with inactivated (γ-irradiated) EB secrete significantly more IL-4 than TNF-α and IFN-γ. This study suggests that the type of immune response (Th1 vs Th2) to *C. trachomatis* may be tissue specific (132).

While there is ample evidence that CD4+ T cells play an integral part in the resolution of *C. trachomatis* and *C. muridarum* infections (34, 36, 39, 60), the role for CD8 + T cell has been controversial even though CD8+ T cells are induced following infection and *Chlamydia*-specific human and mouse CD8+ T cells are cytotoxic for *Chlamydia*-infected target cells (137). A recent study by Murthy *et al*. demonstrated that wt and CD8+ T cell-deficient mice displayed similar clearance of *C. muridarum* following vaginal chlamydial challenge (92). These data support previous studies which demonstrated that CD8+ T cells are not critical for *C. trachomatis* clearance (87, 88, 121). Furthermore, the CD8+ T cell-deficient mice demonstrated reduced oviduct pathology (hydrosalpinx) compared to wt, suggesting a role of CD8+ T cells in chlamydial pathogenesis (92). An interesting study demonstrated that the majority of CD8+ T cells in the cervix before and after a *C. trachomatis* infection do not express perforin (53). Perforin is a cytolytic protein found in the granules of CD8+ T cells

which forms a pore by inserting itself into the cells plasma membrane resulting in lysis of the target cell. Therefore, the lack of perforin in endocervix CD8+ T cells may explain why CD8+ T cells do not play a critical role in the elimination of genital chlamydial infection. Although CD8+ T cells appear not to be critical in resolving a chlamydial infection and may even contribute to chlamydial sequelae, they nonetheless may play a contributory albeit secondary role by regulating other cells and by their own production of IFN-γ (137).

Dendritic Cells

Dendritic cells (DC) are known to be the quintessential antigen presenting cells. Immature DC are highly phagocytic and after internalization of pathogens they degrade the components and present the peptides to T cells via MHC receptors activating the T cells and initiating a cell- mediated and / or humoral immune response. The capacity of DCs to present chlamydial antigens to T cells, secrete Th1 cytokines such as IL-12 and TNF-α and the importance of MHC class molecules in chlamydial infection has been demonstrated both *in vitro* and *in vivo* (64, 84, 87, 99, 122). An early study conducted by Lu and Zhong showed that bone marrow derived dendritic cells (BMDC) pulsed with heat-killed *C. trachomatis* and adoptively transferred into a naive mouse was protective against a subsequent challenge with live *C. trachomatis* in a mouse lung infection model (82). This protection was mediated by a Th1 response further demonstrating a correlation between Th1 skewed immunity and protection against chlamydial infection. In contrast, DCs that were pulsed with recombinant MOMP and adoptively transferred into mice elicited primarily the Th2-associated antibody IgG1 (119). Furthermore, IL-10 (Th2-associated cytokine) KO DC pulsed with UV-inactivated *C. trachomatis* and adoptively transferred activated a high frequency of Th1 cells (47). These data have direct relevance to vaccine development because it indicates that the type of cytokines produced and antigens processed by the DC and presented to CD4+ T cells

is essential in the Th1/Th2 balance of the immune response to *Chlamydia*. There is also evidence that live *Chlamydia* is required for an optimal and protective immune response. Rey-Ladino and colleagues demonstrated that the level of protection induced by DC pulsed with UV-inactivated *C. trachomatis* EB and adoptively transferred into mice was significantly less than in mice that were challenged with live EB pulsed DC (116). A more recent study discovered that murine DCs pulsed with live *C. muridarum* EBs presented 45 MHC class II peptides mapping 13 proteins whereas dead EBs presented only six MHC class II peptides mapping to three proteins (143). However, *C. trachomatis* has developed strategies to limit the presentation of these antigens to T cells by downregulating MHC expression on APC (54). *C. trachomatis* has been shown to inhibit MHC molecules within infected cells through the degradation of the MHC class I transcription factor RFX-5 and the MHC class II transcription factor USF-1 by secreting the chlamydial protease CPAF into the cytosol (29, 111, 145, 146). DC are important to vaccine research because they are the critical links between innate and adaptive immunity. Two recent studies using DC transfected with a recombinant adenovirus carrying *C. trachomatis* MOMP antigen (81) and DC pulsed *ex vivo* with recombinant *C. trachomatis* protease-like factor (rCPAF) (75) illustrate the ability of DC to induce protective immunity against genital *C. trachomatis* and *C.muridarum* challenge respectively.

Macrophages

Studies using both *C. trachomatis* and *C. muridarum* have shown that macrophages are recruited to sites of infection (88) and are capable of phagocytosing *Chlamydia* (8). Macrophages are also a source of both proinflammatory cytokines such as IL-8, IL-6 and TNF-α (6, 141). However, unlike epithelial cells, macrophages are not a hospitable niche for chlamydial intracellular replication illustrated by the fact that compared to epithelial cells only a small fraction of chlamydial RBs are detected in macrophages (124). *C. trachomatis*

197 destruction inside the macrophage has been associated with host cell autophagy, a process by
198 which cells degrade cytoplasmic proteins and organelles (2, 124, 140), and studies have
199 demonstrated that macrophage autophagy can enhance antigen presentation to T cells (22).
200 Furthermore, INF-γ has been shown to enhance both autophagy and upregulation of MHC
201 class molecules in macrophages (2, 15). This is relevant because, in addition to activating
202 primed T cells, there is evidence suggesting that macrophages are able to initiate a humoral
203 response in naive mice (130). Therefore, enhanced upregulation of MHC molecules
204 containing chlamydial antigens may induce T cells to initiate both a cell-mediated and
205 antibody immune response against *Chlamydia*. However, Jendro *et al*. demonstrated that
206 human macrophages infected with *C. trachomatis* can induce T cell apoptosis (61, 62). In
207 addition to efficiently eliminating *Chlamydia* and presenting the peptides to T cells,
208 macrophages may also have an effect on chlamydial infection by inducing T cell death and
209 perpetuating a persistent infection.

210 **B cells/Antibodies**

211 Previous studies demonstrated that anti-*Chlamydia* antibodies correlated with
212 protective immunity against *C. trachomatis* in humans (4, 59) and numerous *C. trachomatis*
213 proteins have been shown to induce antigen specific antibodies (36). However, even though
214 anti-*Chlamydia* antibodies are able to neutralize infection *in vitro* (5, 13) growing evidence
215 show that B cells may not play a critical role in controlling a primary chlamydial infection but
216 are important for a secondary memory response (89, 90). Several mechanisms have been
217 proposed on how B cells contribute to immunity during re-infection. These mechanisms
218 include antibody-mediated neutralization and opsonization (5), antibody-dependent cellular
219 cytotoxicity (ADCC) (86) (a mechanism of cell-mediated immune defense whereby cells that
220 have antibodies attached to their surface are targeted for lysis), and the formation of antigen-

antibody complexes that bind Fc receptors on the APC which then enhances phagocytosis and antigen presentation to the CD4+ T cell (57).

Heat shock proteins (hsp), which are found in both eukaryotic and prokaryotic organisms, are stress-proteins that are involved in the correct folding of intracellular proteins. *C. trachomatis* is known to secrete hsp`s during an infection and antigenic epitopes from the bacterial hsp`s have proven to be strong inducers of cellular and humoral immunity. Chlamydial hps60 exhibits over 70% sequence homology and 100% amino acid homology of four defined epitopes with human hsp60 (3) and several studies have suggested that autoimmunity to human hsp60 is a result of cross reactivity after a chlamydial infection (27, 136). However, a recent study demonstrated an association with tubal factor infertility (TFI) and antibodies to MOMP and hsp60 from *C. trachomatis* but no connection between TFI and antibodies to human hsp60 (49) pointing to an infectious rather than an autoimmune response as the cause of TFI.

In conclusion cell-mediated immunity that activates macrophages, neutrophils and mediators such as IL-12, IFN-γ and TNF-α is required for initial clearance. However, for protective immunity both cell-mediated and humoral immunity are needed including antigen-specific T cells and antibodies that enhance uptake, processing and presentation of chlamydial antigens by DC for a rapid Th1-mediated chlamydial clearance.

VACCINES

Due to increasing rates of mainly asymptomatic *C. trachomatis* infections worldwide and the adverse long term consequences resulting from these infections (ectopic pregnancy, infertility, preterm birth) developing an anti-chlamydial vaccine is paramount. However, a human vaccine that elicits both T cell and B cell immunity has been elusive. Although the two

murine models using *C. trachomatis* and *C. muridarum* are the most common models used for chlamydial vaccine research non-human primates, pigs and guinea pigs have also been utilized (24)**.** Our poor understanding of the immune response in the female genital tract, which is highly regulated by sex hormones during the menstrual cycle (52), the lack of adjuvants that not only optimize the immune response to *Chlamydia* antigens but can target the vaccine-specific-immune responses to the site of infection and limited understanding of what type of chlamydial antigens induce a protective immune response hinder the development of a human *C. trachomatis* vaccine. *C. trachomatis* vaccine has to induce both mucosal and systemic immune responses, but autoimmune cross reactions with human antigens and unregulated inflammation that causes pathology has to be avoided. Table 2 summarizes recent chlamydial antigens, delivery systems, routes of vaccination and infection and the subsequent immune responses elicited.

Intact attenuated organisms

Successful vaccines against ovine enzootic abortions have been available for many years (32). These vaccines consisted of live attenuated or inactivated *C. abortis* strains and provided proof of principle that a successful vaccine against *Chlamydia* was possible in mammals. However, these vaccines did not prevent infectivity and lacked the rigorous immunization schedules, efficacy, safety and toxicity standards required for human vaccine (56, 85). Nonetheless, because of the success of these vaccines, live attenuated *C. trachomatis* bacteria were used as the first human *Chlamydia* vaccines (43). Attenuation was induced by either mutagenesis or by growing the organisms in culture. In the latter approach, after several passages, one or more mutations arise, which may result in a nonvirulent attenuated strain. Vaccines with live organisms are generally considered optimal because they contain virtually all of their antigenic determinants in the correct three dimensional conformation. Moreover,

they replicate similarly to the target pathogen thus promoting the processing and presentation of antigens similar to natural infection and eliciting humoral and cell-mediated immunity. However, using live attenuated organisms for vaccines has drawbacks because large scale production of pure *Chlamydia* is extremely complex and these vaccines need cold storage. Even more importantly, they can also revert to virulent wild-type strains resulting in disease or persistent infection (26).

Initial vaccine human trials using live attenuated *C. trachomatis* led to partial short-lived protection, however some individuals who were re-exposed to *Chlamydia* developed a more severe pathological delayed-type hypersensitivity (DTH) response than those that did not receive the vaccine (43). Because of the safety issues of live vaccines, research switched to organisms that were heat or chemically-inactivated. The major disadvantage of these types of vaccines is the absence of replication and poor induction of cell-mediated immunity, which is critical for the clearance of *Chlamydia*, necessitating the need for re-vaccination and adjuvants. Heat or chemical bacterial inactivation may also release unwanted and detrimental components which can have deleterious effects or degrade protein antigenic determinants thereby reducing the degree of protection. Recently, plasmid-deficient *Chlamydia* strains have been used in vaccine research with conflicting results. O`Connell *et al*. demonstrated that a plasmid-deficient strain of *C. muridarum* (Nigg) that is defective in its ability to accumulate glycogen did not cause inflammatory pathology in mice. Furthermore, the plasmid-deficient bacterium protected mice against a secondary infection with plasmid-competent virulent *C. muridarum* (97). However, a different group demonstrated that mice vaccinated with an attenuated plasmidless *C. trachomatis* (L2R) were not protected from colonization and inflammatory pathology after a secondary challenge with wild-type *C. trachomatis* (serovar D) although there was a reduction in infectious burden at early time points (100).

Subunit antigenic determinants

Another vaccine strategy utilized is the administration of purified antigenic determinants known to elicit an immune response. Subunit vaccines are safer than attenuated or heat/chemically inactivated organisms because they cannot revert to a virulent form and undesirable antigens that might induce immunopathology can be avoided. One of the most studied vaccine candidate for *C. trachomatis* is the structurally and immunologically dominant protein in the chlamydial outer membrane MOMP. This membrane protein contains several conserved CD4+, CD8+, and B cell epitopes (96) . An early study conducted by Pal and colleagues demonstrated that *C. muridarum* COMP (chlamydial outer membrane complex), a chlamydial outer membrane with a cysteine cross-linked protein shell, significantly protected mice against genital challenge whereas MOMP did not (108). Several years later the same group administered purified and refolded preparation of *C. muridarum* MOMP along with Freund`s adjuvant. The refolded MOMP-Freunds adjuvant conferred a significant level of protection in the vaccinated mice against a genital infection demonstrating the importance of adjuvants and a correct MOMP configuration in eliciting a protective immune response (109). Tiffrea *et al.* discovered that a polymer that keeps membrane proteins soluble (amphipol) in aqueous solution was able to stabilize MOMP and enhance its protective ability as a vaccine (128). Another group immunized mice with a *C. trachomatis* MOMP-ISCOM vaccine. ISCOM (immune stimulating complex) which are mainly composed of cholesterol, phospholipids and saponin, are known to induce both a cell-mediated and humoral response when used as vaccine adjuvants. Inoculation with MOMP-ISCOM was able elicit a Th1 antigen-specific response and vaginal infection was cleared within one week (58). A *C. muridarium* MOMP native preparation combined with an adjuvant consisting of nontoxic subunit B cholera toxin conjugated to CpG (CTB-CpG) elicited a significant cell

mediated and antigen-specific antibody response against a pulmonary challenge with *C. muridarum* (18). A non-human primate model was used to demonstrate the efficacy of a vaccine formulated with native MOMP. Rhesus macaques that were immunized intramuscularly and subcutaneously along with the adjuvants CpG-2395 and Montanide ISA 720 produced high levels of Th1 cytokines (INF-γ, TNF-α) and *C. trachomatis*-specific IgG and IgA (19). Drawbacks of subunits vaccines include the fact that extracting, refolding and purifying protein complexes such as MOMP is very expensive and purifications are not standardized so difference in extraction methods may influence the conformation of the protein epitopes and vaccine efficacy.

Recombinant proteins

The advent of recombinant DNA technology has made it possible to produce large quantities of bacterial proteins. Thus, different attempts were made to use rMOMP in *C. trachomatis* vaccine. Unfortunately, producing rMOMP with its native conformational epitopes intact on a large scale is challenging and in some expression systems full-length rMOMP is toxic (78, 147). In 2009, a comparison of vaccines using native or recombinant MOMP demonstrated that the degree of protection obtained with recombinant MOMP was not as robust as that achieved with native MOMP preparation (123). However, other studies using rMOMP with and without adjuvants demonstrated protection against *Chlamydia* (98, 127). In 2011, Kalbina and colleagues designed a chimeric gene construct containing two antigenic regions of MOMP and introduced the construct into a bacteria (*Escherichia coli*) and two plants (*Arabidopsis thaliana*, *Daucus carota*). The construct was successfully expressed *in E. coli*, and stable integration of the transgene was demonstrated in *A. thaliana* and *D. carota* over several generations. The rMOMP purified from *E. coli* was used to produce antibodies in rabbits and these antibodies recognized the proteins in both *E. coli*, *A.*

342 *thaliana*, *D. carota* as well as in inactivated *C. trachomatis* elementary bodies. The stability
343 of the construct in the offspring plants suggests that this system may be useful for large scale
344 production of rMOMP and the authors plan to use the transgenic plants as edible vaccine
345 vectors for laboratory animal experiments (67).

346 Other recombinant proteins besides MOMP have also been shown to be potential
347 vaccine candidates. In 2007, Murphy *et al*. investigated the potential of recombinant CPAF to
348 elicit an immune response that would resolve chlamydial infection. Mice immunized
349 intranasally with rCPAF and IL-12 (Th1 cytokine) demonstrated increased IFN-γ, and
350 minimal IL-4 (Th2 cytokine) production, elevated IgG2a (Th1) and IgA (mucosal) antibody
351 levels, displayed a markedly reduced bacterial burden upon *C. muridarum* genital inoculation
352 and were protected against pathological consequences of *Chlamydia* infection compared with
353 mock immunized mice (91). The same group demonstrated that rCPAF intranasal vaccination
354 may prevent infertility from repeated genital *C. muridarum* infections in mice (93). Mice
355 immunized with a recombinant chlamydial glycogen phosphorylase (GlgP) and intravaginally
356 challenged with live *C. muridarum* elicited a Th1-dominant T cell response that included anti-
357 chlamydial antibodies and reduced hydrosalpinx severity. Additionally, the GlgP-immunized
358 mice exhibited a significant reduction of vaginal shedding on day 14 post-infection (76).
359 Olsen *et al*. utilized two recombinant proteins in a subunit chlamydial vaccine. The fusion
360 protein CTH1 consisted of CT443 (omcB) which is known to elicit both a humoral and cell-
361 mediated response and CT521 (rl 16) a known target for cells during natural infection in
362 humans. Immunization with CTH1 along with the strong Th1 inducing adjuvant CAF01
363 elicited TNF-α, IL-2 and INF-γ production from T cells and high titers of both Th1 (IgG2a)
364 and Th2 (IgG1) CTH1-specific antibodies. The vaccine significantly reduced bacterial
365 shedding after a vaginal challenge with live *C. trachomatis* and *C. muridarum* and protection
366 was demonstrated to be solely CD4+ T cell-mediated in the *C. muridarum* model (102). Lu

and colleagues screened 5 recombinant chlamydial antigens (ABC transporter [ArtJ], outer membrane complex protein B [OmcB], macrophage infectivity potentiator [Mip], inclusion membrane protein [Inc (crpA:TC0726)], and an hypothetical protein), that were previously found to react with sera from intravaginally *C. muridarum* infected mice for their ability to induce protection against chlamydial infection. Only Mip induced pronounced protection which was characterized by Th1-dominant T cell response and anti-Mip antibodies (80).

Plasmid DNA

DNA vaccines work by injecting a plasmid that encodes a specific gene of interest within the host. The product of the gene can then be expressed inducing an immune response. DNA vaccines have several advantages compared with other vaccination strategies. DNA can be easily and inexpensively purified and plasmid vectors can be rapidly constructed and easily tested (79). Additionally, DNA vaccines can encode for multiple epitopes that are in the native three dimensional configuration and avoid the problem associated with attenuated organisms which are able to revert back to virulent forms. However, as with other vaccine strategies, DNA vaccines have some disadvantages. In autoimmune diseases such as lupus anti-DNA antibodies are produced and there is the possibility that bacterial DNA injection could elicit a humoral response that cross-reacts with the host DNA. Also, because DNA encodes for proteins, DNA vaccines cannot be utilized for non-protein based antigens such as polysaccharides or lipids (73) and although likely rare, there is the risk that DNA could integrate into the host chromosome (35). In 1999 Pal and colleagues immunized mice with a DNA vaccine that encoded for the MOMP gene of *C. trachomatis*. When the mice were vaginally challenged with *C. trachomatis* the immune response was modest and the mice were not protected against infection (106). The following year Dong-Ji *et al*. demonstrated that immunization with DNA-MOMP and boosting with MOMP-ISCOM conferred higher

391 protection against *C. trachomatis* when compared with mice that were only immunized with
392 MOMP-ISCOM (28). More recently two studies using a pig model assessed the efficacy of
393 DNA chlamydial vaccines. Schautteet *et al*. combined aerosol-vaginal delivery of DNA
394 vaccine encoding for MOMP co-administered with DNA encoding for three different
395 adjuvants (GM-CSF, *E. coli* enterotoxin subunit A and B). Vaccination induced significant
396 protection against genital *C. trachomatis* challenge although the infection was not completely
397 resolved (117). Ou and colleagues demonstrated that an OmpA-based DNA vaccine against
398 *Chlamydia abortus* in piglets elicited higher antigen-specific IgG antibodies and T cell
399 proliferative response compared with controls (105). Mammalian cells transfected with a
400 plasmid encoding for MOMP epitopes inserted in a human papillomavirus (HPV) major
401 capsid protein L1 was used in a murine model of *C. trachomatis* genital infection.
402 Intramuscular administration elicited a Th1 response characterized by low IL-4 production
403 and antibodies against MOMP (139). All of these recent studies demonstrate the feasibility of
404 DNA-based vaccine and this approach thus deserves further study.
405

406 **OTHER CHLAMYDIAL VACCINES AND DELIVERY SYSTEMS**

407 **Bacterial ghosts**

408 Bacterial ghosts (BGs) are cell envelopes derived from Gram-negative bacteria. BGs
409 are devoid of all cytoplasmic content but have a preserved cellular morphology including all
410 cell surface structures. BGs are non living but retain all of the antigenic components of their
411 living counterparts and the inside of the BG envelope can be loaded with peptides, drugs, or
412 DNA (74). In 2007, a vaccine system in which a DNA plasmid that encoded for *C.*
413 *trachomatis* MOMP and the porin protein (PorB) was inserted into a recombinant *Vibrio*

cholerae ghost (rVCG) was used. Animals that were immunized intramuscularly with the DNA-bacterial ghost vaccine completely resolved a *C. trachomatis* genital infection after two weeks post-infection. The inflammatory response was Th1, characterized by high levels of IgA and IgG2a (55). More recently, Eko and colleagues used the rVCG that contained PorB and chlamydial polymorphic membrane protein-D (PmpD) proteins to evaluate its ability to induce chlamydial immunity. Intramuscular immunization elicited high levels of Th1-associated antibody IgG2a, mucosal-associated antibody IgA, IFN-γ (Th1) and low levels of IL-5 (Th2) in response to an intravaginal *C. muridarum* infection (31).

Biodegradable polymers

PLGA (poly D, L-lactide-co-glycolide) is an FDA approved polysaccharide that can encapsulate peptide, proteins or DNA. PLGA`s are efficiently phagocytosed by DC and macrophages (83, 134) and PLGA antigens are able to be presented on both MHC class I and II molecules thus activating CD4+ and CD8+ T cells (45, 133). Chitosan is a linear polysaccharide derived from the deacetylation of chitin. The glucosamine units of chitosan have a density of amine groups which permits strong electrostatic interactions with proteins and genes. Additionally chitosan is mucoadhesive and has enhanced penetration capacity across mucosal barriers.(10). Both of these nanoparticles are biodegradable, relatively non-toxic and have been used as delivery systems for chlamydial vaccines. Two recent studies using recombinant MOMP encapsulated in PLGA demonstrated enhanced capacity of the peptide to induce Th1 cytokine, cellular and antibody immune response (33, 125). Cambridge *et al*. demonstrated that MOMP was expressed in the muscle tissues and spleens of mice that were intramuscularly injected with chitosan nanoparticles containing recombinant MOMP DNA (14).

Gas vesicles

Gas vesicles are gas containing structures found in some bacteria and Archaea. These protein structures are hollow, rigid, lipid-free, allow diffusion of gases across its membrane, and are able to express peptides from various genes. Studies have shown that in the absence of adjuvants, *Halobacteria* gas vesicles that displayed viral peptides elicited robust long-lived immune response characterized by immunological memory in mice (120). *Halobacteria*-derived gas vesicles that were loaded with gene fragments coding for MOMP, OmcB (outer membrane complex B), and PompB (polymorphic outer membrane B) and expressed on the surface were able to elicit a Th1 cytokine profile in human foreskin fibroblasts *in vitro*. In addition the presence of the recombinant proteins were confirmed by anti-*Chlamydia* antibodies and from *Chlamydia*-positive patient serum suggesting this could be an effective antigen delivery system for a *Chlamydia* vaccine (20).

ADJUVANTS

Adjuvants enhance immunity and one of the main challenges in developing an effective chlamydial vaccine is identifying antigen/adjuvant combinations that elicit a protective immune response *in vivo.* Various adjuvant such as the ones mentioned in this review (e.g. Freund`s adjuvant, ISCOM`s, CTB-CpG, CpG, bacterial ghosts) have been used in chlamydial vaccine research with varying results. Recent research has added other new antigen/adjuvant candidates with encouraging results. A study by Yu and colleagues evaluated the chlamydial protein PmpG and five adjuvants, including three cationic liposome formulations, Montanide ISA720-CpG-ODN1826 and alum. The results demonstrated that the cationic liposomal adjuvants DDA-MPL and DDa-TDB elicited the best protective immune

responses against *C. muridarum*. Additionally, using DDA-MPL as an adjuvant along with 7 different T cell antigens (PmpG, PmpE, Aasf, Rp1F, TC0420, TC0825) conferred equal or better protection than the vaccine antigen MOMP alone (142). This highlights the various opportunities to further improve vaccine candidates by identifying the optimal epitope/adjuvant combination

VACCINATION ROUTES

Vaccine efficacy is not only defined by the type of antigen and adjuvant used but also by the administration route, since lymphocytes primed by antigens in vivo are endowed with specialized homing programs guiding their migration to specific mucosal sites (95). Once naive T cells are primed in a lymph node, a global switch of their homing program occurs which enables them, while trafficking through the blood circulation, to detect chemokines and adhesion molecules which direct them to their tissue destination. Lymphocytes, activated by antigen presentation occurring in lymph nodes draining a mucosal site, acquire specialized homing programs leading them to preferentially migrate to the same or other specific mucosal sites. Of note, T cell homing to the genital mucosa involves either $\alpha 1\beta 1$, $\alpha 4\beta 1$ (110) or $\alpha 4\beta 7$/E selectin (70) in *Chlamydia* infected mice. Both systemic and mucosal immunization routes have been shown to be able to induce both humoral and cell-mediated immune responses in the genital tract with intranasal immunization being often more effective (11, 94). Overall, mucosal immunization routes were more effective at preventing genital challenges with a variety of pathogens (37, 44, 63, 77, 144).

Numerous immunization routes have been used for chlamydial vaccinations including oral (48), intranasal (i.n.) (46), intravaginal (i.vag) (118), subcutaneous (107), intramuscular (30), perivaginal (107), perisacral (107), sublingual v(113) and colonic (16). A study using

purified MOMP with a *Borrelia* surface protein as an adjuvant demonstrated that in two different mouse strains "intramuscular + subcutaneous" and "perivaginal + perisacral" immunization elicited high systemic (IgG) and mucosal (IgA) serum antibodies. In contrast, the mice that received the MOMP-adjuvant i.n. had low IgG and IgA serum antibodies (107). However, a recent study showed that i.n. immunization with rMOMP resulted in MOMP-specific IgA and IgG antibodies in genital tract secretions demonstrating i.n. administration may target immunity to the reproductive tract (23). Several studies comparing the protective ability of various vaccination routes demonstrated that a combined mucosal and systemic inoculation may be optimal. Using rMOMP with the adjuvants CpG and Montanide for systemic route (intramuscular and subcutaneous) and rMOMP with cholera toxin for the mucosal routes (sublingual and colonic), the authors demonstrated that following i.n. *C. trachomatis* challenge the sublingual + intramuscular + subcutaneous group showed the best protection (113). Another group demonstrated that mice immunized by combined mucosal and systemic routes with *C. muridarum* recombinant MOMP plus the adjuvants CpG and Montanide not only elicited the strongest chlamydia-specific humoral and cell-mediated response after vaginal challenge with *C. muridarum* but also protected against infertility (16).

CONCLUSIONS

Chlamydial infection is a public health concern worldwide and a vaccine that stimulates multiple arms of the adaptive immune system and avoids immunopathological consequences would be the best solution for the control of this sexually transmitted disease. Unfortunately, a partial or fully protective vaccine has yet to be developed highlighting the complex nature of the immunobiology mounted against this intracellular parasitic bacterium. The immune response to chlamydial infection is dynamic and involves cells and mediators

from both arms of the host's immune system. Clearance of a chlamydial infection requires a coordinated immune response between innate immune cells such as macrophages, DC and cells important in both cell-mediated and humoral adaptive responses such as CD4+T cells, CD8+ T cells and B cells. Activation and clonal expansion of T cells occurs through cognate interactions with DC that present chlamydial antigens on their MHC molecules and B cells produce anti-chlamydial antibodies through interaction with these clonal T cells. However, persistent infection seems to induce chronic inflammation and tissue damage. A shift from Th1 to Th2 also appears to induce scarring and immune pathology. It is therefore essential to understand these immunological dynamics in order to develop a vaccine that is both effective, long-lasting and does not have the deleterious effects associated with unregulated inflammation. Further research is needed to identify novel adjuvants that enhance the immune response and antigens that induce a protective T cell response and anti-chlamydial antibodies.

A mathematical model developed by Gray and colleagues demonstrated that a fully protective vaccine, administered to adolescents before they are sexually active, would be able to eradicate *Chlamydia* infection in 20 years. In addition, the model predicted that vaccinating 100% of women would have a greater epidemiological impact than vaccinating both sexes (42). Unfortunately there are risks and ethical questions associated with vaccination programs as demonstrated by the first *Chlamydia* vaccine using live attenuated bacterium (43). Thus, research is needed to develop an efficient and safe chlamydial vaccine.

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Table 1. Summary of recent developments in chlamydial research including chlamydial strain/antigen utilized, cell type infected and immune response elicited

Cell type	Chlamydia/Antigen	Immune Response	Ref.
Mouse macrophage cell line (J774) and human macrophages	*C. trachomatis*	• Live and inactivated *Chlamydia* induced elevated IL-8, IL-1β, TNF-α, IL-6.	(3, 24)
Mouse (RAW) and human (THP-1) macrophage cell line	*C. trachomatis*	• Live *Chlamydia* induced autophagy.	(1, 22)
Human macrophages	*C. trachomatis*	• Live *Chlamydia* infected macrophages induced T cell apoptosis.	(10, 11)
Mouse BMDC	C. *muridarum*	• DC pulsed with UV-inactivated *Chlamydia* in vitro secreted elevated levels of IL-12. • DC pulsed with UV-inactivated *Chlamydia* and adoptively transferred into naive mice induced strong protection against live chlamydial lung infection. • IL-12$^{-/-}$ DC failed to induce Th-1 dominant response and did not induce strong protection against chlamydial infection.	(14)
Mouse BMDC	rMOMP	• DC pulsed with rMOMP secreted IL-12 and induced infection-sensitized CD4+T cells to secrete IFN-γ. • DC pulsed with rMOMP and adoptively transferred into naive mice generated a Th2 anti-MOMP immune response.	(21)
Mouse BMDC	*C. trachomatis*	• IL-10$^{-/-}$ DC pulsed with UV-inactivated *Chlamydia* caused early DC maturation, activation, increased ability to process and present antigens and enhanced the rate of Th1 activation.	(7)
Mouse BMDC	*C. muridarum*	• DC incubated with UV-inactivated *Chlamydia* expressed low levels of CD40 and CD80, secreted low levels of proinflammatory cytokines and exhibited reduced recognition by *Chlamydia*-specific CD4+ T cells. • Adoptive transfer of live EB-pulsed DC was more effective that UV Chlamydia at protecting mice against a live intranasal chlamydial challenge.	(20)
Mouse BMDC	*C. muridarum*	• DC pulsed with live EBs presented 45 MHC class II *C. muridarum* peptides mapping to 13 proteins. In contrast DC pulsed with heat or UV-inactivated *Chlamydia* presented only six MHC class II chlamydial peptides mapping to 3 proteins. • Only two epitopes were shared in common between live and inactivated *C. muridarum*.	(25)
Mouse BMDC	Recombinant adenovirus carrying *C. trachomatis* MOMP	• DC exhibited increased CD80 and MHC class II, IL-12 and were able to stimulate CD4+ T cell proliferation and IFN-γ. • Adoptively transferred MOMP transfected DC generated Th1-biased cytokine production, mucosal IgA and protected mice against chlamydial genital tract infection.	(13)
Mouse BMDC	UV *C. muridarum* + CpG or rCPAF + CpG	• DC pulsed with rCPAF + CpG exhibited increased CD86, CD80, CD40, MHC class II, IL-12 but not IL-10 and IL-4. • Mice adoptively immunized with rCPAF + CpG or UV *C. muridarum* + CpG pulsed DC produced elevated IFN-γ, IG1, IgG2a and exhibited reduced *Chlamydia* shedding and reduced oviduct pathology compared to infected mock-immunized mice.	(12)

Mouse T cells	*C. muridarum*	• Athymic nude mice established chronic genital tract infection whereas wild-type mice resolved infection in 20 days.	(19)
Mouse T cells	*C. trachomatis* T cell antigens + AbISCO-100	• Potent CD8+ T response, polyfunctional Th1-polarized CD4+ T cell responses (INF-γ, TNF-α, IL-2) and high protein specific Th1-skewed antibody response (IgG2c). • Adoptive transfer of CD4+ T cells and CD8+ T cells to naive non-immunized mice protected against *C. trachomatis* vaginal challenge whereas passive transfer of immune sera did not.	(18)
Mouse T cells	*C. muridarum* MOMP + CpG and Montanide ISA	• Vaccinated mice were depleted of CD4+ and CD8+ T cells and challenged vaginally with live *C. muridarum*. Depletion of CD4+ T cells, but not CD8+ T cells diminished vaccine-induced protection.	(4)
Mouse CD4+ T cells	*C. trachomatis*	• Genital tract *C. trachomatis* infection stimulated the activation and memory development of *C. trachomatis*-specific CD4+ T cells. • CD4+ T cells are necessary to confer protection against *C. trachomatis* infection.	(6)
Mouse CD4 + T cells	*C. muridarum*	• CD4 T cell clone-induced epithelial NO production was critical for controlling replication. • Most potent CD4+ T cell clones were dependent on T cell degranulation for chlamydial replication control.	(9)
Human CD4+ T cells	*C. trachomatis*	• CD4+ T cells from women with genital tract infection that were pulsed ex vivo with EB secreted significantly more IL-4 than TNF-α and INF-γ.	(23)
Mouse CD8+ T cells	*C. muridarum*	• TNF-α from CD8+ T cells contributed significantly to oviduct pathological sequelae, but not bacterial clearance, following genital chlamydial challenge.	(17)
Human CD8+ T cells	*C. trachomatis*	• Endocervix effector memory CD8+ T cells from *C. trachomatis* infected women expressed low perforin levels.	(8)
Human B cells/Antibodies		• Identified 21 antibody inducing antigens from *C. trachomatis*-infected patients sera.	(5)
Mouse B cells/Antibodies	Recombinant outer membrane vesicles carrying *C. muridarum* HtrA	• Mice immunized with outer membrane vesicles carrying HtrA induced anti-HtrA-specific antibodies that neutralized *C. muridarum* infectivity in vitro.	(2)
Mouse B cells/Antibodies	*C. muridarum* or MOMP monoclocal antibody (mAb)	• Passive immunization with serum from *C. muridarum* infected mice conferred a marked level of protection from *C. muridarum* genital reinfection and shortened the time of infection. • MOMP mAbs conferred significant level of immunity to reinfection and reduced shedding.	(15)
Mouse B cells/Antibodies	rCPAF + CpG	• Both wild-type and B cell deficient (μmT) mice vaccinated intranasally with rCPAF + CpG and challenged with live *C. muridarum* vaginally demonstrated comparable clearance and similar reductions in pathology.	(16)

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Table 2. Summary of recent developments in chlamydial vaccine research

Vaccines	Advantages	Disadvantages	Ag/Adjuvants	Ag Immunization Route	Model/ *Chlamydia* Infection Route	Immune Response	Ref.
Intact *Chlamydia*	• Intact Ag • Native configuration • Replication • Humoral/Cellular immunity	• Requires refrigeration • Potential reverting to virulent strains • Large scale production difficult • Possible transmission to unvaccinated individuals	Plasmid-deficient *Chlamydia* (CM972, CM3.1)		Mouse / i.v.	• Elevated IgG2a (Th1); low levels of IgG1 (Th2) • Mutants do not stimulate TLR2-dependent cytokine production • Infected mice with mutant *Chlamydia* and challenged with wt *Chlamydia* are protected against oviduct disease	(17)
			Plasmid-Deficient *Chlamydia* (L2)		Mouse / i.v	• Elevated IgG2a; low IgG1; no IgA (mucosal) • No pathology in the urogenital tract induced by L2 • Mice vaccinated with plasmid-deficient bacterium were not protected from infection/inflammation with secondary wt chlamydial infection	(19)
Purified Subunits	• Do not revert to virulent strains • Avoids undesirable antigens	• Expensive to produce • Purification not standardized • Difficult to maintain native conformation of antigen complex	MOMP + subunit B cholera toxin conjugated to CpG	i.m. + s.c.	Mouse / i.n.	• Elevated IgG2a, IgG3 (Th1); lower IgG1 • Elevated INF-γ (Th1)	(2)
			MOMP-ISCOM	i.n. or i.m.	Mouse / i.n.	• i.m. induced highest INF-γ and IL-4 (Th2)	(10)
			MOMP + Freund`s adjuvant	i.m + s.c	Mouse / i.v.	• Vortexed MOMP elicited higher IgG2a vs IgG1 • Sonicated MOMP elicited higher IgG1 vs IgG2a	(23)
			MOMP + IC31	i.m + s.c	Mouse / i.n.	• Higher IgG1 than IgG2a	(3)
			MOMP + CpG/Montanide	i.m + s.c	Rhesus macaque	• Elevated IgG ,IgA, INF-γ and TNF-α	(4)
Recombinant Proteins	• High yields • Inexpensive	• Some proteins require post-translational modification • If produced in *E. coli* possibility of endotoxin contamination	rMOMP + Cholera toxin/CpG or CTA1	s.l. or t.c. or i.n.	Mouse / i.n.	• Elevated IFN-γ and TNF-α • i.n. immunization with MOMP + either adjuvant protected mice from infection but not pathology • t.c. immunization with MOMP and CTA1-DD protected mice from pathology but *Chlamydia* burden was same as control mice	(18)
			rMOMP + CpG /Montonide	i.m + s.c.	Mouse / i.n.	• Vaccination protected against fibrotic scarring in lungs • Elevated IgG2a and lower levels of IgG1	(27)
			rCPAF + IL-12	i.n.	Mouse / i.v.	• Increased IFN-γ ; minimal IL-4 • Elevated IgG2a and IgA	(15)
			rCPAF + CpG	i.n.	Mouse / i.v.	• Vaccination significantly prevented infertility	(16)
			rCTH1 + CAF01	s.c.	Mouse / i.v.	• T cell production of TNF-α/IL-2/IFN-γ • anti-CTH1 IgG2a, IgG1 • Protection was solely CD4+T cell-mediated	(20)
			rGlgP + CpG	i.m.	Mouse / i.v.	• Th1-dominant T cell response • Reduced hydrosalpinx severity	(12)
			rMIP	i.m.	Mouse / i.v.	• More IgG2a vs IgG1	(13)

			rCT043 rCT823 + ISCOM and CT144 + ISCOM	i.m. s.c.	Mouse / i.n. Mouse / i.v.	• Elevated IFN-γ and no IL-4 • Reduced hydrosalpinx severity • rCT043 reduces bacterial load in a mouse model of i.n. infection • Elevated INF-γ, TNF-α, IL-2 • No detectable IL-4 and IL-10 • Elevated IgG2c (Th1) but not IgG1	(14) (24)
DNA Vaccines	• Cheap • Easy to produce • Can encode for multiple epitopes • Native conformation of antigenic determinants	• Safety • Possible genome integration • Anti-DNA antibodies • Not possible for non-proteins	DNA MOMP Priming with MOMP and secondary boost with DNA MOMP-ISCOM DNA MOMP + GM-CSF, enterotoxin (*E. coli*) A & B ompA	i.m. i.m. i.n. + i.v. i.m.	Mouse / i.v. Mouse / i.n. Pig / i.v. Pig / i.m.	• Elevated levels of IgG2a and IgG1 • Elevated levels of IgG2a, IgA and IFN-γ • Vaccination induced significant protection against genital challenge • Protection correlated with efficient T cell priming and elevated IgA • anti-MOMP antibodies and low IL-4 production	(22) (6) (25) (21)
Bacterial Ghosts	• Inactivation not required therefore relevant antigenic determinants are not denatured • Easy to produce • Require no refrigeration • Carriage of different antigens, DNA and drugs simultaneously • Recognition and phagocytosis by APC	• Presence of LPS	MOMP & PorB DNA plasmid PmpD & PorB DNA plasmid	i.m. i.m.	Mouse / i.v. Mouse / i.v.	• High levels of IgG2a and IgA • High levels of IgG2a and IgA, IFN-γ and low levels of IL-5 (Th2)	(9) (7)
Biodegradable Polymers	• Biodegradable • Non-toxic • High encapsulation capacity • PLGA`s are efficiently phagocytosed by DC and macrophages • Chitosan has mucosal adhesiveness properties and enhanced penetration across mucosal barrier		rMOMP encapsulated in PLGA Chitosan containing rMOMP DNA	s.c i.m.	Mouse	• Elevated CD4+ and CD8+ T cells • Elevated INF-γ, IL-12; reduced IL-4, IL-10 • Elevated IgG2a; reduced IgG1	(8, 26) (1)

Vaccines from Transgenic Plants	• Low cost production • Ease of use	• Requirement for strong adjuvant	MOMP introduced into *A. thaliana* and *D. carota*				(11)
Gas Vesicles	• Able to express peptides from various genes		Gen fragments coding for MOMP, OmcB, Pomp loaded into *Halobacteria*-derived gas vesicles			• Elicited Th-1 cytokines in human foreskin fibroblasts	(5)

*** i.m. (intramuscular), s.c. (subcutaneous), i.n. (intranasal), s.l. (subligual), t.c. (trancutaneous), i.v. (intravaginal)**

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