Novel vaccine formulations against pertussis offer earlier onset of immunity and provide protection in the presence of maternal antibodies

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ABSTRACT

Whooping cough is a respiratory illness most severe in infants and young children. While the introduction of whole-cell (wP) and acellular pertussis (aP) vaccines has greatly reduced the burden of the disease, pertussis remains a problem in neonates and adolescents. New vaccines are needed that can provide early life and long-lasting protection of infants. Vaccination at an early age, however, is problematic due to the interference with maternally derived antibodies (MatAbs) and the bias towards Th2-type responses following vaccination. Here we report the development of a novel vaccine formulation against pertussis that is highly protective in the presence of MatAbs. We co-formulated pertussis toxoid (PTd) and filamentous hemagglutinin (FHA) with cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN), cationic innate defense regulator (IDR) peptide and polyphosphazene (PP) into microparticle and soluble vaccine formulations and tested them in murine and porcine models in the presence and absence of passive immunity. Vaccines composed of the new adjuvant formulations induced an earlier onset of immunity, higher anti-pertussis IgG2a and IgA titers, and a balanced Th1/Th2-type responses when compared to immunization with Quadracel®, one of the commercially available vaccines for pertussis. Most importantly, the vaccines offered protection against challenge infection in the presence of passively transferred MatAbs.

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1. Introduction

Whooping cough is a respiratory disease caused by infection with *Bordetella pertussis* or *B. parapertussis*. The disease is responsible for severe respiratory symptoms in infants and young children. Complications can include seizures, pneumonia, encephalopathy and death [1]. 20–40 million cases of pertussis and about 200,000–400,000 deaths occur globally each year, most of them in developing countries [2]. Pertussis is most severe in infants and young children, who are either too young to have been vaccinated or have not received their full vaccination series [3]. Even though the incidence of disease has dramatically decreased with the introduction of pertussis vaccines many cases are still reported, even in developed nations [4]. In 2010, the United States and Canada experienced more than 20,000 cases with more than 15 deaths [5,6]. Therefore, more effective vaccines are needed to induce early-life immunity and provide long-lasting protection.

Challenges for neonatal vaccines include the so-called "immaturity" of the infant's immune system, particularly its bias towards mounting T helper 2 (Th2) responses, and the interference with MatAbs. Throughout pregnancy, both the fetus and mother have inhibited T helper 1 (Th1) responses to prevent a fetal-maternal immune rejection that could lead to miscarriage [7]. Thus, neonatal immune responses are typically skewed towards a Th2-type [8,9]. MatAbs transferred from the mother to the offspring can provide short-lived protection against disease, but also interfere with active immunization, which is the reason why in most countries around the world the first immunization against pertussis occurs at two months of age at the earliest. [10]. MatAbs have been demonstrated to interfere with vaccines in a number of ways, including neutralization of the vaccine, phagocytosis of MatAb-coated antigen and inhibition of B cell activation by Fcγ-receptor mediated signals [11]. MatAbs can also mask immunodominant vaccine epitopes thus preventing antigen binding to infant B cells [9,11]. Overall,

however, the ratio of the MatAb:vaccine antigen seems to be critical as it has been observed that antibody responses to vaccines may only be produced once MatAbs have fallen below a specific threshold [9,11]. Interference with active immunization has been observed for measles [12], hemophilus influenzae [13], hepatitis A [14], influenza A [15], tetanus [16], and varicella-zoster [17], and pertussis [8,11].

The goal of the present study was to evaluate a novel vaccine platform in the presence of MatAbs. The adjuvant platform consists of three components that effectively induce both innate and adaptive immunity in the neonate. These components are CpG ODNs [18] that by binding to Toll-like receptor-9 (TLR-9) on dendritic cells (DCs) and B cells promote the production of Th1 responses and pro-inflammatory cytokines [19], synthetic cationic IDR peptides, which are short derivatives of host defense peptides, which induce chemokine production and/or act as chemokines [20], promote wound healing, cell trafficking and modulate the responses of DCs and cells of the adaptive immune response [21] [22] and lastly, PPs, which are synthetic, biodegradable polymers composed of an inorganic backbone of alternating phosphorus (P) and nitrogen (N) atoms and two side groups attached to each P [23]. PPs are inexpensive to produce, can be lyophilized and stored for a long time at room temperature. PPs are potent immunomodulators [24] that can also be formed into microspheres [25] for vaccine delivery.

Formulating the vaccine into microparticles protects it from degradation while increasing its uptake by antigen presenting cells [26–28]. Microparticles deliver the antigen into the phagoendosome of antigen presenting cells where it is processed and loaded into MHC molecules. This can be greatly assisted by the cationic IDR peptides that act as cell penetrating peptides to translocate themselves and cargo into cells [29]. These peptides also recruiting immune cells through stimulation of local chemokine production [30]. Pathogen recognition receptors like TLR9 are present in the phagoendosome. CpG-ODNs are known intracellular TLR9 ligands. Including CpG-ODN in a microparticle vaccine allows for priming the antigen presenting cells into Th1-like responses.

We previously showed that vaccination with this novel adjuvant platform greatly enhanced immune responses to pertussis [31,32]. Here, we proposed to test whether this novel vaccine formulation would be able to overcome interference with MatAbs.

2. Materials and methods

2.1. Animals

BALB/c mice were obtained from Charles River (Montreal, Quebec, Canada). Landrace sows were obtained from the Saskatoon Prairie Swine Centre, University of Saskatchewan. The animals were housed and treated according to the ethical guidelines provided by the Canadian Council for Animal Care and as previously described [31].

2.2. Vaccine components

Genetically-detoxified pertussis toxoid was kindly provided by Novartis, formerly Chiron (Dr. R. Rappouli) and FHA antigen was purchased from List Biological Laboratories, Inc. (Campbell, California). Synthesis of PCEP was contracted out to Idaho National Laboratories, CpG ODN were purchased from Pfizer and IDR peptides were provided by Dr. Robert Hancock, UBC [32]. Quadracel® was purchased from Sanofi Pasteur (Toronto, ON). A human dose of 0.5 ml contains 15 Lf Diphtheria toxoid, 5 Lf Tetanus toxoid, acellular pertussis [20 μg chemically detoxified PT, 20 μg FHA, 3 μg pertactin (PRN), 5 μg fimbriae types 2 and 3

(FIM)], inactivated poliomyelitis vaccine (IPV) 40 D-antigen units type 1 (Mahoney), 8 D-antigen units type 2 (MEF-1) and 32 D-antigen units type 3 (Saukett) with 1.5 mg of aluminum phosphate. For comparison, the antigen dose of the experimental vaccine was calculated to contain a matching dose to the Quadracel® vaccine.

2.3. Vaccination of mice

Dams were subcutaneously (s.c.) immunized between the shoulder blades or intranasally (i.n.) with 1 μg (100 μl) of PTd in PBS (pH 7.2; 1.54 mM KH_2PO_4, 155.17 mM NaCl, 2.71 mM Na_2HPO_4-$7H_2O$; Gibco, Invitrogen; Carlsblad, CA) or the Quadracel® vaccine. Some dams were boosted two weeks following the primary vaccination. Seven or 14 day old mice were vaccinated via numerous routes including s.c (50 μl between the shoulder blades), i.m. (12.5 μl into each quadriceps muscle) or i.n. (12.5 μl into each nostril). Neonatal mice were boosted in the same manner four weeks following the primary vaccination. Vaccines containing CpG ODN and IDR peptide required pre-complexing of the two adjuvants
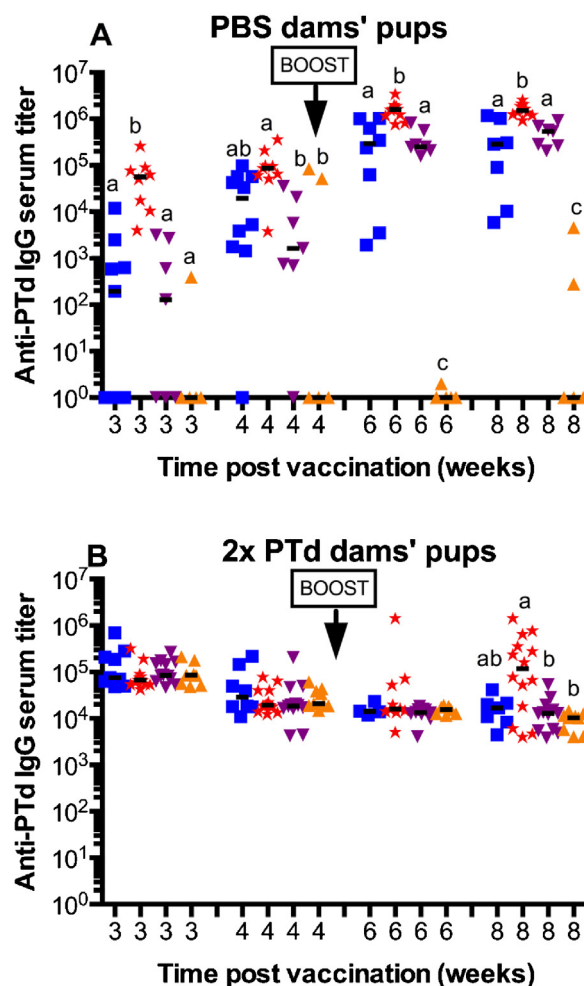
Fig. 1. Induction of anti-PTd IgG antibodies in the serum of mouse pups subcutaneously primed and boosted with Quadracel® vaccine (■), 1 μg PTd plus 2.37 μg CpG 10101, 4.74 μg IDRP 1002, 2.37 μg EP3 soluble vaccine (★), 1 μg PTd (▼) or PBS (▲). Pups were born to PBS-vaccinated dams (A) and dams vaccinated and boosted with PTd (B). Pups were vaccinated at seven days and boosted at thirty-five days of age (four weeks post primary vaccination). Serum samples were analysed using an ELISA assay. Different subscripts indicate significant differences between groups. Within each time point (a) is significantly different than (b) or (c). Letters shared in common between or among the groups indicate no significant difference. (p < 0.05). Bold lines indicate the median.

as previously described [33]. Neonatal mice were vaccinated with 1 μg of both PTd and FHA. All formulations consisted of 2.37 μg of CpG ODN 10101, 4.74 μg of IDR-1002 and 2.37 μg of PCEP (EP3). The vaccines were diluted in PBS.

2.4. Vaccination of pigs

Pregnant sows were i.m. (side of the neck, trapezius muscle) vaccinated with 2×10^{10} CFU of whole, heat-inactivated *B. pertussis* in 2 ml of PBS or PBS alone four weeks before farrowing. At three to five days of life neonatal piglets were i.m. vaccinated (1 ml) with one of the vaccine formulations. The piglets were boosted in the same manner two weeks following the primary vaccination. Neonatal piglets were vaccinated with 10 μg of PTd and FHA each. All formulations consisted of 150 μg of CpG ODN 10101, 300 μg of IDR-1002 and 150 μg of PCEP (EP3).

2.5. Microparticle vaccine preparation

Microparticle vaccines were prepared as previously described by Garlapati et al. [28,33].

2.6. 2. 6 Sample collection

Mouse dams were bled pre and post vaccination. Neonatal mice were bled 2, 4, 6 and 8 weeks (unless otherwise stated) post vaccination. Blood samples were centrifuged ($4547 \times g$) and serum stored at –20 °C. Nasal washes were collected 4 and 7 days post infection by flushing the nasal cavity with 500 μl of PBS. Sows were bled pre and post vaccination. Blood samples were centrifuged at 1349 g for 10 min. Neonatal piglets were bled pre-vaccination as well as 1, 2, 3 and 4 weeks following primary vaccination (unless otherwise stated). Serum was stored at −20 °C.
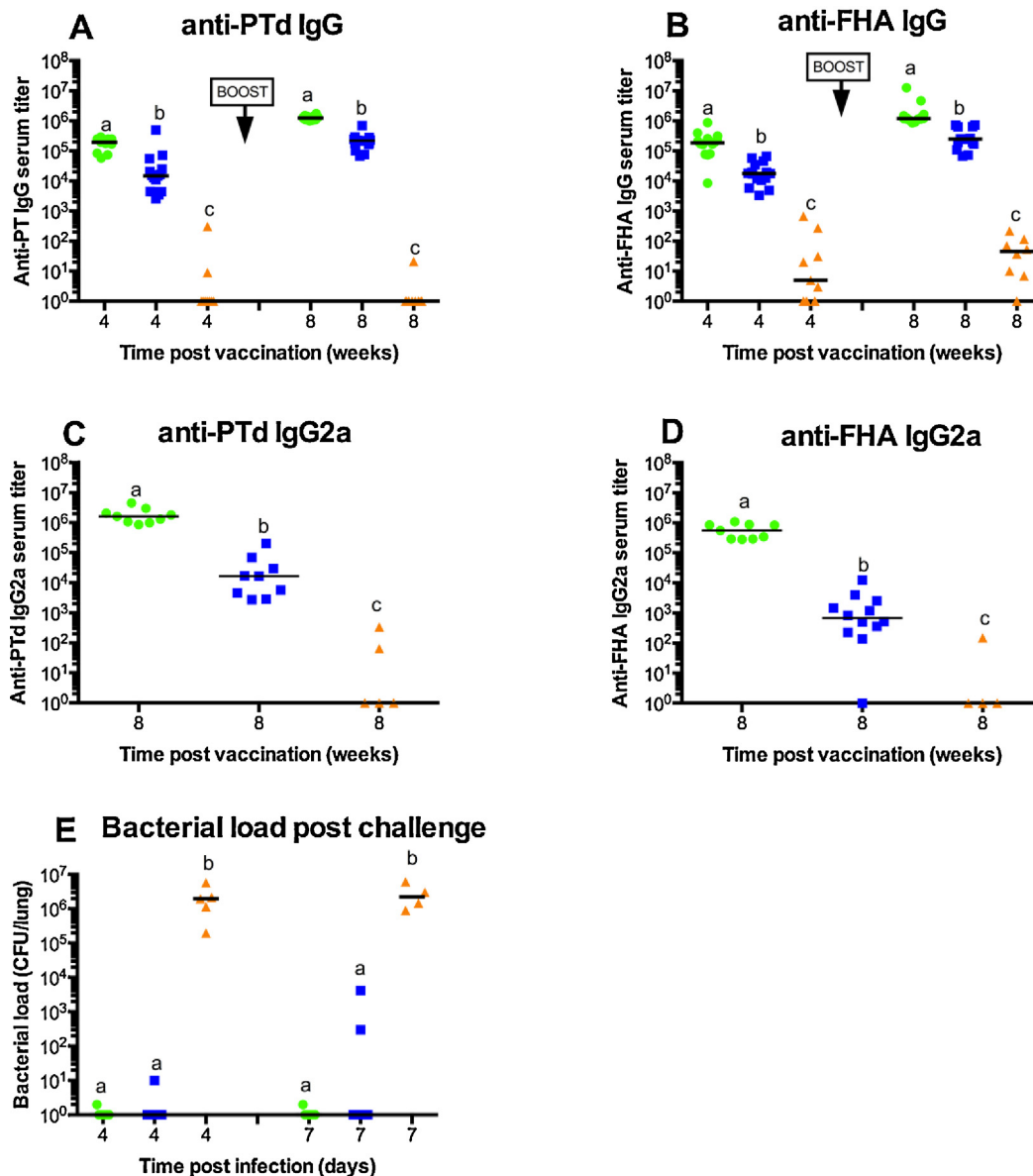
Fig. 2. Induction of anti-PTd and FHA IgG antibodies, anti-PTd and FHA IgG2a antibodies and bacterial load (CFU/lung) following challenge infection in pups intramuscularly primed and boosted with 1 μg PTd and 1 μg FHA plus 2.37 μg CpG 10101, 4.74 μg IDRP 1002, 2.37 μg EP3 microparticle vaccine (●), Quadracel® vaccine (■), or PBS (▲). Pups were born PBS vaccinated dams. Pups were vaccinated at 14 days of life and boosted four weeks later. Serum samples were analysed using an ELISA assay. Pups were challenged with 4×10^6 CFU *B. pertussis* at ten weeks of life and sacrificed four and seven days later. Different subscripts indicate significant differences between groups. Within each time point (a) is significantly different than (b) or (c). Letters shared in common between or among the groups indicate no significant difference ($p < 0.01$). Bold lines indicate the median.

2.7. B. pertussis challenge

Bacterial cultures were prepared as described [34]. 9–10 week-old mice were intranasally challenged with 4×10^6 CFU *B. pertussis*. Half of the pups were sacrificed 4 days post infection and the other half one week post infection (unless otherwise stated). The lungs were removed and placed into SS media until homogenized, diluted and plated onto charcoal blood agar (Becton Dickinson, Sparks, MD) to determine CFU in the lung. Plates were incubated at 37 °C for one week before colonies were counted.
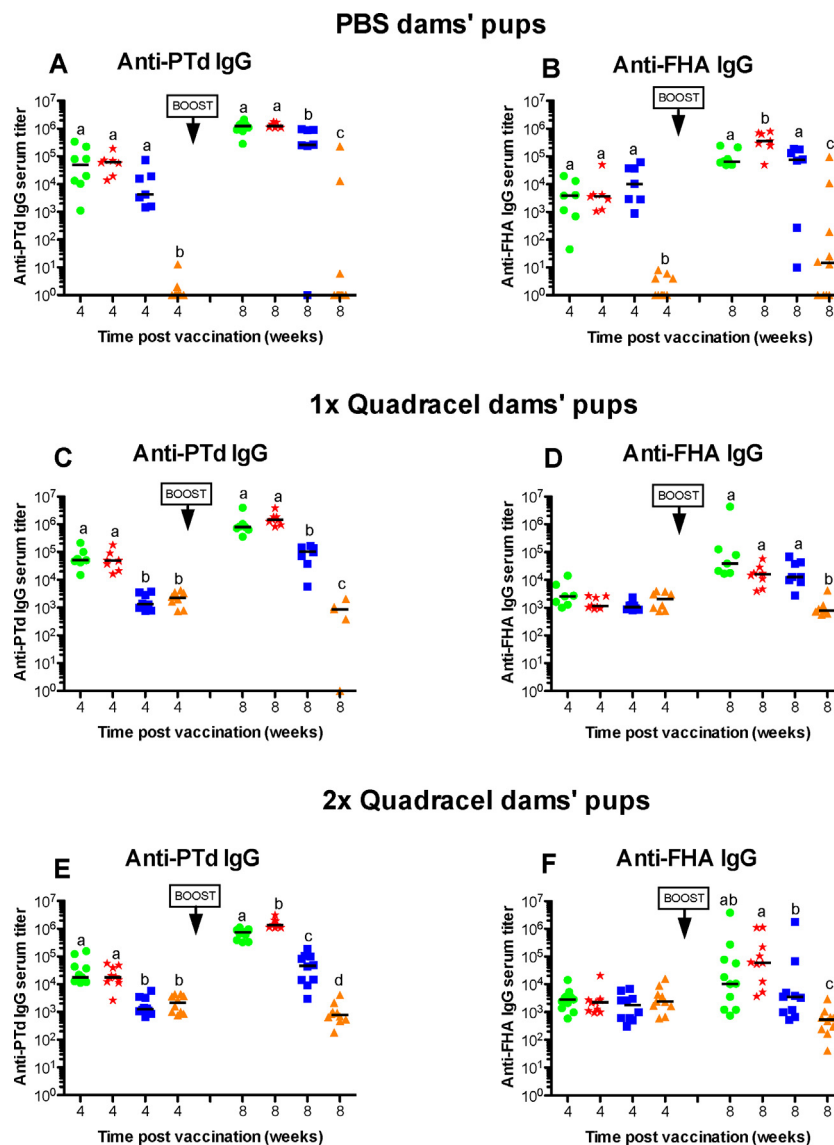
2.8. Anti-PTd and FHA ELISA

Polystyrene microtiter plates (Immulon 2 HB; Thermo, Milford, MA) were coated overnight at 4 °C with 0.25 µg/ml (100 µl per well) PTd or FHA for analysis of murine serum samples. The antigen was diluted in coating buffer (sodium carbonate buffer; 15 mM
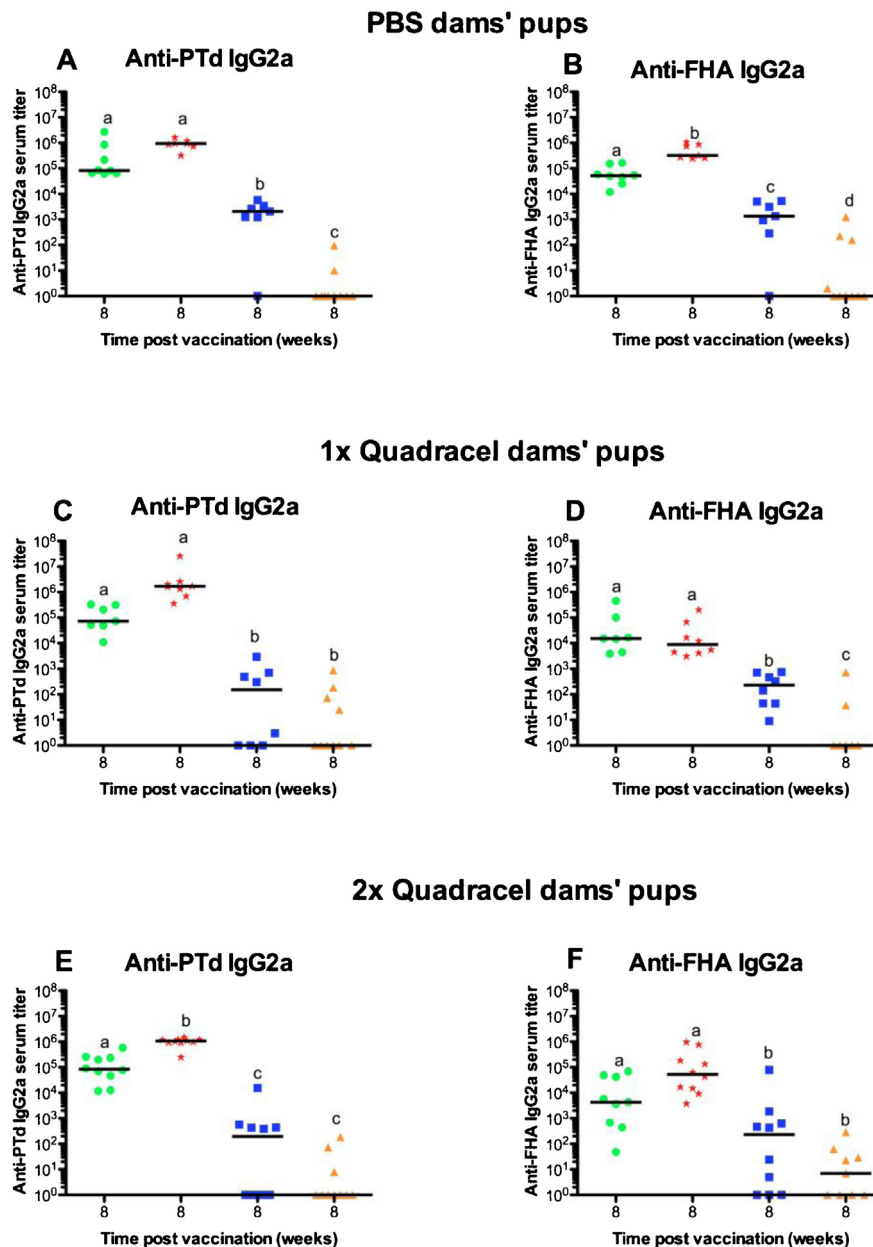
Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6). Sera were diluted four-fold, starting at 1:100 dilution for IgG and IgG2a. For sIgA, ELISA plates were coated with 0.50 µg/ml of antigen and samples added at neat concentration. Biotin-conjugated goat anti-mouse IgG, IgG2a and IgA (1:10,000 dilution; Invitrogen, Camarillo, CA) were used for the detection of PTd- and FHA-specific antibodies. Detection was carried out as previously described [31]. For the detection of porcine anti-PTd and FHA IgG, the plates were coated overnight with 0.5 µg/ml (100 µl per well) of antigen in coating buffer. ELISA was performed as previously described [31].

2.9. Statistical analysis

All statistical analyses were carried out using GraphPad Prism software, version 5.0b. Most experimental data were not normally distributed. Thus, the Mann–Whitney test was used to examine differences between two experimental groups. When experiments

Fig. 3. Induction of anti-PTd and FHA IgG antibodies in the serum of mouse pups intranasally primed and boosted with 1 µg PTd plus 1 µg FHA co-formulated with 2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3 microparticle vaccine (●), 1 µg PTd plus 1 µg FHA co-formulated with 2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3 soluble vaccine (★), Quadracel® vaccine (■) or PBS (▲). Pups were born of PBS-vaccinated dams (A, B), PTd-vaccinated dams (C, D) and PTd-vaccinated and boosted dams (E, F). Pups were vaccinated at 14 days of age and boosted four weeks later. Serum samples were analysed using an ELISA assay. Different subscripts indicate significant differences between groups. Within each time point (a) is significantly different than (b), (c) and (d). Letters shared in common between or among the groups indicate no significant difference ($p < 0.05$). Bold lines indicate the median.

PBS dams' pups

1x Quadracel dams' pups

2x Quadracel dams' pups

Fig. 4. Induction of anti-PTd and FHA IgG2a antibodies in the serum of mouse pups intranasally primed and boosted with 1 μg PTd plus 1 μg FHA co-formulated with 2.37 μg CpG 10101, 4.74 μg IDRP 1002, 2.37 μg EP3 microparticle vaccine (●), 1 μg PTd plus 1 μg FHA co-formulated with 2.37 μg CpG 10101, 4.74 μg IDRP 1002, 2.37 μg EP3 soluble vaccine (★), Quadracel® vaccine (■) or PBS (▲). Pups were born of PBS-vaccinated dams (A, B), PTd-vaccinated dams (C, D) and PTd-vaccinated and boosted dams (E, F). Pups were vaccinated at 14 days of age and boosted four weeks later. Serum samples were analysed using an ELISA assay. Different subscripts indicate significant differences between groups. Within each time point (a) is significantly different than (b), (c) and (d). Letters shared in common between or among the groups indicate no significant difference ($p < 0.05$). Bold lines indicate the median.

involved more than two groups, data were rank transformed and then analyzed by one-way analysis of variance (ANOVA). In those instances where the F ratio was significant, differences among the means of the ranks of the experimental groups were assessed using the Tukey test. Differences were considered statistically significant when $P < 0.05$

3. Results

3.1. Role of novel vaccine adjuvants

To study interference of MatAbs with neonatal vaccination ten dams were immunized a week prior to mating and boosted two weeks later. Seven control dams were treated subcutaneously with

PBS four weeks before delivery. High levels of maternal anti-PTd IgG antibodies were only present in pups born to vaccinated mothers, but not in animals born of PBS treated dams, as shown in Fig. 1. Immunization of newborn mice in the absence of MatAbs with either the Quadracel® vaccine or PTd and novel adjuvant combination (CpG, IDR peptide and PP) soluble vaccine or PTd alone vaccine resulted in the induction of PTd-specific serum antibodies (Fig. 1A). However, three weeks post primary vaccination anti-PTd IgG titers were around 500-fold higher following vaccination with the novel adjuvant combination as compared to titers produced by vaccination with the Quadracel® vaccine (Fig. 1A) indicating an earlier onset of immunity following vaccination with the experimental vaccine. In contrast, serum antibody titers in pups born of vaccinated dams indicated that responses

to both the Quadracel® and PTd alone vaccines were impaired by MatAbs. Responses in these groups were similar to those in the control group (Fig. 1B). Immunization with PTd co-formulated with CpG, IDR peptide and PP resulted in enhanced immune responses following the boost at eight weeks post primary vaccination (Fig. 1B).

3.2. Addition of a second antigen to the novel vaccine formulation increases vaccine efficacy

To improve vaccine efficacy, FHA antigen was added into the experimental formulation. To mask the antigens from vaccine-neutralizing MatAbs, the vaccine was formulated into PP microparticles. Two-week old pups born to PBS vaccinated dams were intramuscularly vaccinated with PTd and FHA co-formulated with CpG, IDR peptide as PP microparticles, Quadracel® or PBS. Four weeks later pups were boosted and challenged at ten weeks with *B. pertussis*. Immunization with the microparticle vaccine induced higher anti-PTd (Fig. 2A) and anti-FHA (Fig. 2B) IgG titers pre and post boost, when compared to vaccination with the Quadracel® vaccine. The same was found for anti-PTd IgG2a (Fig. 2C) and anti-FHA (Fig. 2D) IgG2a. Both pertussis vaccines protected the pups from a challenge with 4×10^6 CFU of *B. pertussis* as demonstrated by the significant reduction of bacteria in mouse lungs four and seven days post infection (Fig. 2E).
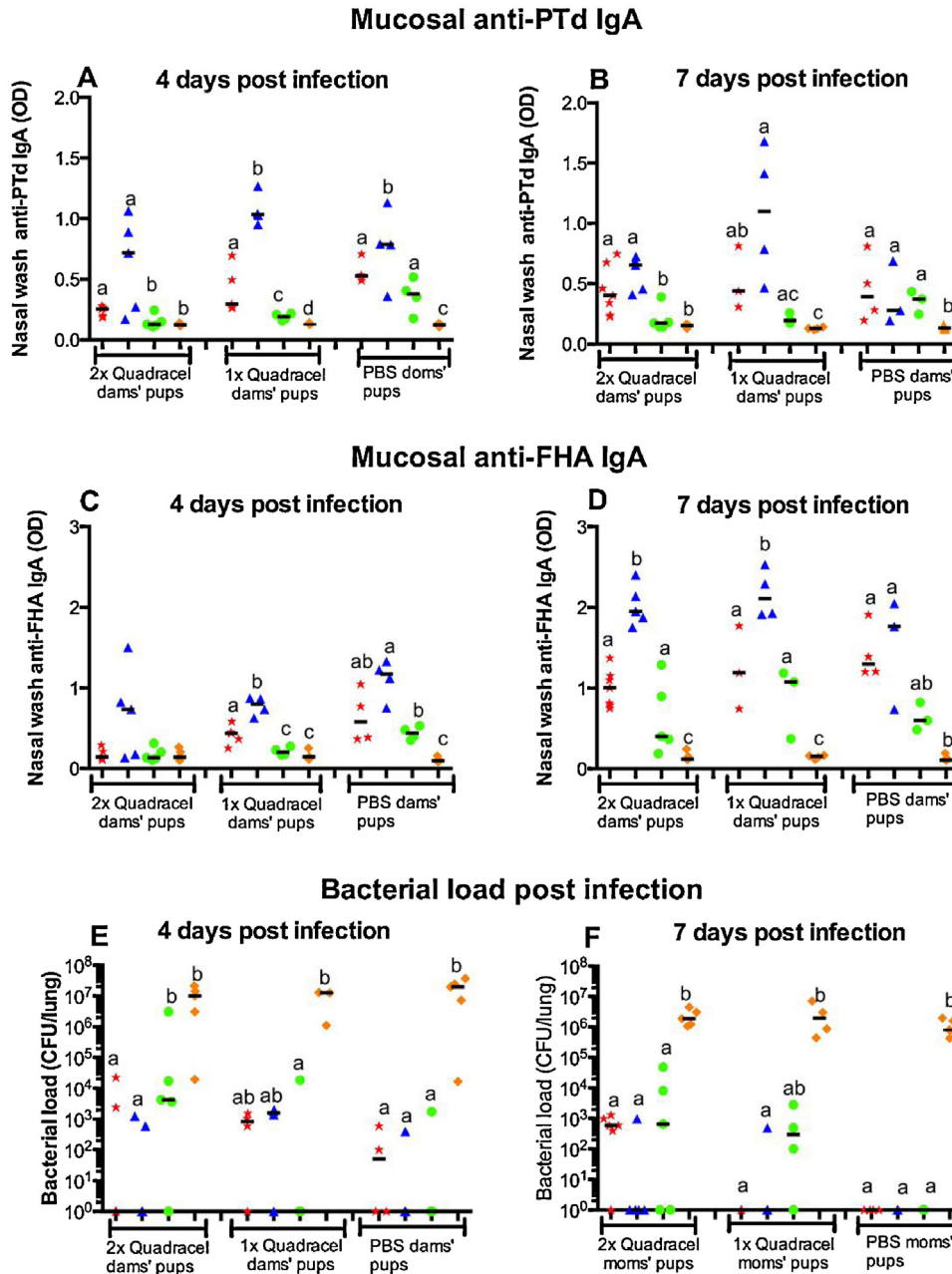
Fig. 5. Induction of anti-PTd and FHA IgA antibodies in the nasal washes of mouse pups intranasally primed and boosted with 1 μg PTd plus 1 μg FHA co-formulated with 2.37 μg CpG 10101, 4.74 μg IDRP 1002, 2.37 μg EP3 microparticle vaccine (●), 1 μg PTd plus 1 μg FHA co-formulated with 2.37 μg CpG 10101, 4.74 μg IDRP 1002, 2.37 μg EP3 soluble vaccine (★), Quadracel® vaccine (■) or PBS (▲). Pups were vaccinated at 14 days of age and boosted four weeks later. Nasal washes were collected four and seven days post infection and analysed using an ELISA assay. Pups were challenged with 4×10^6 CFU *B. pertussis* at ten weeks of life and sacrificed four and seven days later. Different subscripts indicate significant differences between groups. Within each time point (a) is significantly different than (b) and (c). Letters shared in common between or among the groups indicate no significant difference ($p < 0.05$). Bold lines indicate the median.
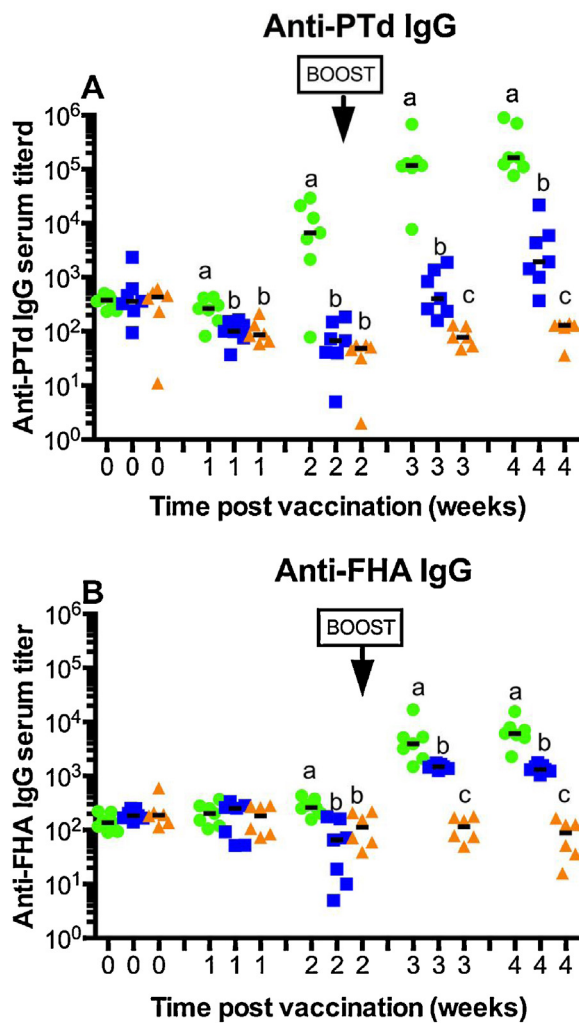
Fig. 6. Induction of anti-PTd and FHA IgG antibodies in the serum of piglets intramuscularly vaccinated and boosted with 10 µg PTd and 10 µg FHA co-formulated with150 µg CpG 10101, 300 µg IDRP 1002 and 150 µg EP3 microparticle vaccine (●), Quadracel® vaccine (■) or PBS (▲). Piglets were born to naïve sow. Piglets were vaccinated at three days of age and boosted two weeks post priming. Serum samples were analysed using an ELISA assay. Different subscripts indicate significant differences between groups. Within each time point (a) is significantly different than (b) and (c). Letters shared in common between or among the groups indicate no significant difference (p < 0.05). Bold lines indicate the median.

The bivalent vaccine formulations were also tested in the presence of passive immunity using intranasal administration. This time, dams were vaccinated with the Quadracel® vaccine. Unlike previously, these animals passed both PTd and FHA antibodies to their offspring. Pups born to these dams were intranasally vaccinated and boosted with the novel microparticle and soluble vaccine formulations composed of PTd and FHA as vaccine antigens. Immune responses of pups born of vaccinated and naïve dams were compared to responses induced by the commercial vaccine. Both the Quadracel® and the experimental vaccine formulations induced high anti-PTd IgG titers in the absence (Fig. 3A) and presence (Fig. 3C and E) of MatAbs. Additionally, all pertussis vaccines induced high concentrations of anti-FHA titres in the absence (Fig. 3B) and presence (Fig. 3D and F) of passive immunity. In comparison to the Quadracel® vaccine, the experimental vaccines, induced higher anti-PTd IgG2a and anti-FHA IgG2a titers in the absence (Fig. 4A and B) and presence of passive immunity (Fig. 4C–F)) eight weeks post primary vaccination. Amongst the three pertussis vaccines, vaccination with soluble vaccine

formulation induced highest sIgA-titers in nasal lavage against PTd (Fig. 5A and B) and FHA (Fig. 5C and D) four and seven days post infection. Both, the microparticle and soluble pertussis vaccine formulations protected the pups following infection (Fig. 5E and F).

Finally, these results were confirmed in the pig model. Piglets born of naïve sows were intramuscularly vaccinated and boosted with PTd and FHA adjuvant combination microparticle vaccine, the Quadracel® vaccine or PBS. ELISA analysis of piglet serum samples revealed that following a boost at two weeks of age the experimental microparticle-formulated vaccine induced higher anti-PTd (Fig. 6A) and anti-FHA IgG (Fig. 6B) antibodies compared to the commercial vaccine two to four weeks post primary vaccination.

4. Discussion

Whooping cough is one of the most prevalent vaccine-preventable diseases [35] and one of the top ten causes of death in children [2]. Improved vaccines are urgently needed that offer an earlier onset as well as longer duration of immunity in the very young. Here, we tested novel soluble and microparticle vaccine formulations based on combination of PTd and FHA combined with a novel adjuvant platform for their ability to induce early-life immunity even in the presence of MatAbs. The use of optimized adjuvants can result in the stimulation of innate and adaptive immunity, leading to stronger, longer lasting immune responses to neonatal vaccination. Combining adjuvants often results in stronger immune responses than using a single adjuvant [36]. It was previously shown co-formulating CpG-ODN with IDRs and PCEP can greatly enhance vaccine immunogenicity [37–39]. Using the murine model, we showed that co-formulating PTd with this adjuvant combination resulted in higher anti-PTd IgG titers and earlier immunity as compared to vaccination with PTd alone or the commercial vaccine Quadracel® composed of five pertussis antigens. PCEPs were used since they are not only strong immune modulators [40] but can be formulated into microspheres and act as a vaccine delivery systems [25].

We formulated the experimental vaccine into microparticles to protect it from degradation as well as recognition of the antigen by MatAbs. Neonatal mice intramuscularly vaccinated with PTd and FHA co-formulated with the novel adjuvant platform microparticle vaccine showed full protection following *B. pertussis* challenge. The vaccine also induced higher anti-PTd and FHA IgG antibodies as compared to the Quadracel® vaccine. Interestingly, the microparticle vaccine formulation induced higher anti-PTd and anti-FHA IgG2a titers, which are indicative of highly sought after Th1 responses during neonatal vaccination.

We also investigated the effect of mucosal vaccines in the presence of MatAbs. Mucosal vaccination has been hypothesized to circumvent interference with MatAbs as these are the sites with restricted transport of MatAbs [41]. Microparticle vaccine delivery has an additional advantage in its use in mucosal delivery. Integration of antigens and adjuvants into microparticles protects the vaccine from degradation at mucosal surfaces. The results demonstrate that intranasally vaccinated neonatal mice induce generous anti-PTd and FHA IgG serum levels even in the presence of high titers of MatAbs. Compared to the Quadracel® vaccine, soluble and microparticle vaccines induced superior anti-PTd and FHA IgG2a antibody titers. It was previously demonstrated that a Th-1 shift is crucial for the resolution of *B. pertussis* infection [42,43]. The efficacy of microparticle and soluble vaccines in the presence of MatAbs was proven by *B. pertussis* clearance following the infection. The vaccines induced similar protection to that of the commercial vaccine, despite having only two of the five pertussis antigens present in the Quadracel® vaccine.

In addition, experiments demonstrated that pups born of dams vaccinated with the Quadracel® vaccine were able to induce high anti-PTd and FHA IgG serum titers following vaccination with the novel vaccines. Vaccination resulted in induction of superior anti-PTd and FHA IgG2a serum titers as compared to the commercially available vaccine. Interestingly, the experimental soluble vaccine produced the highest concentrations of anti-PTd and FHA IgA in the nasal lavages. All three vaccines significantly reduced the bacterial load following vaccination. However, we observed a higher concentration of bacteria in vaccinated pups born of vaccinated dams. This could be explained by a higher concentration of anti-pertussis MatAbs at the time of challenge as (this time) dams were vaccinated with the commercial vaccine unlike previously a single antigen (PTd). In other studies the dams were vaccinated with a single antigen, which resulted in a total clearance of bacteria in vaccinated pups. Furthermore, the microparticle vaccine formulation was also successful in the porcine model. Formulating PTd, FHA and adjuvant combination into PP microparticles induced greater anti-PTd and FHA total IgG than vaccination with the Quadracel® vaccine.

In the present study, it was demonstrated that co-formulating the antigen with a novel adjuvant platform increased antibody production in neonates in the presence of MatAbs. Formulating the vaccine into microparticles further increased antibody production following parenteral immunization. The divalent soluble and microparticle vaccines co-formulated with CpG ODN, IDR peptide and PP protected the neonates from infection despite having only two of the five pertussis antigens present in the in Quadracel® vaccine. The novel vaccine formulations also induced high anti-PTd IgG2a antibodies, indicative of a Th-1 shift, a much desired outcome of newborn vaccines. Formulating the pertussis vaccine into novel combination of adjuvants could reduce the number of doses and/or the antigen amounts needed for protection. Fewer antigens in the vaccine would further reduce the costs associated with vaccine production.

Acknowledgements

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