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Comparison of the nine polymorphic membrane proteins of *Chlamydia trachomatis* for their ability to induce protective immune responses in mice against a *C. muridarum* challenge

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Abstract

Objectives—To test vaccines, formulated with novel antigens, to protect mice against *Chlamydia* infections.

Methods—To determine the ability of polymorphic membrane proteins (Pmps) to induce cross-species protective immune responses, recombinant fragments from all nine *C. trachomatis* serovar E Pmps were used to vaccinate BALB/c mice utilizing CpG-1826 and Montanide ISA 720 as adjuvants. *C. muridarum* recombinant MOMP and PBS, formulated with the same adjuvants, were used as positive and negative controls, respectively. Mice were challenged intranasally with 10^4 inclusion-forming units (IFU) of *C. muridarum*. Animals were weighed daily and at 10 days post-challenge, they were euthanized, their lungs harvested, weighed and the number of chlamydial IFU counted.

Results—Following vaccination the nine Pmps elicited immune responses. Based on body weight changes, or number of IFU recovered from lungs, mice vaccinated with Pmp C, G or H were the best protected. For example, over the 10-day period, the negative control group vaccinated with PBS lost significantly more body weight than mice immunized with PmpC or G ($P < 0.05$). *C. muridarum* MOMP vaccinated mice were better protected against body weight losses than any group immunized with Pmps. Also, the median number of IFU recovered from the lungs of mice vaccinated with PmpC (72×10^6) or PmpH (61×10^6) was significantly less than from mice immunized with PBS (620×10^6; $P < 0.05$). As determined by the number of IFU, all Pmps elicited less protection than *C. muridarum* MOMP (0.078×10^6 IFU; $P < 0.05$).

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Conclusions—This is the first time PmpC has been shown to elicit cross-protection against a respiratory challenge. Additional work with Pmps C, G and H is recommended to determine their ability to protect animal models against genital and ocular challenges. Keywords: C. trachomatis, polymorphic membrane proteins (Pmps), vaccine, C. muridarum, PmpC,

Introduction

Chlamydia trachomatis is the leading cause of bacterial sexually transmitted diseases worldwide [1]. Genital infections affect particularly young individuals [2–4]. Newborns become infected in the birth canal resulting in ocular, respiratory and gastrointestinal infections [4, 5]. Chronic ocular infections, which can result in trachoma, affect individuals in regions with poor sanitary conditions [6, 7]. Antibiotic therapy is available, but due to the high percentage of asymptomatic patients, or inappropriate treatment, long-term sequelae including abdominal pain, infertility, ectopic pregnancy, lymphogranuloma venereum and blindness, can develop [3, 4, 8]. Moreover, *C. trachomatis* is associated with cervical hypertrophy, induction of squamous metaplasia and HIV and HPV infections [9, 10]. Countries that have established screening or disease notification programs have observed an increase in the prevalence of genital infections likely due to a block in the development of natural immunity as a result of the antibiotic therapy [11, 12]. Thus, a vaccine is the best approach to control these infections [13–17].

The *C. trachomatis* major outer membrane protein (MOMP) is currently the most promising antigen for a vaccine [16, 18–22]. However, this protein may only induce serovar/serogroup protection and therefore, there is a need to identify additional antigens with broader protection [17, 23, 24]. Genome sequencing uncovered the presence of a family of polymorphic membrane proteins (Pmps) unique to *Chlamydiales* [25]. In *C. trachomatis* and *C. muridarum*, previously called *C. trachomatis* mouse pneumonitis, there are nine *pmp* genes [26]. Chlamydial Pmps form a group of proteins that have multiple repeats of conserved GGA (I,L,V) and FxxN tetrapeptide motifs in their N-terminus and central regions with MWs ranging from ~100–150 kDa [25, 27–31]. Pmps have three functional domains including a cleavable *sec*-dependent N-terminal signal for translocation through the cytoplasmic membrane, a C-terminal β-barrel sequence for outer membrane translocation, and a passenger domain for secretion or cell surface localization. Based on their structure, they are considered to be type V secretion autotransporters [32]. Indeed, all *C. trachomatis* Pmps are located on the chlamydial cell surface [27, 33–35]. In addition, in their tetrapeptide-harboring regions, all nine *C. trachomatis* Pmps harbor adhesion capacity to their host cells [36]. Interestingly, mutational experiments revealed the two repetitive peptide motifs GGA (I, L, V) and FxxN to be essential for the adhesion function [37]. Presence of recombinant Pmps during the chlamydial adhesion to host cells reduces infection [36]. Furthermore, some Pmps contain poly G tracts that control the phase variable expression of their genes providing means for evading host immune responses [38].

As determined by the presence of serum antibodies, Pmps have been found to be immunogenic in humans and mice infected with *Chlamydia* [39–41]. In 2006, Crane et al. [33] reported that antibodies to PmpD could pan-neutralize *in vitro* all the *C. trachomatis* serovars. Interestingly, antibodies to MOMP and LPS blocked the ability of the PmpD

antibody to neutralize chlamydial infection. The authors suggested that MOMP and LPS could act as decoys to block the binding of neutralizing antibodies to PmpD [33]. Karunakaran et al. [42], using an immunoproteomic approach, discovered T cell epitopes in Pmps (E, F, G and H) of *C. muridarum*. Vaccination of C57BL/6, BALB/c and C3H/HeN mice, with fragments from each of these four proteins, as determined by accelerated vaginal clearance, elicited protection against a challenge with *C. muridarum.* PmpG was the most protective.

Here, to identify which of the nine Pmps can elicit the most robust cross serovar protection mice were vaccinated with fragments of each of the nine *C. trachomatis* serovar E Pmps and were challenged intranasally with *C. muridarum.* Our data shows that PmpC induced the most robust immune responses and was the most protective.

Results

Determination of the humoral immune responses

To determine antibody titers, sera and vaginal washes were collected the day before the intranasal challenge. Antibody titers were determined using *C. muridarum* EBs as the antigen and also the same recombinant Pmp fragments used for immunization. As shown in Table 1, in mice immunized with the Pmps very low IgG geometric mean titers (GMT) against *C. muridarum* EBs were observed, ranging from 635 to 100. In the positive control mice immunized with MOMP the IgG GMT to the *C. muridarum* EBs was 102,000.

As a measure of the Th1 versus Th2 responses the levels of IgG2a and IgG1 were determined (Table 1). Overall the IgG2a/IgG1 ratio for the Pmps was close to 1, except PmpI (3.2), suggesting well-balanced Th1/Th2 responses to EBs. Immunization with MOMP resulted in a biased Th1 response (IgG2a/IgG1= 7.8). Using EBs as the antigen, none of the Pmp elicited detectable levels of IgG or IgA antibodies in the vaginal washes. Mice vaccinated with MOMP had positive IgG (403) and IgA (13) GMT.

Significantly different results were obtained when antibody levels were determined using as antigens the Pmp peptides used for immunization (Table 2). The IgG antibody GMT against the homologous Pmp fragment ranged from 325,000 for PmpF to 8,257,000 for PmpG. For some of the Pmps these titers were equivalent to those obtained in mice immunized with MOMP (6,553,000) using MOMP as the antigen. The IgG2a/IgG1 ratios were overall greater than 1 indicating that most of the Pmp elicited Th1-biased responses to the homologous protein. MOMP also elicited Th1 responses (IgG2a/IgG1 ratio = 6.4). Significant IgG and IgA antibody titers were also obtained in vaginal washes. The IgG titers ranged from 453 (PmpF) to 20,480 (PmpG and PmpI) and the IgA titers from 28 (PmpD and PmpF) to 453 (PmpG). The IgG and IgA titers in vaginal washes to MOMP were 10,240 and 160, respectively.

Characterization of the cellular immune responses

To determine cell mediated immune responses in mice immunized with PmpC, PmpE, PmpG or PmpH spleen T cells were isolated using a nylon column and restimulated with *C. muridarum* EBs. As shown in Table 3, only PmpC (SI=6.3) elicited cell proliferative

responses that were significant when compared with negative PBS control (SI = 4.3; $P < 0.05$). MOMP also elicited significant cell mediated responses (SI 10.9).

Levels of IFN-γ were measured in the supernatants of the EBs-stimulated T-cells. As shown in Table 3, only levels (pg) of IFN-γ from groups of mice immunized with PmpC (38) or PmpE (51) were significantly different from those of the PBS control (< 15; $P < 0.05$). All IFN-γ levels induced by Pmps were lower than those in mice vaccinated with MOMP (555) ($P < 0.05$).

Disease burden and number of *C. muridarum* IFU recovered following the i.n. challenge

Starting the day of challenge mice were weighed for 10 consecutive days when they were euthanized, their lungs harvested, weighted and the number of *C. muridarum* IFU determined. The percentage change in body weight was used as a measurement of the systemic effects of the infection and the weight of the lungs as a parameter of the local inflammatory responses.

Between days 2 and 4 following the challenge all mice lost body weight (Fig. 2). As shown in Table 4 and Fig. 3A, by day 10 p.c. negative control vaccinated with PBS had lost 21.1% of their initial body weight while mice immunized with MOMP, positive control, had only lost 2.3% of their weight. None of the Pmp immunized mice had lost statistically significantly less body weight than the PBS controls ($P > 0.05$). However, when body weight changes were analyzed over the 10-day period using the ANOVA Repeated Measures test, animals vaccinated with PmpC or PmpG had significant differences with the PBS controls ($P < 0.05$). As determined by changes in mean body weight, MOMP elicited better protection than any of the Pmps.

At day 10 post-challenge, the mean weight of the lungs of mice vaccinated with PBS was 0.31 g, while those of mice immunized with MOMP weighted 0.19 g (Table 4, Fig. 3B). The lungs of mice immunized with the Pmps weighed from 0.29 to 0.31 g. None of these weights were statistically significantly different from the PBS immunized animals indicating that robust local inflammatory responses were still present. Therefore, as determined by lungs weight, Pmps did not elicit significant protection.

As shown in Table 4 and Fig. 3C, mice immunized with PmpC (72×10^6) or PmpH (61×10^6) had statistically significantly less IFUs in the lungs than the PBS negative control (620×10^6; $P < 0.05$). The PmpG immunized group (106×10^6) approached significant protection ($P < 0.1$). As determined by the number of IFUs recovered, all Pmps elicited less protection than MOMP (0.078×10^6 ; $P < 0.05$).

As additional parameters to measure protection and immunity levels of IFN-γ and *C. muridarum* specific IgA were determined in lungs supernatants. As expected, high levels of IFN-γ (6,214 pg/ml) were observed in mice immunized with PBS. Significantly lower levels of IFN-γ, compared with the PBS group, were measured in mice immunized with PmpB, PmpC, PmpD, PmpE and PmpF, although all the Pmp had significantly higher levels of IFN-γ than mice vaccinated with MOMP (789; $P < 0.05$).

PmpC (0.33), PmpG (0.31) and PmpH (0.31) vaccinated animals had significantly higher levels of *C. muridarum*-specific IgA than the PBS immunized group (0.26; $P < 0.05$). All Pmps immunized mice had lower IgA levels than MOMP vaccinated animals (0.66; $P < 0.05$).

Discussion

The aim of this study was to compare the ability of each of the nine *C. trachomatis* Pmps to induce cross-species protective immune responses in mice against an intranasal challenge with *C. muridarum*. Our data shows that, as determined by changes in body weight and number of IFU recovered from the lungs, the best protection was obtained with Pmp C. The other Pmps elicited limited or no protection. To our knowledge this is the first time that all nine *C. trachomatis* Pmps have been tested in parallel for their ability to cross-protect in an animal model. The novel discovery that PmpC is the most protective should be further explored.

Brunham's research group has done extensive work on the immunology and protective ability of the *C. muridarum* Pmps. For example, Karunakaran et al. [42] showed that dendritic cells infected with *C. muridarum* presented PmpG and PmpF peptides on their MHC class II receptors. *Chlamydia*-specific purified CD4+ T cells produced IFN-γ following co-culture with the *C. muridarum* infected dendritic cells suggesting T cell recognition of the MHCII bound Pmps [43]. Subsequently, Yu et al. [44] demonstrated that mice, after receiving dendritic cells pulsed with PmpG or PmpF, were protected against intranasal and genital challenges with *C. muridarum*. The same group of investigators also immunized mice with PmpG, PmpE/F and MOMP or with PmpE, PmpF, PmpG or PmpH and showed significant decreases in vaginal shedding. Splenocytes from these mice restimulated with the same Pmps demonstrated that PmpG and PmpH induced robust production of IFN-γ while there was a low or no production after stimulation with PmpF or PmpE, respectively. Furthermore, the PmpG peptide (303–311; YVDPAAAGG), an epitope not present in *C. trachomatis*, protected mice against respiratory and vaginal challenges with *C. muridarum* [45]. Following a vaginal challenge with *C. muridarum* a polyvalent vaccine formulated with PmpEFGH and MOMP, adjuvanted with DDA/MPL, as determined by vaginal shedding, elicited a more robust protection that vaccination with PmpEFGH, PmpG or MOMP alone [46]. In addition to working with *C. muridarum*, Karunakaran et al. [47] also immunized mice with a polyvalent vaccine formulated with PmpEFGH from *C. trachomatis* serovar D and three MOMPs from serovars D, J and F. The mice were challenged transcervically and, as determined by the number of *C. trachomatis* serovar D IFU recovered, animals vaccinated with the combination of the four Pmps and the three MOMPs, were better protected than those vaccinated with either the Pmps, or the three MOMPs alone. Interestingly, Inic-Kanada et al. reported that PmpC of *C. trachomatis* serovar B elicited partial protection in guinea pigs against an ocular challenge with *C. caviae* [48, 49].

Here, we sought to characterize the humoral and cell mediated immune responses elicited in mice following vaccination with each of the nine Pmps from *C. trachomatis* serovar E, the most prevalent urogenital tract serovar, and to determine their ability to induce cross

protection against a *C. muridarum* intranasal challenge. The Pmps sequence conservation among the *C. trachomatis* serovars ranges from ~80% for PmpF to ~99% for PmpD [28, 30]. Comparison of the sequences of *C. muridarum* Pmps with those of *C. trachomatis* shows that conservation ranges from ~50% for PmpC to 80% for PmpA [25]. The degree of amino acid identity between the Pmps fragments of *C. trachomatis* serovar E utilized in this study and the same regions in *C. muridarum* ranged from 60–80% and harbored at least two of the repetitive peptide motifs GGA (I, L, V) and FxxN known to be essential for adhesion (Fig. 1). Based on changes in body weight and number of *C. muridarum* IFU recovered from the lungs, the most robust protection was observed with PmpC followed by PmpH. The protection correlated with higher cell mediated immune responses and levels of IgA-*C. muridarum* specific titers in lungs and levels of IgA-anti-Pmps in the vaginal washes. Protection did not correlate with the degree of amino acid identity between the Pmps fragments of *C. trachomatis* and *C. muridarum* utilized in this study.

In addition to sequence homology, another factor that can influence the ability of a particular Pmp to elicit immune responses and protection may be the time of expression and the quantity of the Pmp in EBs and on the EBs cell surface. Characterization of the expression levels showed that Pmps, such as A, D and I, are mainly transcribed in the middle of the developmental cycle (~ 18 h post-infection) of *C. trachomatis* while the others, B, C, E, F, G and H, are transcribed at the end of the cycle (32–48 hrs. post-infection) [27, 28]. Based on a quantitative proteomic analysis of the RBs and EBs from serovar L2, Saka et al. [50] concluded that PmpG was the most abundant Pmp (26% of all the Pmps), followed by E (18%), B and H (17% each) and C (11%) in EBs. Pmp A and I were not detected in EBs but were present in RBs. PmpD was the most abundant Pmp in RBs (40%) but only accounted for 1% of the Pmps detected in EBs. Our findings suggest that Pmps have to be present in EBs to elicit protection since the three protective Pmps are all among the five most abundant Pmps present in EBs.

Accessibility to the immune system and therefore the ability to elicit antibodies and cell mediated immune responses, is a critical factor for the antigen to protect. Using 159 sera from women with PID, infected young females and male patients, Tan et al. [39] found that PmpB, PmpC, PmpD and PmpI were the most frequently reactive and elicited more robust immune responses than PmpA and PmpE. Importantly differences in reactivity were observed between adolescent females and those with PID and also in males versus female samples. It was suggested that Pmps may be differently regulated in the various chlamydial serovars or that PmpC and PmpD could be strongly antigenic in adolescent females. Antibodies to PmpB and PmpI were more prevalent in patients with PID suggesting that these two proteins may be involved in pathogenesis. Also, in a study by Taylor et al. [51], PmpA and PmpI were found to be associated with upper tract genital infection.

To assess which Pmp could elicit the most robust protective immune responses, we vaccinated mice with fragments of each of the nine *C. trachomatis* serovar E Pmps, and the adjuvant combination CPG plus Montanide, known to induce Th1-biased immune responses [18]. *C. muridarum* MOMP and PBS, delivered with the same adjuvants, were used as positive and negative controls, respectively. Antibody responses to the Pmps, using *C. muridarum* EBs as the antigen, were weak, suggesting that either the Pmps are not easily

accessible on the surface of the EBs or that the amount of protein present in EBs is limited. When the same recombinant antigens used for vaccination were used as antigens in an ELISA the antibody titers were very high for all nine Pmps indicating robust humoral antibody responses. *C. muridarum* EBs were also used to restimulate T-cells from mice vaccinated with PmpC, PmpE, PmpG or PmpH. Only mice vaccinated with PmpC elicited proliferative responses significantly higher than the PBS control. PmpC, PmpE and PmpG induced significant levels of IFN-γ in spleen supernatants when compared with PBS. As determined by changes in body weight and number of IFU recovered from the lungs, PmpC and PmpH were the most protective antigens. The other Pmps induced limited or no protection.

Phylogenetic analysis of *pmpC,* performed by Gomez et al. [52], for all the *C. trachomatis* serovars showed that the most prevalent genital serovars E, F and Da form a distinct clade. In these serovars, *pmpC* contained two putative insertion sequences (IS)-like elements, with 10 and 15-bp direct repeats respectively, while the other genital serovars only carried one IS-like element. The sequence identity of PmpC for the E, F and Da serovars is 98–100% and therefore, inclusion of this antigen in a human vaccine could have a major impact on the prevalence of chlamydial infections. In support of the possibility of cross-serovar protection induced by PmpC, Inic-Kanada et al. [48] have recently shown that mice vaccinated in the ocular mucosa with PmpC from *C. trachomatis* serovar E developed humoral and cell mediated immune responses that cross-reacted with serovar B.

In conclusion, PmpC, PmpG and PmpH should be further explored as vaccine candidates. The phase variation of Pmps suggests that including at least two of them in a vaccine will be prudent. To account for the allelic variation of MOMP including this protein from the serovars most commonly found in humans should further increase the effectiveness of the vaccine [46]. It is important to emphasize, that even a vaccine with moderate efficacy, 50% protection over a 10 year period, could have a major impact on the epidemiology of *C. trachomatis* infections [13].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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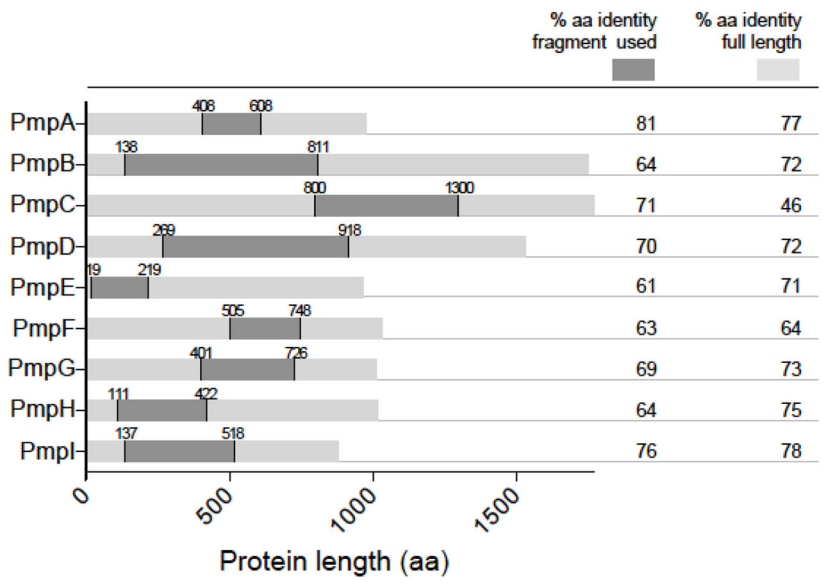

Fig. 1. Diagram showing percentage amino acid identity between *C. trachomatis* serovar E (strain DK-20) and *C. muridarum* (strain Nigg II) for the full length Pmps and the protein fragments used for this study

The numbers above each Pmp identify the first and the last amino acid of the protein fragment used in this study.

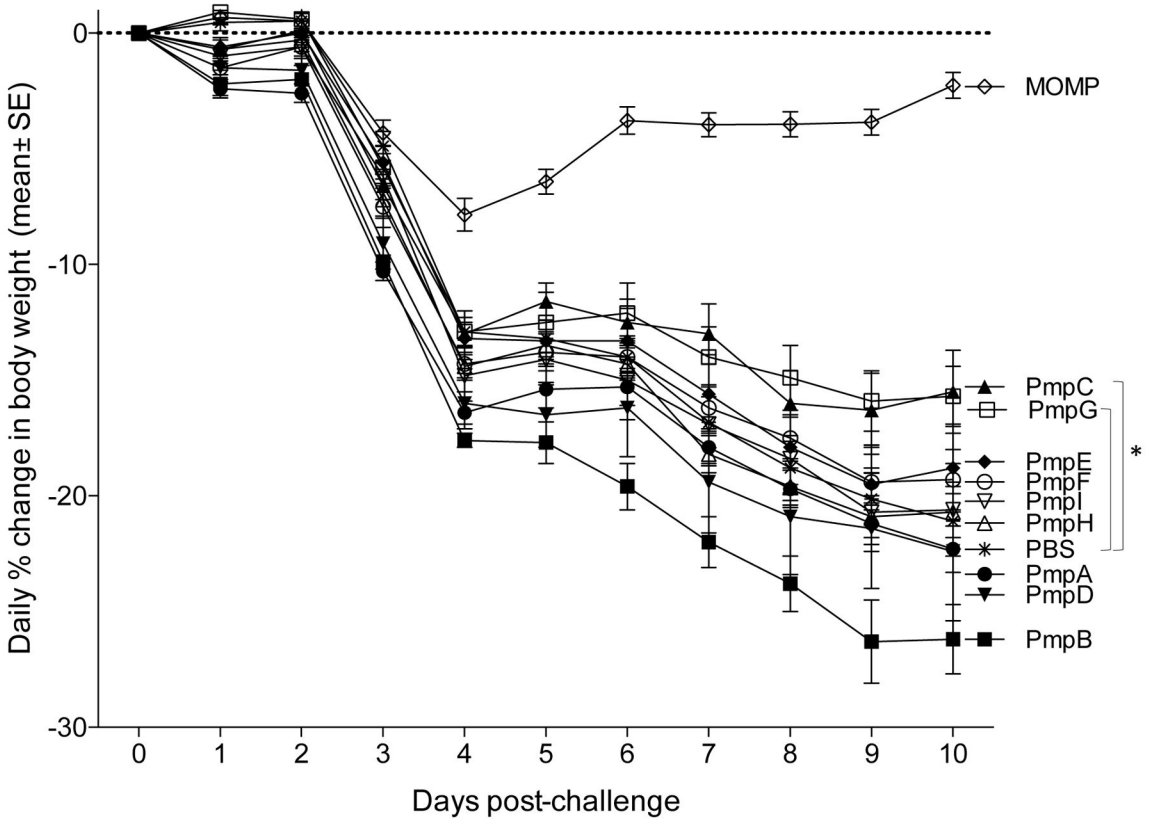

Fig. 2. Daily changes in body weight following the i.n. challenge with *C.* muridarum

Mean percentage changes in daily mean body weight following the i.n. challenge (*, $P < 0.05$ by the Repeated Measures ANOVA).

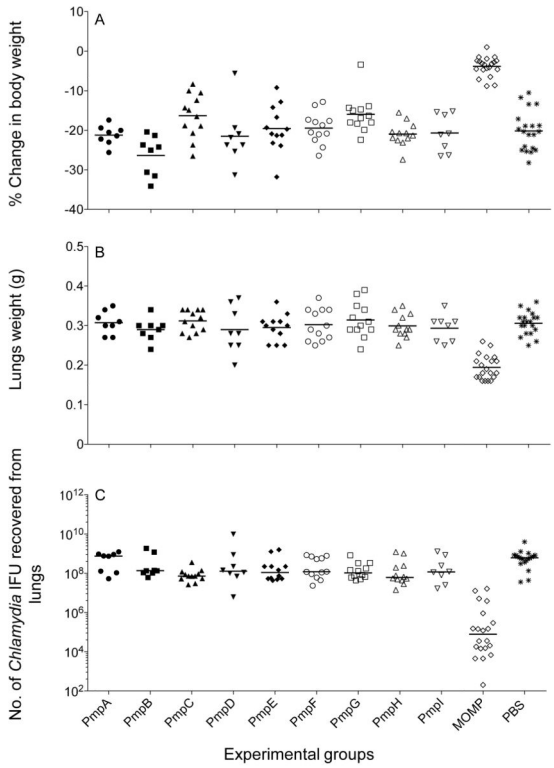

Fig. 3. Disease burden at day 10 following the i.n. challenge with *C. muridarum*

A. Percentage change in body weight at 10 days following the i.n. challenge. The mean is shown as a horizontal line. Each symbol represents a single animal.

B. Lungs weight (g) at day 10 after the i.n. challenge. The mean is shown as a horizontal line. Each symbol represents a single animal.

C. Number of *C. muridarum* IFU recovered from the lungs at day 10 after the i.n. challenge. The median is shown as a horizontal line. Each symbol represents a single animal.

Table 1

Sera and vaginal washes antibody geometric mean titers (GMT) (range) to *C. muridarum* EB the day before the i.n. challenge.

	Serum anti-*C. muridarum* EB				Vaginal wash anti-*C. muridarum* EB	
Antigen	**IgG**	**IgG2a**	**IgG1**	**IgG2a/IgG1 ratio**	**IgG**	**IgA**
PmpA	100 (100–100)[b]	<100 (<100-<100)[b]	<100 (<100-<100)[b]	-	<10 (<10–<10)[b]	<10 (<10–<10)[b]
PmpB	317 (100–800)[a,b]	635 (100–3,200)[a,b]	317 (<100–12,800)[b]	2.0	<10 (<10–<10)[b]	<10 (<10–<10)[b]
PmpC	126 (100–200)[b]	126 (100–200)[b]	<100 (<100–100)[b]	1.3	<10 (<10–<10)[b]	<10 (<10–<10)[b]
PmpD	635 (400–800)[a,b]	252 (100–400)[a,b]	1,008 (200–3,200)[a]	0.3	<10 (<10–<10)[b]	<10 (<10—<10)[b]
PmpE	141 (100–200)[a,b]	126 (<100–100)[b]	112 (<100–800)[b]	1.1	<10 (<10–<10)[b]	<10 (<10–<10)[b]
PmpF	100 (<100–100)[b]	<100 (<100–<100)[b]	<100 (<100–<100)[b]	–	<10 (<10–<10)[b]	<10 (<10–<10)[b]
PmpG	159 (<100–800)[a,b]	141 (<100–1,600)[b]	<100 (<100–100)[b]	1.4	<10 (<10–<10) [b]	<10 (<10–<10)[b]
PmpH	317 (100–800)[a,b]	317 (100–800)[a,b]	178 (<100–1,600)[b]	1.8	<10 (<10–<10)[b]	<10 (<10–<10)[b]
PmpI	252 (200–400)[a,b]	317 (200–400)[a,b]	<100 (<100–<100)[b]	3.2	<10 (<10–<10)[b]	<10 (<10–<10)[b]
MOMP	102 (51–205) x 10^3	102 (51–205) x10^3	13 (32–51) x 10^3	7.8	403 (320–640)	13 (10–20)
PBS	<100 (<100–<100)	<100 (<100–<100)	<100 (<100–<100)	–	<10 (<10–<10)	<10 (<10–<10)

[a] Significant (P<0.05) by ANOVA using Dunnett's multiple comparison test compared to PBS immunized group.

[b] Significant (P<0.05) by ANOVA using Dunnett's multiple comparison test compared to MOMP immunized group.

Table 2

Sera and vaginal washes antibody geometric mean titers (GMT) (range) to homologous Pmp the day before the i.n. challenge.

	Serum anti–Pmp titer (x10^3)				Vaginal wash anti-Pmp titer	
Antigen	**IgG**	**IgG2a**	**IgG1**	**IgG2a/IgG1 ratio**	**IgG**	**IgA**
PmpA	516 (409–819)	129 (102–204)	204 (102–409)	0.6	2,560 (1,280–5,120)	40 (20–80)
PmpB	6,553 (6,553–6,553)	3,276 (3,276–3,276)	1,638 (819–3,276)	2.0	10,240 (10,240–10,240)	226 (80–640)
PmpC	3,276 (3,276–3,276)	2,600 (1,638–3,276)	819 (819–819)	3.2	7,241 (5,120–10,240)	226 (80–640)
PmpD	1,300 (819–1,638)	650 (409–819)	325(204–819)	2.0	2,560 (1,280—5,120)	28 (10–80)
PmpE	1,638 (1,638–1,638)	819 (819–819)	204 (204–204)	4.0	2,560 (1,280–5,120)	80 (40–160)
PmpF	325 (204–409)	102 (51.2–204)	129 (102–204)	0.8	453 (320–640)	28 (20–40)
PmpG	8,257 (6,553–13,107)	3,276 (3,276–3,276)	1,638 (819–3,276)	2.0	20,480 (10,240–40,960)	453 (320–640)
PmpH	4,128 (3,276–6,553)	1,638 (1,638–1,638)	1,032 (819–1,638)	1.6	10,240 (5,120–20,480)	160 (80–320)
PmpI	3,276 (3,276–3,276)	1,638 (819–3,276)	325 (204–409)	5.0	20,480 (10,240–40,960)	226 (80–640)
MOMP[a]	6,553 (6,553–6,553)	2,600 (1,638–3,276)	409 (409–409)	6.4	10,240 (5,120–20,480)	160 (80–320)
PBS[a]	<0.1 (<0.1–<0.1)	<0.1 (<0.1–<0.1)	<0.1 (<0.1–<0.1)	-	<10 (<10–<10)	<10 (<10–<10)

[a]Serum tested against *C. muridarum* rMOMP.

Table 3

In vitro T-cells proliferative responses and cytokine production from stimulated T-cells the day before the i.n. challenge.

	Mean CPM ± 1SE				Mean IFN-γ concentration (pg/ml) ±1SE	
	Cm EB[a]		Con A[b]			
Antigen	**Δ CPM**	**SI**	**Δ CPM**	**SI**	***Cm* EB**	**Con A**
PmpC	1,725 ± 196[c,d]	6.3 ± 0.6[c,d]	59,422 ± 15,834	183 ± 48	38 ± 11[c,d]	10,333 ± 3,469
PmpE	1,627 ± 211[d]	2.0 ± 0.3[d]	30,046 ± 2,145	57 ± 4	51 ± 19[c,d]	11,496 ± 1,544
PmpG	1,336 ± 267[d]	5.0 ± 0.8[d]	26,623 ± 1,409	80 ± 4.2	<15[d]	16,718 ± 3,914
PmpH	558 ± 76[d]	2.8 ± 0.2[d]	30,728 ± 2,321	97 ± 7.3	<15[d]	9,481 ± 1,334
MOMP	3,829 ± 791	10.9 ± 2.1	27,916 ± 2,080	73 ± 5	555 ± 188	21,250 ± 2,389
PBS	967 ± 125	4.3 ± 0.4	38,301 ± 3,008	132 ± 10	<15	15,329 ± 2,606

[a]The ratio of *C. muridarum* EB to APC was 5:1.

[b]Concanavalin A (5μg/ml) was used as positive control.

[c]Significant (P<0.05) by ANOVA using Dunnett's multiple comparison test compared to PBS immunized group.

[d]Significant (P<0.05) by ANOVA using Dunnett's multiple comparison test compared to MOMP immunized group.

Table 4

Disease burden, yields of IFU, and IFN-γ and *C. muridarum*-specific IgA in lung's homogenates at 10 d.p.c.

Antigen	% Change in body wt (mean ± 1SE)	Lungs wt (g) (mean ± 1SE)	Median # IFU recovered from lungs (min-max) x10^6	IFN-γ (pg/ml) (mean ±1SE)	IgA (A_{405}) (mean ±1SE)
PmpA	−22.3 ± 1.0[b]	0.31 ± 0.01[b]	755 (53–1,253)[d]	4,968 ± 618[b]	0.27 ± 0.01[b]
PmpB	−26.2 ± 1.5[b]	0.29 ± 0.01[b]	134 (61–1,853)[d]	3,911 ± 611[a,b]	0.25 ± 0.01[b]
PmpC	−15.5 ± 1.8[b]	0.31 ± 0.01[b]	72 (26–362)[c,d]	3,080 ± 571[a,b]	0.33 ± 0.02[a,b]
PmpD	−22.4 ± 3.0[b]	0.29 ± 0.02[b]	128 (6–9,756)[d]	4,121 ± 814[a,b]	0.27 ± 0.03[b]
PmpE	−18.8 ± 1.9[b]	0.30 ± 0.01[b]	111 (43–1,586)[d]	4,164 ± 699[a,b]	0.30 ± 0.02[b]
PmpF	−19.3 ± 1.3[b]	0.30 ± 0.01[b]	120 (23–853)[d]	4,225 ± 689[a,b]	0.29 ± 0.02[b]
PmpG	−15.7 ± 1.3[b]	0.31 ± 0.01[b]	106 (44–815)[d,e]	5,621 ± 718 [b]	0.31 ± 0.02[a,b]
PmpH	−20.7 ± 1.1[b]	0.30 ± 0.01[b]	61 (14–1,223)[c,d]	5,332 ± 610[b]	0.31 ± 0.01[a,b]
PmpI	−20.6 ± 2.0[b]	0.29 ± 0.01[b]	117 (17–1,289)[d]	4,851 ± 523[b]	0.27 ± 0.02[b]
MOMP	−2.3 ± 0.56	0.19 ± 0.01	0.078 (0.0002–16)	789 ±168	0.66 ± 0.16
PBS	−21.1 ± 1.2	0.31 ± 0.01	620 (36–4,028)	6,214 ± 581	0.26 ± 0.01

[a]Significant (P<0.05) by ANOVA using Dunnett's multiple comparison test compared to PBS immunized group.

[b]Significant (P<0.05) by ANOVA using Dunnett's multiple comparison test compared to MOMP immunized group.

[c]Significant (P<0.05) by Kruskal-Wallis test compared to PBS immunized group.

[d]Significant (P<0.05) by Kruskal-Wallis test compared to MOMP immunized group.

[e]Significant (P<0.1) by Kruskal-Wallis test compared to PBS immunized group.