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Improved protection against *Chlamydia muridarum* using the native major outer membrane protein trapped in Resiquimod-carrying amphipols and effects in protection with addition of a Th1 and a Th2 adjuvant

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Abstract

A new vaccine formulated with the *Chlamydia muridarum* native major outer membrane protein (nMOMP) and amphipols was assessed in an intranasal (i.n.) challenge mouse model. nMOMP was trapped either in amphipol A8-35 (nMOMP/A8-35) or in A8-35 conjugated with Resiquimod (nMOMP/Resiq-A8-35), a TLR7/8 agonist added as adjuvant. The effects of free Resiquimod and/or additional adjuvants, Montanide ISA 720 (TLR independent) and CpG-1826 (TLR9 agonist), were also evaluated. Immunization with nMOMP/A8-35 alone administered i.n. was used as negative adjuvant-control group, whereas immunizations with C. muridarum elementary bodies (EBs) and MEM buffer, administered i.n., were used as positive and negative controls, respectively. Vaccinated mice were challenged i.n. with *C. muridarum* and changes in body weight, lungs weight and recovery of Chlamydia from the lungs were evaluated. All the experimental groups showed protection when compared with the negative control group. Resiquimod alone produced weak humoral and cellular immune responses, but both Montanide and CpG-1826 showed significant increases in both responses. The addition of CpG-1826 alone switched immune responses to be Th1-biased. The most robust protection was elicited in mice immunized with the three adjuvants and conjugated Resiquimod. Increased protection induced by the Resiquimod covalently linked to A8-35, in the presence of Montanide and CpG-1826

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Declaration of interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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was established based on a set of parameters: 1) the ability of the antibodies to neutralize *C. muridarum*; 2) the increased proliferation of T-cells in vitro accompanied by higher production of IFN-γ, IL-6 and IL-17; 3) the decreased body weight loss over the 10 days after challenge; and 4) the number of IFUs recovered from the lungs at day 10 post challenge. In conclusion, a vaccine formulated with the *C. muridarum* nMOMP bound to amphipols conjugated with Resiquimod enhances protective immune responses that can be further improved by the addition of Montanide and CpG-1826.

Keywords

Chlamydia muridarum ; major outer membrane protein; vaccine; Resiquimod; TLR7/8 adjuvants; amphipols; A8-35

INTRODUCTION

Chlamydia trachomatis is the most common bacterial sexually transmitted pathogen worldwide [1]. It is estimated that more than three million new infections occur annually in USA [2]. *C. trachomatis* also infects the eyes, which can result in blinding trachoma, the most common cause of preventable blindness [3]. In addition, it can also produce respiratory and gastrointestinal infections [4]. Severe primary and recurrent genital *C. trachomatis* infections can lead to acute and chronic diseases including cervicitis, urethritis, pelvic inflammatory disease, ectopic pregnancy and infertility [5–7]. In addition, the high prevalence of asymptomatic genital infections in women (75%) and men (50%) makes it difficult to diagnose and treat these patients [4, 8]. Screening patients at risk and treating them with antibiotics have not yielded positive results [9]. Likely antibiotic treatment interferes with the development of adaptive immunity and results in an increase susceptibility to reinfections [10]. Therefore, the implementation of a vaccine is the best approach to eradicate these pathogens [11–14].

In the 1960's, several groups of investigators tested live and inactivated whole organism vaccines in humans and non-human primates to protect against trachoma [3, 15]. Although no vaccine was implemented, important conclusions emerged from these trials. Certain groups of vaccinated individuals were protected for short periods of time (1–3 years) and the protection was serovar/serogroup specific. In addition, in some vaccinated individuals reexposure to *C. trachomatis* resulted in a hypersensitivity reaction in their eyes or increased susceptibility to reinfection. Although the cause of the hypersensitivity reaction is still under investigation, the possibility of a chlamydial component, such as the 60-kDa heat-shock protein, being responsible for this negative reaction was considered [16]. Thus, the search for a subunit vaccine was initiated.

DNA sequencing of the *C. trachomatis* genome and phylogenetic analysis concluded that the major outer membrane protein (MOMP) was likely the antigen that induced the serovar/serogroup protection observed during the trachoma vaccine trials [17, 18]. Subunit vaccines using recombinant MOMP, MOMP peptides or DNA plasmids expressing MOMP induced limited protection [19–21]. Therefore, the tri-dimensional conformation of MOMP, or a post-translational modification, important for protection was considered. As a result, a

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detergent-extracted trimeric form of native MOMP (nMOMP) was evaluated as a potential vaccine [22]. Strong protective immune responses were elicited in mice against genital and respiratory challenges and in non-human primates against an ocular challenge when nMOMP was used as the antigen [23–27]. The potential toxic and denaturing effects of detergents and the need to work above their critical micelle concentration to prevent protein aggregation, lead to the search for different surfactants to keep nMOMP water-soluble and more suitable for vaccine formulation [28]. To stabilize membrane proteins in aqueous solution, Tribet et al. developed amphipathic polymers, better known under the name of amphipols (APols) [29]. To date, more than three dozen membrane proteins including the *Escherichia coli* OmpF, a protein structurally similar to MOMP, have been kept soluble in their native conformation using APol A8-35 [30, 31]. APols interact with the hydrophobic transmembrane region of the protein while leaving the extramembrane regions accessible. In the present study, following extraction and purification using detergents, the *C. muridarum* nMOMP was transferred to APol A8-35 to maintain its structure and solubility in an aqueous solution.

Subunit vaccines formulated with highly purified proteins used as antigens require the addition of adjuvants to boost local innate and adaptive immune responses [32]. Co-delivery of adjuvant along with the antigen increases the efficacy of vaccines [33]. This approach can be achieved with the use of adjuvants chemically linked to APols. A wide range of compounds has been covalently linked to the backbone of APols without affecting their solubility nor their ability to keep membrane proteins water-soluble [31]. These modifications include the binding of affinity tags (hexa-histidine, imidazole, biotine), fluorescent dyes (NBD, rhodamine, fluorescein, Alexa Fluor 647) and adjuvants (EP67 and CpG-1826) yielding functionalized APols [30, 34–36]. In the present study, we covalently grafted Resiquimod, a TRL7/8 agonist, to A8-35, yielding Resiq-A8-35, which was subsequently used to keep *C. muridarum* nMOMP water-soluble. Vaccination of mice with this formulation elicited more robust protective responses than when nMOMP/A8-35 was delivered along with free Resiquimod. In addition, to further improve the protection induced by the vaccine, two other adjuvants Montanide ISA 720 VG, a non-TLR adjuvant, and CpG-1826, a TLR-9 agonist, were subsequently included in the vaccine formulations and tested for their ability to protect mice against a respiratory challenge with *C. muridarum*.

MATERIALS AND METHODS

See Supplementary Material

RESULTS

Conjugation of A8-35 with Resiquimod

The synthesis of A8-35 functionalized with Resiquimod (Resiq-A8-35) consists in the random grafting on polyacrylic acid backbone of isopropylamine, octylamine and Resiquimod *via* the formation of amide bonds. Briefly, Resiquimod was introduced with octylamine at the first step of polyacrylic acid backbone modification. After the second step of modification with isopropylamine, the Resiq-A8-35 was obtained at a final yield (80%) comparable with a typical yield recovered after A8-35 synthesis.

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The grafting ratio of Resiquimod was estimated to be 1.6 mol% as determined by ^{13}C and ^{1}H-NMR spectroscopy and UV-visible spectrophotometry. This percentage is more than three times weaker than expected. The grafting ratio of isopropylamine was also weaker than expected (21.6 mol% instead of 40 mol%). Nevertheless, the solution behavior of Resiq-A8-35 was assessed by SEC analysis (Fig. 1A). The superimposable chromatographic profiles of A8-35 and Resiq-A8-35 monitored at 220 nm show the unchanged ability of the Resiquimod-labeled A8-35 to self-organize into homogeneous particles. Based on the grafting ratio of Resiquimod and considering 9 polymers per APol particle, the number of Resiquimod per particle was found to be close to 5 and the particle mass of Resiq-A8-35 to ~41.6 kDa, corresponding to a moderate increase in particle mass (+~4%).

The ability of Resiq-A8-35 to keep a membrane protein soluble in aqueous solution was tested with the monomeric porin OmpX from *E. coli*. Compared to A8-35, Resiq-A8-35 presented the same capability at keeping OmpX soluble and homogeneous at similar protein/APol mass ratio indicating that the presence of Resiquimod covalently linked to the polymer's backbone does not affect the properties of A8-35 (data not shown). Conversely, we tested whether the Resiq-A8-35 would influence the structure or thermal stability of nMOMP. Figure 1B (inset) shows a peak at 216 nm indicating beta-strand secondary structure with an intensity similar to what is expected for nMOMP [28]. The intensity at 216 nm as a function of temperature shows that the secondary structure changes very little from 25–79 °C.

Antibody responses in serum and vaginal washes following immunization

Three groups of female BALB/c mice were immunized with the following preparations: nMOMP/A8-35 only or supplemented with Resiquimod either tethered to A8-35 or free. In addition, three other groups of mice were immunized with the same formulations plus Montanide and another set of three groups of mice received Montanide plus CpG-1826 as adjuvants. A positive control group of mice was immunized i.n. with live *C. muridarum* EBs and a negative control group of animals was inoculated with MEM buffer. Serum samples and vaginal washes for testing humoral responses were collected the day before the challenge with *C. muridarum*.

As shown in Table 1, no significantly different IgG GMT were observed whether Resiquimod was tethered to A8-35 or free. Progressively higher *C. muridarum* specific antibody GMT in serum was detected when additional adjuvants were included in the formulation. For example, Montanide and Montanide plus CpG-1826, present in both nMOMP/Resiq-A8-35 and nMOMP/A8-35 plus free Resiquimod formulations, increased the antibody levels in mice by around one and two orders of magnitude, respectively. Vaccines formulated with APol-conjugated Resiquimod had more Th2-biased responses than those containing free Resiquimod.

To evaluate the predominance of Th1 *versus* Th2 responses, the IgG2a/IgG1 ratios in serum were determined. Mice immunized with nMOMP/A8-35 alone or with nMOMP/A8-35 conjugated or not to Resiquimod, all had Th2-biased immune responses. Addition of Montanide to the three vaccine formulations maintained similar IgG2a/IgG1 ratios. A

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major shift towards Th1 immune response was observed when CpG-1826 was added, as was determined for the *C. muridarum* EBs control group.

No differences in neutralization titers were observed between mice immunized with free *versus* APol-conjugated Resiquimod (Table 1). Increasingly higher neutralization titers were observed when additional adjuvants were included in the vaccine preparations. For example, the addition of Montanide to nMOMP/Resiq-A8-35 formulation increased the titer by 2.5 folds, whereas the addition of Montanide and CpG-1826 induced an increase by 20 folds, which was four times higher than the neutralization titer obtained for the positive control group. Similar increases were observed in preparations containing free Resiquimod.

Low IgG and IgA antibody titers were detected in vaginal washes. As expected, IgG titers in vaginal washes paralleled those determined in serum. The more adjuvants were included in the vaccine formulation, the higher the IgG titer was. IgA titers also gradually increased with the addition of adjuvants. In all these groups, the IgG GMT was higher than the IgA GMT. In contrast, in mice immunized with live EBs, the IgG and IgA GMT in vaginal washes were 40 and 92, respectively.

Mapping of MOMP epitopes recognized by antibodies

To identify MOMP epitopes recognized by antibodies induced by vaccination, ELISA plates coated with 25-mers MOMP overlapping peptides were probed with serum collected the day before the i.n. challenge (Fig. 2). Sera from mice vaccinated with nMOMP/A8-35 plus free Resiquimod recognized the same epitopes as mice immunized with live EBs, *i.e.* peptides corresponding to four variable domains of MOMP (VD1, VD2, VD3, VD4) and one constant domain (CD5). In comparison, sera from nMOMP/Resiq-A8-35 or nMOMP/A8-35 did not recognized VD3 but the nMOMP/Resiq-A8-35 sera probed positive with CD5. In the presence of the Montanide, all groups had more robust and broad binding to VDs. In addition, sera from mice immunized with nMOMP/A8-35+Resiquimod+Montanide reacted also with CD2. The addition of CpG-1826 further intensified the antibody binding and extended the number of peptides recognized to CD2. The broader and most robust B-cell epitopes recognition was obtained with mice vaccinated with nMOMP/A8-35+Resiquimod+Montanide+CpG-1826.

T-cell responses of immunized mice

Vaccinated mice were euthanized and their spleens harvested the day before the i.n. challenge. T-cells were separated and stimulated with *C. muridarum* EBs at a ratio of 1:1 to APC + T cells. Culture medium was used as a negative control and ConA as a positive control. As shown in Table 2, T-cell responses, even when Montanide was included in the formulation, were similar in mice vaccinated with APol-conjugated or free Resiquimod as determined by cell proliferation and levels of production of three cytokines, IFN-γ, IL-6 and IL-17. Similarly, no significant differences were found between mice vaccinated with Resiquimod only *versus* Resiquimod+Montanide. Furthermore, none of these groups differs from the controls immunized with nMOMP/A8-35 with or without Montanide. Significant increases in these parameters were observed when CpG-1826 was added to the three vaccine formulations. For example, in the presence of the three adjuvants, immunization

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with APol-conjugated Resiquimod elicited two times more IFN-γ production compared to free Resiquimod. Similar differences were observed between these two groups for the levels of IL-6 (305 versus 182 pg/mL) and IL-17 (177 versus 87 pg/mL), respectively. Positive control mice vaccinated with live EBs had the highest T-cell proliferation and IFN-γ levels.

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

To measure the systemic effects of the *C. muridarum* i.n. infection, the body weight of each mouse was recorded for 10 days post challenge (p.c.) (Fig. 3). Except for the control group of mice immunized i.n. with live *C. muridarum* EBs, all other groups rapidly lost weight from D2 to D4 p.c. The three groups of mice immunized with either Resiquimod conjugated to APol (nMOMP/Resiq-A8-35) or free (nMOMP/A8-35+Resiquimod), or nMOMP/A8-35 only, lost the most weight by D4 p.c. (around 8.9%). Mice immunized with the addition of Montanide lost between 6.3% and 7.7% of their initial body weight, while including CpG-1826 decreased the body weight losses to 4.8% - 5.2%. After D4 p.c. all groups, except the MEM control group, gained weight. Mice immunized with nMOMP/Resiq-A8-35+Montanide+CpG-1826 recovered weight earlier and faster than any other experimental group.

At D10 p.c., the only group of mice with a percent of body weight loss similar to the positive control group of mice immunized with *C. muridarum* EBs (+4.06±1.00%) was the nMOMP/Resiq-A8-35+Montanide+CpG-1826 vaccinated group (+1.26±0.95%) (Fig. 4A and Table 3). Furthermore, only the addition of Montanide+CpG-1826 to the APol-conjugated Resiquimod significantly decreased the body weight loss when compared to nMOMP/Resiq-A8-35 group (−3.96±0.92%), whereas the addition of only Montanide did not improve body weight loss. As expected, the negative control group of mice inoculated with MEM buffer lost the most body weigh by D10 p.c.

Lungs weight at D10 p.c.

To evaluate the local inflammatory responses, the weight of the lungs was determined at D10 p.c. (Fig. 4B and Table 3). Overall, no major differences were found among most of the experimental groups. Significant differences in the mean weight of the lungs were observed between the adjuvant control group of mice immunized with nMOMP/A8-35 and all the other groups of mice, except the nMOMP/A8-35+Resiquimod immunized group. Furthermore, only mice vaccinated with nMOMP/A8-35 were different from the *C. muridarum* EBs immunized group. All experimental groups had lower lungs weight than the MEM negative control group.

Burden of *C. muridarum* in the lungs at D10 p.c.

At D10 p.c., mice were euthanized and the number of *C. muridarum* IFU determined in the lungs. We observed a gradual decrease in the number of IFU recovered from the lungs as more adjuvants were included in the vaccine formulations Fig. 4C). The most robust protection was observed in mice immunized with the three adjuvants. No significant differences were observed between groups immunized with APol-conjugated or free Resiquimod ($P > 0.05$). However, the median number of IFU recovered from lungs of the nMOMP/Resiq-A8-35+Montanide+CpG-1826 immunized group was similar to the positive

control group, whereas that of the nMOMP/A8-35+Resiquimod+Montanide+CpG-1826 immunized group was significantly higher ($P < 0.05$). Statistically less IFU's were recovered from all the immunization groups when compared to the MEM negative control group.

Local immune response in the lungs at D10 p.c.

To identify local immune parameters that correlate with protection, we measured the levels of *C. muridarum*-specific IgA and IFN-γ in lungs homogenates harvested at D10 p.c. (Figs. 5A and 5B). The amount of IFN-γ in the lungs decreases when the level of protection conferred by the vaccination rises probably due to lower replication of *C. muridarum*. The negative control group of mice immunized with MEM had the highest IFN-γ value, whereas the groups vaccinated using two or three adjuvants, as well as the positive EBs control group, had IFN-γ values below the limit of detection (15 pg/mL). The mean levels of IFN-γ present in mice immunized with free and APol-conjugated Resiquimod were not different among themselves but were statistically lower than the amount of IFN-γ present in animals immunized without adjuvant.

The mean OD_{405} values of IgA in lungs supernatants from mice vaccinated with *C. muridarum* EBs and MEM were the highest and the lowest, respectively, and both values differ statistically from all the other values obtained for immunized groups. A gradual increase in *C. muridarum*-specific IgA production in the lungs was elicited by the additional adjuvants in the vaccine. For example, the presence of Montanide or Montanide+CpG-1826 in nMOMP/Resiq-A8-35 formulation increases the levels of IgA present in immunized mice compared to animal vaccinated with nMOMP/Resiq-A8-35 only. No significant differences were observed whether Resiquimod was conjugated to A8-35 or free.

DISCUSSION

The implementation of subunit vaccines has highlighted the need to evaluate the use of adjuvants in modern vaccinology. To determine the effects of delivering the antigen and the adjuvant together, we covalently linked Resiquimod to APol A8-35 and used the resulting conjugate, Resiq-A8-35, to keep *C. muridarum* nMOMP water-soluble. CD results show that the structure and stability of the protein are not affected by the presence of Resquimod attached to A8-35. To elicit increases in Th2 and Th1 responses respectively, Montanide ISA 720 VG and CpG-1826 were subsequently included in the vaccine. Mice were immunized by a combination of mucosal followed by systemic routes. Animals vaccinated using A8-35-conjugated Resiquimod were better protected against a respiratory challenge with *C. muridarum* than animals immunized with free Resiquimod. Addition of Montanide further enhanced protective immune responses. The most robust protection was achieved when the three adjuvants, namely A8-35-conjugated Resiquimod, Montanide and CpG-1826, were included in the vaccine. These observations support and expand those reported in a previous study where A8-35 conjugated to the adjuvant EP67 was used in vaccine formulations [36].

The *C. muridarum* native integral membrane protein nMOMP purified in detergents can be used in vaccine formulations [22]. However, detergents have toxic and denaturing effects that can be detrimental in a vaccine. APols, which were developed as an alternative to detergents to keep membrane proteins soluble in aqueous solutions present several

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advantages over detergents for vaccine applications [30, 37]. APols have a very low critical aggregation concentration [38] and, when adsorbed to a membrane protein, do not dissociate following dilution [39], preventing most likely protein aggregation in circulating fluids like blood. The nMOMP/APol formulation should make for a safer delivery of the antigen to target cells while maintaining better the protein structure than a detergent preparation [31]. Indeed, a greater protection was observed after exchanging detergents for A8-35 [28]. A possible explanation would be a better accessibility of the antigenic domains of nMOMP to the immune system when A8-35 creates a stable layer belt surrounding the hydrophobic regions of the protein [40]. Molecular dynamics simulations of *E. coli* OmpX reveal indeed a more restricted dynamics of both the hydrophobic beta-barrel and the hydrophilic loops [41]. Furthermore, MOMP/A8-35 complexes are also highly stable at high temperatures, during freeze/thaw cycles and long-term room temperature storage [42]. An additional important characteristic of APols is their ability to be labeled or functionalized without changing their physical or chemical properties [31]. Most labeled APols to date are derivatives of A8-35, which have been grafted with a variety of compounds including other adjuvants such as oligodeoxynucleotides and EP67 [36, 43].

Resiquimod (imidazoquinoline compound R-848) is a guanosine derivative and a synthetic TLR7/8 agonist, which activates dendritic and B cells [44]. Resiquimod induces Th1 cell immunity and antibody production. It has been used as a topically active immune response modifier that promotes secretion of Th1 cytokines, including IFN-γ, IFN-α, IL-12 and TNF-α in human blood cultured mononuclear cells. Co-delivery of Resiquimod with antigen enhanced protection against several infections including Leishmania and mycobacteria [45].

The expected enhancement of Th1 responses elicited by Resiquimod was not observed in this study. Several factors may account for this finding. nMOMP has a mild Th2 adjuvant effect and this may have masked the Th1 response expected following immunization with Resiquimod [46]. The effects of Resiquimod are dependent on several variables including species of animal, dose and delivery route. For example, immunizations with HIV Gag proteins, using unconjugated Resiquimod, enhanced T-cell responses in rhesus macaques but not in mice [47]. In the present study, APols grafted with Resiquimod carried the equivalent of 1.5 μg/dose/immunization. This amount is 13 times less than the 20 μg/dose/immunization used by Weeratna et al. [44] to evaluate an HBsAg vaccine in BALB/c mice. The latter induced a Th1-biased immune response, but failed to produce detectable adjuvant activity in humoral or cell-mediated response when administered either i.m. or s.c. A dose-dependent response was also assessed by Vasilakos et al. [48] in BALB/c mice, using between 0.01 and 10 mg/kg of Resiquimod. The dose of 1 mg/kg, corresponding to 20 μg/dose/immunization was found to be the most efficient in decreasing the levels of IgE, and a minimal of 2 μg/dose/immunization was required to induce a IgG2a response.

Montanide, a non-TLR adjuvant, containing metabolizable oil and a surfactant system that allows water-in-oil emulsions, has been used in subunit vaccines against *Leishmania infantum, Plasmodium spp*. and in Phase I and 2 HIV-1 clinical trials. An nMOMP vaccine, using Montanide, in combination with CpG-1826, was found to elicit protection in mice against intranasal and genital challenges and in non-human primates against ocular challenges with *Chlamydia* [23, 49]. The addition of Montanide to the APol-conjugated

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and free Resiquimod formulations, maintained Th2-biased immune responses, significantly increased ELISA titers, broadened and enhanced the recognition of B-cell MOMP linear peptides and neutralizing antibody titers in serum, and did not increased cellular immune responses. Although there was a trend to improve protection against disease burden and yields of *C. muridarum* IFUs from the lungs, no significant differences were observed when compared with the corresponding groups vaccinated only with Resiquimod.

CpG-1826, an oligonucleotide, is a TLR9 agonist that induces strong Th1-biased immune responses [50]. This effect is accomplished by the synergy between TLR9 and B-cell receptors, stimulating preferentially the antigen-specific B cells, the DC maturation and differentiation with Th1 cells activation, the enhanced cytotoxic T-lymphocytes generation even without CD4-T-cell help and the inhibition of B-cell apoptosis [50]. In the present experiments, addition of CpG-1826 had the most profound effects on the humoral and cellular immune responses and in protection. ELISA and neutralizing antibody titers increased by almost 10-fold and the IgG2a/IgG1 levels switched, indicative of strong Th1 responses. Antibodies to several MOMP peptides in both VDs and CDs broadened and intensified. This was confirmed by a sharp increase in cell-mediated immune responses as indicated by T-cell proliferation and levels of IFN-γ in supernatants from *C. muridarum* EBs-stimulated T-cells. In the group immunized with free Resiquimod, the IgG2a/IgG1 ratio was the highest but the T-cell proliferative responses and levels of IFN-γ were the lowest, suggesting a differential effect of the free Resiquimod in the humoral *versus* cell mediated immune responses. Importantly, marked improvement was observed in disease burden and yields of *C. muridarum* IFU from the lungs. Although no significant differences were observed between APol-conjugated and free Resiquimod, based on the number of *C. muridarum* IFU recovered from the lungs, the conjugated group was not significantly different from the EBs immunized group.

A potential shortcoming of MOMP as a vaccine antigen is the presence of various alleles of this protein corresponding to the different *C. trachomatis* serovars [15]. As shown here, immunization with EB elicits almost exclusively antibody responses to the VD of MOMP. The results adding adjvuants to the MOMP preparation are encouraging since we observed broadening of immune responses to conserved cryptic B-cell epitopes located in the CDs of MOMP when including Resiquimod, Montanide and CpG-1826. Similar vaccine limitations exist with other pathogens, including *Plasmodium* sp., influenza A virus and HIV-1 [47, 51, 52]. Khurana et al. [51] showed that induction of a broader range of antibodies by MF59 correlated with cross-reactive immune responses in adults and improved protective efficacy of the vaccine in ferrets challenged with a heterovariant influenza H5N1 strain. Testing adjuvant combinations with *C. trachomatis* vaccines should help broaden cross-serovar protection.

In a previous study where *C. muridarum* nMOMP was kept in solution with A8-35 conjugated to the adjuvant EP67, the addition of Montanide significantly increased the levels of neutralizing antibodies, whereas no additional increase was observed with the subsequent addition of CpG-1826 [36]. This is in contrast to what we observed in the present study. Here, addition of Montanide resulted in a non-significant increase of levels of neutralizing antibodies, whereas the inclusion of CpG-1826 produced significant increase in

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their levels in the three vaccine formulations tested. The mechanisms by which Resiquimod, but not EP67, inhibited production of neutralizing antibodies that was overcome by the addition of CpG-1826 were not investigated. However, this finding further underlines the need for testing each vaccine since variations in the formulations can have profound effects on the immune responses and protection. Compared to EP67-conjugated A8-35, the use of Resiquimod-conjugated A8-35 in combination with Montanide and CpG-1826 leads to a better protection as regard several parameters measured at 10 days p.c., like change in body weight and number of IFU recovered from the lungs, with both parameters similar to the positive control group of mice. Since APols are freely miscible one with another and both EP67-[36] and CpG-1826 [43] are available, it would be interesting to examine the level of protection afforded by various combinations of these functionalized APols, in the presence or absence of other adjuvants.

The differences in the ability of Resiquimod and CpG-1826 to elicit Th1 immune responses observed in this study confirm previous results. CpG-1826 and Resiquimod can be expected to have different effects due to a dissimilar distribution of their respective TLR receptors on the immune cells. For example, Weeratna et al. [44] found a superior effect of CpG-1826 in augmenting humoral and cellular immune response when compared to Resiquimod due to a masked effect, as TLR9 and TLR7 are expressed on the same murine cells. Also, there is a marked difference in molecular weight between CpG-1826 (6,500 g/mol) and Resiquimod (314 g/mol). The difference in size of these molecules could explain differences in bio-distribution, free Resiquimod ending predominantly in the circulation due to its small size (detected in serum at 5 min post inoculation), whereas CpG-1826 is collected in lymph nodes. In mouse models, the adjuvant effect of CpG-1826 was unsurpassed at inducing Th-1 type responses, even in the presence of adjuvants that promote a Th-2 bias [44]. In addition, CpG-1826 is an effective mucosal adjuvant [50]. The enhanced ability of CpG-1826 to produce levels of IL-12 that are maintained for longer periods as well as the differences in kinetics in cytokine production when compared to Resiquimod explained this variation in spite of their very similar mode of action [48].

In conclusion, a vaccine formulated with *C. muridarum* nMOMP, trapped either in APol A8-35 or in A8-35 conjugated with Resiquimod elicited protective immune responses in mice against a respiratory challenge. Protection was enhanced by the co-delivery of antigen and adjuvants using APols. Addition of Montanide and CpG-1826 to the vaccine formulation further enhanced protection. The most robust protection was induced by the formulation containing in nMOMP trapped with A8-35 conjugated with Resiquimod, plus Montanide and CpG-1826. Further studies are recommended to characterize the use of APols to co-deliver membrane antigens with vaccine adjuvants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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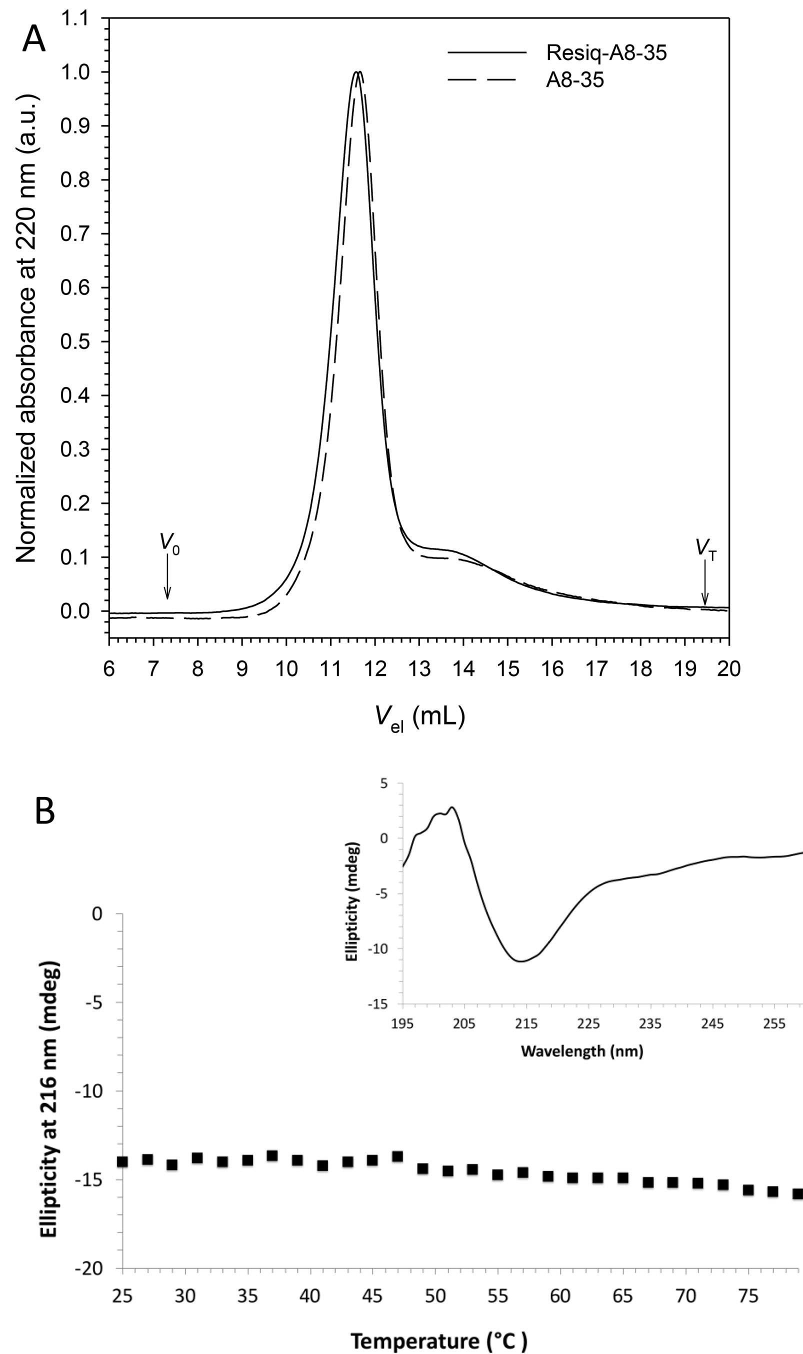

Figure 1: Resiq-A8-35 analysis.

A. Size exclusion chromatography analysis of Resiq-A8-35 particles. Elution profiles of Resiq-A8-35 and A8-35 (control) injected onto a Superose 12 column at 5 mg/mL in 20 mM Tris/HCl, 100 mM NaCl, pH 8.0. Detection was carried out at 220 nm. The profiles were normalized to the maximum absorbance of the elution peaks. V_0 stands for the void volume and V_T for the total volume of the column. B. Characterization of the stability of nMOMP/Resiq-A8-35 complexes by circular dichroism. Spectra of 0.3 mg/mL nMOMP/Resiq-A8-35 in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 were incubated at temperatures ranging

from 25 to 79 °C and ellipticity was measured at 216 nm. A full wavelength spectrum from 195 to 260 nm was measured at 25 °C (inset).

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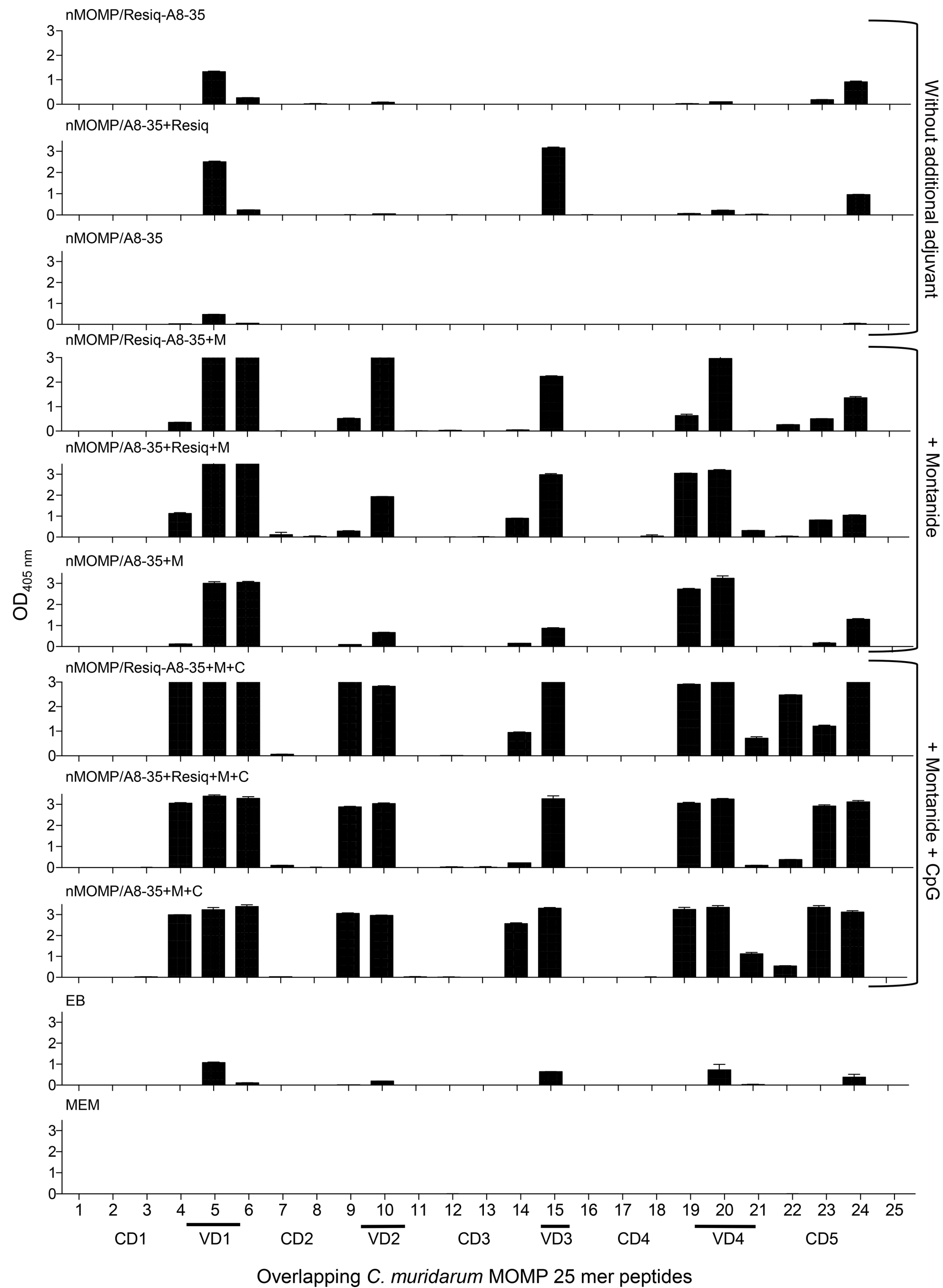

Figure 2: Binding of serum antibodies to synthetic *C. muridarum* MOMP peptides.

Serum samples from mice immunized with three different formulations (nMOMP/Resiq-A8-35, nMOMP/A8-35+Resiq, nMOMP/A8-35) without or with the presence of Montanide, or Montanide plus CpG-1826, and serum samples from control animal groups (mice immunized with *C. muridarum* EBs and MEM buffer) were collected the day before the intravaginal challenge and their reactivities to 25-mer peptides corresponding to the *C. muridarum* mature MOMP were analyzed by ELISA.

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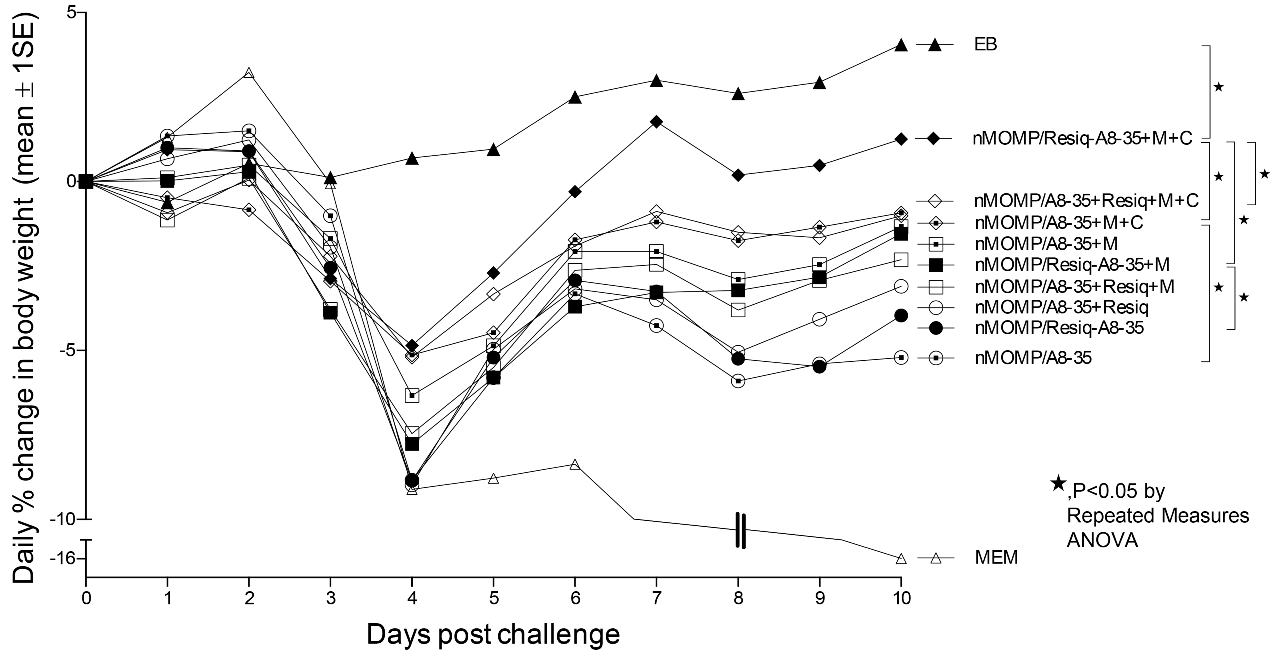

Figure 3: Daily percentage change in mean body weight following the i.n. challenge.

Vaccinated mice were challenged i.n. with 10^4 IFUs of *C. muridarum* and changes in body weight were determined daily for a period of 10 days. As positive controls mice were immunized i.n. with live *C. muridarum* elementary bodies (EBs) and the negative controls received i.n. minimal essential medium (MEM) (*, P< 0.05 by the Repeated Measures ANOVA).

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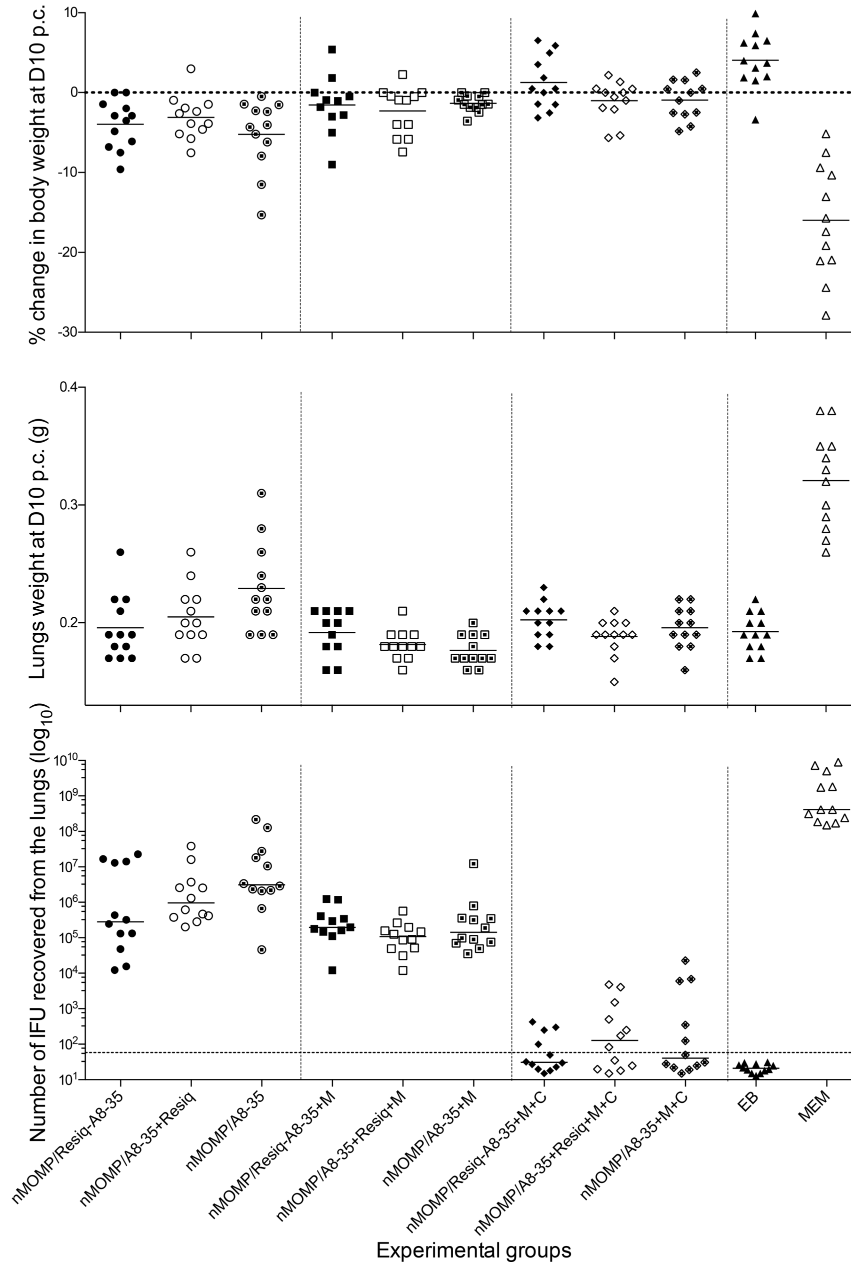

Figure 4: Systemic and local disease burden following the i.n. challenge with *C. muridarum*.
Mice vaccinated with *C. muridarum* nMOMP in A8-35, and in the presence of different adjuvant combinations, were challenged i.n. with 10^4 IFUs of *C. muridarum.* At D10 p.c., mice were euthanized and weighed, their lungs collected, weighted and homogenized, and the number of *C. muridarum* IFUs in the lungs determined using monolayers of HeLa cells.
A. 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.
B. Lung weight (g) at D10 after the i.n. challenge. The mean is shown as a horizontal line. Each symbol represents a single animal.
C. 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.

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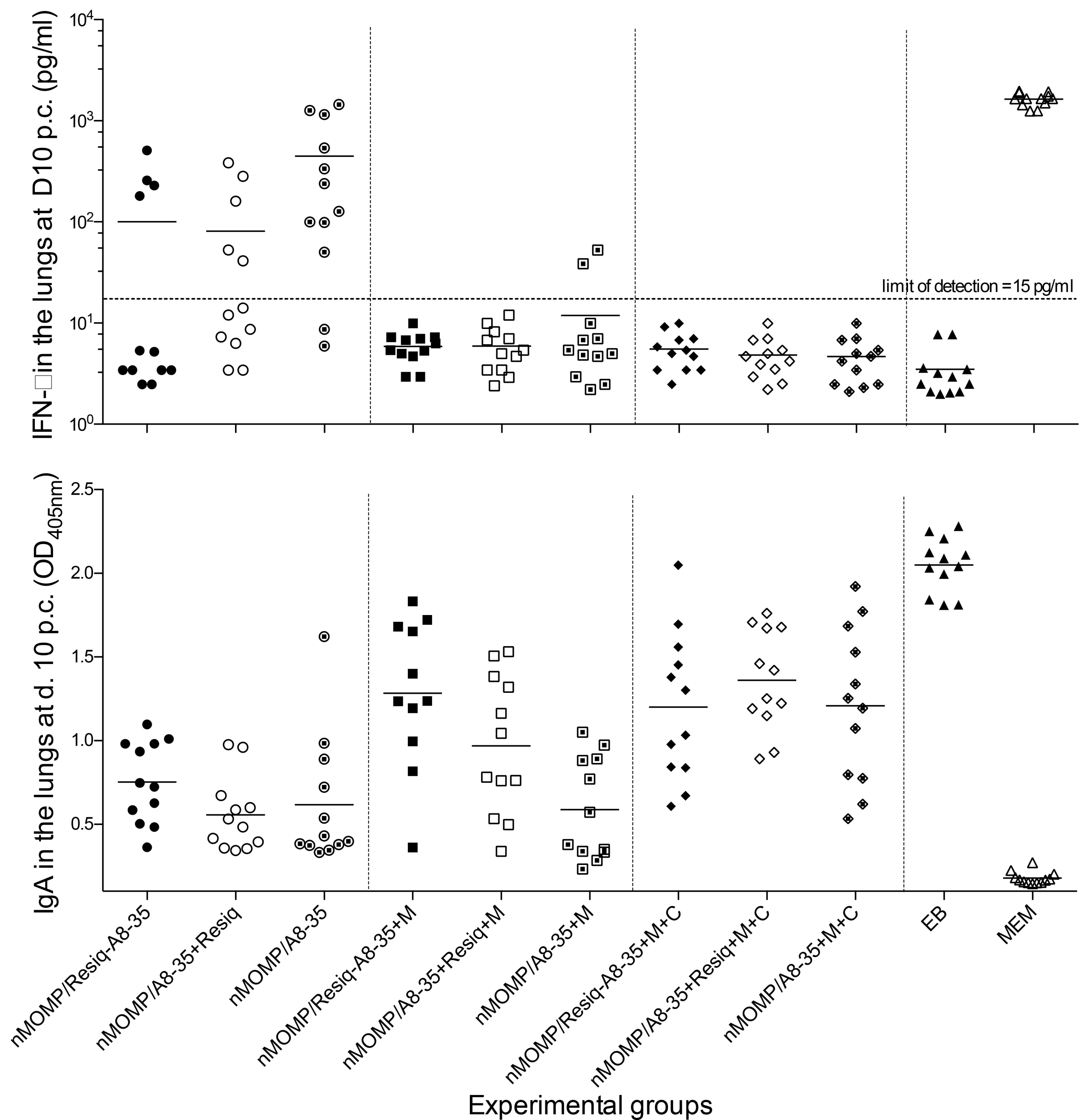

Figure 5: Levels of IFN-γ and titers of *C. muridarum*-specific IgA in the lungs at D10 p.c.

At 10 days following the *C. muridarum* i.n. challenge, vaccinated mice were euthanized, their lungs collected, homogenized, centrifuged and the supernatants were used to determine the levels of IFN-γ and *C. muridarum* specific IgA. Mice immunized with *C. muridarum* EBs and MEM were used as positive and negative controls, respectively. A. Levels of IFN-γ (pg/mL) detected in the lungs at D10 after the i.n. challenge. B. Levels of *C. muridarum*-specific IgA (OD_{405}) detected in the lungs at D10 following the i.n. challenge. On each panel the mean is shown as a horizontal line and each symbol represents a single animal.

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Table 1:

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

Vaccine	Serum						Vaginal washes	
	IgG	IgG1	IgG2a	IgG2a/IgG1	IgA	Neutralization titer	IgG	IgA
nMOMP/Resiq-A8-35	2,851[a,b,c,d] (800–6,400)	4,703[a,b,c,d] (1,600–12,800)	317[a,b,d] (100–1,600)	0.07	126[b] (<100–200)	317[b,d,g] (200–800)	17[b,d,g] (10–20)	<10
nMOMP/A8-35+Resiq	2,016[a,b,e,f] (800–3,200)	2,743[a,e] (1,600–6,400)	686[a,b,e,f] (100–1,600)	0.25	139[b,f] (<100–400)	159[b,e,f,g] (100–200)	10[b,e,f] (10–10)	<10
nMOMP/A8-35	1,270 (800–1,600)	1,089 (400–6,400)	233 (<100–1,600)	0.21	141 (<100–400)	<50 (<50–50)	<10	<10
nMOMP/Resiq-A8-35+M	16,127[a,d,g] (12,800–51,200)	21,945[a,b,g,h] (12,800–25,600)	294[a,b,d,e,h] (<100–3,200)	0.01	283[a,b] (200–400)	800[d,g] (400–1,600)	20[a,d,g] (20–40)	<10
nMOMP/A8-35+Resiq+M	22,807[a,f,g] (12,800–51,200)	10,973[a,b,f,g] (6,400–25,600)	3,200[a,g,f] (800–6,400)	0.29	224[a,b] (200–800)	800[b,g] (800–800)	30[a,f,g] (20–80)	<10
nMOMP/A8-35+M	22,807 (6,400–102,400)	5,080 (800–12,800)	3,456 (400–51,200)	0.68	356 (200–800)	635 (200–1,600)	53 (40–80)	20 (10–80)
nMOMP/Resiq-A8-35+M+C	204,800[a,b,g] (51,200–819,200)	21,945[a,b,f,g,i] (1,600–204,800)	110,598[a,b,g] (51,200–409,600)	5	317[a,b] (100–3,200)	6,400[b,g] (6,400–6,400)	184[a,b,g] (80–320)	10[b,g] (10–10)
nMOMP/A8-35+Resiq+M+C	289,631[a,b,g] (102,400–819,200)	1,176[a,h] (200–6,400)	162,550[a,b,g] (102,400–409,600)	138	504[a] (200–1,600)	3,200[g] (1,600–3,200)	557[a,b,g] (320–1,280)	20[a,b,g] (10–40)
nMOMP/A8-35+M+C	409,600 (102,400–819,200)	4,032 (400–12,800)	119,453 (51,200–409,600)	30	504 (400–800)	4,032 (3,200–6,400)	557 (320–640)	10 (<10–20)
Cm EB	14,703 (12,800–25,600)	1,234 (800–3,200)	10,973 (6,400–12,800)	9	1,393 (800–3,200)	1,600 (1,600–1,600)	40 (40–40)	92 (40–160)
MEM	<100	<100	<100	-	<100	-	<10	<10

[a]P<0.05 by One-way ANOVA compared to MEM immunized group.

[b]P<0.05 by One-way ANOVA compared to *Cm* EB immunized group.

[c]P<0.05 by One-way ANOVA compared to nMOMP/Resiq-A8-35/+M immunized group.

[d]P<0.05 by One-way ANOVA compared to nMOMP/Resiq-A8-35+M+C immunized group.

[e]P<0.05 by One-way ANOVA compared to nMOMP/A8-35+Resiq+M immunized group.

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[f] $P<0.05$ by One-way ANOVA compared to nMOMP/A8-35+Resiq+M+C immunized group.

[g] $P<0.05$ by One-way ANOVA compared to nMOMP/A8-35 immunized group.

[h] $P<0.05$ by One-way ANOVA compared to nMOMP/A8-35+M immunized group.

[i] $P<0.05$ by One-way ANOVA compared to nMOMP/A8-35+M+C immunized group.

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Table 2:

In vitro T cell proliferative response and cytokine production from the day before challenge

Vaccine	cpm* (mean ±1SE)	SI# (mean ±1SE)	cpm to ConA (mean ±1SE)	IFN-γ (pg/ml) (mean ±1SE)	IL-6 (pg/ml) (mean ±1SE)	IL-17^ (pg/ml) (mean ±1SE)
nMOMP/Resiq-A8-35	96±30[a,b,c,d]	1.38±0.12[a,b,c,d]	62,694±5,721	47±9[a,d]	60±12[a,b,c,d]	<4±0.3
nMOMP/A8-35+Resiq	694±149[a,f,g]	2.84±0.40[a,e,f]	45,357±3,198	89±42[a,e]	29±4[a,e]	<4±1
nMOMP/A8-35	835±186	3.33±0.52	51,114±5,530	36±6	29±5	6±2
nMOMP/Resiq-A8-35+M	723±133[a,d,f]	4.29±0.61[a,d,f,g]	51,642±3,941	56±14[a,d]	45±13[a,d]	15±10[d]
nMOMP/A8-35+Resiq+M	293±42[a,e]	2.15±0.16[a,e]	31,699±1,686	39±9[a,e]	35±9[a,e]	4±1[e]
nMOMP/A8-35+M	591±170	3.19±0.63	50,499±2,930	33±12	28±2	5±2
nMOMP/Resiq-A8-35+M+C	3,443±507[a,e,f]	11.73±1.58[a,b,f]	32,586±1,831	1,691±263[a,b,e,f]	305±44[b,e,f]	177±31[a,e,f]
nMOMP/A8-35+Resiq+M+C	1,831±214[a,f]	8.57±0.88[a,f]	38,245±4,709	755±201[a,b,f]	182±33[b,f]	87±19
nMOMP/A8-35+M+C	2,467±527	9.09±1.73	47,099±3,369	1,178±266	216±38	124±47
Cm EB	12,832±1,872	20.53±2.85	32,017±2,544	2,922±67	266±23	31±2
MEM	157±83	1.33±0.18	47,326±4,656	25±11	11±6	<4±1

* cpm: difference in counts per minute between EB stimulated and medium stimulated T-cells. *C. muridarum* EB were added at a ratio of 1:1 to APC + T cells.

SI: Stimulation Index: Ratio of CPM in supernatants from EB stimulated T-cells over CPM from medium stimulated T-cells.

^ IL-17 limit of detection is 4pg/ml

[a] $P<0.05$ by One-way ANOVA compared to *Cm* EB immunized group.

[b] $P<0.05$ by One-way ANOVA compared to nMOMP/A8-35 immunized group.

[c] $P<0.05$ by One-way ANOVA compared to nMOMP/A8-35+Resiq immunized group.

[d] $P<0.05$ by One-way ANOVA compared to nMOMP/Resiq-A8-35/+M+C immunized group.

[e] $P<0.05$ by One-way ANOVA compared to nMOMP/A8-35+Resiq+M+C immunized group.

[f] $P<0.05$ by One-way ANOVA compared to MEM immunized group.

[g] $P<0.05$ by One-way ANOVA compared to nMOMP/A8-35+Resiq+M immunized group.

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Table 3:

Disease burden, yields of *Chlamydia* IFU, and IFN-γ and *C. muridarum*-specific IgA in lung's homogenates at D10 p.c.

Vaccine	% Change in body weight (mean ±1SE)	Lungs weight (g) (mean ± 1SE)	Median number IFU recovered from lungs (min-max) ×10^3	IFN-γ* (pg/ml) (mean ±1SE)	IgA (A_{405}) (mean ±1SE)
nMOMP/Resiq-A8-35	−3.96±0.92[a,b,c]	0.20±0.01[a,d]	283 (12–22,570)[e,f,g]	100±47[a,d]	0.753±0.054[a,b,c,k]
nMOMP/A8-35+Resiq	−3.10±0.78[a,b]	0.21±0.01[a]	997 (204–38,295)[e,f,h,i]	81±37[a,d]	0.557±0.045[a,b,l,m]
nMOMP/A8-35	−5.21±1.29	0.23±0.01	3,117 (46–216,450)	447±154	0.617±0.079
nMOMP/Resiq-A8-35+M	−1.54±1.06[a,b]	0.19±0.01[a,d]	196 (12–1,240)[e,f,g]	<15	1.284±0.102[a,b,d]
nMOMP/A8-35+Resiq+M	−2.31±0.86[a,b]	0.18±0.00[a,d]	108 (12–570)[e,f,j]	<15	0.969±0.090[a,b,m]
nMOMP/A8-35+M	−1.33±0.30	0.18±0.00	143 (36–12,210)	<15	0.588±0.065
nMOMP/Resiq-A8-35+M+C	+1.26±0.95[a,d]	0.20±0.00[a,d]	BLD (BLD-0.30)[f,j]	<15	1.201±0.095[a,b,d]
nMOMP/A8-35+Resiq+M+C	−1.01±0.70[a,b,d]	0.19±0.01[a,d]	0.12 (BLD-4.81)[e,f,j]	<15	1.361±0.067[a,b,d]
nMOMP/A8-35+M+C	−0.93±0.69	0.20±0.01	BLD (BLD-22)	<15	1.208±0.096
Cm EB	+4.06±1.00	0.19±0.01	BLD (BLD-BLD)	<15	2.050±0.036
MEM	−15.99±2.04	0.32±0.01	410,700 (149,850–8,833,500)	1,636±70	0.179±0.008

BLD: below limit of detection (< 50 *Cm* IFU/lungs mouse).

*Limit of detection for IFN-γ is 15pg/ml.

[a] P<0.05 by One-way ANOVA compared to MEM immunized group.

[b] P<0.05 by One-way ANOVA compared to *Cm* EB immunized group.

[c] P<0.05 by One-way ANOVA compared to nMOMP/Resiq-A8-35+M+C immunized group.

[d] P<0.05 by One-way ANOVA compared to nMOMP/A8-35 immunized group.

[e] P<0.05 by Kruskal-Wallis test compared to *Cm* EB immunized group.

[f] P<0.05 by Kruskal-Wallis test compared to MEM immunized group.

[g] P<0.05 by Kruskal-Wallis test compared to nMOMP/Resiq-A8-35/+M+C immunized group.

[h] P<0.05 by Kruskal-Wallis test compared to nMOMP/A8-35+Resiq+M immunized group.

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[i] P<0.05 by Kruskal-Wallis test compared to nMOMP/A8-35+Resiq+M+C immunized group.

[j] P<0.05 by Kruskal-Wallis test compared to nMOMP/A8-35 immunized group.

[k] P<0.05 by One-way ANOVA compared to nMOMP/Resiq-A8-35+M immunized group.

[l] P<0.05 by One-way ANOVA compared to nMOMP/A8-35+Resiq+M immunized group.

[m] P<0.05 by One-way ANOVA compared to nMOMP/A8-35+Resiq+M+C immunized group.