



Immunization with PCEP microparticles containing pertussis toxoid, CpG ODN and a synthetic innate defense regulator peptide induces protective immunity against pertussis

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ABSTRACT

We investigated the efficacy of a novel microparticle (MP) based vaccine formulation consisting of pertussis toxoid (PTd), polyphosphazene (PCEP), CpG ODN 10101 and synthetic cationic innate defense regulator peptide 1002 (IDR) against *Bordetella pertussis* in mice. We studied whether encapsulation of these IDR-CpG ODN complexes into polyphosphazene-based microparticles further enhanced their immunomodulatory activity compared to soluble formulations containing PCEP (SOL), or without PCEP (AQ). *In vitro* stimulation of murine macrophages showed MP induced significantly higher levels of pro-inflammatory cytokines. When assessed in a *B. pertussis* infection challenge model, a single immunization with MP formulation led to significantly lower bacterial loads compared to other formulations and non-vaccinated animals. ELISPOT of splenocytes showed that MP group mice had significantly higher number of antigen-specific IL-17 secreting cells. The cytokine profile in lung homogenates of MP group mice after challenge showed significantly higher amounts of MCP-1, TNF- α , IFN- γ , IL-12 and IL-17 and significantly lowered IL-10 levels suggesting a strong Th1 shift. Protection was observed against challenge infection with *B. pertussis*. On the other hand protective immune responses elicited in Quadracel[®] immunized mice were Th2 skewed. Hence, we conclude that formulation of PTd, PCEP, CpG ODN and IDR into MP generates a protective immune response in mice against pertussis emphasizing the potential of MP as a delivery vehicle for the potential development of single-shot vaccines.

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1. Introduction

Effective immunization largely depends on the consideration of immunogenic vaccine antigens and effective adjuvants. Most live attenuated or killed vaccines have been replaced by subunit vaccines, which are safer but typically are less immunogenic and thus require the presence of strong adjuvants that can induce an early onset of immunity, long duration, and if needed, a shift in the type of the response. Furthermore, the use of effective adjuvant platforms can also help to reduce the number of immu-

nizations required, ideally to a single immunization only. Adjuvants include a large group of molecules that can be divided into delivery systems and immune modulators. Most often immune stimulants are derived from pathogen associated molecular patterns (PAMPs) also termed as 'danger signals' like bacterial unmethylated CpG, LPS, flagellin and viral double stranded RNA to name a few. These PAMPs are recognized by cells of the innate immune system, including antigen presenting cells, which express specific pathogen recognition receptors (PRRs) such as Toll like receptors (TLRs).

In the present study, we evaluated a novel vaccine platform containing CpG ODNs, polyphosphazenes and cationic innate defense regulator peptide (IDR) 1002. CpG ODNs have been studied extensively in regards to their immune stimulatory activities and are well characterized as vaccine adjuvant in both preclinical and clinical studies [1]. CpG ODN act through TLR9, expressed on human plasmacytoid DCs and B-cells [2], and favor induction of a

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pro-inflammatory Th1 immune response. Thus, CpG ODN has been used as adjuvants to promote a Th1 or mixed Th1/Th2 response in experimental vaccines against various diseases [3,4]. Interestingly, CpG ODNs have shown greater adjuvant activity when co-administered with other adjuvants [5,6]. In the present study, CpG ODNs were co-formulated with synthetic innate defense regulator (IDR) peptides, which have well documented selective immune stimulatory activities that include protection against infections, chemokine induction leading to the recruitment of leukocytes, wound healing, modulation of apoptosis, and anti-inflammatory activities [7,8]. IDRs are synthetic mimics of host defense peptides, which represent important components of the innate immune system and these peptides also enhance and modulate adaptive immune responses [9,10]. We previously demonstrated this adjuvantation with a pertussis vaccine [11].

Polyphosphazenes are an emerging class of well-defined macromolecules that combine immune stimulatory activity and dose-sparing effects with the ease of their assembly into supra-molecular MP structures to achieve optimal delivery [12]. Several studies have demonstrated the adjuvant activity of polyphosphazenes including formulation with IDR and CpG ODN: however, all of these studies used soluble polyphosphazenes. Here, we report the development of polyphosphazene microparticles as a means to create depots at the site of injection, facilitate uptake by antigen-presenting cells, and potentially allow delivery via the mucosal surfaces [13].

2. Materials and methods

2.1. Antigen and adjuvants

PTd was kindly provided by Novartis vaccines (Sienna, Italy). Poly [di (sodium carboxylatoethylphenoxy) phosphazene] (PCEP) of MW 10^8 g/mol was synthesized at Idaho National Laboratory, Idaho Falls, ID, USA. Phosphorothioate-stabilized single stranded CpG ODN (TCGTCGTTTTCGCGCGCGCCG) was provided by Pfizer (Ottawa, ON, CAN). IDR peptide (VQRWLIVWRIRK) was synthesized at GENSCRIPT, USA Inc. (Piscataway, NJ, USA).

2.2. Formulation and scanning electron microscopy

The CpG ODN 10101 and IDR 1002 were complexed in a ratio of 1:2 (w/w) at 37 °C for 30 min and PCEP was added along with the PTd antigen to obtain the SOL formulation resulting in a ratio of 1:2:1 (w/w/w) ratio of PCEP:IDR:CpG ODN. The AQ formulations were made as above but without PCEP. The MPs were formulated by the drop-wise addition of 0.2% of NaCl to the SOL formulation described above, incubated for 20 min at RT and this emulsion was added to 8.8% CaCl₂ and stirred for 10 min. The MP was collected by centrifugation at $1340 \times g$ for 10 min and washed with de-ionized water, and collected by centrifugation as described above. The supernatants and washes were collected, pooled and the amount of unincorporated CpG ODN was estimated by QUBIT[®] ssDNA assay kit (Invitrogen), the unincorporated IDR was estimated by HPLC, and the PTd by QUBIT[®] protein assay kit (Invitrogen). The formulations were stored at 4 °C. The encapsulation efficiency was estimated as, $E = \frac{(\text{total amount of analyte} - \text{amount of analyte in the supernatant and washes})}{(\text{total amount of analyte})} \times 100$ where analyte is either PTd, CpG-ODN or IDR 1002. The surface morphology and size of the MP was analyzed by scanning electron microscopy (SEM; JM4500, Jeol, Japan) at 1000 \times , 5000 \times and 20,000 \times magnification and the images were processed by using ImageJ freeware (www.rsweb.nih.gov/ij/).

2.3. In vitro stimulation of mouse J774 macrophages with PCEP-IDR-CpG ODN formulations

Mouse J774 cells (ATCC, VA, USA) were seeded at 2×10^6 cells in DMEM (Sigma D5546) supplemented with 10% fetal bovine serum in 24-well tissue culture plates (FALCON[™]; Beckton, Dickinson and Company) and the formulations were overlaid on the cells in triplicates and incubated at 5% CO₂ at 37 °C for 48 h. The formulations used were: (1) MP-CpG ODN-IDR (MP-complexed), (2) mixture of MP-IDR and MP-CpG ODN (MP-uncomplexed), (3) PCEP + CpG ODN + IDR (SOL-complexed), (4) CpG ODN + IDR (AQ-complexed), (5) *E. coli* lipopolysaccharide (LPS) and (6) medium alone. The above formulations contained 10 µg of PCEP, 10 µg CpG ODN and 10 µg or 20 µg of IDR per well. The supernatants were collected by centrifugation at $8500 \times g$ for 10 min to obtain cell-free supernatants and stored by freezing at -20 °C. The amount of secreted MCP-1, TNF- α , IL-6 and IL-12p40 were assayed using sandwich ELISA DUOSET[®] (Research and Diagnostics Systems, MN, USA) as per the manufacturer's instructions.

2.4. Immunization of mice

Cohorts of 6–8 week old female BALB/c mice were obtained from Charles River Laboratories (St. Constant, QC). All experiments were conducted in accordance with the ethical guidelines by the University of Saskatchewan and the Canadian Council for Animal Care. The mice ($n = 12$ per group) were given a single immunization by subcutaneous injection on the back with formulations containing 10 µg of PCEP, 20 µg of IDR 1002, 10 µg CpG ODN 10101 as SOL, MP or AQ formulations, with Quadracel[®] (Sanofi-Pasteur) diluted to 1 µg of PTd per animal and one group received only phosphate buffered saline pH 7.4 (PBS). The mice were immunized on day 1 and serum was separated from blood collected by tail vein puncture on days 14 and 28 after immunization.

2.5. B. pertussis challenge

B. pertussis Tohoma-1 strain were streaked onto charcoal agar plates supplemented with 10% sheep blood (CBA) and incubated at 37 °C for 48 h to obtain single colonies. A few single colonies were subsequently spread onto fresh CBA plates and incubated as above. After 48 h, plates were overlaid with 300 µl of 1% casamino acids, bacteria were scraped off into the casamino acid solution and 200 µl of the suspension was used to inoculate fresh CBA plates. These were incubated and harvested as described above and transferred into 2 ml of SS medium and quantified using a spectrophotometer. Bacterial concentration was adjusted to 5×10^6 /200 µl and administered intranasally. After 2 h, 2 animals from each group were humanely euthanized and their lungs were collected and homogenized in 1 ml of SS medium and 10-fold dilutions were plated on CBA agar plates to determine the number of viable bacteria. Lungs from 5 mice per group were collected at days 3 and 7 after challenge and processed as described above. The lung homogenates were stored in 0.1 mg/ml of PMSF at -20 °C and used to examine MCP-1, TNF- α , IL-12p40, and IFN- γ cytokine production and to evaluate total IgG and IgA antigen-specific antibody responses.

2.6. Analysis of PTd specific antibody titers

Antigen specific total IgG, IgG1, IgG2a and IgA immune responses were determined by end-point ELISA using methods previously described [14]. Briefly, 100 µl of pertussis toxin (PT, Sigma-Aldrich Inc., CA, USA; 0.25 µg/ml) in carbonate coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) was added to each well. Wells were washed 6 times with Tris-buffered saline pH 7.3 (TBS) containing 0.05% TWEEN[™] 20 (TBS-T). Diluted mouse serum

samples (for IgG1 and IgG2a) or lung homogenates (IgG and IgA) were added to the wells at 100 μ l/well and incubated for 1 h at room temperature. Wells were washed again with TBS-T and biotinylated goat-anti mouse IgG, IgG1, IgG2a, and IgA antibodies (Caltag Laboratories, CA, USA) were added to wells (1/5000) at 100 μ l/well and incubated for 1 h at room temperature. Wells were washed and alkaline phosphatase conjugated with streptavidin (Cedarlane Laboratories, ON, CAN) diluted 1/5000, was added in each well followed by 1 h incubation at room temperature. Wells were washed 8 times in double distilled water (ddH₂O). Di(Tris) *p*-nitrophenyl phosphate (PNPP) (Sigma–Aldrich Inc.) was diluted 1/100 in substrate buffer (1 mM of MgCl₂, 200 mM of Tris–HCl, pH 9.8) and 100 μ l/well was added. The reaction was allowed to develop for 15 min, and absorbance was read as optical density (OD) at 405 nm in a Microplate Reader (Bio-Rad Laboratories Inc., CA, USA). Results are reported as titers, which are the reciprocal of the highest dilution that gave a positive OD reading. A positive titer was defined as an OD reading that was at least two times greater than the values for a negative sample obtained from naive mice.

2.7. Isolation of splenocytes

Spleens were collected 3 and 7 days after challenge and placed in cold, minimal essential medium (GIBCO®, Carlesbad, CA, USA). The spleens were sieved through a 40 μ m nylon cell strainer (BD FALCON, San Jose, CA, USA) using scissors and a syringe plunger. 1 ml of sterile NH₄Cl lysis buffer was added to the cell suspension to lyse the erythrocytes for 1 min and lysis was stopped by immediately topping up the 15 ml tube with MEM. The splenocytes were washed once with MEM medium and resuspended in complete AIM V medium (incomplete AIM V, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 1 \times antibiotic pen strep, 1% FBS, 20 μ M L-glutamine, 50 μ M 2-mercaptoethanol) to a final concentration of 1 \times 10⁷ cells/ml. Cells were counted using a MULTISIZER™ 3 COULTER COUNTER® (Beckman Coulter, ON, Canada) according to the manufacturer's instructions. Cell concentrations were determined using software provided by the manufacturer.

2.8. Detection of PTd-specific cytokine-producing cells by ELISPOT

Nitrocellulose microtiter plates (Whatman, Florham Park, NJ, USA) were coated with 1.25 μ g/ml purified rat anti-mouse IL-4 and IFN- γ capture monoclonal antibodies (BD Biosciences, Mississauga, ON, Canada) in coating buffer for 16 h at 4 °C. Plates were washed and blocked with complete AIM V medium (GIBCO) in a 37 °C incubator. Splenocytes (1 \times 10⁶ cells/well) were added in triplicates. PTd antigen (1 μ g/well) was added and incubated at 37 °C for 18 h. Cell suspensions were removed and 1.25 μ g/ml purified biotinylated rat anti-mouse IL-4 and IFN- γ monoclonal antibodies (BD Biosciences) diluted in PBS and 0.1% Tween-20 (PBST) at 1.25 μ g/ml were added to each plate and incubated for 16 h at 4 °C. Plates were washed with PBST and a streptavidin alkaline phosphatase/glycerol solution was added to the plates at 1/500 dilution in PBST for 1.5 h at room temperature. The plates were washed 8 times with ddH₂O and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (NBT/BCIP) (Sigma) insoluble alkaline substrate solution was added to all plates for 5 min at RT. Plates were finally washed with ddH₂O and left to dry at RT. Spots were counted manually using a Stemo 2000 inverted light stereomicroscope (Zeiss, Toronto, ON, Canada).

2.9. Statistical analysis

The data were analyzed and graphed using GraphPad Prism version 5.01 for Windows®, (GraphPad Software Inc., San Diego, CA, USA, www.graphpad.com). The data were not normally

distributed and hence statistical significance was tested using the Kruskal–Wallis test. When the results were significant, differences among the individual medians were examined using the Mann–Whitney test. Significant effects were declared when $P < 0.05$.

3. Results

3.1. MP characterization and scanning electron microscopy

The incorporation efficiency of PTd in the MPs was estimated to be around 78% for PTd and 95% for CpG and HDP. Previous studies showed that particles less than 10 μ m are preferentially taken up by APC [12,15,16]. As such, SEM of MPs that comprised of PCEP with CpG ODN, and IDR-1002 was performed to ensure that the resulting size of the particles was compatible with uptake into APC to ensure that an effective dosage of antigen would be processed. Our previous studies of encapsulated CpG ODN using the same methodology not only showed that the MPs generated were less than 10 μ m, but also revealed 99% uptake into murine macrophages [12,15]. Indeed, the addition of IDR-1002 into the MP was consistent with these previous findings revealing particles ranging in size from 0.5 to 5 μ m in diameter (Fig. 1A and B). At higher magnification (20,000 \times), a close inspection of the surface of these MP revealed that it was not smooth; instead, the surface of these MP seem to be composed of smaller nanoparticle structures (Fig. 1C).

3.2. In vitro release of chemokines and cytokines from mouse macrophages treated with CpG ODN and IDR in MP and soluble formulations

To assess the efficacy of MP formulation, we compared the levels of the pro-inflammatory cytokines TNF α , IL-6 and IL-12p40 in murine J774 macrophages treated with CpG ODN-IDR (AQ), PCEP-CpG ODN-IDR (SOL) and MP co-encapsulating PCEP-CpG ODN-IDR. Other than measuring pro-inflammatory responses, we also looked for the chemokine MCP-1, a chemotactic agent for monocytes/macrophages, T cells, NK cells, and neutrophils, since it was previously shown that both CpG ODN and the IDR-HH2 alone enhanced MCP-1 production [17], while their complexes demonstrated a synergistic increase in production [11]. The induction of MCP-1 was strongest with the SOL formulation compared to the MP formulation (Fig. 2A) co-encapsulating CpG ODN-IDR complexes or CpG ODN and HDP delivered in uncomplexed MP. The release of pro-inflammatory cytokines TNF- α and IL-6 was significantly higher in MP treated macrophages than AQ or SOL formulation treated groups (Fig. 2B and D). The IL-12p40 levels were two-fold higher in the MP than SOL or AQ formulation treated groups (Fig. 2C). LPS was used as a positive control to demonstrate the viability of the cells. Based on these results, we conclude that the MP delivery induced higher levels of pro-inflammatory cytokines in mouse macrophages.

3.3. Systemic antibody induction following parenteral immunization with PTd antigen entrapped in MP or in solution

Encouraged by an enhanced pro-inflammatory *in vitro* response, the adjuvant formulation of PCEP:IDR:CpG ODN was tested in an *in vivo* mouse pertussis challenge model with the inclusion of PTd antigen in SOL (PCEP-IDR-CpG ODN-PTd), MP (PCEP-IDR-CpG ODN-PTd) and AQ (IDR-CpG ODN-PTd) formulations and compared with commercial acellular pertussis vaccine containing PTd, FHA, fimbrial antigens and with alum adjuvant (Quadrace1®). To examine the effects of MPs *in vivo*, we assessed the levels of IgG1 and IgG2a PTd-specific antibody levels in sera at 14 and 28 days post immunization. At day 14, it was observed that animals

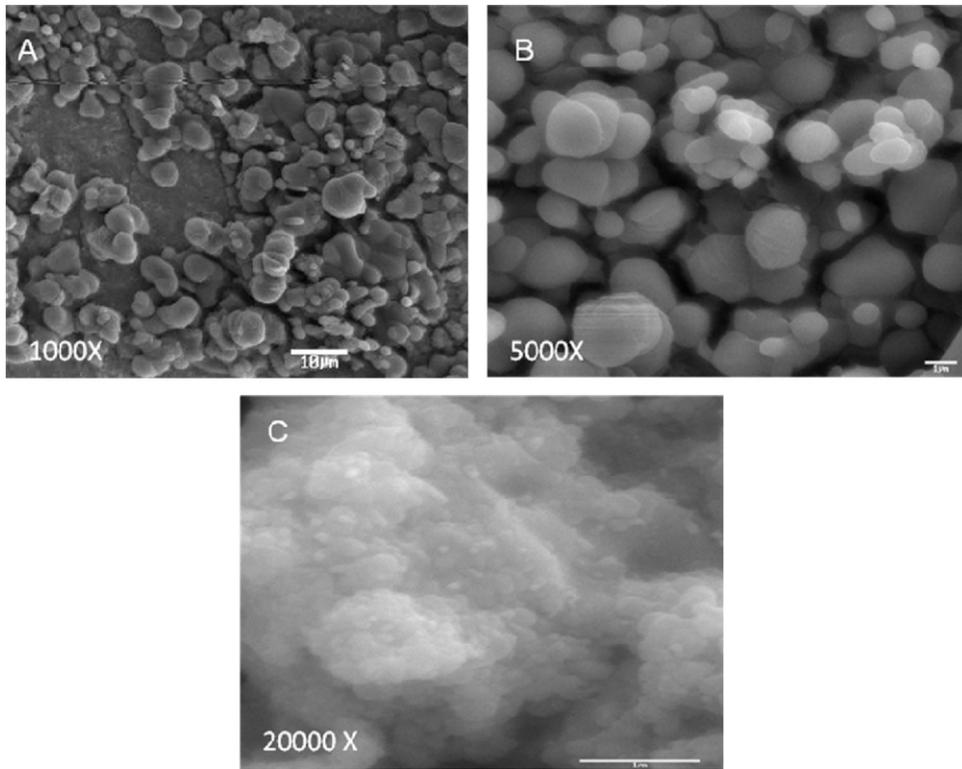


Fig. 1. Scanning electron micrographs of PCEP MPs encapsulating CpG ODN-IDR-PTd. The lyophilized MP was examined at magnification of 1000× (A), 5000× (B) and 20,000× (C).

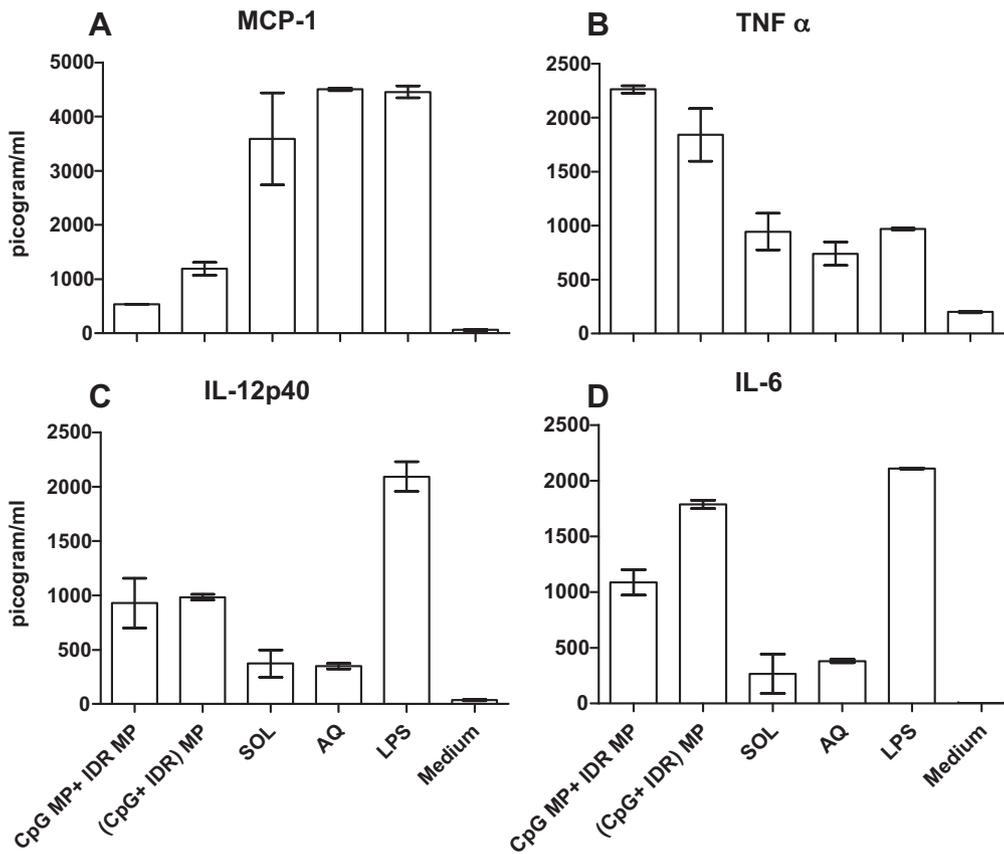


Fig. 2. *In vitro* cytokine secretion by mouse J774 macrophages (1×10^6 cells) pulsed with PCEP MP encapsulating 10 µg CpG ODN and 20 µg IDR individually, or MP co-encapsulating CpG ODN-IDR complexes, SOL (PCEP+ CpG ODN+ IDR), AQ (CpG ODN+ IDR) along with LPS (5 µg) and media alone for 48 h in duplicate. The bars represent median values with interquartile range.

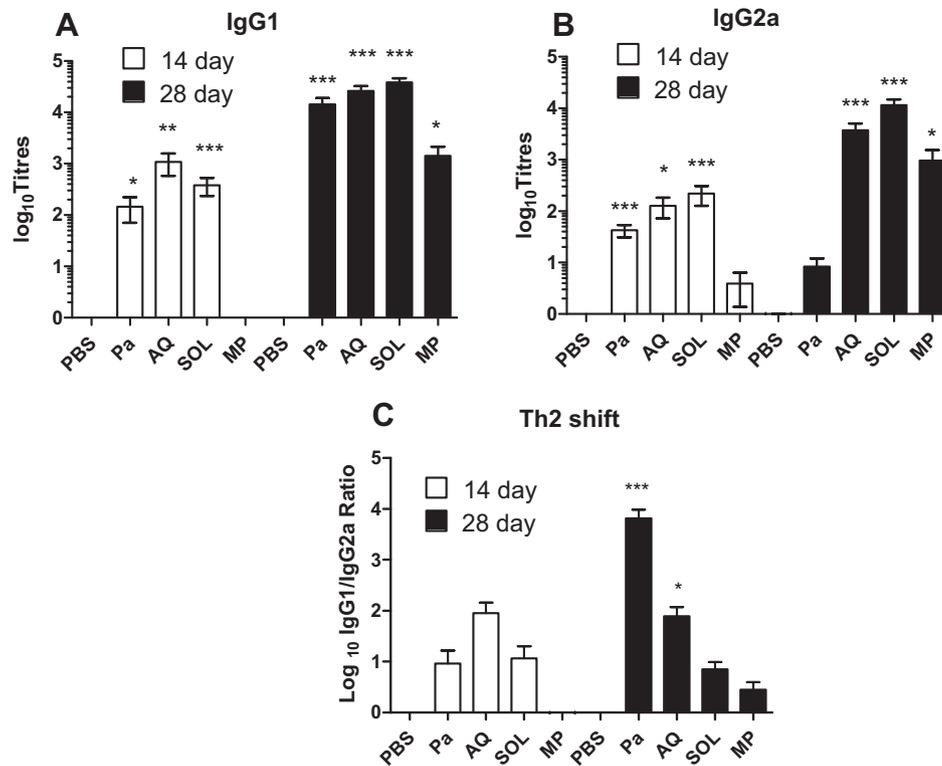


Fig. 3. PTd specific IgG1 (A) and IgG2a (B) isotype antibody levels in serum of mice ($n = 12$) immunized only once on day 0 with Pa (QuadracelTM), PBS, AQ(PTd-CpG ODN-IDR), SOL(PCEP-PTd-CpG ODN-IDR) and MP(PCEP-PTd-CpG ODN-IDR) and sera collected on days 14 and 28 and IgG1 and IgG2a isotype antibodies estimated by ELISA. (C) The median of the IgG1/IgG2a ratio of individual animals showing the degree of Th2 shift in antibody responses. The bars represent the median of antibody titers with interquartile range. Statistical significance at each time point from PBS control group was analyzed by Kruskal–Wallis test followed by Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

immunized with Quadracel[®], SOL and AQ formulations showed significantly higher amounts of IgG1 antibodies than the negative control (Fig. 3A). At day 28, the IgG1 levels were similar in groups of mice immunized with Quadracel[®] SOL and AQ formulations (Fig. 3A). The levels of IgG1 in the MP group were 10 times lower than other groups. At day 28, IgG2a levels were significantly higher in SOL group than all the other groups, and were about 10 times less in both AQ and MP groups. Expectedly, Quadracel[®] induced only weak IgG2a responses (Fig. 3B). In contrast, IgG2a responses were almost non-detectable in the Quadracel group, and about 100–1000 fold higher in mice immunized with microparticle-based or soluble adjuvant formulations. The ratio of IgG2a and IgG1 was 1.58 in mice immunized with MP and was lower than 1 in mice immunized with Quadracel[®], AQ and SOL formulations (Fig. 3C). Interestingly the ratio of IgG2a and IgG1 titers was more than 1000-fold lower in mice immunized with Quadracel[®] indicating a Th2 skew in this group.

3.4. Induction of T-cell responses by ELISPOT of splenocytes

To confirm if the antigen-specific antibody response was consistent with a cell-mediated response, the splenocytes of challenged mice were stimulated with PTd to assay the number of cells secreting IFN- γ and IL-4 by ELISPOT assay. The absolute number of IFN- γ spots in the SOL and MP formulations were significantly higher as compared to Quadracel[®] and AQ formulations (Fig. 4A). In contrast, the absolute number of IL-4 spots were higher in the Quadracel[®] group indicating that the presence of CpG and/or IDR in AQ, SOL, MP group shifted the immune response towards a Th1-type which was more clear when the ratio of IFN- γ and IL-4 spots were examined (Fig. 4C). While the ratio of 0.48 for Quadracel[®] reflected a predominantly Th2 response, the ratio was 0.8 and 1.0 for AQ and SOL groups, demonstrating that the presence of CpG ODN-IDR adjuvant

complexes in the formulations induced more of a Th1 response. Importantly, the MP group had a ratio of 1.78, indicating a strong Th1 shift. We also looked at Th17 responses as it has been documented that IL-17 mediates the clearance of pathogens from airway epithelium [18,19]. The number of IL-17 spots detected was statistically significantly higher in the MP group (Fig. 4D).

3.5. Bacterial clearance from lungs and IgA/IgG levels after intranasal *B. pertussis* challenge

Ultimately, whether an immune response is through induction of antibodies or cytokines, the best indicator for vaccine efficacy is its ability to clear the infectious agent, in this case *B. pertussis*. This was tested in an intranasal challenge with *B. pertussis*. Mice immunized with the microparticle-based adjuvant formulation displayed about 100 fold lower bacterial burden at day 7 post infections. Similar to mice immunized with Quadracel (Fig. 5). In contrast, the mice in SOL and AQ groups failed to reduce the bacterial burden in the lungs and had similar bacterial burden as in the control group. Co-encapsulation of SOL components in MP enhanced their protective efficacy. One of the most interesting observations in this study was the levels of IgG and IgA antibodies in the lungs after challenge. The levels of both PTd specific IgA and IgG in the MP group were significantly higher than all other groups (Fig. 6).

3.6. Cytokine production in lungs after challenge

The levels of MCP-1 in the lung homogenates were higher in both SOL and MP group in comparison to Quadracel[®] or AQ formulations at day 3 after challenge (Fig. 7A). After 7 days we detected twice the amount of MCP-1 in the MP group compared to the SOL group. Hence the persistence of MCP-1 was extended after challenge in the MP group. Analysis of TNF- α , IL-10, IFN- γ and IL-12p40

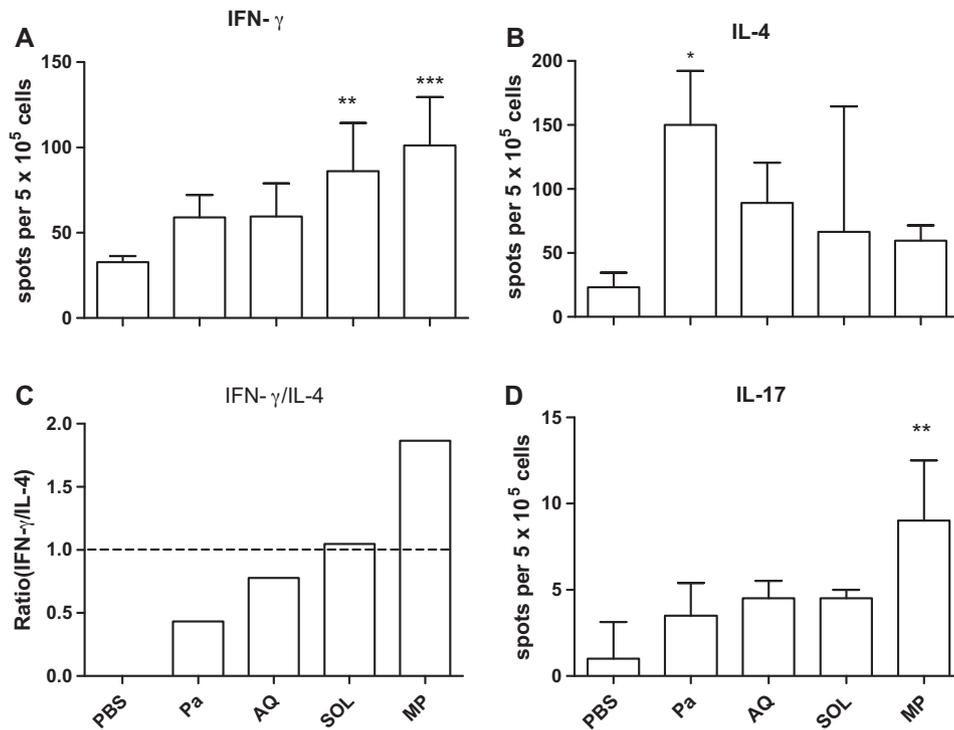


Fig. 4. PTd-specific T-cell responses of splenocytes from mice ($n = 12$). The mice were challenged intranasally with 5×10^6 bacteria four weeks after the groups of mice were immunized subcutaneously with Pa(QuadracelTM), or with Aq(CpG ODN-IDR-PTd), SOL(PCEP-CpG ODN-IDR-PTd) and MP(PCEP-CpG ODN-IDR-PTd) formulations. The bars represent the median number of spots with interquartile range. Statistical significance from PBS control group was analyzed by Kruskal–Wallis test followed by Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The ratios Th1/Th2 were calculated from median number of spots of IFN- γ /IL-4 for each group.

cytokines showed that immunization with MP induced a predominantly Th1-type response in the lungs (Fig. 7B–E). Quadracel[®] produced a predominantly Th2-type of response. The levels of IL-10 were lower in all groups other than Quadracel[®] but surprisingly the levels rebounded to that of Quadracel[®] at day 7 in SOL. Furthermore, IL-17 levels in lungs from Quadracel[®] and MP immunized mice were significantly higher than Aq or SOL groups (Fig. 7F). We conclude that immunization with MP induced higher levels of Th1 and Th17 type cytokines, while immunization with Quadracel[®] induced more Th2 type cytokines.

4. Discussion

In this study we found that a single subcutaneous immunization with MPs co-encapsulating CpG ODN, IDR and PCEP along with PTd provided better protection against pertussis than these components given in soluble formulation. The co-encapsulation of the adjuvants and the antigen in MP provided a significantly higher Th1 and Th17 type response in the lung in spite of lower systemic humoral responses.

Multi-component vaccine formulations require an effective delivery system for co-delivery of all components to the immune cells and tissues to generate a desired response. As such, in the present work we used the polyphosphazene adjuvant PCEP in com-

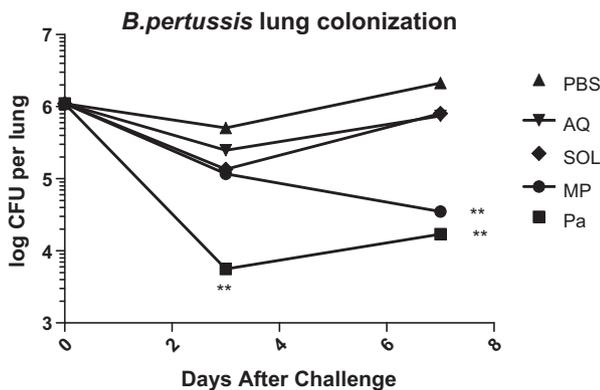


Fig. 5. Course of *B. pertussis* respiratory infection in immunized and control mice. The mice were immunized subcutaneously with Pa(QuadracelTM), or with Aq(CpG ODN-IDR-PTd), SOL(PCEP-CpG ODN-IDR-PTd) and MP(PCEP-CpG ODN-IDR-PTd) and challenged on 28 d and viable counts of bacteria in the lungs were assessed at 0, 3 and 7 d after the challenge. The results are reported as median CFU per lung estimated for individual lungs from five mice from each group. Statistical significance between the immunized and placebo groups was analyzed by Kruskal–Wallis test followed by Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

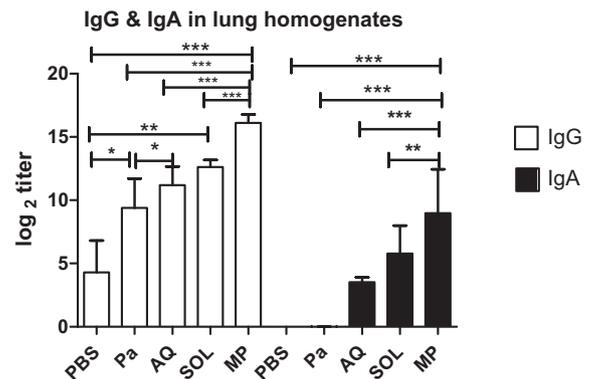


Fig. 6. PTd specific IgG and SIgA antibody levels from lung homogenates in mice ($n = 12$) immunized once on day 0 and challenged with 5×10^6 CFU of *B. pertussis* bacteria on day 28. Mice were killed on day 3 and day 7 after challenge. The bars represent median values of antibody titers with interquartile range. Statistical significance between groups at each time point with the PBS group was analyzed by Kruskal–Wallis test followed by Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

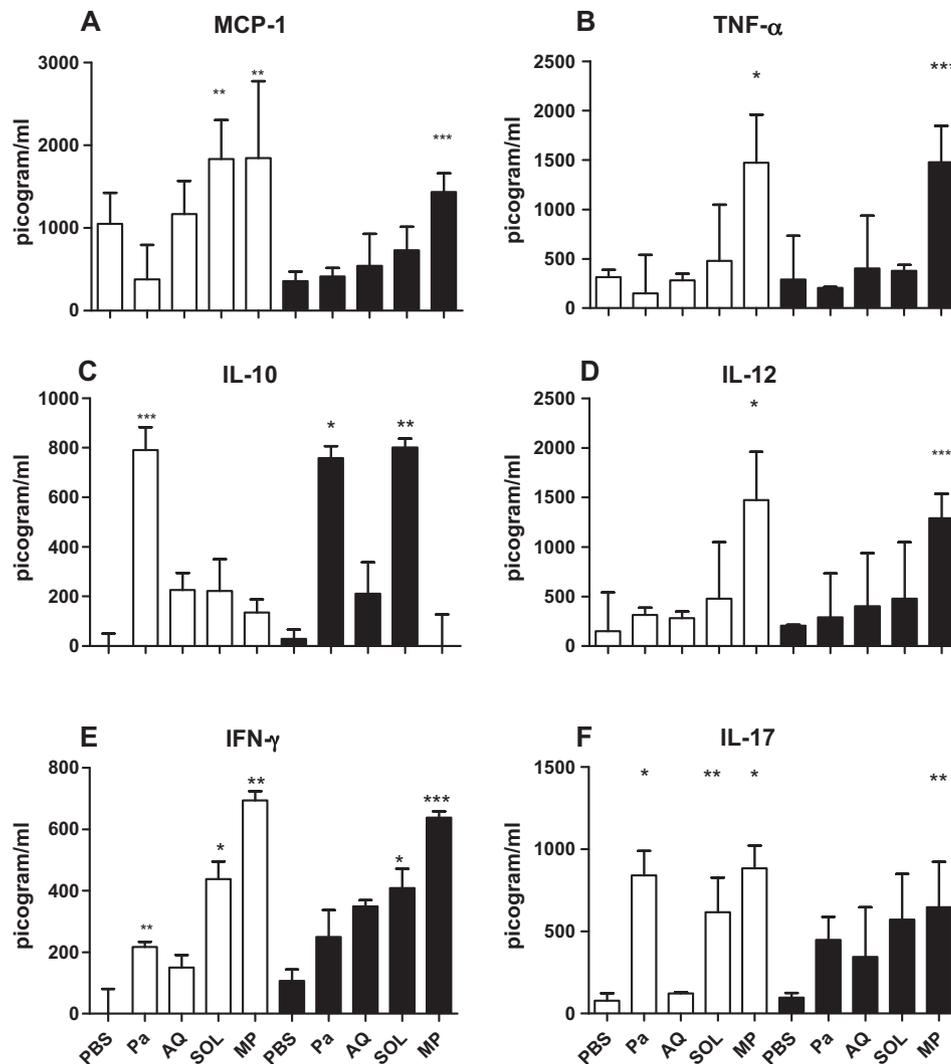


Fig. 7. Cytokines in lung homogenates analyzed by ELISA at days 3 (empty bars) and 7 (filled bars) after challenge in groups of mice immunized subcutaneously with QuadraceTM (Pa), PBS, AQ(CpG ODN-IDR-PTd), SOL(PCEP-CpG ODN-IDR-PTd) and MP(PCEP-CpG ODN-IDR-PTd) formulations. The bars represent median values of each cytokine with inter-quartile range. Statistical significance of each group with the PBS control at each time point was analyzed by Kruskal–Wallis test followed by Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

bination with complexes of CpG ODN and IDR for delivering PTd as a model antigen against pertussis. The formulation was delivered in two ways, either as a soluble ad-mixture of all the components (SOL) or co-delivered in MPs in which PCEP itself was used as an encapsulating agent without the need for additional component for encapsulation. Here, we found that the MP group had about 100 times lower bacterial burden in the lungs compared to non-immunized mice.

The advantage of using MP as a tool is that particulate delivery increases vaccine stability and uptake of the antigen to the MHC class I and class II compartments resulting in induction of both cell-mediated and humoral immune responses [20]. Historically, poly(lactic-co-glycolic acid) (PLGA), MPs and/or nanoparticles have been investigated extensively as delivery systems. PLGA MPs have been moderately successful in delivering various protein antigens, DNA, siRNA as well as cytokines. Unfortunately, their usefulness is limited by the lack of stability, complex preparation methods, high capital investment, and the use of organic solvents which may compromise the immunogenicity of antigens and may be potential carcinogens. However, polyphosphazene MPs are prepared by a simple step coacervation with NaCl and ionic cross-linking with CaCl₂ [21]. This methodology can be commercially scalable and

does not require complex manufacturing equipment, elevated temperatures, risk of aerosol generation or the use of organic solvents. The release kinetics of antigen and adjuvants from MP can be controlled to pulsatile or sustained release, a characteristic that makes single-shot vaccines a real possibility [22].

Mice vaccinated with MPs had significantly reduced bacterial burden though they had 10-fold lower antibody responses. The protection levels were similar to that of Quadrace which contains four additional antigens. These results are consistent with clinical trials demonstrating that five-, three- and most two component vaccines are more efficacious than a monocomponent chemically detoxified PTd vaccine [23]. Clearly, our formulation could be improved by the inclusion of additional pertussis antigens.

Protection against pertussis is mediated by both humoral and cell-mediated immunity and evidence suggests that cell-mediated immunity is critical for protection [24]. For example, protection is maintained among children whose antibody levels drop below the level of detection over time [25] suggesting that cell-mediated immunity is an important component of protection. Cell-mediated immune responses remain measurable substantially longer than antibodies to the same antigens, particularly PTd, and the cell-mediated immune responses to initial doses of pertussis vaccines

are believed to correlate better with long-term immunity than antibody responses [23]. Here we demonstrated a microparticle-based vaccine adjuvanted with CpG-ODN IDR and polyphosphazenes induce a strong shift towards Th1 type responses.

To address why animals immunized with MPs were more efficacious in bacterial clearance, we looked at the levels of IgG and IgA antibodies in the lung homogenates after challenge. To our surprise we found that their levels were the highest in MP groups which may have enhanced macrophage killing of antibody-opsonized bacteria. It has been reported in the literature that IgG opsonized *B. pertussis* was efficiently phagocytosed by human polymorphonuclear cells (PMN) mediated by the PMN IgG FcγRIIa and FcγRIIIb receptors [23]. Similarly, bacteria opsonized with IgA triggered similar PMN activation via FcαR. In the same study it was also shown that simultaneous opsonization of bacteria with both IgA and IgG led to enhanced bacterial clearance compared to either of the isotypes alone. It was also reported that FcαRI-mediated binding, phagocytosis, and bacterial killing by PMN originating from human FcαRI-transgenic mice as this receptor is absent in mice [26]. However, the absence of this receptor does not prevent the binding of IgA to mouse PMN [27] and suggest alternative receptors on PMN for opsonization via IgA. Hence, we postulate that immunization with MPs induced significantly higher levels of IgG and IgA in the lungs, which subsequently contributed to enhanced bacterial killing.

The IgG and IgA in the lungs were higher in MP group than SOL though the serum antibody levels were lower in MP group. This may be because of enhanced priming by the MP than by SOL formulation leading to increased levels of local antibody response in the lungs after challenge in the former. These can be further supported by higher levels of serum antibody levels observed after a booster immunization (unpublished results) than in a single shot as described in the present study. This may be due to better B-cell memory induced by MP formulation.

Earlier studies on the mechanisms that prevent replication, dissemination and eventual clearance of *B. pertussis* from the respiratory tract appear to reflect the dual extra- and intracellular location of the bacteria in the host and require the distinct but coordinated functions of the cellular and humoral arms of the immune responses for optimal protection [28]. The levels of pro-inflammatory cytokines TNF-α, IL-12p40 and the chemokine MCP-1 were significantly higher only in the lungs of mice in the MP group. This could have been likely due to the adjuvant effect of CpG ODN and IDR peptide in the formulation, respectively. We believe that the MP-complexed formulation showed higher pro-inflammatory response compared to the SOL and AQ formulations because of possible better synergy due to delivery of PTd, CpG ODN and IDR peptide in the MP formulation to the same APC. This synergy is reflected by our *in vitro* study where in mouse macrophages, PCEP MP formulation containing CpG ODN and IDR peptides produced higher pro-inflammatory response as complexed or uncomplexed using PCEP:IDR:CpG ODN ratio of 1:2:1.

The higher amount of pro-inflammatory cytokines in the lungs is known to regulate the selective induction of Th1 cells and secretion of cytokines such as IFN-γ (Th1) and IL-17 (Th17). Cytokines secreted by Th1 cells, especially IFN-γ, provide help for opsonizing antibody production and activate macrophages and neutrophils to take up and kill intracellular *B. pertussis* bacteria. The Th1 responses are characteristics of immune responses in children and mice immunized with whole cell pertussis vaccine (Pw) [29,30]. The acellular pertussis vaccines, however, are devoid of bacterial toxins that stimulate pro-inflammatory cytokines but consists of components like FHA, which stimulate IL-10 production and consequently have anti-inflammatory activity and preferentially induce Th2 cells. Th2 cells provide help to B-cells to secrete IgE and murine IgG1 antibodies, which neutralize toxins and prevent adherence of bacteria in the respiratory tract. The levels of IL-10 were significantly

higher in Quadracel® group with very low amount of IFN-γ and IL-12p40, signifying a predominantly Th2 response. Both SOL and MP generated significantly higher amounts of IL-12p40 and IFN-γ and lower amount of IL-10 showing a clear Th1 shift. Interestingly, 7 days after challenge, the IL-10 levels rebounded in the SOL group to levels comparable to that of Quadracel®. Thus, the MP formulation seems to maintain the Th1 response for a longer duration than SOL formulation.

In summary, we demonstrated that immunization with PTd encapsulated into microparticles and adjuvanted with CpG ODN and IDR induced strong Th1 responses and partial protection against challenge with *B. pertussis*. From here on, future studies will determine whether inclusion of additional antigens like Pertactin and/or FHA in our formulations may result in enhanced protection comparable to commercial acellular or cellular vaccines in a single shot model.

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