Human Vaccines 6:10, 676-680; October 2010; © 2010 Landes Bioscience

Development of a *Chlamydia trachomatis* T cell vaccine

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The immune correlates of protection for most of the currently used vaccines are based on long-lived humoral immunity. Vaccines based on humoral immunity alone are unlikely to protect against infections caused by intracellular pathogens and today's most pressing infectious diseases of public health importance are caused by intracellular infections that not only include *Chlamydia trachomatis* but also tuberculosis, malaria and HIV/AIDS. For these infections, vaccines that induce cellular immune responses are essential. Major impediments in developing such vaccines include difficulty in identifying relevant T cell antigens and delivering them in ways that elicit protective cellular immunity. In turn this is compounded by the complexity and plasticity of T cell developmental pathways that often correlate with specific aspects of protective immunity. Genomics and proteomics now provide tools to allow unbiased selection of candidate T cell antigens. This review mainly focuses on an immunoproteomic approach used in our laboratory to identify Chlamydia T cell antigens and how these T cell antigens can be developed into a future human Chlamydia vaccine.

Key words: chlamydia, vaccine, immunoproteomics, MHC, T cell, dendritic cell, antigen, adjuvant

Submitted: 04/28/10

Accepted: 05/07/10

Previously published online: www.landesbioscience.com/journals/vaccines/article/12299

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Why is a Chlamydia Vaccine Needed?

Public health programs have targeted *Chlamydia trachomatis* as a major problem mainly because of the ability of the organism to cause long-term disease sequelae such as blindness, infertility and ectopic pregnancy. The World Health Organization estimates that over 85 million cases of trachoma and 92 million cases of sexually transmitted *C. trachomatis* infection globally occur each year. *C. trachomatis* is the most common reportable communicable disease in Canada and the United States. The principle components of the control program for sexually transmitted *C. trachomatis* include case detection, antimicrobial treatment and contact tracing. In jurisdictions where programs have been systematically introduced, *C. trachomatis* case rates have substantially fallen. This has been the experience in British Columbia (BC), Canada where case rates were reduced by more than 50% between 1991 and 1997 following the introduction of a control program.[1] However, BC like other parts of Canada and several Scandinavian countries has subsequently seen a substantial rise in rates[2] concomitant with a substantial rise in reinfection rates.[1] We have hypothesized that control programs based on case identification and treatment interfere with the effects of immunity on population susceptibility to infection and termed this the "arrested immunity hypothesis". In effect, the control program appears to increase the incidence of infection by increasing susceptibility to reinfection. Interestingly parallel results have also been reported for trachoma control programs based on antimicrobial drug therapy.[3] These data suggest that the current treatment-based approach to *C. trachomatis* transmission is likely to provide incomplete control and that an effective vaccine may be the best strategy.

Chlamydia vaccine research was first initiated shortly after the initial isolation of *C. trachomatis* from cases of trachoma in the early 1960s.[4] Trials used different strains (serovars) of *C. trachomatis* as

whole inactivated bacterial cells termed elementary bodies administered intramuscularly with oil-in-water adjuvants to children at risk for trachoma. Vaccines were also tested in primate models of experimental ocular infection.[5] In aggregate, the studies generated results that have endured as the fundamental paradigm of *C. trachomatis* vaccinology. Critical observations concluded that partial protection occurs in both humans and primates but is short lived; protection is strain (serovar) specific; and infection following vaccination in primates is associated with worse inflammation.[6,7] The organism was speculated to be composed of antigens that elicited protective immunity and others that engendered immunopathology. Thus, these trials yielded important lessons for the modern era of Chlamydia vaccinology, namely that an effective *C. trachomatis* vaccine would need to be molecularly defined and engender long-lived protective immune responses. Since then Chlamydia research has focused on understanding the details of how cellular immune responses are generated and maintained in vivo during Chlamydia infection both in humans and in mice.

IFNγ-Mediated T_h1 Immune Response is Essential for Chlamydia Clearance

Although Chlamydia is clearly able to persist in hosts after the initial development of an adaptive immune response, there is strong evidence for immunity. Bailey et al.[8] have collected some of the best clinical data during studies of trachoma where they observed that host resistance dramatically increases with age and that clearance of infection correlated with enhanced cell-mediated immune responses.[9] We have studied resistance to *C. trachomatis* infection among a unique cohort of highly exposed sex workers in Nairobi, Kenya and collected immunoepidemiologic evidence for immunity. Women whose peripheral blood lymphocytes had IFNγ responses to Chlamydia antigen were highly resistant to infection whereas women who made interleukin-10 (IL-10) responses had a nearly 6 fold increased risk of acquiring infection.[10] Women with low CD4 T cell counts due to HIV had lower IFNγ responses and a greater risk of developing chlamydial pelvic inflammatory disease.[11] Collectively the clinical data show that the immune correlates for protection against *C. trachomatis* infection are cell-mediated IFNγ responses; IL-10 responses correlate with susceptibility.

The above clinical data correlating susceptibility and resistance with cell-mediated immune responses mirror data obtained in the mouse model.[12] Mice and humans are clearly different but mouse biology has proven surprisingly informative of human biology especially with respect to the molecular basis for disease. This has been true for murine *C. muridarum* infection informing the immunobiology of human *C. trachomatis* infection. *C. muridarum* is a mouse adapted strain of Chlamydia whose genome is highly syntenic to that of *C. trachomatis* and which differs in composition in only six genes.[13] In the *C. muridarum* murine model, clearance and resistance to infection were correlated with CD4 T cell immune responses[14] and IL-10 antagonized protective immunity.[15] B cells and antibody also played a role in protection against reinfection although that role strongly depended on CD4 T cell-mediated adaptive changes that occurred in the local genital tract during primary infection.[16]

Overall both the clinical and experimental data demonstrate that cell-mediated IFNγ immune responses are correlated with clearance of Chlamydia infection and resistance to reinfection. Genomics has uncovered the total repertoire within which protective Chlamydia antigens are to be found; and the new tools of proteomics offer heretofore-unavailable sensitivity in detecting immunologically relevant antigens. Furthermore, there has been substantial progress in rationalizing the whole process for the development of a vaccine based upon cellular immunity. In particular, the essential roles for dendritic cells (DCs) and MHC molecules in cell-mediated immunity have been elucidated in molecular detail.

Dendritic Cell Based Immunoproteomic Discovery of Chlamydia T Cell Antigens

DCs are at the centre of the initiation of immune responses by naïve T cells and appear to be particularly important to the development of Chlamydia immunity. DCs capture antigen in the periphery and migrate to regional lymph nodes where they present processed antigen on MHC molecules to naïve T cells. DCs undergo a maturation process during migration to lymph nodes and express new surface molecules that act as co-stimulants to naïve T cells causing activation and polarization of the cytokine secretion pattern. Therefore, in addition to containing appropriate molecular antigens, a successful Chlamydia vaccine will need to activate professional antigen-presenting cells and polarize T cell responses to the protective type cytokine secretion pattern and generate long-lived T cell memory. Initial work from our laboratory demonstrated that expression of GM-CSF—a cytokine known to mobilize DCs—in the mouse airway compartment significantly enhanced systemic T_h1 cellular immune responses following immunization with inactivated *C. muridarum*.[17] This suggested that DCs recruited by GM-CSF contributed to the development of a T_h1 immune response. Further in vitro observations demonstrated that DCs exposed to live versus to UV-irradiated *C. muridarum* develop distinct phenotypes, such that DCs exposed to live Chlamydia become mature and effectively present antigen to Chlamydia-specific CD4 T cells, while DCs exposed to UV-irradiated Chlamydia were not immunologically mature.[18] Since DCs are essential to induce Chlamydia immunity via presentation of chlamydial antigens to naïve T cells, we hypothesized that Chlamydia peptide antigens presented by the surface MHC molecules of Chlamydia infected DCs could be explored as potential source for the identification of T cell antigens.

An approach called immunoproteomics, in which peptides presented by MHC molecules are identified by tandem mass spectrometry (MS/MS),[19,20] allows genomic information to guide the delineation of the complete T cell immunoproteome of the organism. These methods have been applied to several immunological problems[21] but instrument sensitivity has prevented its applicability to pathogens. Recent advancements in MS/MS technology now provide sensitivity limits near one

femtomole (fmol) and are able to measure peptide masses to within one part-per-million accuracy.[22] This brings the detection technology into a range compatible with the levels of microbial peptides that can reasonably be purified from MHC molecules presented on the surface of antigen presenting cells such as DCs.

The immunoproteomic approach that we used to identify Chlamydia T cell antigens involved multiple steps (**Fig. 1**). First, bone marrow cells isolated from the femurs and tibias of mice are grown in the presence of GM-CSF and IL-4. These bone marrow derived DCs (BM-DCs) are pulsed with Chlamydia. Pulsed BM-DCs are lysed and MHC molecules isolated using allele-specific anti-MHC monoclonal antibody affinity columns. Purified MHC molecules are then washed and peptides eluted with acetic acid and separated from high molecular weight material by ultrafiltration through 5-kDa cut-off membranes. The purified MHC-bound peptides are initially analyzed qualitatively using an LTQ-OrbitrapXL (Thermo Electron) online coupled to a nanoflow HPLC using a nanospray ionization source. The mass spectrometer is set to fragment the five most intense multiply-charged ions per cycle. Fragment spectra are extracted using DTASuperCharge (http://msquant.sourceforge.net) and searched using the Mascot algorithm against a database comprised of the protein sequences from *C. muridarum*. DC adoptive transfer method is employed to deliver peptides that are recognized by Chlamydia specific CD4 T cells to identify those that are protective. The selected peptides are further characterized by cloning the parent proteins of these MHC-binding peptides as vaccine candidates and tested in a genital tract mouse model.

We used the immunoproteomic approach to identify 13 Chlamydia peptides derived from 8 novel epitopes presented by MHC class II molecules from BM-DCs infected with Chlamydia (**Table 1**).[23] These MHC class II-bound peptides were recognized in vitro by Chlamydia specific CD4 T cells harvested from immune mice recovered from Chlamydia infection and adoptive transfer of DCs pulsed ex vivo with the peptides partially protected mice against intranasal and genital tract Chlamydia infection. We further investigated these peptides by cloning recombinant proteins corresponding to the MHC binding peptides. Recombinant Chlamydia proteins were also found to be recognized in vitro by immune T cells suggesting that these proteins can be processed to generate the immunologically relevant peptides. These proteins were also able to protect mice against Chlamydia infection in vivo as shown by DC adoptive transfer experiments.[24] Based on these results, three of the 8 source proteins (RplF, PmpG and PmpE/F) were deemed suitable for further evaluation. However, only PmpE/F and PmpG were selected for further animal studies as RplF has significant homology to the human homolog and thus may not be suitable as a human vaccine.

The immunoproteomic approach described above directly identifies T cell epitopes presented by antigen presenting cells resulting in a vast improvement in the positive validation rate. Another advantage in using immunoproteomics is that the peptides identified are the result of physiological processing and presentation pathways and are based on both the affinity for the MHC molecules as well as the frequency of their presentation.

Pathogens contain a large number of possible antigens for immune responses but only a few immunodominant antigens are typically recognized following infection or immunization. The reasons for immunodominance are complex and can be intrinsic to the targets (i.e., differences in affinity for immune receptors) or extrinsic (i.e., competition that suppresses the response to one target in favor of another).[25] We hypothesized that the antigens we identified through the immunoproteomic approach are immunodominant and can be recognized in varying MHC backgrounds. We tested whether the peptides (originally identified in C57BL/6) and their source proteins are also recognized by mouse strains other than C57BL/6. As expected, proteins and peptides were readily recognized by T cells from C57BL/6 mice. Proteins, but not peptides, were also recognized by Chlamydia-specific T cells from Balb/c and C3H/HeN mice (unpublished data). These data suggest that there are T cell epitopes within the identified proteins that are presented by MHC haplotypes other than I-A^b and that these Chlamydia proteins are immunologically recognized in genetically different strains of mice, arguing that they are immunodominant.

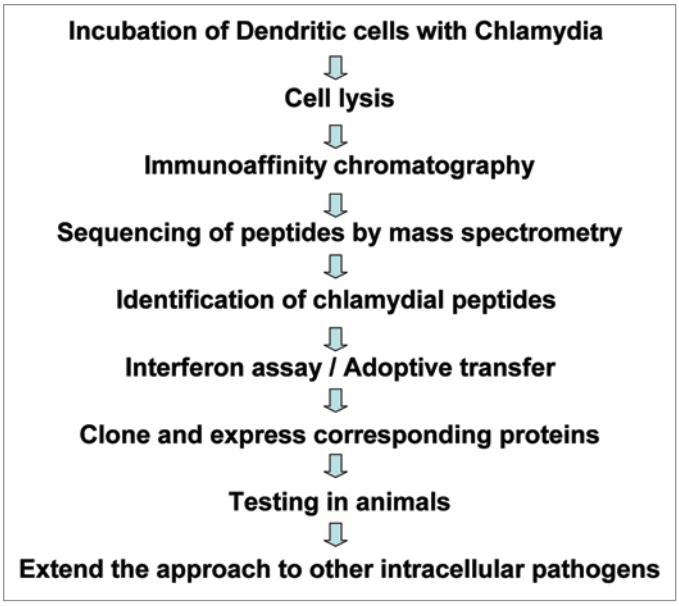

Figure 1. Schematic depiction of the sequence of steps involved in the immunoproteomic approach for Chlamydia T cell vaccine development.

Delivery Systems and Immunomodulators

Since a Chlamydia vaccine cannot be practically delivered via adoptive transfer of

Table 1. *Chlamydia* T cell antigens identified by immunoproteomic approach

Peptide sequence	Source protein	Abbreviation
KGNEVFVSPAAHIIDRPG	Ribosomal protein L6	RplF
SPGQTNYAAAKAGIIGFS	3-oxoacyl-(acyl carrier protein) reductase	FabG
KLDGVSSPAVQESISE	Anti-anti-sigma factor	Aasf
ASPIYVDPAAAGGQPPA	Polymorphic membrane protein G	PmpG-1
DLNVTGPKIQTDVD	Hypothetical protein TC0420	TC0420
IGQEITEPLANTVIA	ATP-dependent Clp protease, proteolytic subunit	ClpP-1
AFHLFASPAANYIHTG	Polymorphic membrane protein F	PmpE/F-2
MTTVHAATATQSVVD	Glyceraldehyde 3-phosphate dehydrogenase	Gap

DCs, it is essential to identify a delivery system together with an immunomodulator that elicits protective cell-mediated immunity exhibiting a T_h1 bias. The three major adjuvant systems that we have evaluated include DDA/TDB (Dimethyldioctadecylammonium Bromide/D-(+)-trehalose 6,6'-dibehenate), AbISCO (an effective immune stimulating complex) and CpG ODN (CpG oligonucleotide). Among the three adjuvants tested, CpG ODN formulation was not able to engender protection against Chlamydia infection at any level in vaccinated mice and the AbISCO formulation conferred only moderate protection. Strikingly, studies using DDA/TDB substantially improved the protective efficacy of chlamydial protein antigens in murine models of genital tract *C. muridarum* infection.[26]

DDA/TDB is a recently discovered adjuvant that consists of cationic liposomes as delivery system and a synthetic mycobacterial cord factor as immunomodulator. One of the important observations that we made during the evaluation of DDA/TDB in our adjuvant preparations is that it induced T_h17 in addition to strong T_h1 immune response.[26] It is known that TDB selectively activates the FcRγ-Syk-Card9 pathway in antigen presenting cells to induce a unique innate immune activation program that directs protective T_h1 and T_h17 immunity after subunit *Mycobacterium tuberculosis* vaccination[27] demonstrating that TDB adjuvant with this mode of action is distinct from TLR-triggering adjuvants such as CpG engaging MyD88.[28] TDB was recently shown to use the monocyte-inducible C-type lectin (Mincle) as its cell receptor on the surface of APCs.[29]

A reason why the T_h17 Chlamydia antigen-specific response was not found in mice vaccinated with CpG formulation might be that CpG may inhibit T_h17 differentiation through IL-12 and IFNγ.[30] Based on these observations, we speculate that an adjuvant that triggers a T_h17 response in addition to T_h1 may be the optimum choice for Chlamydia vaccine. Interestingly, a recent study from our laboratory also showed that differences in T_h1/T_h17 responses could explain differences in susceptibility and resistance to *C. muridarum* infection in Balb/c and C57BL/6 mice.[31]

We evaluated protection against Chlamydia infection mice immunized with both individual proteins as well as in combination formulated with different adjuvants. The protein combination consist of PmpG, PmpE/F and MOMP (MOMP is the major outer membrane protein and a reference chlamydial antigen used in many vaccine immunological studies) gave promising results approaching the levels of protection observed in positive controls using live Chlamydia infection.[26] The superior protection obtained in the protein combination group compared with individual protein group suggests that a successful Chlamydia vaccine will need to be composed of multiple proteins in order to provide broad coverage in an outbred population, to cross protect against multiple variants of *C. trachomatis* as well as to maximize immunogenicity.

Since all the protection results described above were observed in C57BL/6 mouse, the strain in which the antigens were originally discovered by immunoproteomics, we also tested the protection efficacy of multiple Chlamydia protein antigens and DDA/TDB in Balb/c mice that has a different MHC genetic background. Remarkably the vaccine combination engendered significant protection in Balb/c mice suggesting the T cell antigens identified through the immunoproteomic approach are truly immunodominant.

Future Work

Even though vaccine formulated with three Chlamydia T cell antigens and DDA/TDB adjuvant generated significant protection against infection, it remains important to evaluate protection against tissue pathology. Importantly, mice primed by a prior intranasal infection were completely protected against the development of oviductal pathology following intravaginal challenge with Chlamydia. We therefore have initiated experiments with different adjuvant formulations together with multiple antigens in mice to examine for pathology as determined by visually apparent hydrosalpinx in oviducts. It remains important to discover the mechanism behind the different outcomes for oviductal pathology following infection-induced versus vaccine-induced immunity. Clearly an ongoing challenge for Chlamydia vaccine research remains the discovery of strategies that maximize the protective effects of immune T cells while simultaneously preventing such cells from causing immune-mediated tissue damage. Finally it also appears likely that the immunoproteomic approach we are exploring in Chlamydia vaccine research may be useful for other problematic intracellular pathogens such as tuberculosis, malaria and HIV for which vaccine solutions are desperately sought.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (grant no. R01AI076483).

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