Vaccine 35 (2017) 1705–1711

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

The cationic liposomal adjuvants CAF01 and CAF09 formulated with the major outer membrane protein elicit robust protection in mice against a *Chlamydia muridarum* respiratory challenge

Sukumar Pal [a], Delia F. Tifrea [a], Frank Follmann [b], Peter Andersen [b], Luis M. de la Maza [a,*]

[a] Department of Pathology and Laboratory Medicine, Medical Sciences I, Room D440, University of California, Irvine, Irvine, CA 92697-4800, USA
[b] Department of Infectious Disease Immunology, Adjuvant Research, Staten Serum Institute, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 20 January 2016
Received in revised form 20 January 2017
Accepted 9 February 2017
Available online 24 February 2017

Keywords:
Cationic liposomal adjuvants
CAF01
CAF09
Chlamydia vaccine
Mouse model
MOMP

ABSTRACT

Two cationic liposomal adjuvants CAF01 and CAF09 were formulated with the native or the recombinant *Chlamydia muridarum* major outer membrane protein (nMOMP and rMOMP). BALB/c mice were immunized with the four vaccine formulations using the subcutaneous followed by the intranasal (i.n.) routes. As positive controls mice were inoculated i.n. with live *C. muridarum* and negative controls received i.n. minimal essential medium (MEM). Four weeks after the last immunization mice were challenged i.n. with 10^4 inclusion forming units (IFU) of *C. muridarum*. Following the challenge the mice were weighed daily. At 10 days post-challenge the mice were euthanized, their lungs weighed and the number of *C. muridarum* IFU determined. Serum collected the day before the challenge showed that all four groups of mice immunized with CAF01, or CAF09 and MOMP had significant *C. muridarum*-specific antibody titers. As determined by a T-cell lymphoproliferative assay, these four groups of mice also mounted robust cell mediated immune responses with high production of IFN-γ and IL17 and low levels of IL-4. Following the challenge the four groups of mice lost significantly less body weight than the MEM-immunized group. Lungs of mice vaccinated with CAF01, or CAF09, and nMOMP were significantly lighter than those from mice immunized using rMOMP. The number of IFU recovered from the lungs of mice vaccinated with CAF01, or CAF09, and nMOMP was similar to the number of IFU recovered from mice immunized with live EB. Mice that received rMOMP had significantly higher numbers of IFU than other groups. In conclusion, CAF01 and CAF09 elicited very robust protective humoral and cellular immune responses and were equally effective at adjuntavizing the *C. muridarum* MOMP. Mice vaccinated with nMOMP were significantly better protected than those immunized with rMOMP, indicative of the importance of the structural conformation of this antigen in protection.

1. Introduction

Chlamydia trachomatis infections occur worldwide producing genital, ocular, respiratory and gastrointestinal diseases [1–4]. Attempts to control chlamydial infections using screening programs have failed [5,6]. The search for a vaccine was initiated years ago since even, a low efficacy vaccine, could have a major impact on the epidemiology of these infections [7–9]. The major outer membrane protein (MOMP) is the leading antigen for a subunit vaccine [10–15]. MOMP has minimal intrinsic adjuvanticity and therefore, there is a need to include adjuvants in the vaccine [16,17]. Control of a *Chlamydia muridarum* infection requires cell mediated immune responses, likely controlled by IFN-γ secreting Th1 cells, and neutralizing antibodies [11,14,18,19]. Here, we tested two cationic adjuvants (CAF01 and CAF09), which elicit strong Th1 immune responses, for their ability to protect against *Chlamydia* [20–22]. CAF01 contains the immune stimulating synthetic glycolipic trehalose-dibehenate (TDB) incorporated into cationic dimethyldioctadecylammonium bromide (DDA) liposomes. TDB signals through the CLEC receptor Mincle and induces Th1/Th17 memory responses together with high antibody titers in mice [20,23]. CAF01 delivered by parenteral prime/mucosal boost routes also induces secretory IgA [24,25]. CAF09 consists of DDA liposomes stabilized with monomycoloyl glycerol (MMG)-1 combined with Poly (I:C), a TLR3 ligand. MMG-1 stimulates human DC's and the delivery of Poly I:C is facilitated by the liposomal formulation [26]. CAF09 induces strong Th1 responses with high

* Corresponding author.
E-mail address: lmdelama@uci.edu (L.M. de la Maza).

http://dx.doi.org/10.1016/j.vaccine.2017.02.020
0264-410X/

antibody levels and has also been demonstrated to cross prime CD8 T-cell responses [22]. Several investigators have evaluated the efficacy of MOMP as a vaccine antigen, using various adjuvants, and observed different levels of protection against respiratory and genital challenges [13,14,18,27–31]. We formulated CAF01 and CAF09 with the native, or the recombinant, MOMP (nMOMP or rMOMP) with the goal of determining which of these adjuvant/antigen combinations is the most effective at protecting mice against an intranasal (i.n.) challenge with *C. muridarum*.

2. Materials and methods

See supplemental material.

3. Results

3.1. Humoral immune responses following vaccination

To determine the humoral immune responses in vaccinated mice, serum samples were collected the day before the i.n. challenge and *C. muridarum*-specific antibodies determined using EB as the antigen (Table 1). Positive controls immunized i.n. with live EB had an IgG geometric mean titer (GMT) of 51,200 (range: 51,200–51,200) while negative controls inoculated i.n. with MEM had a titer below the level of detection (<100). Animals vaccinated with CAF01 and nMOMP had similar IgG levels (GMT: 19,027; range 4000–64,000) to those immunized with CAF09 (GMT: 19,027; range 8000–32,000). Mice vaccinated using CAF01 (GMT: 6727; range 2000–32,000), or CAF09 (GMT: 9514: range 8000–18,000) and rMOMP also had similar IgG titers.

To determine whether the various vaccine formulations elicited Th1 or Th2-biased humoral immune responses the IgG2a/IgG1 ratios were calculated. Mice immunized with either CAF01 (ratio: 10,159:2691 = 3.8), or CAF09 (ratio: 6400:3200 = 2.0) and nMOMP, had Th1-biased responses while those vaccinated with rMOMP had Th2 responses (CAF01: ratio 400:1345 = 0.29), or a balanced Th1/Th2 response (CAF09; ratio 1,600:1600 = 1).

In vitro neutralizing antibody levels where determined in sera the day before challenge (Table 1). Positive controls immunized with EB had a neutralizing GMT of 3200 (range 1600–6400) while mice inoculated with MEM, as negative controls, had a titer BLD (<50). Animals vaccinated with CAF01, or CAF09 and nMOMP had GMT of 126 (range: 100–200) and 200 (range: 100–800), respectively, while mice receiving CAF01, or CAF09 and rMOMP had neutralizing titers BLD (<50).

Antibody levels were also determined in vaginal washes the day before challenge (Table 1). Controls immunized with EB had IgG and IgA GMT of 269 (range: 160–320) and 320 (320–320), respectively, while mice inoculated with MEM had no detectable antibodies (<10). Significantly lower IgA levels were detected in mice immunized with CAF01 (26; range 20–40) than CAF09 (135; range 80–160) and nMOMP ($P < 0.05$). Levels of IgG were also low in mice immunized using CAF01 (13; range < 10–20), or CAF09 (80; range 80–80) and nMOMP ($P < 0.05$). Vaginal wash antibodies induced by CAF09 were statistically significantly higher than those produced by CAF01. Animals vaccinated with rMOMP had no detectable levels of *C. muridarum*-specific IgG or IgA in vaginal washes independent of the adjuvant used.

To determine what specific epitopes elicited antibody responses, serum samples were probed with 25 aa overlapping *C. muridarum* MOMP peptides (Fig. 1). Antibodies from controls immunized with live EB recognized peptides located almost exclusively to the four variable domains (VD) and constant domain (CD) 5 while no reactivity was obtained with sera from mice inoculated with MEM. Animals immunized with CAF01, or CAF09 and nMOMP, in comparison to those immunized using rMOMP, elicited broader repertoires of antibodies that included all VD and CD5. Mice vaccinated with CAF01 and rMOMP mounted antibody responses mainly to VD1 and CD5 while those immunized using CAF09 also had antibodies to VD3.

In conclusion, based on these findings, except in vaginal washes, no significant differences in antibody responses were observed between mice immunized with CAF01 versus CAF09. Broader and more robust antibody responses were observed in mice vaccinated with nMOMP versus rMOMP.

3.2. Cellular immune responses following vaccination

As a parameter of the *C. muridarum*-specific cellular immune responses, proliferation was determined using nylon-wool purified spleen T-cells (Table 2). The stimulation index (SI) of T-cells from mice vaccinated with live EB was 18.6 ± 2.3, while in animals inoculated with MEM the SI was 2.5 ± 0.2. Mice immunized with CAF01 and nMOMP or rMOMP had SI of 26.9 ± 2.7 and 30.6 ± 2.5, respectively, both significantly higher than the MEM control ($P < 0.05$). Similarly, animals vaccinated using CAF09 and nMOMP, or rMOMP, had SI of 40.5 ± 3.6 and 34.8 ± 6.1, respectively, both significantly higher than the MEM control ($P < 0.05$).

Mean IFN-γ levels (pg/ml), as a measure of a Th1 response, were determined in supernatants from EB-stimulated T-cells (Table 2). Positive controls immunized with EB had high quantities (5470 ± 146) while those inoculated with MEM had low levels of IFN-γ (76 ± 27). In mice vaccinated with CAF01 and nMOMP (3528 ± 945), or rMOMP (5293 ± 191), or with CAF09 and nMOMP (5416 ± 59), or rMOMP (4521 ± 690) levels of IFN-γ were equivalent to those observed in controls immunized with EB. Mean levels of IL-4 (pg/ml), a marker of Th2 responses, were also present but at much lower levels ranging from (16 ± 7) in mice immunized with CAF01 and nMOMP and (26 ± 14) in animals receiving CAF09 and rMOMP, supportive of strong Th1 responses in all groups immunized with both CAF adjuvants. High levels of IL-17 were elicited in the four samples from mice immunized with CAF01 or CAF09. However, these levels were higher than those elicited by

Table 1
Humoral immune responses in sera and vaginal washes the day before challenge.

	Serum geometric mean titer (GMT) (range)			Serum neutralizing	Vaginal washes GMT (range)	
Vaccine	IgG	IgG2a	IgG1	GMT (range)	IgA	IgG
CAF01/nMOMP	19,027 (4000–64,000)[a]	10,159 (3200–51,200)[a,b]	2691 (400–12,800)[a]	126 (100–200)[a]	26 (20–40)[a]	13 (<10–20)[a]
CAF01/rMOMP	6727 (2000–32,000)[a]	400 (100–1600)[a]	1345 (200–6400)[a]	<50 (<50–<50)	<10 (<10–<10)	<10 (<10–10)
CAF09/nMOMP	19,027 (8000–32,000)[a]	6400 (1600–12,800)[a]	3200 (1600–6400)[a]	200 (100–800)[a]	135 (80–160)[a,c]	80 (80–80)[a,c]
CAF09/rMOMP	9514 (8000–16,000)[a]	1600 (400–3200)[a]	1600 (100–6400)[a]	<50 (<50–<50)	<10 (<10–<10)	10 (10–10)
Cm EB	51,200 (51,200–51,200)	72,408 (51,200–102,400)	3200 (1600–6400)	3200 (1600–6400)	320 (320–320)	269 (160–320)
MEM	<100	<100	<100	<50 (<50–<50)	<10 (<10–<10)	<10 (<10–<10)

[a] $P < 0.05$ by Student's *t* test compared to MEM control group.
[b] $P < 0.05$ by Student's *t* test compared to CAF01/rMOMP group.
[c] $P < 0.05$ by Student's *t* test compared to CAF01/nMOMP group.

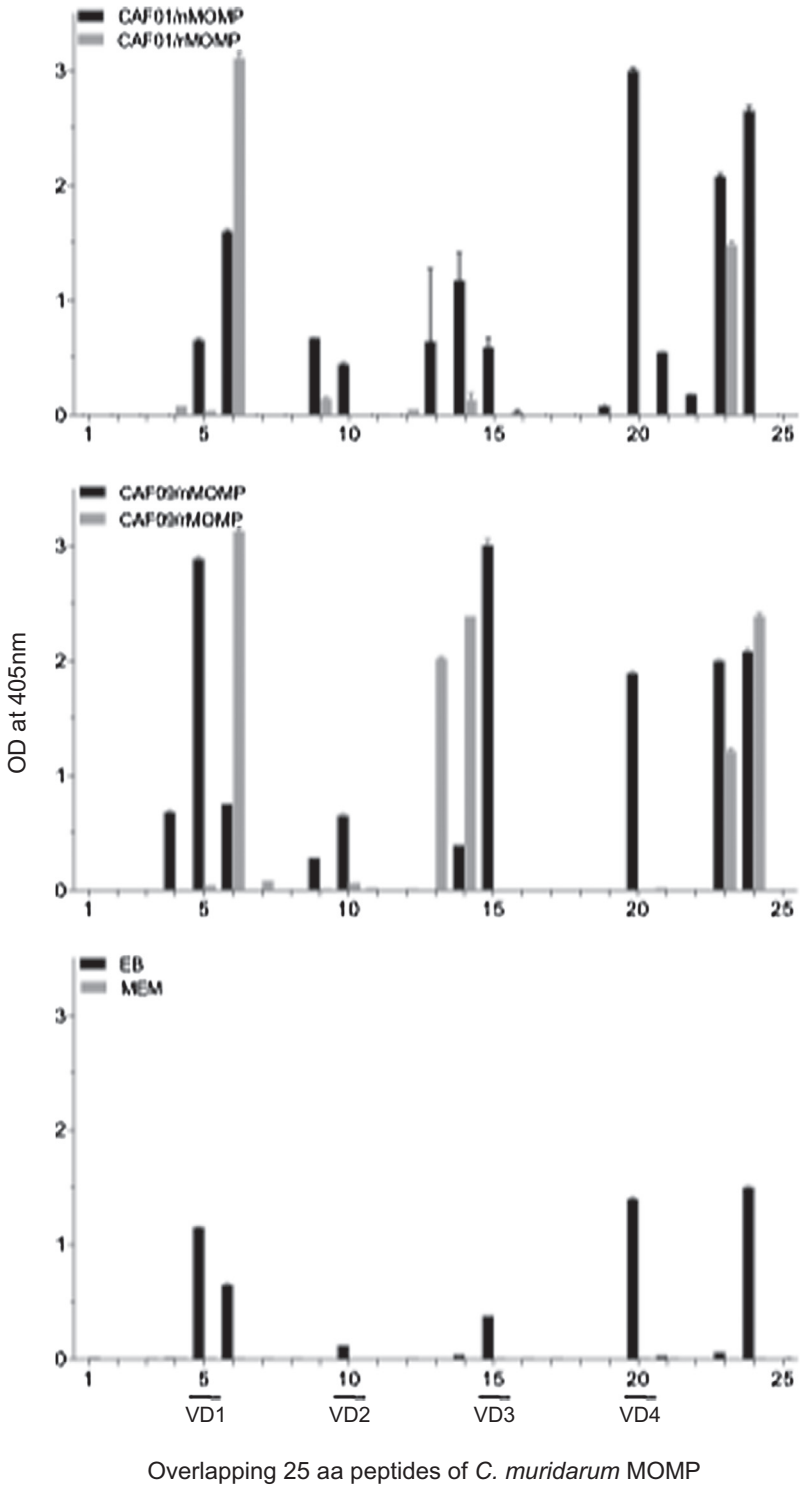

Fig. 1. Binding of serum antibodies to synthetic *C. muridarum* MOMP peptides. Serum samples from immunized mice were collected the day before the i.n. challenge and their reactivity to 25-mer peptides corresponding to the *C. muridarum* mature MOMP were analyzed by ELISA.

stimulation with EB and therefore, cannot be used as a parameter indicative of protection.

We conclude that the cellular immune responses were similar in mice immunized with CAF01 or CAF09. Also, no significant differences were observed between groups vaccinated with nMOMP versus rMOMP.

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

As a measurement of the systemic effect of the infection, the body weight was determined for 10 days following the i.n. challenge. All mice, except those immunized i.n. with EB, lost weight for the first 2–4 days post challenge (d.p.c.) (Fig. 2). Subsequently,

Table 2
In vitro T cell proliferative response and cytokine production from stimulated T cells the day before challenge.

	Δ cpm mean ± 1SE		Stimulation index mean ± 1SE		IFN-γ (pg/ml) mean ± 1SE		IL-17 (pg/ml) mean ± 1SE		IL-4 (pg/ml) mean ± 1SE	
Vaccine	*Cm* EB	Con A	*Cm* EB	Con A	*Cm* EB	Con A	*Cm* EB	Con A	*Cm* EB	Con A
CAF01/nMOMP	20,779 ± 2178[a,b,c]	60,875 ± 1228	26.9 ± 2.7[a,b]	76.9 ± 2.9	3528 ± 945[a]	5287 ± 191	736 ± 138[a,b]	658 ± 142	16 ± 7	103 ± 14
CAF01/rMOMP	12,889 ± 1074[a,b,d]	80,005 ± 6647	30.6 ± 2.5[a,b]	184.9 ± 15.3	5293 ± 191[a]	5363 ± 206	660 ± 136[a,b]	1899 ± 404	20 ± 10	349 ± 137
CAF09/nMOMP	29,173 ± 2656[a,b,c,d]	84,479 ± 8972	40.5 ± 3.6[a,b,c,d]	115.5 ± 12.2	5416 ± 59[a]	5409 ± 132	1046 ± 15[a,b,c,d]	2024 ± 437	22 ± 11	117 ± 14
CAF09/rMOMP	34,239 ± 6183[a,b,c,d]	61,227 ± 3780	34.8 ± 6.1[a,b]	61.4 ± 3.7	4521 ± 690[a]	5735 ± 63	655 ± 171[a,b]	1939 ± 514	26 ± 14	191 ± 63
Cm EB	8207 ± 1057	43,743 ± 7339	18.6 ± 2.3	94.9 ± 15.7	5470 ± 146	5730 ± 136	108 ± 18	1073 ± 421	15 ± 3	135 ± 23
MEM	696 ± 79	41,661 ± 4632	2.5 ± 0.2	93.2 ± 10.2	76 ± 27	5142 ± 286	<4	450 ± 256	<4	119 ± 15

Cm EB = *C. muridarum* EB were incubated with irradiated splenocytes at a ratio of 5:1 for preparing antigen presenting cells.
Con A = Concanavalin A was used as positive stimulant at a concentration of 5 μg/ml.
[a] $P < 0.05$ by the Student's *t* test compared to MEM control group.
[b] $P < 0.05$ by the Student's *t* test compared to EB group.
[c] $P < 0.05$ by the Student's *t* test compared to CAF01/rMOMP group.
[d] $P < 0.05$ by the Student's *t* test compared to CAF01/nMOMP group.

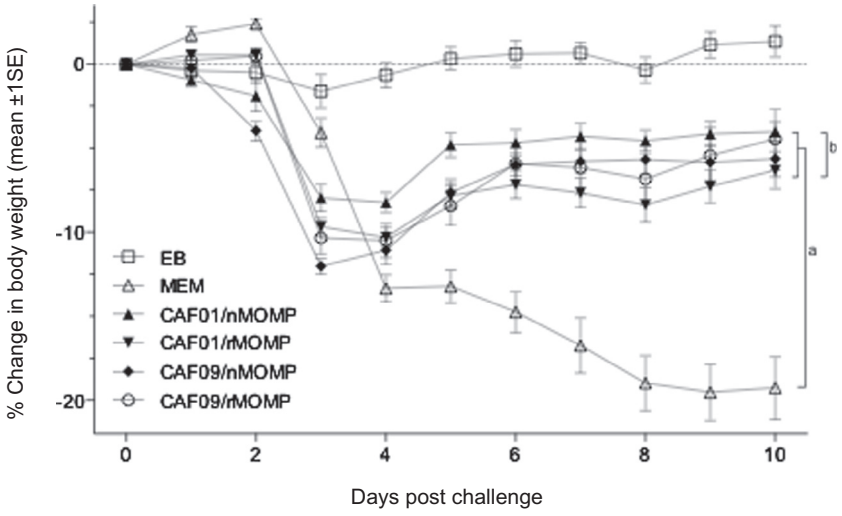

Fig. 2. Daily percentage change in body weight (mean ± 1SE) following the i.n. challenge with *C. muridarum*. [a]: $P < 0.05$ by the repeated measures ANOVA test when comparing CAF01/rMOMP, CAF01/nMOMP, CAF09/rMOMP or CAF09/nMOMP versus the MEM group. [b]: $P < 0.05$ by the repeated measures ANOVA test when comparing CAF01/nMOMP versus CAF01/rMOMP, CAF01/nMOMP versus CAF09/nMOMP and CAF01/nMOMP versus CAF09/rMOMP.

mice vaccinated with CAF01, or CAF09 and nMOMP, or rMOMP slowly regained most of their initial body weight in contrast to controls immunized with MEM ($P < 0.05$). As determined by the repeated measures ANOVA test, the cumulative body weight changes over the 10-day period were significantly ($P < 0.05$) different between mice immunized with CAF01 plus nMOMP and the other three experimental groups. No significant differences were observed between the CAF09 nMOMP versus rMOMP group ($P > 0.05$).

By 10 d.p.c., mice immunized with CAF01 and nMOMP, or rMOMP, weighted −4.02 ± 0.59% and −6.32 ± 1.11% less, respectively, than their initial body weight (Fig. 3A and Table 3). Similarly, mice immunized with CAF09 and nMOMP, or rMOMP, weighted −5.63 ± 1.06% and −4.46 ± 1.81% less, respectively than their initial body weight. None of these body weight changes are significantly different from each other ($P > 0.05$). These body weight changes at 10 d.p.c., however, are significantly different when compared mice inoculated with MEM (−19.27 ± 1.86%) ($P < 0.05$). Therefore, both CAF01 and CAF09, formulated with nMOMP or rMOMP, were similarly efficient at protecting mice from the systemic effects of *C. muridarum* infection.

3.4. Lungs weight

As a parameter of local inflammatory responses, the lungs weight was determined at 10 d.p.c. (Fig. 3B and Table 3) The mean weight of the lungs (g) in the two groups of mice vaccinated with CAF01 (0.23 ± 0.01), or CAF09 (0.25 ± 0.01) and nMOMP was significantly lower than MEM immunized controls (0.30 ± 0.01; $P < 0.05$) but not significantly different among themselves ($P > 0.05$). In animals vaccinated with CAF01 (0.33 ± 0.01), or CAF09 (0.32 ± 0.02), and rMOMP lungs weights were not significantly different among them ($P > 0.05$) and were similar to the MEM group. These results indicate that both CAF01 and CAF09 adjuvants were equally effective at inducing immune responses that helped cleared the local infection in the lungs. nMOMP was more effective than rMOMP at controlling local inflammatory responses.

3.5. Burden of C. muridarum infection in the lungs

Ten days after the i.n. challenge, mice were euthanized and their lungs cultured for *C. muridarum* (Fig 3C and Table 3). The median number of IFU recovered from lungs of mice vaccinated

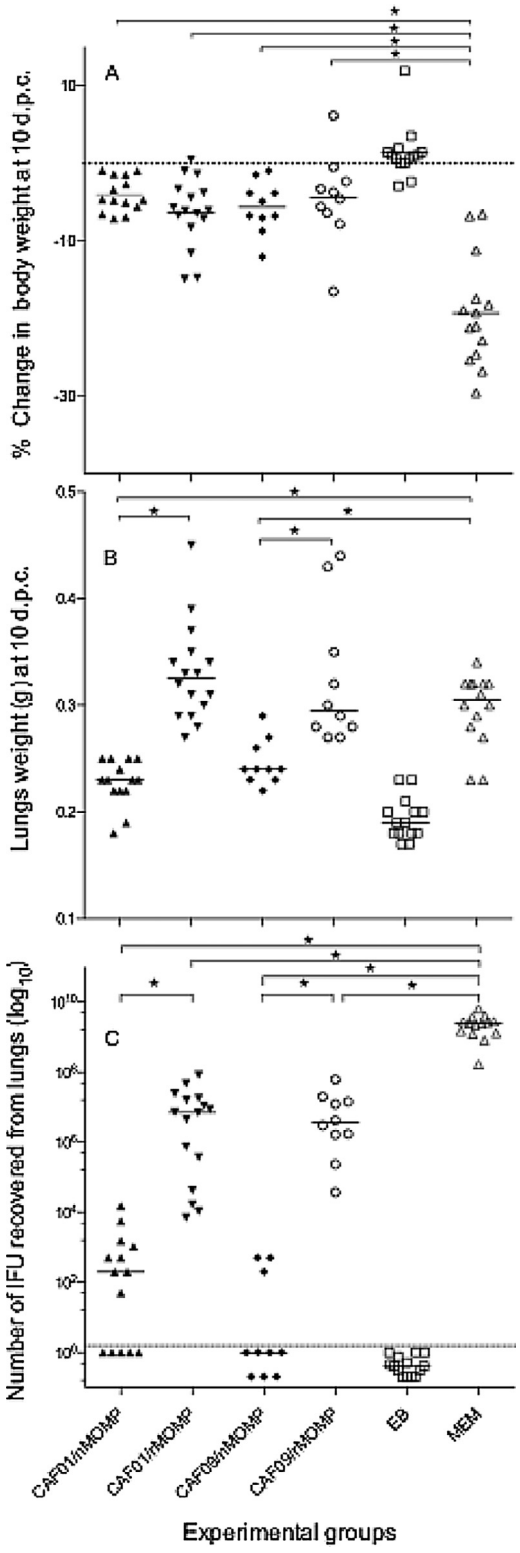

Fig. 3. (A) Percentage change in mean body weight at 10 d. following the i.n. challenge with *C. muridarum*. The mean is shown as a horizontal line. Each symbol represents a single animal. *: $P < 0.05$ when compared to MEM-immunized group. (B) Lungs weight at 10 d. following the i.n. challenge with *C. muridarum*. The mean is shown as a horizontal line. Each symbol represents a single animal. *: $P < 0.05$ when compared to other-immunized group. (C) Number of IFU recovered from the lungs at 10 d. following the i.n. challenge with *C. muridarum*. The median is shown as a horizontal line. Each symbol represents a single animal. *: $P < 0.05$ when compared to other-immunized group.

with CAF01 and nMOMP was 0.0002 (range: BLD–0.015) $\times 10^6$ and from those immunized with CAF09 was BLD (range: BLD–0.0005) $\times 10^6$. These two groups were not significantly different among themselves. In comparison with the positive control inoculated with live EB, BLD (range BLD-BLD), the CAF09 group was not significantly different ($P > 0.05$) while the CAF01 group was ($P < 0.05$). The median number of IFU recovered from mice immunized with CAF01 and rMOMP was 7.1 (range: 0.007–82.8) $\times 10^6$ and those immunized with CAF09 3.6 (range: 0.037–61.9) $\times 10^6$. The two groups immunized with rMOMP were not significantly different among themselves ($P > 0.05$) but were significantly different when compared with controls inoculated with EB ($P < 0.05$). The number of IFU in the lungs of mice vaccinated with CAF01, or CAF09 using nMOMP or rMOMP, was significant lower than in mice immunized with MEM (median: 2463; range: 165–28,317) $\times 10^6$ IFU ($P < 0.05$). Therefore, protection against the local infection was similar for CAF01 and CAF09. nMOMP was more effective than rMOMP at clearing the lung infection.

3.6. Local immune responses in the lungs at 10 d.p.c

To evaluate local immune responses, the lung homogenates supernatants were collected at 10 d.p.c. and levels of IFN-γ, IL-4 and *C. muridarum*-specific IgA were determined (Table 3). The mean levels of IFN-γ (pg/ml) in mice vaccinated with CAF01, or CAF09 and nMOMP were the same as those immunized with live EB (<15) indicative that, by 10 d.p.c., these animals had controlled the *C. muridarum* infection. These values are significantly different from those of mice immunized CAF01 (871 ± 216), or CAF09 (965 ± 315) and rMOMP, or MEM (2199 ± 238), that still had high amounts of *C. muridarum* IFU in their lungs ($P < 0.05$). No specific trends were observed in levels of IL-4 suggesting that this cytokine does not play a critical role in chlamydial infection or, that by 10 d. p.c., it has almost reached basal levels in all four groups.

The amounts of *C. muridarum*-specific IgA (OD_{405}) followed the opposite trend than the levels of IFN-γ. Mice vaccinated with CAF01, or CAF09, all had higher levels of IgA than controls inoculated with MEM. Well-protected animals, in particular those vaccinated with CAF01 (2.69 ± 0.11), or CAF09 (2.32 ± 0.15) and nMOMP and the controls immunized with EB (3.05 ± 0.04) had very high levels of IgA.

In summary, both CAF01 and CAF09 were similarly effective at controlling the local infection in the lungs. nMOMP was more efficacious than rMOMP at eliciting protective immune responses.

4. Discussion

The adjuvants CAF01 and CAF09, mixed with *C. muridarum* nMOMP or rMOMP, were compared for their ability to elicit protective immune responses in BALB/c mice against an i.n. challenge. Qualitative and quantitative similar humoral and cellular immune responses were elicited by both adjuvants. As determined by changes in body weight, lungs weight and number of *C. muridarum* IFU recovered from lungs, both adjuvants induced comparable protection. With both adjuvants, more robust protection was elicited with nMOMP than with rMOMP.

Here, CAF01 and CAF09 were formulated with the *C. muridarum* nMOMP, or rMOMP and BALB/c mice were immunized by the s.c. followed by the i.n. routes and challenged i.n. with 10^4 IFU of *C. muridarum*. Significant *C. muridarum*-specific total IgG antibody titers were detected in serum collected the day before the challenge from the four groups of mice. IgG antibody titers were higher in mice immunized with nMOMP than rMOMP. Both CAF01 and

Table 3
Disease burden, yields of *Chlamydia* IFU, and levels of IFN-γ, IL-4 and *C. muridarum*-specific IgA in lungs' homogenates at 10 d.p.c.

Vaccine	% Change in body weight (mean ± 1 SE)	Lungs weight (g) (mean ± 1 SE)	Median number IFU recovered from lungs (min–max) $\times 10^6$	IFN-γ (pg/ml) (mean ± 1 SE)	IL-4 (pg/ml) (mean ± 1 SE)	IgA (A_{405}) (mean ± 1 SE)
CAF01/nMOMP	−4.02 ± 0.59[a,b]	0.23 ± 0.01[a,b,c]	0.0002 (BLD-0.015)[e,f,g]	<15[a,b,c,i]	21 ± 1	2.69 ± 0.11[a,b,c,i]
CAF01/rMOMP	−6.32 ± 1.11[a,b]	0.33 ± 0.01[b]	7.1 (0.007–82.8)[e,f]	871 ± 216[a,b]	28 ± 1	1.09 ± 0.06[a,b,d]
CAF09/nMOMP	−5.63 ± 1.06[a,b]	0.25 ± 0.01[a,b,d]	BLD (BLD-0.0005)[e,h]	<15[a,b,d]	20 ± 0.3	2.32 ± 0.15[a,b]
CAF09/rMOMP	−4.46 ± 1.81[a,b]	0.32 ± 0.02[b]	3.6 (0.037–61.9)[e,f]	965 ± 315[a,b]	21 ± 0.9	1.22 ± 0.07[a,b]
Cm EB	1.35 ± 0.92	0.19 ± 0.01	BLD (BLD-BLD)	<15	34 ± 12	3.05 ± 0.04
MEM	−19.27 ± 1.86	0.30 ± 0.01	2463 (165–28,317)	2199 ± 238	20 ± 0.3	0.54 ± 0.06

BLD: below limit of detection (<50 *Chlamydia* IFU/lungs mouse).
[a] $P < 0.05$ by Student's *t* test compared with MEM group.
[b] $P < 0.05$ by Student's *t* test compared with EB group.
[c] $P < 0.05$ by Student's *t* test compared with CAF01/rMOMP group.
[d] $P < 0.05$ by Student's *t* test compared with CAF09/rMOMP group.
[e] $P < 0.05$ by the Mann-Whitney *U* test compared to MEM group.
[f] $P < 0.05$ by the Mann-Whitney *U* test compared to EB group.
[g] $P < 0.05$ by the Mann-Whitney *U* test compared to CAF01/rMOMP group.
[h] $P < 0.05$ by the Mann-Whitney *U* test compared to CAF09/rMOMP group.
[i] $P < 0.05$ by Student's *t* test compared with CAF09/nMOMP group.

CAF09 favor Th1 responses [14,19,22]. To determine if the vaccinations elicited Th1, or Th2–biased responses, the IgG2a/IgG1 ratios were determined. Interestingly, both adjuvants favored Th1 immune responses when formulated with nMOMP (IgG2/IgG1 ratios: ∼2–4) while, with rMOMP, responses were more balanced (IgG2/IgG1 ratios: ∼0.3–1). Massari et al. [16] have shown that nMOMP proteosomes signal via TLR-2. Adjuvants that signal via TLR2 induce mostly Th2-type immune responses [17]. Due to the structural heterogeneity, proteosomes cannot be produced with rMOMP and therefore, cannot be tested for adjuvant activity. Thus, we need to assume that rMOMP also has adjuvant activity and induces more robust Th2 responses than nMOMP. CAF01 and CAF09 formulated with nMOMP, but not with rMOMP, elicited neutralizing antibodies that likely accounted for better protection.

Using synthetic peptides we determined MOMP domains recognized by IgG antibodies. As expected, most antibodies bound to the four VDs, although CD5 was also recognized [32]. No major differences were observed between mice immunized with CAF01 versus CAF09 and rMOMP although mice receiving CAF01 only recognized epitopes in VD1 and CD5 while those immunized using CAF09 also recognized VD3 epitopes. Mice vaccinated using nMOMP, with both adjuvants, mounted broader antibody responses that included all the VDs, than those immunized with rMOMP. This may account for the better protection obtained with nMOMP since neutralizing B-cell epitopes are mainly located in VDs while T-cell epitopes map mainly to CDs [32–34].

Based on a T-cell lymphoproliferative assay and production of IFN-γ, all four-vaccine formulations elicited robust Th-1 cell mediated immune responses. Interestingly, these responses were equivalent to those induced in positive control mice immunized with live EB. These results are very encouraging since in general, using other adjuvants, we have found the cellular immune responses to be weaker for vaccines formulated with MOMP, in particular rMOMP, than those of animals immunized with live EB [35]. These results therefore, confirm the ability of both CAF01 and CAF09 to elicit strong Th1-biased immune responses in mice using antigens from various pathogens including malaria, *Mycobacterium tuberculosis* and *C. muridarum* [20,23,36–38].

As shown by body weight loss, the four groups of mice, immunized with CAF01 or CAF09 and MOMP, were protected against the i.n. *C. muridarum* challenge when compared with the MEM-immunized group. Weight loss occurred mainly on days 2 to 3 p.c. During this time period, the *C. muridarum* infectious challenge likely goes through the first round of replication and systemic dissemination. The body weight then slowly increased over the next 6 d. indicative of control of the infection. In contrast, negative controls vaccinated with MEM continuously lost weight during the 10 d. of observation. As expected, positive controls immunized with EB had minimal body weight loss by 3 d.p.c. and then regained their initial weight.

Lungs weight showed that only mice vaccinated with CAF01, or CAF09, and nMOMP were protected. This suggests that while mice vaccinated with nMOMP had already controlled the local growth of *C. muridarum* by 10 d.p.c., those immunized with rMOMP still had significant inflammation. This was confirmed when the bacterial load was determined in lungs. As shown by the number of *C. muridarum* IFU recovered from the lungs, vaccination with CAF01 or CAF09 and nMOMP, elicited equivalent protection to EB. Groups that received rMOMP were also protected but had significantly higher numbers of IFU than those vaccinated with nMOMP. Levels of IFN-γ in lungs supernatants, used as an indication of local inflammatory responses, paralleled these results. Groups of mice with high numbers of IFU in lungs had high levels of IFN-γ while those with low IFU number had low IFN-γ levels. Titers of *C. muridarum* specific IgA followed the opposite pattern suggesting that this local immune response has an important role in protection. The somewhat stronger IgA by the CAF01 formulation compared to CAF09, is likely relate to the ability of CAF01 to promote IL-17 responses that have been reported to accelerate the recruitment of IgA producing B cells and upregulate polymeric immunoglobulin receptor (pIgR) expression in the mucosal epithelium of importance for IgA secretion [24,25,39].

In summary, CAF01 and CAF09, formulated with MOMP, elicited similarly strong protective humoral and cell mediated immune responses in mice against a *C. muridarum* i.n. challenge. nMOMP induced a more robust protection than rMOMP. Since the cellular immune responses were equivalent in mice vaccinated with nMOMP versus rMOMP while antibody levels were overall higher, qualitatively broader and had more neutralizing activity in animals immunized with nMOMP than rMOMP, we conclude that antibody responses may account for some of the differences in protection between the two MOMP preparations. These findings support experiments, in which a critical role for antibodies in protection against a chlamydial challenge was determined [14,18,19,40]. The more robust protection induced by nMOMP versus rMOMP, expands studies in which other adjuvants were used, and confirms the critical role in protection played by the structural conformation of MOMP [35,41].

Acknowledgements

This work was supported by Public Health Service grant AI092129 from the National Institute of Allergy and Infectious Diseases.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.02.020.

References

[1] Chlamydia screening among sexually active young female enrollees of health plans–United States, 2000–2007. MMWR Morb Mortal Wkly Rep. vol. 58; 2009. p. 362–5.
[2] Miller WC, Ford CA, Morris M, Handcock MS, Schmitz JL, Hobbs MM, et al. Prevalence of chlamydial and gonococcal infections among young adults in the United States. JAMA 2004;291:2229–36.
[3] Schachter J, Dawson CR. Human chlamydial infections. Littleton (Mass): PSG Pub. Co.; 1978.
[4] Darville T. Recognition and treatment of chlamydial infections from birth to adolescence. Adv Exp Med Biol 2013;764:109–22.
[5] Gotz H, Lindback J, Ripa T, Arneborn M, Ramsted K, Ekdahl K. Is the increase in notifications of *Chlamydia trachomatis* infections in Sweden the result of changes in prevalence, sampling frequency or diagnostic methods? Scand J Infect Dis 2002;34:28–34.
[6] Brunham RC, Pourbohloul B, Mak S, White R, Rekart ML. The unexpected impact of a *Chlamydia trachomatis* infection control program on susceptibility to reinfection. J Infect Dis 2005;192:1836–44.
[7] Grayston JT, Woolridge RL, Wang S. Trachoma vaccine studies on Taiwan. Ann N Y Acad Sci 1962;98:352–67.
[8] Grayston JT, Wang SP. The potential for vaccine against infection of the genital tract with *Chlamydia trachomatis*. Sex Transm Dis 1978;5:73–7.
[9] de la Maza MA, de la Maza LM. A new computer model for estimating the impact of vaccination protocols and its application to the study of *Chlamydia trachomatis* genital infections. Vaccine 1995;13:119–27.
[10] de la Maza LM, Peterson EM. Vaccines for *Chlamydia trachomatis* infections. Curr Opin Investig Drugs 2002;3:980–6.
[11] Farris CM, Morrison RP. Vaccination against Chlamydia genital infection utilizing the murine *C. muridarum* model. Infect Immun 2011;79:986–96.
[12] Mabey DC, Hu V, Bailey RL, Burton MJ, Holland MJ. Towards a safe and effective chlamydial vaccine: lessons from the eye. Vaccine 2014;32:1572–8.
[13] Pal S, Peterson EM, de la Maza LM. Vaccination with the *Chlamydia trachomatis* major outer membrane protein can elicit an immune response as protective as that resulting from inoculation with live bacteria. Infect Immun 2005;73:8153–60.
[14] Olsen AW, Follmann F, Erneholm K, Rosenkrands I, Andersen P. Protection against *Chlamydia trachomatis* infection and upper genital tract pathological changes by vaccine-promoted neutralizing antibodies directed to the VD4 of the major outer membrane protein. J Infect Dis 2015.
[15] Tifrea DF, Pal S, Popot JL, Cocco MJ, de la Maza LM. Increased immunoaccessibility of MOMP epitopes in a vaccine formulated with amphipols may account for the very robust protection elicited against a vaginal challenge with *Chlamydia muridarum*. J Immunol 2014;192:5201–13.
[16] Massari P, Toussi DN, Tifrea DF, de la Maza LM. Toll-like receptor 2-dependent activity of native major outer membrane protein proteosomes of *Chlamydia trachomatis*. Infect Immun 2013;81:303–10.
[17] Toussi DN, Massari P. Immune adjuvant effect of molecularly-defined toll-like receptor ligands. Vaccines 2014;2:323–53.
[18] Farris CM, Morrison SG, Morrison RP. CD4+ T cells and antibody are required for optimal major outer membrane protein vaccine-induced immunity to *Chlamydia muridarum* genital infection. Infect Immun 2010;78:4374–83.
[19] Olsen AW, Andersen P, Follmann F. Characterization of protective immune responses promoted by human antigen targets in a urogenital *Chlamydia trachomatis* mouse model. Vaccine 2014;32:685–92.
[20] Agger EM, Rosenkrands I, Hansen J, Brahimi K, Vandahl BS, Aagaard C, et al. Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. PLoS One 2008;3:e3116.
[21] Gram GJ, Karlsson I, Agger EM, Andersen P, Fomsgaard A. A novel liposome-based adjuvant CAF01 for induction of CD8(+) cytotoxic T-lymphocytes (CTL) to HIV-1 minimal CTL peptides in HLA-A*0201 transgenic mice. PLoS One 2009;4:e6950.
[22] Korsholm KS, Hansen J, Karlsen K, Filskov J, Mikkelsen M, Lindenstrom T, et al. Induction of CD8+ T-cell responses against subunit antigens by the novel cationic liposomal CAF09 adjuvant. Vaccine 2014;32:3927–35.
[23] Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, et al. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. J Immunol 2009;182:8047–55.
[24] Lorenzen E, Follmann F, Bøje S, Erneholm K, Olsen AW, Agerholm JS, et al. Intramuscular priming and intranasal boosting induce strong genital immunity through secretory IgA in minipigs infected with Chlamydia trachomatis. Front Immunol 2015;6:e628.
[25] Christensen D, Mortensen R, Rosenkrands I, Dietrich J, Andersen P. Vaccine-induced Th17 cells are established as resident memory cells in the lung and promote local IgA responses. Mucosal Immunol 2017;10:260–70.
[26] Andersen CA, Rosenkrands I, Olsen AW, Nordly P, Christensen D, Lang R, et al. Novel generation mycobacterial adjuvant based on liposome-encapsulated monomycoloyl glycerol from *Mycobacterium bovis* bacillus Calmette-Guerin. J Immunol 2009;183:2294–302.
[27] Su H, Messer R, Whitmire W, Fischer E, Portis JC, Caldwell HD. Vaccination against chlamydial genital tract infection after immunization with dendritic cells pulsed ex vivo with nonviable *Chlamydiae*. J Exp Med 1998;188:809–18.
[28] Su H, Parnell M, Caldwell HD. Protective efficacy of a parenterally administered MOMP-derived synthetic oligopeptide vaccine in a murine model of *Chlamydia trachomatis* genital tract infection: serum neutralizing IgG antibodies do not protect against chlamydial genital tract infection. Vaccine 1995;13:1023–32.
[29] Pal S, Barnhart KM, Wei Q, Abai AM, Peterson EM, de la Maza LM. Vaccination of mice with DNA plasmids coding for the *Chlamydia trachomatis* major outer membrane protein elicits an immune response but fails to protect against a genital challenge. Vaccine 1999;17:459–65.
[30] Dong-Ji Z, Yang X, Shen C, Lu H, Murdin A, Brunham RC. Priming with *Chlamydia trachomatis* major outer membrane protein (MOMP) DNA followed by MOMP ISCOM boosting enhances protection and is associated with increased immunoglobulin A and Th1 cellular immune responses. Infect Immun 2000;68:3074–8.
[31] Igietseme JU, Murdin A. Induction of protective immunity against *Chlamydia trachomatis* genital infection by a vaccine based on major outer membrane protein-lipophilic immune response-stimulating complexes. Infect Immun 2000;68:6798–806.
[32] Stephens RS, Wagar EA, Schoolnik GK. High-resolution mapping of serovar-specific and common antigenic determinants of the major outer membrane protein of *Chlamydia trachomatis*. J Exp Med 1988;167:817–31.
[33] Baehr W, Zhang YX, Joseph T, Su H, Nano FE, Everett KD, et al. Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. Proc Natl Acad Sci USA 1988;85:4000–4.
[34] Su H, Morrison RP, Watkins NG, Caldwell HD. Identification and characterization of T helper cell epitopes of the major outer membrane protein of *Chlamydia trachomatis*. J Exp Med 1990;172:203–12.
[35] Sun G, Pal S, Weiland J, Peterson EM, de la Maza LM. Protection against an intranasal challenge by vaccines formulated with native and recombinant preparations of the *Chlamydia trachomatis* major outer membrane protein. Vaccine 2009;27:5020–5.
[36] Hansen J, Jensen KT, Follmann F, Agger EM, Theisen M, Andersen P. Liposome delivery of *Chlamydia muridarum* major outer membrane protein primes a Th1 response that protects against genital chlamydial infection in a mouse model. J Infect Dis 2008;198:758–67.
[37] Christensen D, Foged C, Rosenkrands I, Lundberg CV, Andersen P, Agger EM, et al. CAF01 liposomes as a mucosal vaccine adjuvant: in vitro and in vivo investigations. Int J Pharm 2010;390:19–24.
[38] Yu H, Karunakaran KP, Jiang X, Shen C, Andersen P, Brunham RC. *Chlamydia muridarum* T cell antigens and adjuvants that induce protective immunity in mice. Infect Immun 2012;80:1510–8.
[39] Jaffar Z, Ferrini ME, Herritt LA, Roberts K. Cutting edge: lung mucosal Th17-mediated responses induce polymeric Ig receptor expression by the airway epithelium and elevate secretory IgA levels. J Immunol 2009;182:4507–11.
[40] Morrison SG, Morrison RP. A predominant role for antibody in acquired immunity to chlamydial genital tract reinfection. J Immunol 2005;175:7536–42.
[41] Sun G, Pal S, Sarcon AK, Kim S, Sugawara E, Nikaido H, et al. Structural and functional analyses of the major outer membrane protein of *Chlamydia trachomatis*. J Bacteriol 2007;189:6222–35.