High-throughput proteomic screening identifies *Chlamydia trachomatis* antigens that are capable of eliciting T cell and antibody responses that provide protection against vaginal challenge

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Introduction

Chlamydia infection remains the most frequently reported sexually transmitted disease and is a significant public health concern due to its often asymptomatic presence and potentially serious long term consequences to reproductive health and fertility. The most productive step toward control and spread of disease would be the development of an effective prophylactic vaccine. The ideal vaccine would be comprised of Chlamydia antigens that can stimulate both T cell and robust antibody responses. Several B cell antigens have been shown to be protective *in vivo*, yet vaccine success may be improved through the identification and inclusion of additional T cell antigens. A screening platform that individually evaluates both $CD4^+$ and $CD8^+$ T cell responses to every *Chlamydia trachomatis* protein was developed and used to identify the antigen specificity of T cells derived from C57BL/6 mice intraperitoneally infected with *C. trachomatis* serovar D or LGVII. A total of 18 antigens were identified with five selected for further evaluation based upon several factors including the protein identity, predicted location within bacteria, as well as the ability to produce recombinant protein of sufficient quantity. Preliminary immunogenicity studies were conducted with purified recombinant CT111, CT144, CT242, CT687 and CT823 combined with the ISCOM adjuvant AbISCO-100. Antigen specific T cell immunogenicity was determined by IFN-γ ELISPOT, as well as IL-2 and TNF-α production. Of the 5 proteins tested, all elicited both $CD4^+$ and $CD8^+$ T cells when immunized with AbISCO-100 as compared to immunization with a Chlamydia protein that was not identified as a T cell antigen. Further characterization of the immune response determined that antibodies were also generated and in some cases skewed toward a T_H1 response. The proteins were also evaluated in a murine vaginal challenge model; the most promising of these, CT144 and CT823, were selected for more extensive evaluation and are presented here. Immunization with CT144 or CT823 with AbISCO-100 reproducibly reduced the vaginal bacterial burden and percent of animals infected. In addition, immunization with CT823 and AbISCO-100 induced long term protection, >90 days post boost. The results of these studies provides the basis for further work in identifying the protective T cell antigens present in human Chlamydia patients.

Methods

Protein expression and purification. Genes of interest were cloned into the pET45b vector containing an N-terminal 6X-HIS tag and transformed into *E. coli* (DE3) for protein expression. Proteins were purified by Ni^{+2} chromatography and formulated into a final buffer of 20 mM Tris-HCl, 150 mM NaCl. Purity was determined by SDS-PAGE to be >70% and endotoxin level by LAL was ~1 EU/μg.

Immunization. Test formulations were prepared by direct addition of recombinant Chlamydia proteins with the ISCOM adjuvant AbISCO-100 (Isconova, Uppsala Sweden). Protein doses were tested at 5-10 μg per dose and the adjuvant remained constant at 12 μg per dose and administered s.c. in the scruff of the neck. Animals were primed on Day 0 and boosted on day 7.

T cell immunogenicity by IFN-γ ELISPOT. C57BL/6 female mice (three/group) were immunized as described above. Splenocytes were harvested on day 14 and $CD4^+$ and $CD8^+$ T cell populations were enriched using Miltenyi magnetic bead isolation. T cells were plated at 200,000 per well on ELISPOT membranes pre-coated with Mabtech mouse IFN-γ capture antibody. Antigen presenting cells (APC) were prepared from T cell depleted splenocytes from unimmunized mice and pulsed with antigen for 2 hours. For $CD4^+$ T cell stimulation, APC were pulsed with 20 μg/mL of recombinant protein. APC pulsing for $CD8^+$ T cell stimulation required that the protein be premixed with equal volumes of AbISCO-100. APC were then washed and added to the T cells at 100,000 per well and the plates incubated for 18 hours at 37 C. Supernatants were harvested for cytokine analysis and the ELISPOT plates were developed using the Vector Biolabs Vectastain ABC Peroxidase Kit and BD Biosciences AEC substrate development reagents. Spot forming units (SFU) were quantified using an automated ELISPOT reader system (Carl Zeiss) with KS Elispot Software.

Methods

Cytokine Bead Array. Supernatants from the IFN-γ ELISPOT were harvested and assayed for TNF-α, IL-2, IL-10 and IL-17 using the mouse T_H1/T_H2 Cytokine Bead Array Kit (BD Biosciences). Briefly, the supernatants incubated with beads conjugated to anti-cytokine monoclonal antibodies and phycoerythrin-conjugated antibodies. Each cytokine bead-detector combination has a distinct fluorescent profile which was quantified by flow cytometry. The quantity of each cytokine was determined by comparison of fluorescent intensities between test samples and the respective standard curve.

Protein ELISA. ELISA plates were coated with protein at 5 μg/mL (CT144) and 9 μg/mL (CT823) in 0.1 M carbonate coating buffer pH 9.5 overnight at 4 C. Plates were washed with TBS-Tween 20 (TBS-T) and blocked with TBS-T + 1% BSA for 1 hour. Serum samples were serially diluted and incubated for 2 hours at room temperature. Goat anti-mouse alkaline phosphatase conjugated IgG, IgG1 (Jackson Immunology) and IgG2c (Southern Biotech) antibodies were diluted 1:10,000 and 1:4000, respectively, and incubated for 1 hour. Plates were developed with PNPP (Sigmafast, Sigma-Aldrich) stopped with 3N NaOH and read at 405 nm. Endpoint titers were calculated to be the dilution where serum Ig levels equaled twice the plate background.

Chlamydia vaginal challenge. *Chlamydia trachomatis* serovar D (D/UW-3/CX) elementary bodies (EB) were propagated in McCoy cells and purified by sucrose gradient. UV irradiated elementary bodies (UVEB) were prepared by 1 hour exposure to UV light followed by MicroBCA protein assay (Thermo Scientific). C57BL/6 mice (8 per group) were immunized with protein + AbISCO-100 as described above or 20 μg UVEB + AbISCO as a positive vaccination control. Depo-Provera (1.25 mg) was administered s.c. 10 and 3 days prior to vaginal challenge with $5x10^5$ IFU of purified EB. Vaginal swabs were collected on days 3, 7, 14 and 21 post-infection. Bacterial burden was determined by direct culture on McCoy monolayers, stained with anti-Chlamydia-FITC antibody (Millipore) and inclusions counted under epi-fluorescence microscopy.

Local T cell antigen recognition. C57BL/6 and Balb/c mice were infected intravaginally with Chlamydia as described above. Fourteen days post-infection the draining lymph nodes (DLN) were harvested and $CD4^+$ and $CD8^+$ T cells were isolated by magnetic bead isolation (Miltenyi) and IFN-γ ELISPOT was conducted as described above.

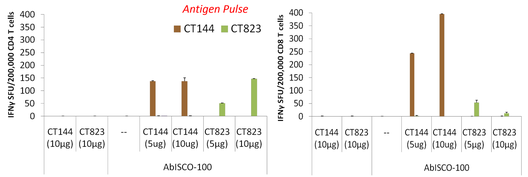
Results

5 selected antigens represent CD4 and CD8 responses and multiple locations. Local antigen-specific T cell recognition persists across MHC type.

Gene number	T cell recognition in screen	Predicted function and location in bacteria	Antigen recognition in DLN			
			Balb/c		C57BL/6	
			CD4	CD8	CD4	CD8
CT111	CD8	groES chaperonin/ cytoplasm	+++	+	-	-
CT144	CD4	Hypothetical / unknown	++	+	+	-
CT242	CD8	OmpH-like / outer membrane	+	++	+	-
CT687	CD8	yfh0_1cysteine desulfurase/ cytoplasm	+	+	+	-
CT823	CD8	DO serine protease / periplasm	++	++	+	+*

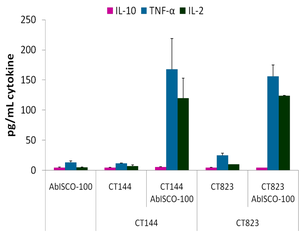
T cells from reproductive tract draining lymph nodes of vaginally infected mice specifically recognize selected antigens. T cell recognition is not restricted to the MHC type, as the screen was performed using C57BL/6 yet Balb/c T cells recognize all 5 proteins at robust frequency while responses in C57BL/6 are poor to negative.
Average of duplicate experiments.
+++ >100 IFN-γ SFU/200,000 T cells + 5-50 control IFN-γ SFU/200,000 T cells
++ 50-100 IFN-γ SFU/200,000 T cells - no SFU detected
* Data from one study

Immunization with recombinant proteins induces polyfunctional T cell responses and T_H1 antibodies



IFN-γ T cell responses to CT144 and CT823 are induced by protein immunization.

Splenic T cells from mice immunized with CT144 or CT823 with AbISCO-100 adjuvant were harvested and the antigen specific T cell frequencies were determined by ELISPOT. Both proteins induced robust antigen-specific T cell responses in multiple experiments with AbISCO-100 as well as other test adjuvants (data not shown). No IFNγ was produced by T cells stimulated with APC's alone or pulsed with a Chlamydia protein not identified in the T cell screens. Data are shown as the mean spot forming units (SD) per 200,000 sorted T cells from pooled splenocytes of three mice per group.

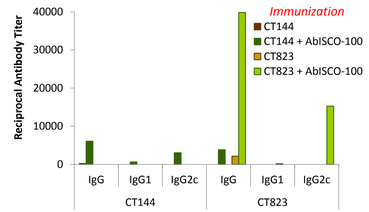


T cell responses to CT144 and CT823 are polyfunctional

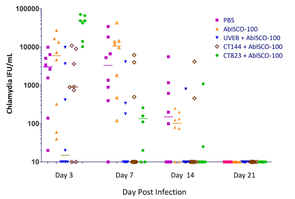
Cell-free supernatants were harvested from ELISPOT plates and evaluated by cytometric bead array for polyfunctional cytokines. Both TNF-α and IL-2 were produced in response to immunization. IL-4, IL-6 and IL-17 were also evaluated and were not detected in any sample. This profile is consistent with a polyfunctional T_H1 response. Data are shown as the mean cytokine concentration in triplicate wells SD.

T_H1 antibodies are detected in serum of immunized animals

Blood was collected by cardiac puncture on day 14. Serum was assayed for antigen specific IgG, IgG1 and IgG2c antibodies by ELISA. When adjuvant was present during immunization, both antigens induced detectable IgG and IgG2c antibodies indicating a T_H1 response. Data are shown as the endpoint titer defined as the highest dilution greater than twice the background.



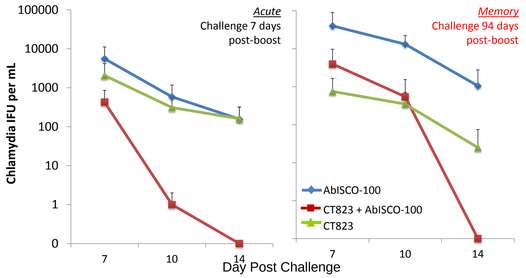
Immunization with recombinant proteins protects against vaginal challenge



Immunization with CT144 and CT823 accelerates bacterial clearance.

C57BL/6 mice were immunized with CT144 or CT823 and AbISCO-100 on Day 0 and Day 7. Mice were challenged Day 14 with $5x10^5$ IFU of *C. trachomatis* serovar D and monitored by genital swab culture. Immunization with protein plus adjuvant reduced bacterial burden on day 7 and cleared infection more rapidly than controls of PBS or adjuvant alone. Data are shown as the IFU/mL, horizontal lines indicate the median for the group. Protection by immunization with CT144 and CT823 + AbISCO-100 is statistically significant compared with immunization with adjuvant alone ($p<0.05$) at all time points as determined by two-way ANOVA analysis .

CT823 immunization induces long-lived protective memory



Immunization with CT823 plus AbISCO-100 induces a protective memory response.

C57BL/6 mice (8 per group) were immunized twice, seven days apart, with CT823 plus AbISCO-100 and challenged either on day 7 post-boost or allowed to rest for 94 days prior to challenge. Animals immunized with CT823 + AbISCO cleared their infections more rapidly both in the acute and memory phases of the experiment.

Conclusions

1. Full proteomic screen of Chlamydia proteins yielded antigens that are recognized by draining lymph node T cells from vaginally infected mice.
2. Recombinant protein antigens CT144 and CT823 administered subcutaneously with AbISCO-100 induce polyfunctional T cell responses with a T_H1 profile.
3. Antibody can be detected as early as 7 days post-boost, also demonstrating a T_H1 bias.
4. Immunization with either CT144 or CT823 with adjuvant can accelerate Chlamydia clearance from the reproductive tract after vaginal challenge.
5. Protection induced by immunization with CT823 + AbISCO-100 is long-lived.
6. Identification of protective T cell antigens in mice leads to development of methods for identifying the protective T cell antigens present in human Chlamydia patients.