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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

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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

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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.