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Journal

Microbes and infection, 18(4)

ISSN

1286-4579

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Publication Date

2016-04-01

DOI

10.1016/j.micinf.2015.12.002

Peer reviewed

Microbes and Infection 18 (2016) 245–253

www.elsevier.com/locate/micinf

Original article

Computational modeling of TC0583 as a putative component of the *Chlamydia muridarum* V-type ATP synthase complex and assessment of its protective capabilities as a vaccine antigen

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Received 1 September 2015; accepted 1 December 2015
Available online 17 December 2015

Abstract

Numerous *Chlamydia trachomatis* proteins have been identified as potential subunit vaccines, of which the major outer-membrane protein (MOMP) has, so far, proven the most efficacious. Recently, subunit A of the V-type ATP synthase (ATPase; TC0582) complex was shown to elicit partial protection against infection. Computational modeling of a neighboring gene revealed a novel subunit of the V-type ATPase (TC0583). To determine if this newly identified subunit could induce protection and/or enhance the partial protection provided by subunit A alone, challenge studies were performed using a combination of these recombinant proteins. The TC0583 subunit alone and concurrently with TC0582, was used to vaccinate BALB/c mice utilizing CpG-1826 and Montanide ISA 720 VG as adjuvants. Vaccinated animals were challenged intranasally with *Chlamydia muridarum* and the course of the infection was followed. Mice immunized with individual antigens showed minimal alleviation of body weight reduction; however, mice immunized with TC0583 and TC0582 in combination, displayed weight loss levels close to those observed with MOMP. Importantly, immunization with a combination of recombinant subunit proteins reduced chlamydial inclusion forming units by approximately a log-fold. These protection levels support that, these highly conserved *Chlamydia* proteins, in combination with other antigens, may serve as potential vaccine candidates.

Keywords: *Chlamydia trachomatis*; Vaccine; Antigens; V-type ATP synthase; Mouse

1. Introduction

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen and the etiological agent of trachoma, the most common cause of preventable blindness in regions of the developing world [1–3]. In addition, this pathogen causes respiratory and gastrointestinal infections [2,4]. Attempts to control these organisms using screening programs and antibiotic treatment have so far failed. In regions that have implemented these programs, an increase in the number of *C. trachomatis* genital infections cases has been reported [5,6]. Therefore, to control *Chlamydia*, a vaccine is likely the most efficacious approach. Decades ago, whole organism vaccines, tested to protect against trachoma, were accompanied by a hypersensitivity reaction in some immunized individuals upon re-exposure to this pathogen [2,7,8]. Although the cause of this hypersensitivity reaction is still under investigation, the possibility that an antigenic component of *Chlamydia* mediated this adverse outcome stimulated the search for a subunit vaccine [9–13].

Analysis of the humoral immune response in mice infected with *Chlamydia muridarum* (previously called *C. trachomatis* mouse pneumonitis) showed that protein TC0582 was an immunodominant antigen [14]. Furthermore, mice vaccinated with TC0582 were significantly protected against an intranasal

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http://dx.doi.org/10.1016/j.micinf.2015.12.002
1286-4579/

challenge with *C. muridarum* [15]. TC0582 was annotated as subunit A (AtpA) of a putative V-type ATP synthase encoded by *Chlamydia* and shares greater than 50% sequence identity with its closest homologs (e.g. the bacterial spirochetes *Borrelia* and *Treponema*). V-type ATP synthase (ATPase) is an inner membrane associated macromolecular assembly that couples the transfer of protons, or sodium cations, across membranes with the synthesis, or hydrolysis, of ATP [16]. *Chlamydia* encode several proteins with a high degree of sequence similarity to various subunits of the bacterial V-type ATPase complex [17]. Analysis of the *C. muridarum* genome organization [18] and transcriptional landscape of *C. trachomatis* indicated that six of these subunits (A, B, D, E, K and I) were transcribed within a single operon [19]. Intriguingly, this operon also includes a protein of unknown function (TC0583). BLAST analysis with either ortholog failed to identify any statistically significant hit outside of *Chlamydia*. However, protein structure homology modeling suggests that TC0583 shares a great deal of structural similarity with V-type ATPase subunit C (AtpC). Subunit C is critical for the function of the ATPase and is localized within the bacterial cytoplasm. As part of an ongoing protein structure and function analysis of TC0583, it was hypothesized that TC0583 could be effective as a vaccine antigen and furthermore may enhance the immunoprotective capabilities of TC0582. To test these premises, mice were vaccinated with TC0582, TC0583, or a combination of both proteins and challenged with *C. muridarum*.

2. Materials and methods

2.1. Growth of C. muridarum stocks

The *C. muridarum* (MoPn; strain Nigg II; ATCC VR 123) was purchased from the American Type Culture Collection (Manassas, VA, USA). Stocks of *Chlamydia* elementary bodies (EB) were prepared as previously described [20].

2.2. Cloning, expression and purification of recombinant C. muridarum TC0583, TC0582 and MOMP

The entire open reading frame of TC0583 (residues 1–266), from *C. muridarum* genomic DNA, was amplified via PCR and subcloned into SspI-digested pTBMalE through ligation independent cloning [21] using the following primers: F: 5′- CTGTACTTCCAATCCAATATTATGAATCAATATTATTTT-3′ and R: 5′- CCGTTATCCACTTCCAATATTCTACCATTTGATTCCTTTCTC-3'. The pTBMalE vector encodes both N-terminal His_6 and maltose binding protein (MBP) tags. After DNA sequence verification, the vector was transformed into competent *Escherichia coli* (Rosetta 2(DE3) pLysS). Cells were grown to an OD_{600} of ~1.5 at 37 °C within Terrific Broth supplemented with ampicillin (100 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}). Turbid cultures were rapidly cooled in an ice bath for 5 min and protein expression was induced overnight at 16 °C by the addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) to a 1 mM final concentration. Centrifugation was used to harvest bacterial cells, followed by sonication in lysis buffer [20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 10 mM imidazole]. The cell homogenate was clarified by centrifugation and the soluble His_6/MBP-tagged protein was collected in the supernatant. Sequential purification of the target protein was performed on Ni^{2+}-NTA-Sepharose and MBPTrap HP (GE Lifesciences) columns, respectively. The purified protein was concentrated to 6 mg ml^{-1} in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 20 mM maltose and stored at 4 °C.

The TC0582 gene was amplified with *Pfu* Turbo DNA Polymerase (Stratagene, La Jolla, CA) using the following primers: F: 5′-C ATGCCATGGTAGCAACTTCAAAAGA-3′ and R: 5′-ATAGTTTAGCGGCCGCCGTCTGCACCATTTTGC-3′ and the DNA was cloned into the pET-45b vector (Novagen, Gibbstown, NJ) as previously described [15] The TC0582 His-tagged protein was extracted and purified from *E. coli* inclusion bodies using the Invitrogen ProBond™ (Carlsbad, CA). The MOMP gene, without the signal sequence, was amplified, expressed and MOMP purified as previously described [22]. Before immunization TC0582 and MOMP were dialyzed against PBS (pH 7.4) with 0.05% Z3-14 and stored at −80 °C [23,15]. Using the limulus amoebocyte assay (BioWhittaker, Inc., Walkersville, MD), the recombinant antigens were found to have less than 0.05 EU of LPS/mg of protein.

2.3. Computational modeling of TC0583, multiple sequence alignment and figure modeling

The entire amino acid sequence (residues 1–266) of *C. muridarum* TC0583 was submitted to the protein structure prediction platform I-TASSER [24–26] for computational modeling. The Local-Global Alignment method [27] was used to superimpose three-dimensional structures and all representative images were generated using PyMol [28]. ClustalW [29] was utilized for multiple sequence alignments, which were then aligned with secondary structure elements using ESPRIPT [30].

2.4. Vaccination protocols

Three-weeks-old female BALB/c (H-2^d) mice (Charles River Laboratories; Wilmington, MA) were housed at the University of California, Irvine, Vivarium. The University of California, IACUC approved all animal protocols.

The adjuvants CpG-1826 (TriLink, San Diego, CA; 10 μg/mouse/immunization) and Montanide ISA 720 VG (SEPPIC inc., Fairfield, NJ; 70% of total vaccine volume) were directly mixed with antigens (TC0582, TC0583 or MOMP: 10 μg of each antigen/mouse/immunization) or antigen combinations (TC0582 + TC0583: 10 μg of each antigen/mouse/immunization) [31,32]. Montanide ISA 720 VG was administered only by systemic routes. The quantification of TC0583 was done subtracting the amount corresponding to the MBP tag.

Mice were immunized twice intranasally (i.n.), followed twice by the intramuscular plus subcutaneous (i.m./s.c.)

routes, at 2-week intervals. An adjuvant control group was immunized with CpG-1826 and Montanide ISA 720 VG in PBS (PBS-C/M). A negative control group was inoculated once i.n. with 20 μl of minimal essential medium (MEM). As positive vaccine controls, mice were immunized i.n. once with 10^4 inclusion forming units (IFU) of *C. muridarum*. As a positive recombinant antigen control mice were vaccinated with rMOMP and the two adjuvants [33,31]. All mice were challenged i.n. with 10^4 IFU of *C. muridarum* four weeks after the last immunization.

2.5. Characterization of the humoral and cell mediated immune responses following vaccination

To measure *C. muridarum*-specific antibodies, following vaccination, blood was collected from each mouse the day before the challenge. ELISAs were performed as previously described with EB, or recombinant proteins, as antigens [32,22]. To assess the T-cell memory response a lymphoproliferative assay was performed before the i.n. challenge as described [34,33]. Briefly, spleens were harvested and disrupted. T cell-enriched lymphocytes were isolated through a nylon wool column and were cultured with accessory cells for antigen presentation (prepared by irradiating (3000 rad; ^{137}Cs) syngeneic unseparated splenocytes) and incubated with UV-inactivated EB at a ratio of 1:10. Concanavalin A (Sigma–Aldrich; St. Louis, MO) was used as a positive stimulant and tissue culture media served as a negative control. At the end of 96 h of incubation, 1.0 μCi of [*methyl*-^{3}H] thymidine (47 Ci/mmol; Amersham, Arlington Heights, IL) were added to each well and the uptake of the [^{3}H] thymidine was measured after 18 h using a scintillation counter (Beckman Instruments, Fullerton, CA). Levels of IFN-γ and IL-6 in supernatants from splenic T cells stimulated with UV-inactivated EB for 48 h were determined using ELISA kits BD Pharmingen, San Diego, CA [22].

2.6. Evaluation of disease burden and infection following the i.n. challenge

After the i.n. challenge, mice were weighed daily for 10 days [35,22]. At day 10 post-challenge (D10 p.c.) mice were euthanized, their lungs weighed, homogenized and serial 10-fold dilutions were used to infect Hela-229 cells. Following centrifugation the plates were incubated for 30 h at 37 °C in a 5% CO_2 incubator. Inclusions were visualized with a cocktail of *Chlamydia*-specific monoclonal antibodies and were counted using a light microscope [33]. The lower limit of detection was 50 IFU/mouse lungs.

2.7. Determination of the local immune responses in the lungs at D10 p.c

To assess the local immune responses in the lungs at the time of euthanasia, the titers of *C. muridarum*-specific IgA and levels of IFN-γ were determined by ELISAs using a 1:2 dilution of the supernatants of homogenized lungs from each mouse as previously described [34].

2.8. Statistical analyses

The One-way ANOVA with the Dunnett's multiple comparisons test, Kruskal–Wallis test or the Repeated Measures ANOVA, were employed to determine the significance of the differences among groups.

3. Results

3.1. Computational modeling of TC0583 suggests it is a member of the putative C. muridarum V-type ATP synthase complex

All members of *Chlamydia* encode an apparent V-type ATPase through six functionally annotated genes (subunits K, I, B, D, A and E encoded by genes CT304-CT308 and CT310, respectively, using *C. trachomatis* serovar D annotation [17]) that are transcribed within a single operon [36–38,19]. Within this set of co-transcribed genes is a single protein of unknown function (CT309) that belongs to DUF2764. BLAST analysis of the CT309 amino acid sequence fails to identify any statistically significant targets (E value $< 1.0e^{-10}$) outside of *Chlamydia*. Homologs to CT309 are found throughout all members of *Chlamydia* including within *C. muridarum*, where TC0583 shares 89% sequence identity.

Genes found within a co-transcribed operon often encode proteins that function within the same pathway, which indicates that TC0583 could potentially interact with the V-type ATPase. To gain support for this hypothesis, structural modeling of TC0583 was performed. The highest confidence computational model generated by I-TASSER (e.g. *C*-score > -1.5 [39]) is comprised of an all α-helical fold assembled into a tapered cylinder (Fig. 1) with a *C*-score of −0.89 [24–26]. The protein structure homology server DALI [40] was then used to predict the top structural and functional homologs to the TC0583 model. The highest scoring hit (Z-score = 31.2) was the V-type ATPase subunit C from *Thermus thermophilus* (*Tt*AtpC; PDB ID: 1R5Z [41]). Structural alignment of TC0583 and *Tt*AptC (Fig. 1) indicates a highly similar topology (RMSD 1.71 Å over 248/266 Cα atoms), despite sharing only 11.7% sequence identity across the entire polypeptide. The C-subunit is a peripheral membrane component of the V-type ATPase that is proposed to physically link the V_0 and V_1 domains [41]. Further insights into the potential molecular function of TC0583 are limited by the lack of C-subunit structures (*Tt*AtpC is currently the only one) and the absence of TC0583 sequence-level homologs outside *Chlamydia*.

3.2. Characterization of the humoral and cellular immune responses following vaccination

Following vaccination the humoral and the cellular immune responses were characterized the day before the i.n. challenge. Antibodies to *C. muridarum* were assessed by an ELISA using EB as the antigen. As shown in Table 1, mice immunized with TC0583 had very low serum IgG antibody titers to EB (159

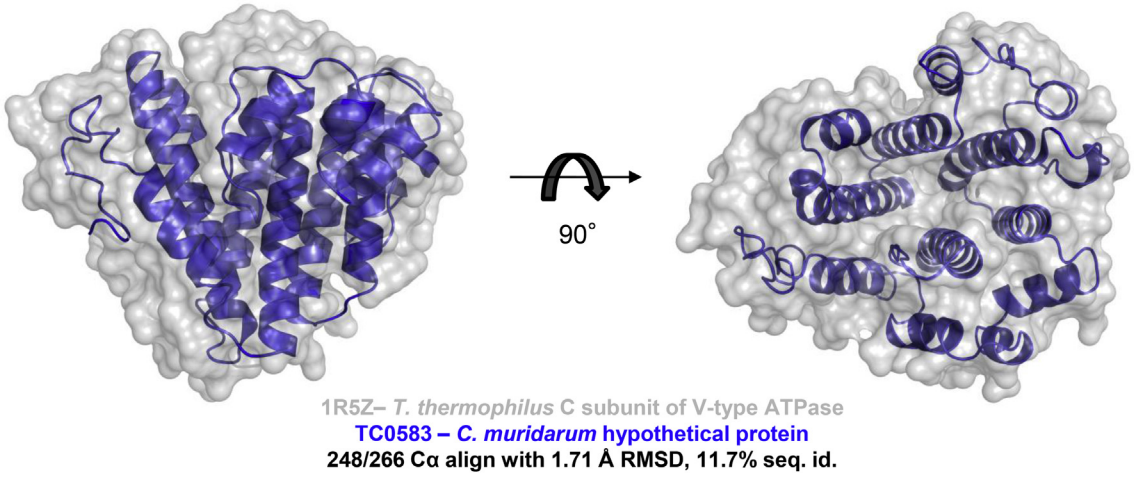

Fig. 1. **Computational modeling of TC0583 suggests it is V-type ATPase subunit C**. Computational model of TC0583 from *C. muridarum* (generated by I-TASSER [24–26]) is shown in cartoon ribbon format (colored blue). Crystal structure of *T. thermophilus* V-type ATPase subunit C (PDB ID: 1R5Z) is shown as electron surface representation and colored gray. TC0583 aligns to the *T. thermophilus* V-type ATPase subunit C with an RMSD of 1.71 Å across 248/266 modeled Cα residues, despite only 11.7% sequence identity between the two proteins.

GMT; range: 100–200) while those immunized with TC0582 had no measurable *C. muridarum* specific IgG antibodies (<100). Mice receiving a combination of both TC0582 + TC0583 had an IgG GMT of 340 (100–800). Serum from mice vaccinated with rMOMP, or EB, had high IgG antibody titers, 129,016 (102,400–204,800) and 72,408 (51,200–102,400), respectively. Anti-rMOMP IgG antibodies were only detected in animals immunized with rMOMP (258,032; 204,800–409,600) or EB (3200; 3200–3200). The two negative control groups, immunized with PBS-C/M, or MEM, had no *C. muridarum* specific IgG antibodies.

Following absorption of the sera with recombinant MBP, from mice immunized with TC0583 or TC0582 + TC0583, antibodies were also measured using recombinant proteins as antigens (Table 1). Animals vaccinated with TC0583 had IgG antibody levels against this protein (3200; 1600–6400) that were similar to those mice immunized with TC0582 + TC0583 (2263; 1600–3200). Mice immunized with TC0582 had high levels of IgG to this antigen (162,550; 102,400–204,800) and animals immunized with TC0582 + TC0583 also had high IgG antibody levels (64,508; 51,200–102,400) to TC0582. Positive control mice inoculated i.n. with live EB had a low antibody titer to TC0582 (283; 100–400) and no detectable anti-TC0583 antibodies (<100).

Mice immunized with TC0583, TC0582 or TC583 + TC582, showed significantly higher T-cell proliferative responses to EB (3092 ± 3487; SI 5.1 ± 3.3), (1213 ± 1042; SI 4.4 ± 2.9) and (2035 ± 1342; SI 6.3 ± 2.7), respectively, compared to control animals vaccinated with PBS C/M (229 ± 119; SI 2.2 ± 0.6) ($P < 0.05$; Table 2). Mice vaccinated with rMOMP had strong proliferative responses (2205 ± 122; SI 16.5 ± 3.7). The most robust lymphoproliferative responses were observed in control mice inoculated i.n. with live EB (5248 ± 1161; SI 39.4 ± 8.5).

Low levels of IFN-γ (pg/ml), in supernatants from EB-stimulated splenocytes, were found in mice vaccinated with TC0583 (299 ± 73), TC0582 (226 ± 59), or the TC582 + TC583 combination (93 ± 7) (Table 2). None of these results were statistically significantly different from each other but TC0583 and TC0582 immunized groups were significantly higher compared to negative controls group immunized with PBS-C/M (49 ± 45). In mice vaccinated with

Table 1
Serum IgG antibody geometric mean titer (GMT) (range) the day before the i.n. challenge.

Experimental groups	Anti-*C. muridarum* EB	Anti-rMOMP	Anti-TC0583	Anti-TC0582
TC0583	159[a,b,c] (100–200)	<100 (<100–<100)	3200[a,b,c] (1600–6400)	<100 (<100–<100)
TC0582	<100[b,d,e] (<100–<100)	<100 (<100–<100)	<100 (<100–<100)	162,550[a,b,d,e] (102,400–204,800)
TC0582 + TC0583	340[a,b,c] (100–800)	<100 (<100–<100)	2263[a,b,c] (1600–3200)	64,508[a,b,c,d] (51,200–102,400)
rMOMP	129,016[a,c,d,e] (102,400–204,800)	258,032[a,c,d,e] (204,800–409,600)	<100 (<100–<100)	<100 (<100–<100)
PBS-C/M	<100 (<100–<100)	<100 (<100–<100)	<100 (<100–<100)	<100 (<100–<100)
EB	72,408 (51,200–102,400)	3200 (3200–3200)	<100 (<100–<100)	283 (100–400)
MEM	<100 (<100–<100)	<100 (<100–<100)	<100 (<100–<100)	<100 (<100–<100)

[a] $P < 0.05$ by One-way ANOVA when compared to PBS-C/M immunized group.
[b] $P < 0.05$ by One-way ANOVA when compared to rMOMP immunized group.
[c] $P < 0.05$ by One-way ANOVA when compared to TC0582 immunized group.
[d] $P < 0.05$ by One-way ANOVA when compared to TC0583 immunized group.
[e] $P < 0.05$ by One-way ANOVA when compared to TC0582 + TC0583 immunized group.

Table 2
In vitro splenic T-cells proliferative responses and IFN-γ and IL-6 levels detected in the supernatants of proliferating splenic T-cells stimulated with UV-treated EB.

Experimental groups	ΔCPM	SI	IFN-γ (pg/ml)	IL-6 (pg/ml)
TC0583	3092 ± 3487[a,b]	5.1 ± 3.3[a]	299 ± 73[a,c]	121 ± 18[a,b]
TC0582	1213 ± 1042[a]	4.4 ± 2.9	226 ± 59[a,c]	59 ± 6[a,c]
TC0582 + TC0583	2035 ± 1342[a]	6.3 ± 2.7[a]	93 ± 7[c]	60 ± 5[a,c]
rMOMP	2205 ± 122[a]	16.5 ± 3.7[a]	4406 ± 766[a]	126 ± 8[a]
PBS-C/M	229 ± 119	2.2 ± 0.6	49 ± 45	22 ± 6
EB	5248 ± 1161	39.4 ± 8.5	29,274 ± 4283	221 ± 37
MEM	546 ± 591	1.9 ± 0.7	<15 ± 4	<10 ± 5

A ratio of 10:1, EB to splenocytes, was used.
ΔCPM: difference in counts per minute between EB stimulated and medium stimulated T-cells.
SI: Stimulation Index: Ratio of CPM in supernatants from EB stimulated T-cells over CPM from medium stimulated T-cells.
[a] $P < 0.05$ by One-way ANOVA when compared PBS-C/M immunized group.
[b] $P < 0.05$ by One-way ANOVA when compared to TC0582 + TC0583 immunized group.
[c] $P < 0.05$ by One-way ANOVA when compared to rMOMP immunized group.

rMOMP, levels of IFN-γ in supernatants (4406 ± 766) were significantly higher than in animals immunized with TC0582, TC0583 or a combination of these two antigens ($P < 0.05$). The highest level of IFN-γ was detected in supernatants from mice vaccinated with EB (29,274 ± 4283).

Low levels of IL-6 were measured in spleen supernatants from all groups of mice (Table 2). However, the levels of IL-6 in mice immunized with TC0583, TC0582, or the combination, were significantly higher than in the negative control group ($P < 0.05$).

3.3. Changes in body weight of mice following the C. muridarum *i.n. challenge*

As a measure of the systemic effects of the *C. muridarum* i.n. challenge, the body weight was monitored. Except for control animals immunized i.n. with live EB, all other groups of mice rapidly lost weight from D2 to D4 p.c. (Fig. 2A). Mice immunized with TC0583, TC0582, PBS C/M, or MEM-0, lost between 10 and 12% of their initial body weight while animals vaccinated with rMOMP, or the TC0582 + TC0583 combination, lost between 4 and 6% by D4 p.c. Subsequently, mice immunized with TC0583, or TC0582, lost another 4% and 2%, respectively, of their initial weight, while mice vaccinated with TC0582 + TC0583, or rMOMP, maintained their weight. Over the 10 day period mice immunized with TC0583, TC0582, TC0582 + TC0583, or rMOMP, had lost less body weight than the controls immunized with PBS-C/M ($P < 0.05$). Animals immunized with TC0582 + TC0583 lost less body weight than those immunized with TC0583 or TC0582 ($P < 0.05$). No significant difference in body weight loss over the 10 day period was observed in mice immunized with TC0582 + TC0583 when compared with the group vaccinated with rMOMP ($P > 0.05$).

As shown in Fig. 2B and Table 3, at D10 p.c., the mean body weight losses of mice vaccinated with TC0582 (−11.97 ± 6.23%) or TC0582 + TC0583 (−5.91 ± 6.80%), were significantly different from the group immunized with PBS-C/M (−18.68 ± 6.62%) ($P < 0.05$). The body weight losses were not significantly different between TC0583 (−14.65 ± 7.75%) and TC0582, but were significantly higher than the TC0582 + TC0583, or rMOMP, vaccinated groups ($P < 0.05$). The body weight loss of mice vaccinated with TC0582 + TC0583, or rMOMP (−3.46 ± 4.32%), were not statistically different ($P > 0.05$) from each other.

3.4. Lungs weight at D10 p.c

As a measure of the local inflammatory responses, the weight of the lungs (g) was determined at the time of

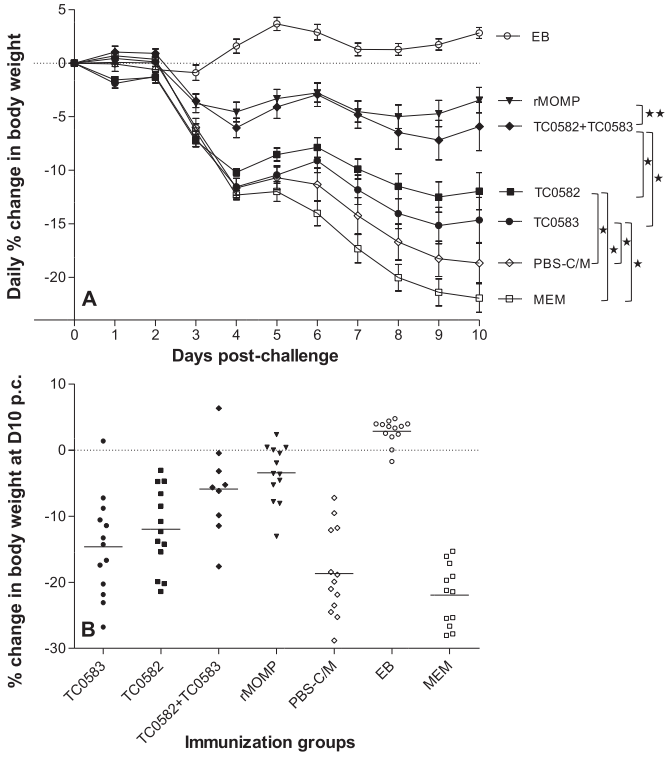

Fig. 2. **Systemic disease burden following the i.n. challenge with *C. muridarum*.** A. Daily percentage change in mean body weight following the i.n. challenge (*, $P < 0.05$ and **, $P > 0.05$ by the Repeated Measures ANOVA). B. Percentage change in mean body weight at D10 following the i.n. challenge. The mean is shown as a horizontal line. Each symbol represents a single animal.

Table 3
Disease burden and yields of *C. muridarum* recovered from the lungs at day 10 p.c.

Experimental groups	% Change in body weight [mean ±1SE]	Lungs weight (g) [mean ±1SD]	No. IFU X10^6 [median (range)]
TC0583	−14.65 ± 7.75[a,b]	0.31 ± 0.03[a]	103 (5–1034)[f,g]
TC0582	−11.97 ± 6.23[a,b,c]	0.32 ± 0.05[a]	66 (9–1261)[f,g]
TC0582 + TC0583	−5.91 ± 6.80[c,d]	0.29 ± 0.04[a,e]	44 (0.31–604)[f,g]
rMOMP	−3.46 ± 4.32[c]	0.22 ± 0.04[c]	0.07 (0.02–159)[f]
PBS-C/M	−18.68 ± 6.62	0.33 ± 0.04	747 (96–9740)
EB	2.81 ± 1.84	0.19 ± 0.02	BLD (BLD – BLD)[h]
MEM	−21.94 ± 4.60	0.32 ± 0.03	906 (438–11,778)

[a] $P < 0.05$ by One-way ANOVA when compared to rMOMP immunized group.
[b] $P < 0.05$ by One-way ANOVA when compared to TC0582 + TC0583 immunized group.
[c] $P < 0.05$ by One-way ANOVA when compared to PBS-C/M immunized group.
[d] $P < 0.05$ by One-way ANOVA when compared to TC0583 immunized group.
[e] $P < 0.1$ by One-way ANOVA when compared to PBS-C/M immunized group.
[f] $P < 0.05$ by Kruskal–Wallis test when compared to PBS-C/M immunized group.
[g] $P < 0.05$ by Kruskal–Wallis test when compared to rMOMP immunized group.
[h] BLD: below the limit of detection (50 IFU/mouse lungs).

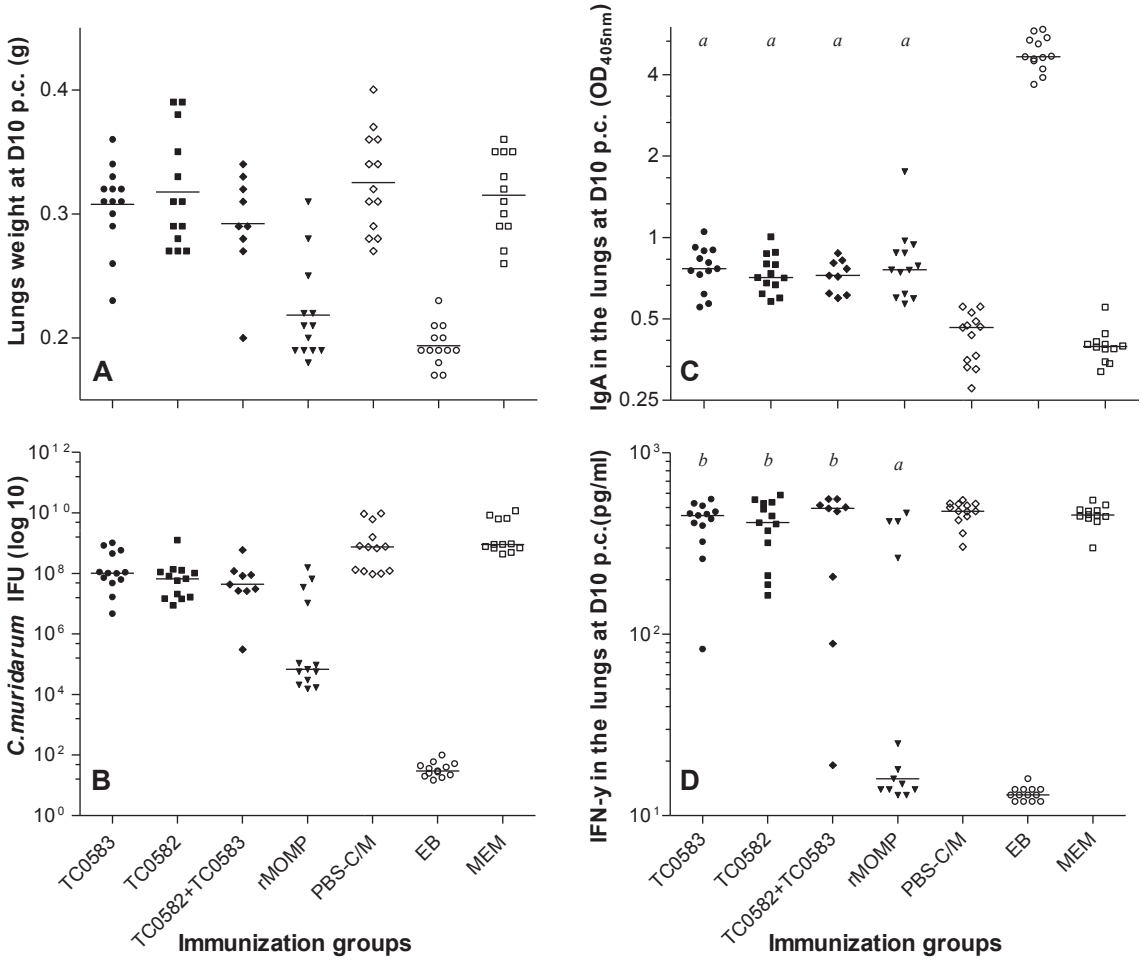

Fig. 3. **Local disease burden, titers of *C. muridarum*-specific IgA and levels of IFN-γ present in the lungs at D10 p.c.** A. Lung weight (g) at D10 after the i.n. challenge. The mean is shown as a horizontal line. Each symbol represents a single animal. B. Number of *Chlamydia* IFU recovered from the lungs at D10 after the i.n. challenge. The median is shown as a horizontal line. Each symbol represents a single animal. C. Levels of *C. muridarum*-specific IgA (OD_{405}) detected in the lungs at D10 following the i.n. challenge. The mean is shown as a horizontal line. Each symbol represents a single animal. D. Amounts of IFN-γ (pg/ml) detected in the lungs at D10 after the i.n. challenge. The mean is shown as a horizontal line. Each symbol represents a single animal. *[a]*: $P < 0.05$ by One-way ANOVA when compared to PBS-C/M immunized group. *[b]*: $P < 0.05$ by One-way ANOVA when compared to rMOMP immunized group.

euthanasia (D10 p.c.). As shown in Fig. 3A and Table 3, no significant differences ($P > 0.05$) in the mean weight of the lungs were observed between the groups of mice immunized with TC0583 (0.31 ± 0.03), TC0582 (0.32 ± 0.05) and TC0582 + TC0583 (0.29 ± 0.04). The weight of the lungs in these three groups was not significantly different from the negative control mice immunized with PBS-C/M (0.33 ± 0.04) ($P > 0.05$) although for the group vaccinated with TC0582 + TC0583 there was a trend ($P > 0.1$). However, they were statistically significantly different from those of rMOMP (0.22 ± 0.04) immunized mice ($P < 0.05$). The weight of the lungs from mice vaccinated with rMOMP was not different from that of the positive control group immunized with EB (0.19 ± 0.02) ($P > 0.05$).

3.5. Burden of C. muridarum infection in the lungs

The median (range) number of *C. muridarum* IFU recovered from lungs of mice vaccinated with TC0583 was 103 (5–1034) × 10^6 IFU, not statistically different from the number of IFU recovered from lungs of mice immunized with TC0582 [66 (9–1261) × 10^6 IFU], or the TC0582 + TC0583 combination [44 (0.31–604) × 10^6 IFU]; ($P > 0.05$) (Fig. 3B and Table 3). These were statistically different than the number of IFU recovered from the negative control group immunized with PBS-C/M [747 (96–9740) × 10^6 IFU], ($P < 0.05$). The most robust protection, among the animals receiving a subunit vaccine, was observed in mice vaccinated with the control rMOMP [0.07 (0.02–159) × 10^6 IFU]; ($P < 0.05$ when compared with the other three groups immunized with recombinant antigens). Animals immunized with live EB, had negative cultures [below the limit of detection (BLD): 50 IFU/mouse lungs].

3.6. Local immune responses in lungs at D10 p.c

To determine immune local parameters that correlate with protection we measured the levels of *C. muridarum*-specific IgA and IFN-γ in supernatants from lungs harvested at D10 p.c. (Fig. 3C and D). The mean OD_{405} values of *Chlamydia*-specific IgA in the lungs of mice vaccinated with TC0583 (0.781 ± 0.145), TC0582 (0.742 ± 0.125), or TC0582 + TC0583 (0.727 ± 0.100), were not significantly different among themselves, or the rMOMP control group (0.835 ± 0.307), but were significantly higher than those of the negative control immunized with PBS-C/M (0.432 ± 0.093) ($P < 0.05$). As expected, mice inoculated with EB had the highest *C. muridarum*-specific IgA levels (4.814 ± 0.692) in the lungs.

The mean levels of IFN-γ (pg/ml) present in the lung supernatants at D10 p.c. in mice immunized with TC0583 (412 ± 127), TC0582 (401 ± 144), TC0582 + TC0583 (380 ± 214), PBS-C/M (470 ± 71), or with MEM (456 ± 61) were not statistically different among them ($P > 0.05$). The amounts of IFN-γ in mice vaccinated with rMOMP (132 ± 186), or EB (13 ± 1), were statistically significantly lower than the negative control groups ($P < 0.05$).

4. Discussion

Our results demonstrated that vaccines formulated with TC0582, TC0583, or a combination of both, and a combination of adjuvants that favor a Th1-biased response, elicited significant protective humoral and cellular immune responses as determined by changes in body weight, weight of the lungs and number of *C. muridarum* IFU recovered from the lungs.

Chlamydia do not solely rely on the host cell for production of energetic substrates and, for an obligate intracellular pathogen, encode a surprising array of metabolic pathways [42,17,43]. Despite the apparent presence of a complete V-type ATPase, it seems likely that this macromolecular complex is responsible for generating the transmembrane electrochemical potential gradient in *Chlamydia* (e.g. ATP hydrolysis rather than synthesis) as obvious homologs to the majority of the electron transport chain are absent. Computational modeling suggests that hypothetical protein TC0583 (or CT309 in *C. trachomatis*) is likely V-type ATPase subunit C. Previous vaccination studies, with different subunits of the *C. muridarum* V-type ATPase, indicated protection could be achieved with TC0582 (subunit A) with the caveat that there is sequence identity with the equivalent mouse homologs [15]. However, Cheng et al. [15] have shown that sera from mice vaccinated with TC0582, or with EB, react with TC0582 but not with the mouse AtpA protein and therefore, involvement of this antigen in pathogenesis by production of autoantibodies is unlikely. Rotary proton/sodium-translocating ATPases are conserved throughout all kingdoms of life [14,15,44,45,37,46]. These insights along with the absence of sequence similarity between TC0583 and eukaryotic V-type ATPases indicated this protein warranted further analysis as a potential vaccine candidate.

Attempts to control the worldwide epidemic of *C. trachomatis* genital infections using antibiotics have so far failed. Vaccines are likely to be a more effective approach at controlling these infections [11,10,9,47]. In this study, we determined the ability of two subunit components of a predicted *Chlamydia* V-type ATPase to protect BALB/c mice against an i.n. challenge with *C. muridarum.* TC0582 and TC0583 are putative subunits (A and C, respectively) of the V-type ATPase inner membrane associated macromolecular assembly.

Following immunization the humoral and cell-mediated immune responses were determined. Mice immunized with TC0582, or TC0583, or a combination of TC0582 and TC0583, developed very low or not detectable levels of antibodies when using *C. muridarum* EB as the antigen. This was anticipated since these proteins are expressed at very low levels in EB and are not surface exposed [46]. This premise was supported when recombinant proteins, rather than EB, were used as antigens in the ELISA. In this case high antibody titers were measured in the groups of mice vaccinated with single and combination of both antigens. Antibody titers were higher in animals immunized with TC0582 than TC0583. When the combination of both antigens was used to immunize the antibody titer was also higher against TC0582. These findings support the previous observation that TC0582 is a

more dominant antigen than TC0583. As determined by a lymphoproliferative assay, using EB to stimulate spleen T-cells, the three groups of mice immunized with TC0582, TC0583, or a combination of both, mounted significant cell mediated immune responses. Furthermore, measurement of the levels of IFN-γ in supernatants from stimulated T-cells, were higher in these three groups than in the negative control animals. Interestingly, the TC0582 + TC0583 combination elicited better protection than the individual antigens although the levels of IFN-γ were not higher than for each individual antigen. Other immunological components, such as TNF-α, could account for this protection [48]. Finco et al. [49] used 120 recombinant proteins from *C. trachomatis* serovar D to screen sera from *Chlamydia* infected patients. Of the 79 proteins that were recognized by human antibodies, five (CT119, CT372, CT443, CT681 and CT823), also induced CD4+/IFN-γ in mice infected with *C. trachomatis*, indicative of cell-mediated immune responses. As shown here, TC0582 and TC0583, at least in mice, can also induce humoral and cell mediated immune responses when formulated with adjuvants that favor a Th1 response.

Four weeks after the last immunization mice were challenged i.n. with *C. muridarum*. As determined by changes in body weight and number of IFU recovered from the lungs TC0582 and TC0583 elicited partial protective immune responses. We have previously tested TC0580, TC0581 and TC0582 by themselves, or in combination, for their ability to protect mice against an i.n. challenge with *C. muridarum* and found TC0581 and TC0582 to be protective [15]. Combinations of these antigens were also protective but the level of protection was not additive [15]. We therefore, were very interested in determining if a vaccine formulated with TC0582 and TC0583 could have additive effects. Our results show that, based on changes in body weight, the combination of TC0582 and TC0583 had additive effects. Although, based on both weight of the lungs and the number of IFU recovered, there was a trend in the mice immunized with the combination antigens to have better protection, the differences were not statistically significant when compared with the results obtained in the groups vaccinated with a single antigen. Additive effects have been reported for chlamydial antigens, including some that were not protective on their own, while other have resulted in neutral effects. For example, Finco et al. [49] observed an additive effect by combining protective, or none protective antigens. In contrast, Yu et al. [50], like us, reported neutral effects when using various antigen combinations. Based on these observations, to assess the efficacy a chlamydial multisubunit vaccine, will require testing several combinations of candidate antigens.

A potential shortcoming of using components of the V-type ATPases macromolecular structures is the possible sequence homology with the equivalent eukaryotic complex. BLAST analysis of TC0583 failed to identify significant homology with any eukaryotic or prokaryotic protein outside of *Chlamydia*. On the other hand, protein structure homology modeling suggests that TC0583 has structural similarity with V-type ATPase subunit C (AtpC). The lack of sequence homology of TC0583 to any known eukaryotic protein can be an advantage over the use of TC0582 as an antigen in a vaccine. TC0582 has ~40% amino acid sequence identity with the eukaryotic ATP synthase subunit A. Therefore, vaccination with TC0582 could potentially induce antibodies that cross-reacted with the eukaryotic synthase subunit and elicit an adverse reaction. In the mouse model, so far tested, this does not appear to be the case but could be a shortcoming when vaccinating humans with a wide variety of genetic backgrounds [15].

In conclusion, vaccination of mice with TC0583 elicited moderate immune responses that were partially protective against an intranasal challenge with *C. muridarum*. TC0583 in combination with TC0582 further enhanced the protection. TC0583 is highly conserved among all the *C. trachomatis* serovars, has no sequence homology to a host protein and therefore, should be considered as a potential candidate of a multisubunit vaccine.

Conflict of interest

No conflicts of interests.

Acknowledgments

This work was supported by Public Health Service grant AI067888 (dlM), AI092129 (dlM), GM110761 (MB), and AI079083 (PSH) from the National Institutes of Health.

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