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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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ABSTRACT

A comprehensive proteomic screening technology was previously used to characterize T cell responses to *Chlamydia trachomatis* infection. In this study, we demonstrated that T cells specific for protein antigens identified through this comprehensive technology home to the site of infection after mucosal challenge with *C. trachomatis*. In addition, T cell responses to these proteins were elicited in multiple genetic backgrounds. Two protein antigens, CT823 and CT144, were evaluated as vaccine candidates. When administered with AbISCO-100 adjuvant, these antigens stimulated potent $CD8^+$ T cell responses, polyfunctional T_H1-polarized $CD4^+$ T cell responses, and high titer protein-specific T_H1-skewed antibody responses. Vaccination with either antigen with AbISCO-100 provided long-lived protection against intravaginal challenge with *C. trachomatis.* Adoptive transfer of immune T cells also conferred protection in the challenge model whereas passive transfer of immune serum did not, indicating the critical role for T cell responses in control of this infection. The ability of these antigens to induce potent immune responses and provide long-lived protection in response to challenge provides a basis for the rational design of a *C. trachomatis* subunit vaccine.

1. Introduction

Chlamydia trachomatis is the most common sexually transmitted bacterium and creates over $2B in health care costs annually in the United States [1,2]. The infection is often asymptomatic, resulting in delayed treatment and frequent sequelae including pelvic inflammatory disease, ectopic pregnancy, and infertility. Consequently, development of a prophylactic vaccine represents a promising strategy for control.

While the role of $CD8^+$ T cells remains somewhat controversial, T_H1 type $CD4^+$ T cells and B cells appear critical for the control of infection [3–10], however the development of a safe and effective live attenuated *Chlamydia* vaccine remains elusive. Another approach that may be successful is the creation of a subunit vaccine. A successful subunit vaccine will need to drive T cell activation in response to primary infection, as well as induce a robust antibody response, necessary for protection against repeated exposure.

The identification of T cell specific antigens that could provide broad coverage in a human population has been difficult due to significant diversity of HLA alleles. Antigen targets have been identified using a variety of methods, however, these methods have not been comprehensive [11–14]. Thus far, subunit vaccine candidates targeting T cells have proven to be only partially protective, reinforcing that efficacy may require combinations of B and T cell antigens, as well as superior delivery vehicles or adjuvants that can prime effective immune responses.

Previously, using a high-throughput method to characterize T cell responses to the complete *C. trachomatis* proteome, we identified 13 antigens that induced either $CD4^+$ or $CD8^+$ T cell responses in experimentally infected mice [15,16]. Two antigens, designated CT144 and CT823, administered with the adjuvant AbISCO-100, induced potent T cell responses and elicited measurable protection against murine vaginal challenge [16]. Here we report additional characterization of these antigens for potential effectiveness as vaccine candidates.

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2. Materials and methods

2.1. Protein expression and purification

Genes of the desired proteins were cloned into the pET45b vector and transformed into *Escherichia coli* (ArcticExpress(TM) DE3) for protein expression. Proteins were purified by Ni^{2+} chromatography, and dialyzed into 20 mM Tris–HCl, 150 mM NaCl buffer, pH 8.0. Purity was determined to be >70% by SDS-PAGE, and endotoxin levels were confirmed to be <1 EU/μg by a limulus amebocyte lysate (LAL) assay (Biowhittaker Inc., Walkersville MD).

2.2. Animals and immunizations

Female C57BL/6 and BALB/c mice were purchased at 6–8 weeks of age from Charles River Laboratories (Wilmington, MA). All animal procedures were conducted with the approval of the institutional IACUC. Vaccine formulations in all experiments were comprised of 10 μg recombinant *Chlamydia* proteins with 12 μg of the ISCOM adjuvant, AbISCO-100 (Isconova, Uppsala, Sweden), formulated in PBS. As a positive control, animals were immunized with 20 μg UV-inactivated EB (UVEB) and 12 μg of AbISCO-100. Animals were vaccinated subcutaneously in the scruff of the neck twice, seven days apart.

2.3. Bacterial stocks and Chlamydia quantification

C. trachomatis serovar D (D/UW-3/CX) bacteria were propagated in McCoy cells, and elementary bodies (EB) were purified by RenoCal-76 gradient centrifugation (Bracco Diagnostics, Princeton, NJ) and stored in SPG buffer (0.25 M sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid). Titers of purified stocks and swab samples were quantified by direct culture on McCoy cell monolayers. 44 h post-infection, the monolayers were then fixed in 100% methanol, and stained with FITC labeled anti-*Chlamydia* antibody (Millipore); inclusions were counted for determination of IFU. UVEBs were prepared by UV irradiation for 1 h. The protein concentration was determined by MicroBCA (Thermo Scientific).

2.4. Vaginal challenge

C57BL/6 mice were immunized as described on days 0 and 7. Depo-Provera (1.25 mg) was administered subcutaneously in the flank on days 4 and 11, and then mice were infected by intravaginal deposition of 5×10^5 IFU *Chlamydia* onto the ectocervix on day 14. Vaginal samples were obtained using Dacron swabs on days 3, 7, 10, 14, and 21 following challenge (corresponding to days 17, 21, 24, 28, and 35 of the study). The bacterial burden was determined by direct culture.

2.5. T cell immunogenicity by IFN-γ ELISPOT

Splenic T cell suspensions were enriched for $CD4^+$ and $CD8^+$ T cells by magnetic bead isolation (Miltenyi, Auburn, CA). T cells were plated at a density of 200,000 cells/well on PVDF membranes pre-coated with anti-mouse IFN-γ capture antibody. (Mabtech, Mariemont, OH). Antigen presenting cells (APC) were prepared from T cell-depleted splenocytes of unimmunized mice and pulsed with antigen for 2 h at 37 °C. For $CD4^+$ T cell stimulation, APC were pulsed with 20 μg/mL of recombinant protein, pulsing for $CD8^+$ T cell stimulation required premixing of the protein with AbISCO-100 prior to addition. Antigen-loaded APC were washed and added to the T cells at 100,000/well, followed by incubation for 18 h at 37 °C. Supernatants were harvested for cytokine analysis by Cytometric Bead Array, and ELISPOT plates were developed using the Vector Biolabs Vectastain ABC Peroxidase Kit (Philadelphia, PA) and AEC substrate-development reagents (BD Biosciences, Franklin Lakes, NJ). Spot forming units (SFU) were quantified by ZellNet Consulting (Fort Lee, NJ) using an automated ELISPOT reader system (Carl Zeiss) with KS ELISPOT software.

2.6. Measurement of cytokines

Supernatants from the IFN-γ ELISPOT were analyzed for levels of TNF-α, IL-2, IL-10, IL-4, IL-6, and IL-17A using a mouse T_H1/T_H2/IL-17 Cytometric Bead Array (BD Biosciences) following the manufacturer's instructions.

2.7. Measurement of IgG, IgG1, and IgG2c

Protein- and EB-specific serum antibody titers were determined by ELISA. Blood, collected by either retro-orbital sampling or terminal cardiac puncture, was processed and serum stored at −80 °C. ELISA plates were coated overnight at 4 °C with 5 μg/mL CT144, or 9 μg/mL CT823, respectively, in 0.1 M carbonate buffer, pH 9.5; or at 37 °C with 5 μg/mL live EB in 0.5 M Na_2CO_3, pH 8.2. For protein ELISA, plates were washed with TBS + 0.05% Tween-20 (TBS-T) and blocked with TBS-T + 1% bovine serum albumin for 1 h. Serum samples were serially diluted and incubated in the antigen-coated wells for 2 h at room temperature. Plates were then washed and probed for 1 h with goat anti-mouse alkaline-phosphatase (AP)-conjugated anti-IgG (Sigma), -IgG1 (Jackson Immunology) diluted 1:10,000, or -IgG2c (Southern Biotech) diluted 1:4000. For EB capture, plates were blocked with 10% human AB serum for 1 h, after which serum samples, serially diluted in 2% human AB serum, were added and incubated for 2 h at 37 °C. The plates were washed with TBS-T, and probed with biotinylated goat anti-mouse antibody (Jackson Immunology), diluted 1:1000 in 2% human AB serum, for 1 h. Detection of AP activity for both assays was achieved by the addition of p-Nitrophenyl phosphate (pNPP; Sigmafast, Sigma–Aldrich); the reaction was stopped with 3 N NaOH, and read at 405 nm. Endpoint titers were calculated by extrapolation of the linear portion of the serial dilutions, with the endpoint defined as the dilution at which the linear portion of the curve intersects with background, which was 2 times the value of the negative control (*i.e.* serum from a naïve mouse).

2.8. Local T cell antigen recognition

10–15 BALB/c mice per group were intravaginally infected with 1×10^6 IFU purified *C. trachomatis* serovar D EB. 14 days post-infection, lateral iliac, aortic lumbar and sacral draining lymph nodes (DLN) were harvested and antigen specific T cell responses were determined by IFNγ ELISPOT assay on sorted $CD4^+$ or $CD8^+$ T cells as described.

2.9. Transfer of T cells and antiserum

Donor C57BL/6 mice (9 per group) were vaccinated twice with CT823 (10 μg), CT144 (10 μg), or UVEB (20 μg), formulated with AbISCO-100, on days 0 and 7. Splenocytes and whole blood were harvested on day 14. Whole blood was allowed to clot at room temperature, and centrifuged at 3000 rpm for 10 min. The sera were harvested, pooled, and kept on ice until transfer. Spleens were homogenized, red blood cells removed by ACK lysis, then T cells were enriched by magnetic bead isolation. T cells were resuspended in pyrogen-free PBS at a concentration of 2.5×10^7 cells/mL. Immune T cells and serum were separately transferred, by intravenous injection (200 μL), into naïve mice that had been given 2.5 mg Depo-Provera subcutaneously 6 days prior. The mice were challenged with 1×10^6 IFU *C. trachomatis* serovar D 18 h post transfer and the course of infection was monitored as described. The

Table 1
T cells are primed to novel antigens after genital infection.

Antigen name	DLN T cell response (IFN-γ SFU/200,000 T cells)		Protein name and predicted function
	$CD4^+$	$CD8^+$	
CT111*	174 ± 10	42 ± 8	GroES – chaperonin, stress response protein (HSP10)
CT144**	27 ± 9	<10 SFU	Hypothetical protein – function unknown
CT242*	<10 SFU	29 ± 3	OmpH like – probable outer membrane protein
CT687*	<10 SFU	38 ± 4	yfhO_1 – cysteine desulfurase, amino acid metabolism
CT823*	75 ± 16	37 ± 12	htrA – serine protease

Select antigens identified via *Chlamydia* full proteomic screens, with their protein designation and predicted functions indicated. CT144 was identified as a $CD4^+$ T cell antigen (**), all other proteins were identified as $CD8^+$ T cell antigens (*) in the proteomic library screen. Draining lymph node (DLN) T cells were harvested from intravaginally infected BALB/c mice, and IFN-γ ELISPOT was performed with the indicated recombinant *Chlamydia* proteins as restimulation antigens. T cell frequencies are expressed as the mean ± SD IFN-γ SFU per 200,000 T cells in triplicate wells. 10 SFU was the maximal response to OVA, the negative control protein in the assays. Data are representative of three separate experiments.

antigen-specific responses of the T cells and sera were assayed by IFN-γ ELISPOT and IgG ELISA.

2.10. Statistical analysis

T cell ELISPOT data were analyzed by 2-way Student's *T* tests using Prism software. For analysis of challenge data, 2-way ANOVA was performed. The IFU/mL values were modeled as a function of treatment and time. Where significance was achieved, Bonferroni post-tests were performed.

3. Results

3.1. Antigen-specific stimulation of T cells in multiple MHC backgrounds

C. trachomatis serovar D proteomic screens of T cells derived from C57BL/6 mice intraperitoneally infected with *C. trachomatis* serovar D identified 13 antigens, five of which were chosen for further study as possible vaccine candidates [16]. Because these T cells were responding to *Chlamydia* exposure from an artificial route of infection in a single strain of mouse, studies were performed to determine whether T cells with the same specificities home to the draining lymph nodes (DLN) following a genital infection in a different MHC background. BALB/c mice were intravaginally infected with 1×10^6 purified EB from *C. trachomatis* serovar D. Draining lymph nodes (DLN) were collected on day 14 post-infection, and $CD4^+$ and $CD8^+$ T cells isolated, after which antigen-specific IFN-γ production was determined by *ex vivo* ELISPOT analysis. In the original screens of peritoneally infected mice, CT144 was identified as a $CD4^+$ T cell antigen, and the other four proteins were identified as $CD8^+$ T cell antigens. In the DLN studies, shown in Table 1, all five antigens were recognized by $CD4^+$ or $CD8^+$ T cells isolated from the DLN of intravaginally infected mice. The T cell subset that responded was consistent between peritoneal and genital infection, with the exception of responses to CT111 and CT823, which were recognized by both T cell subsets in the DLN but only by $CD8^+$ T cells in the peritoneal model. The greatest magnitude of response was measured against CT111 in the $CD4^+$ T cell subset. Thus, while all the antigens identified in the original C57BL/6 T cell screens of the *Chlamydia* proteomic library were detected in DLN-derived T cells from intravaginally infected BALB/c mice, the relative intensity of the $CD4^+$ and $CD8^+$ T cell responses did not necessarily reflect the subset from which they were originally detected.

3.2. Evaluation of T cell activation and antibody production in response to protein immunization

Previous studies in our laboratory revealed that immunization with either CT144 or CT823, formulated with adjuvant, was capable of promoting T cell responses and eliciting protection against *Chlamydia* infection in C57BL/6 mice [16]. Since IFN-γ has been implicated as a critical modulator of chlamydial infection [17], ELISPOT analysis was performed to measure the frequency of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in immunized mice. Fig. 1A confirms previous data demonstrating that the frequency of antigen-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, obtained from immunized mice in the presence of adjuvant, was significantly higher compared to that of PBS or un-adjuvanted controls [16]. A polyfunctional T cell response, including the secretion of IL-2, IFN-γ, and TNF-α, is critical for mounting an effective response to infection [18]. To determine the scope of antigen specific responses to CT144 and CT823, respectively, Cytometric Bead Array analysis was performed on supernatants obtained from the $CD4^+$ IFN-γ ELISPOT assay. Polyfunctional, T_H1-dominant $CD4^+$ T cells were identified, demonstrated by significant secretion of TNF-α and IL-2 (Fig. 1B) in addition to IFN-γ (Fig. 1A), in the absence of appreciable T_H2 cytokines, including IL-10 (Fig. 1B). While the array also measured IL-17, IL-4, and IL-6, concentrations were below the level of detection (data not shown).

T cell cytokine production is known to affect IgG-isotype switching; T_H1 induces IgG2a and IgG2c, while T_H2 favors IgG1 production [19]. To examine the influence of immunization with either CT144 or CT823 on specific Ig isotype generation, sera obtained from vaccinated mice were evaluated by protein ELISA. As shown in Fig. 1C, immunization with CT823 + AbISCO-100 resulted in a marked increase in IgG2c secretion, but not IgG1. Similarly, with CT144 vaccination the IgG1 to IgG2c ratio is less than 1 (0.18) indicating a T_H1 dominance. These results correlate with our earlier observation of a T_H1-dominant $CD4^+$ T cell phenotype in the vaccinated animals.

3.3. Vaccination with recombinant protein protects mice against Chlamydia challenge

To more closely examine the kinetics of protection in immunized mice, C57BL/6 mice were immunized with CT144, CT823, or UVEB in the presence of adjuvant followed by intravaginal infection with *Chlamydia*. The bacterial burden in the ectocervix was measured on days 3, 7, 10, 14 and 21 post-challenge. As shown in Table 2, by day 10 post-challenge, CT823-immunized mice showed over a log reduction in cervico-vaginal bacterial burden compared to control mice (117 ± 26 *vs.* 1466 ± 541 IFU, respectively). Immunization with the positive control, UVEB plus AbISCO-100, promoted rapid clearance of bacteria in mice with a four-fold reduction in bacterial burden as early as day 3 post-infection, and only 53% of animals were still infected by day 7. This early reduction was not seen in CT823-immunized animals. Mice immunized with CT144 showed a different profile, with approximate equivalent reduction in bacterial burden as the UVEB-immunized animals on day 3, but then plateaued at a more modest half-log reduction in bacterial burden by day 7, and then a 2–3 fold

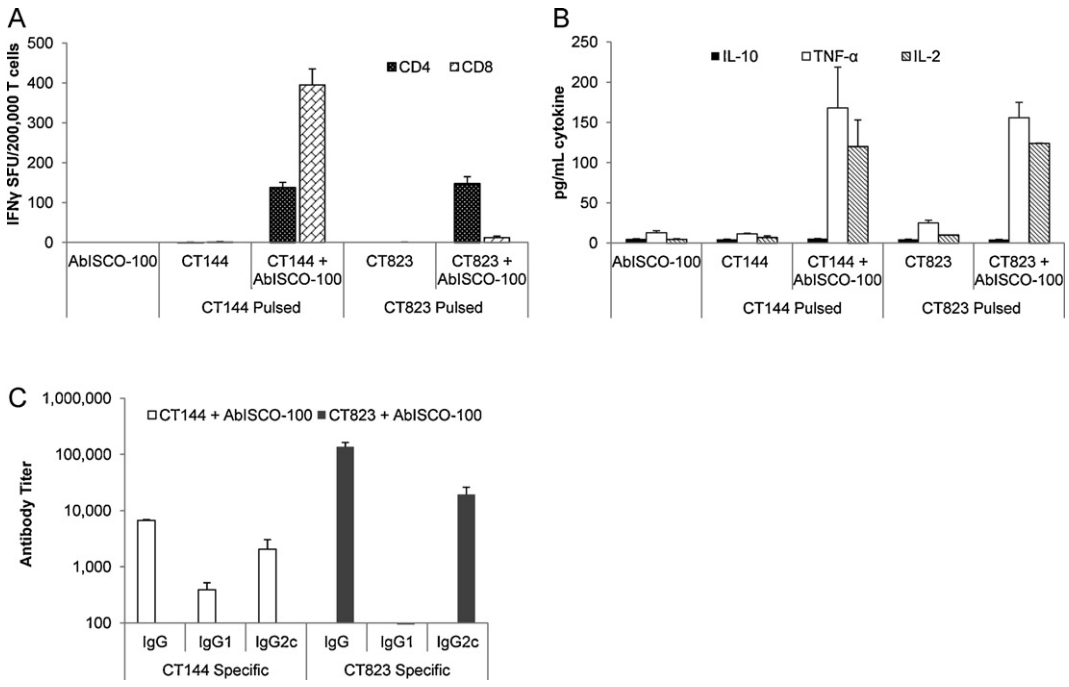

Fig. 1. Vaccination with recombinant *Chlamydia* proteins CT144 and CT823 with AbISCO-100 induces T_H1 biased T cell and antibody responses. Mice were subcutaneously vaccinated with either CT144 or CT823 plus AbISCO-100 twice, seven days apart, and then splenocytes and blood harvested for evaluation of antigen-specific immunity seven days after the second immunization. (A) IFNγ ELISPOT analysis. Data are shown as the mean ± SD IFNγ SFU per 200,000 T cells in triplicate wells. Solid bars indicate the sorted $CD4^+$ T cell response, and hatched bars represent the sorted $CD8^+$ T cell response. All antigen plus adjuvant responses are statistically significant ($p < 0.05$) as determined by two tailed *T*-test, compared to antigen- or adjuvant-only controls. (B) Cytometric Bead Array. Supernatants from $CD4^+$ T cell ELISPOT plates were assayed for the presence of IL-2, TNF-α, IL-10, IL-6, IL-4 and IL-17. Cytokine levels of IL-2, TNF-α, IL-10 are depicted, and represented as the mean cytokine concentration of triplicate wells ± SD. Animals vaccinated with either CT144 or CT823 plus AbISCO-100 produced statistically significant levels of TNF-α and IL-2 ($p < 0.05$ by two tailed *T*-test) as compared to protein alone or adjuvant alone immunized animals. IL-10 responses were not significant between groups and IL-6, IL-4 and IL-17 were undetectable. (C) Protein specific IgG, IgG1 and IgG2c antibodies quantified by ELISA. Data are shown as the endpoint titer per group ± SD of duplicate wells. Calculation of endpoint titers is described in the materials and methods. Immunogenicity data are representative of 3 replicate experiments.

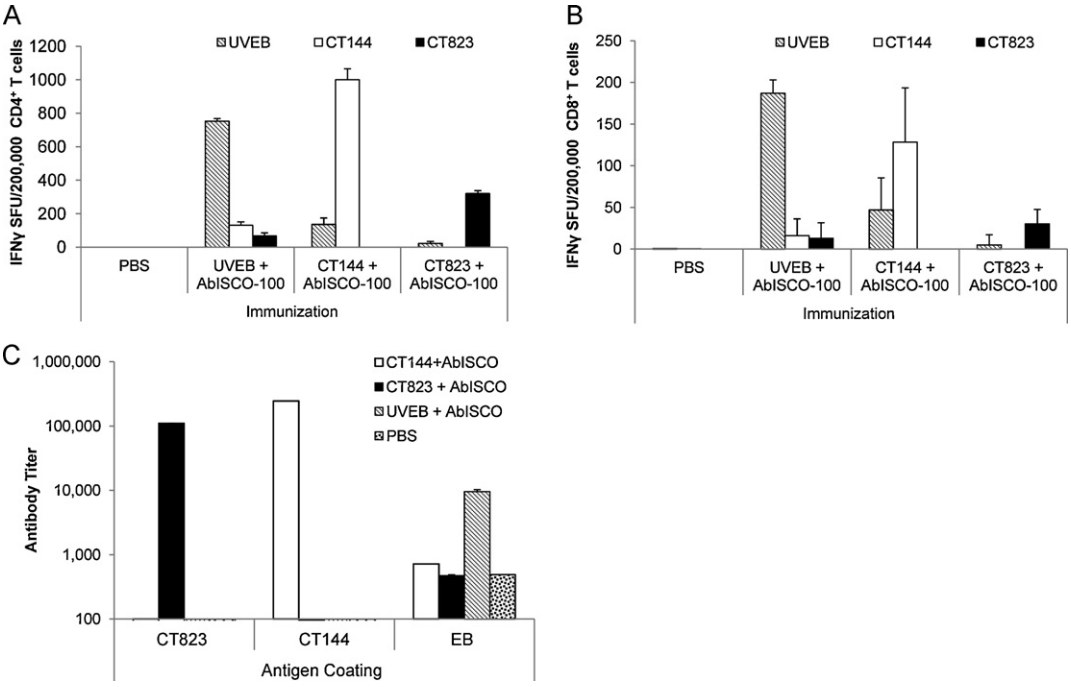

Fig. 2. Immunogenicity characterization of T cells and serum generated for transfer to naïve mice. Donor mice were immunized twice with CT823, CT144, or UVEB, plus AbISCO-100 on days 0 and 7. T cells and serum were harvested on day 14. T cell frequencies of both $CD4^+$ (A) and $CD8^+$ (B) T cells were monitored by IFN-γ ELISPOT. T cell frequencies are expressed as the mean ± SD IFN-γ SFU per 200,000 T cells in triplicate wells. (C) IgG antibody responses. Pooled serum from donor mice were evaluated by ELISA to recombinant proteins used for immunization or live EB. Data are shown as endpoint titer per group. For ELISA, the *x*-axis indicates the recombinant protein or EB used as the capture antigen and the bars represent the immunization group.

Table 2
Vaccination with CT144 and CT823 with AbISCO-100 protects against vaginal challenge.

Chlamydia ectocervical challenge									
	Mean IFU ± SEM (Median)					% animals infected			
	Day 3	Day 7	Day 10	Day 14	Day 21	Day 3	Day 7	Day 10	Day 14	Day 21
CT823 + AbISCO-100[a]	5069 ± 1270 (1706)	1254 ± 459 (106)	117 ± 26 (0)	212 ± 56 (0)	0 (0)	100	69	25	25	0
CT144 + AbISCO-100[b]	1437 ± 343 (900)	863 ± 322 (33)	549 ± 194 (50)	343 ± 142 (0)	0 (0)	80	60	53	20	0
UVEB + AbISCO-100[c]	1538 ± 516 (67)	425 ± 137 (100)	170 ± 61 (0)	69 ± 29 (0)	0 (0)	67	53	47	27	0
PBS control[d]	6014 ± 1421 (3310)	4574 ± 1411 (514)	1466 ± 541 (156)	643 ± 204 (105)	0 (0)	100	77	77	59	0

C57BL/6 mice were immunized with chlamydial proteins CT144 or CT823 or UVEB with AbISCO-100 on days 0 and 7 followed by intravaginal challenge with 5×10^5 IFU *C. trachomatis* serovar D on day 14. Control groups were immunized with either PBS or protein without adjuvant as multiple studies have shown no differences between these groups. Infection was monitored by ectocervical sampling to determine the bacterial load by culture on days 3, 7, 10, 14, and 21 post-infection. The results are the mean *Chlamydia* IFU ± SE, with the Median response in parenthesis, in swabs from 2 experiments, as indicated. The percent of infected animals at the same time points and the numbers of animals per study are noted.
The data represent the combination of at least 2 repeat studies for each group. 2-way ANOVA was used to compare experimental groups to PBS control groups.
[a] $n = 16$, $p = 0.047$.
[b] $n = 15$, $p = 0.0097$.
[c] $n = 15$, $p = 0.0048$; $p < 0.01$ on day 7 (Bonferroni post-test).
[d] Combined PBS control groups of 4 experiments, $n = 22$.

reduction throughout the rest of the monitoring period, compared to controls. The percent of animals infected on day 7, however, was close to the UVEB controls and mirrored the kinetics of clearance in UVEB-immunized animals (Table 2). All animals had cleared *C. trachomatis* bacteria from the lower tract by day 21 post-infection.

3.4. Adoptive transfer of immune T cells to naïve, non-immunized animals protects against chlamydial challenge

To determine if cell-mediated or humoral responses were mediating the observed protection against *Chlamydia* challenge in vaccinated mice, we performed adoptive transfer of immune T cells or passive transfer of sera to naïve animals. Splenic T cells and immune serum were obtained from donor mice that had been immunized with either positive control UVEB or respective recombinant *Chlamydia* proteins, and AbISCO-100. Serum and T cells obtained from the donor mice were subjected to antigen specific antibody ELISA and IFN-γ ELISPOT to confirm and quantify their respective antigen specific responses. As expected, vaccination induced antigen-specific IFN-γ production from $CD4^+$ and $CD8^+$ T cells (Fig. 2A and B), as well as IgG production (Fig. 2C).

When evaluated in the challenge model, adoptive transfer of 5×10^6 immune T cells into recipient animals had the most significant impact on day 7 post-infection (Fig. 3A). The initial increase in bacterial burden from days 3 to 7 followed by a gradual clearance in infection over time, follows the typical pattern seen in this model [20,21]. On day 7 after challenge, mice that were recipients of UVEB, CT144 or CT823-specific T cells demonstrated a statistically significant reduction in bacterial burden compared to the control animals ($p < 0.001$). In addition, the reduction of bacterial burden in animals that received CT823-specific T cells was not statistically different from animals that received the positive control UVEB-specific T cells. In contrast to the adoptive transfer of immune T cells, passive transfer of immune serum from any of the protein- or UVEB-immunized groups did not significantly affect the course of *Chlamydia* infection, when compared to animals transferred with control serum from the mock-immunized group (PBS; Fig. 3B). All animals cleared their infection by day 21 post challenge.

3.5. Protection against infection conferred by recombinant Chlamydia proteins is durable

Since CT823 administered with adjuvant demonstrated significant protection against intravaginal challenge with *C. trachomatis*, we next wanted to assess the durability of the response. Mice were vaccinated twice with CT823 plus AbISCO-100, followed by intravaginal challenge on either day 7 (acute response) or on day 94 (memory response) after the second immunization. As shown in Fig. 4, vaccination with CT823 plus adjuvant induced a potent memory response, resulting in complete clearance of detectable *Chlamydia* by day 14 post-challenge; the remainder of animals cleared their infection by day 21 post-challenge. The kinetics of clearance were consistent, regardless of whether the animals were challenged immediately following the secondary immunization (left panel) or greater than three months later (right panel).

4. Discussion

In this study, we demonstrated that antigens identified through screens of T cells in the periphery are relevant in the context of mucosal infection. To identify the antigens, we utilized a comprehensive screening technology in which every full-length protein from a pathogen can be screened in an unbiased way to identify $CD4^+$ or $CD8^+$ T cell antigens in a population of exposed or infected subjects [16]. In previous experiments we identified T cells responding to antigens CT144 and CT823 after peritoneal infection

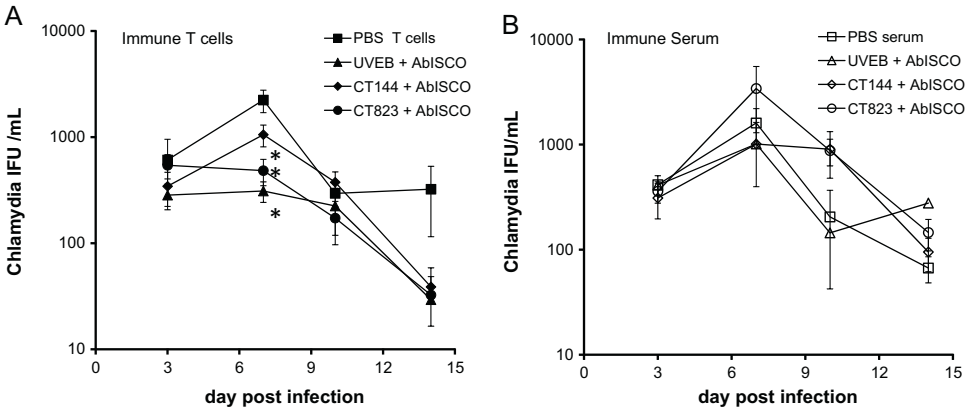

Fig. 3. Adoptive transfer of CT823-, CT144-, and UVEB-immune T cells provides protection against intravaginal challenge of naïve mice. Depo-Provera-treated naïve mice were adoptively or passively transferred with T cells or serum from donor mice that were immunized twice with CT823, CT144, or UVEB, with AbISCO-100, or sham immunized with PBS. 18 h later, recipient mice were challenged with 1×10^6 IFU *C. trachomatis* EB and monitored for infection. Time course of clearance was monitored by culture in animals adoptively transferred with 5×10^6 T cells (A) or passively transferred with immune serum (B). Data are shown as the mean IFU per mL of 8 mice per group ± SE. Groups receiving immune T cells from animals immunized with either UVEB or CT823 and adjuvant had a statistically significant reduction in bacterial burden compared to PBS control, $p = 0.003$ or 0.007 respectively (2-way ANOVA). Animals receiving T cells from CT144 and adjuvant immunized mice also had a reduction in bacterial burden that was approaching statistical significance by the same test, $p = 0.057$. On day 7, all groups that received transferred immune T cells had statistically significant reductions in bacterial burden compared to the group that received naïve T cells (*, $p < 0.01$ Bonferroni post-test). There were no statistical differences between passively transferred groups. All groups cleared infection by day 21.

in C57BL/6 mice, and now we have identified T cells responding to these same antigens in the draining lymph nodes of intravaginally challenged BALB/c and C57BL/6 mice (data not shown). Furthermore, the antigen specificity was not restricted to a single MHC type, although the T cell phenotype that responded to the antigens may differ depending on genetic background. These findings suggest that screening peripheral T cells yields antigens that are relevant in a mucosal infection and are broadly applicable across MHC types, a desired quality in developing vaccines for diverse human populations.

Vaccination with the recombinant proteins CT823 and CT144 when combined with AbISCO-100 induced potent, polyfunctional T cell responses and high-titer antibody with a bias toward a T_H1 subtype. Of the two antigens, CT144 has a predicted transmembrane domain, and would therefore be presumed to be surface expressed and accessible to antibody and potentially a strong candidate for inclusion into a subunit vaccine. Immunization with both proteins conferred partial protection, as evidenced by more rapid clearance of bacteria in immunized mice. In the case of CT823 immunization, protection was long-lived; a desired characteristic of a successful vaccine.

The clearance kinetics differed between animals immunized with the positive control UVEB, compared to vaccination with T cell antigens. This suggests that antibodies specific for the EB may play a role in the early reduction of bacterial burden and infection. This is supported by the observation that when UVEB-immune T cells were adoptively transferred to naïve mice prior to challenge, the kinetics of clearance resembled that of the groups receiving T cells specific for the antigen that is not predicted to be surface expressed (CT823). The passive transfer of sera derived from animals vaccinated with UVEB + AbISCO-100 was not protective. However, this does not conclusively rule out the contribution of antibody to protection. It is possible that the titer of antibody transferred was insufficient to protect against a high dose challenge. Alternatively, the combination of T cell and antibody responses may be required for clearance of infection. Nevertheless, additional work needs to be done to examine the role of secretory IgA in this model, as well as to identify if a high IgG titer can be correlated with protection.

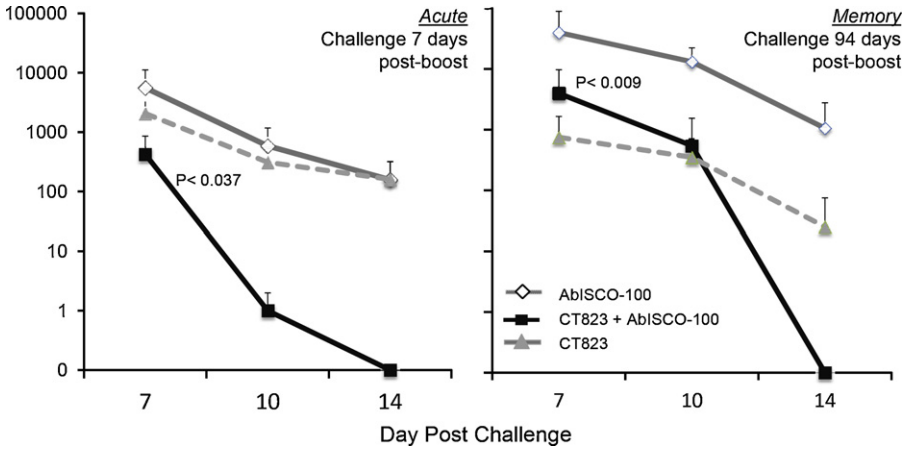

Fig. 4. Vaccination with CT823 + AbISCO-100 elicits long-lived protection against *Chlamydial* challenge. Mice were immunized on days 0 and 7 with CT823 ± AbISCO-100, followed by intravaginal challenge either on day 7 (left panel) or day 94 (right panel) post-second immunization. *Chlamydia* recovered from the ectocervix was monitored by culture on days 7, 10, 14, post-challenge. Groups immunized with CT823 + AbISCO-100 had statistically significant reduction in bacterial burden in both acute and memory studies, $p < 0.037$ and 0.099 respectively as determined by 2-way ANOVA conducted on $\log_2$ transformed data. Symbols represent the mean *Chlamydia* IFU/mL of four animals per group ± SD. All groups cleared infection by day 21.

In addition to accelerating bacterial clearance a second requirement of a *Chlamydia* vaccine is the prevention of upper tract pathology. Pathology was not examined in these studies since infection of mice with serovar D rarely results in the drastic morphological changes associated with *C. muridarum* infection. However, additional work can be done to determine if vaccination with CT144 and CT823 can reduce or prevent the ascension of bacteria to the upper tract and reduce pathology. To this end, we are developing a real-time PCR assay to monitor bacterial burden throughout the reproductive tract. In addition, homologs of these vaccine candidates can be tested in other animal models where significant pathology is routinely observed, to evaluate the impact of immunization.

The protection seen with the T cell antigens CT823 and CT144 suggests that a multivalent vaccine will be required for complete protection. This may include additional T cell antigens and/or targets of antibody such as MOMP [22,23], PmpD [24], or CPAF [25]. To identify additional T cell targets, the comprehensive library screening method is being applied to human samples from donors with natural exposure and defined clinical outcomes. As we have shown, screening of peripheral T cells can identify protective T cell antigens, and the screening of full proteins eliminates the MHC-dependence often associated with T cell antigen discovery efforts. The comprehensive identification of unique antigens that are capable of stimulating both T and B cell responses in humans will further advance the development of a successful prophylactic *Chlamydia* vaccine.

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