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Antibody responses in adult and neonatal BALB/c mice to immunization with novel *Bordetella pertussis* vaccine formulations

Aleksandra Gracia[a], Monika Polewicz[a], Scott A. Halperin[c], Robert E.W. Hancock[d], Andrew A. Potter[a], Lorne A. Babiuk[b], Volker Gerdts[a,*]

[a] *Vaccine and Infectious Disease Organization and Department of Veterinary Microbiology, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 5E3*
[b] *University of Alberta, Edmonton, Alberta, Canada*
[c] *Canadian Center for Vaccinology and the Department of Pediatrics and Microbiology & Immunology, Dalhousie University, Halifax, Nova Scotia, Canada*
[d] *Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada*

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ABSTRACT

A balanced or Th-1 type immune response is required for effective clearance of many pathogens such as *Bordetella pertussis*, the causative agent of whooping cough. Since current acellular pertussis vaccines induce limited Th-1 type immune responses, novel vaccine formulations are needed to induce protective immunity in the infant in the earliest stages of life. Here, we developed a novel vaccine platform consisting of genetically detoxified pertussis toxoid (PTd) with multiple adjuvant components including CpG oligodeoxynucleotides, polyphosphazenes, and cationic innate defence regulator peptides. Co-formulation with these immunomodulators increased the serum IgG2a and IgG1 antibody titres in adult mice when compared to immunization with each of the selected adjuvants or immunization with PTd antigen alone. When used in combination, these adjuvants were able to induce a superior IgG2a response in both adult and neonatal mice, when compared to antigen alone or commercial vaccines. The increased response observed when using this adjuvant formulation was also initiated earlier and, moreover, was maintained over a period of greater than 22 months. The adjuvant platform also showed an ability to induce an immune response in a greater number of mice as compared to antigen alone. This suggests that this uniquely adjuvanted vaccine induces a stronger and more balanced immune response with an earlier onset of this response than vaccination with PTd antigen alone.

1. Introduction

Whooping cough is an acute, highly communicable infection caused by the Gram-negative bacterium *Bordetella pertussis*. It is a major childhood illness in the developing world and even in several industrialized nations [1–3]. Pertussis causes significant morbidity and mortality mostly in infants and young children, who are either too young to have been vaccinated (<two months old), or who have not received their full series of vaccinations [1,3,4]. Although it is reported that >80% of people in industrialized nations have been vaccinated against pertussis, there has been a resurgence of the disease within the past two decades [2–4].

B. pertussis is generally considered to be an extracellular pathogen that localizes at the epithelial cell surface and thus it was previously believed that humoral immunity and a Th2-type response would mediate protection, as is usually the case for extracellular bacteria [5]. Although antibodies can be protective against infection, as shown by passive antibody transfer, experiments in mice have shown that circulating antibodies are not necessary for maintenance of vaccine-mediated immunity [6,7]. Since *B. pertussis* is also able to invade human and mouse lung macrophages, full clearance of this pathogen requires an additional strong cell-mediated immune response (CMI), with antibody alone being insufficient [5–8]. Studies of pertussis infection in human infants and mice indicate that a strong Th-1 response, specifically mediated by IFN-γ, is required to resolve infections [7,9,10]. Polarizing the T-cell response after vaccination towards a Th-1 type response has been shown to improve the effectiveness of acellular pertussis vaccines [7]. Indeed, respiratory-challenge protection has been shown in the absence of a detectable antibody response, but is correlated with a strong Th-1 response [6].

The neonatal immune response is functionally skewed towards a Th-2 type bias [11,12], in part due to the repression of Th-1 responses in the mother and fetus during pregnancy, and compromised antigen presentation. This immune bias can pre-

* Corresponding author. Tel.: +1 306 966 1513; fax: +1 306 966 7478.
E-mail address: volker.gerdts@usask.ca (V. Gerdts).

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clude successful infant immunization when a pathogen, such as pertussis, requires the induction of a balanced or Th1-type immune response through vaccination. Recent studies in mice have indicated that appropriate adjuvantation, such as simultaneous stimulation of multiple Toll-like receptors (TLR), can augment the immune response in neonates and may compensate for a sub-optimal immune system [13]. Thus, to achieve the best possible immune response to vaccination in neonates, multiple adjuvants working synergistically through multiple mechanisms may be required. In the present study we have evaluated a combination of novel adjuvants based on three distinct types of immunomodulators, which together promote a more balanced or Th-1 type response.

Cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN) are intracellular TLR-9 ligands and potent activators of the innate and adaptive immune systems of many species (reviewed in [14]). CpG ODNs promote a Th-1 biased pro-inflammatory response with increased IL-1, IL-6, IL-18, IFN-γ and IL-12 cytokine production; these then induce the maturation of APCs (reviewed in [14,15]). CpG ODN have been used as adjuvants to promote switching from a Th-2 response to a Th-1 response in neonatal mice [15]. Since TLR signalling and activation is necessary for DC maturation and B cell activation, marked effects on antibody production would also be expected, making CpG ODN an excellent candidate adjuvant for a vaccine aimed at shifting the immune response towards the Th-1 direction [16].

Polyphosphazenes (PPs) are synthetic polymers with an inorganic backbone of alternating phosphorus (P) and nitrogen (N) atoms and two side groups attached to each P (reviewed in [17]). PPs can form microspheres to encapsulate possible vaccine antigens [18,19]. Two PPs in particular have been frequently examined as potential adjuvants, polyphosphazene polyelectrolyte, poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) and poly[di(sodium carboxylatophenoxy)phosphazene] (PCPP). PCEP is a strong adjuvant that, when compared with PCPP in a murine model, was found to be an even better enhancer of antigen-specific Th-1 and Th-2 immune responses. PCEP modified the immune response from a Th-2 bias to a mixed Th-1/Th-2 response, with both PPs being vastly more potent adjuvants than alum [20].

Cationic innate defence regulator peptides (IDR) are naturally occurring innate host defence molecules found in animals, insects, and plants. Although originally studied for their (often-weak) direct antimicrobial activities, they have a number of immunomodulatory properties. These include recruitment and selective activation of immune cells including dendritic cells (DC) through mechanisms independent of TLRs, synergy with CpG ODNs, and an ability to protect against a variety of bacteria in mouse infection models (reviewed in [21,22])[23]. IDRs are evolutionarily conserved and involved in the interface between innate and adaptive immunity [24,25]. At physiological concentrations, IDRs can induce the chemotaxis of immune effector cells, through chemokine induction or leukocyte recruitment, to the site of infection, allowing innate, then adaptive immune responses to be generated [26–29]. Evidence exists that IDRs such as cathelicidins and defensins can function as potent adjuvants and promote antigen-specific cellular and humoral immune responses in murine models [30].

The present study was undertaken to determine whether a novel combination of vaccine adjuvants can shift the immune response to *B. pertussis* in both adult and neonatal mice towards a balanced or more Th-1 type response. The results suggest that this vaccine adjuvant technology can be viewed as a platform for future neonatal vaccines directed against pathogens that require a strong cell-mediated immune response for full clearance.

2. Materials and methods

2.1. Animals

Adult male and female BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). All mice were housed and cared for in the Animal Care facilities at VIDO, University of Saskatchewan (Saskatoon, SK). 8-week old females were used for all adult mouse studies, and mice were bred in the Animal Care facilities at VIDO, University of Saskatchewan, to obtain neonatal mice. Neonates were immunized at 7 days and housed with their dams until weaning at four weeks of age. All animal experiments were performed in agreement with the guidelines proposed by the University of Saskatchewan and the Canadian Council for Animal Care.

2.2. Immunization of mice

Adult mice were immunized subcutaneously between the shoulder blades with either 50 μl or 100 μl of vaccine, or sterile phosphate-buffered saline (PBS) (Gibco, Invitrogen; Carlsblad, CA), and boosted in the same manner after four weeks unless otherwise indicated. Neonatal mice also received 50 μl of the respective formulations or sterile PBS subcutaneously between the shoulder blades and were boosted in the same manner after four weeks. Experimental groups requiring pre-complexing of the IDR and CpG ODN adjuvants had IDR and CpG ODN components co-incubated in 1.5 ml Eppendorf tubes (VWR; West Chester, PA) at 37 °C in a block heater for 30 min prior to the addition of any other vaccine components.

2.3. Vaccine components

Genetically detoxified PTd was provided by Novartis, formerly Chiron (Siena, Italy) [31]. Received batches were centrifuged at 1350 × *g* for 10 min before storing at −20 °C in aliquots. Frozen aliquots were thawed at room temperature and then centrifuged at 1350 × *g* for 10 min. The supernatents were removed and subsequently stored at 4 °C for use in the vaccine formulations. Three IDRs, IDR-HH2, IDR-HH18, and IDR-HH1002, were synthesized using a standard solid phase FMOC method [29]. De-protected IDRs were cleaved from the resin and purified by reverse phase HPLC prior to lyophilization. Lyophilized IDRs were resuspended in de-ionized water prior to dilution in PBS for use. CpG ODN were purchased from Coley Pharmaceuticals (now Pfizer). The polyphosphazenes PCEP (VIDO-EP#3) and PCPP (VIDO-PP#4) were synthesized as described previously [20] with minor modifications. The synthesis was performed by the Idaho National Laboratory (Idaho Falls, ID), under the supervision of Dr. John Klaehn. Polyphosphazenes were received in lyophilized form and stored at 4 °C in the dark, then dissolved for use in Dulbecco's PBS (1.54 mM KH_2PO_4, 155.17 mM NaCl, 2.71 mM Na_2HPO_4–$7H_2O$, Sigma–Aldrich, MO) by gentle shaking for 36 h at room temperature (RT). After resuspension, polyphosphazenes were stored in aliquots at RT in the dark.

Quadracel™ was purchased from Sanofi Pasteur (Toronto, ON). The human dosage of 0.5 ml, contains 15 Lf Diphtheria toxoid, 5 Lf Tetanus toxoid, acellular pertussis [20 μg chemically detoxified pertussis toxoid (PT), 20 μg filamentous haemagglutinin (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 aluminum phosphate as adjuvant. For immunization of mice, the volume administered was adjusted to contain a dose of chemically detoxified PT identical to that of the genetically detoxified PTd in the novel vaccine formulations studied here.

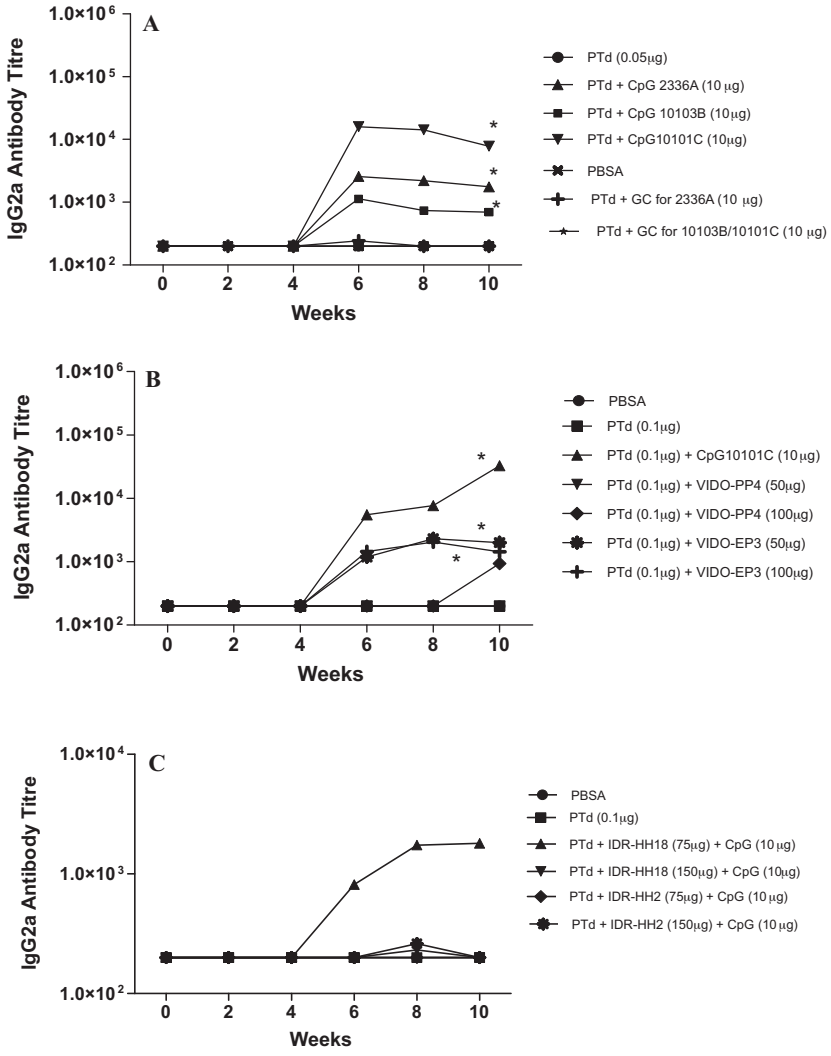

Fig. 1. PTd-specific serum IgG2a Ab responses to immunization with various adjuvants. Adult BALB/c mice ($n = 8$ or 10 mice per group) were immunized with PTd antigen alone, PBS, or antigen combined with various dosages of CpG ODN, IDR, or PP at 8 weeks of age. An identical secondary immunization was given at 4 weeks. Sera were collected immediately prior to the initial immunization and every 2 weeks thereafter for 10 weeks. (**A**) Statistically significant differences occurred between each class of CpG ODN relative to PTd ($p < 0.05$) treated mice. (**B**) Statistically significant differences between CpG ODN with respect to PTd ($p < 0.05$) treated mice occurred, and between 50 μg of VIDO-PP4 when compared to 50 μg or 100 μg of VIDO-EP#3 ($p < 0.05$). (**C**) The only statistically significant differences occurred between CpG ODN combined with 75 μg IDR-HH18 compared to PBSA ($p < 0.05$) treated mice.

2.4. ELISA analysis of PTd-specific Abs

Blood was collected through tail bleeding at two week intervals, starting at zero weeks (at the time of the initial immunization) for adults, and four weeks post-initial immunization for neonates. Serum was then collected by centrifugation of the blood in a microcentrifuge at $5940 \times g$ for 5 min, and this was used to perform ELISAs.

Polystyrene microtiter plates (Immulon 2 HB; Thermo, Milford, MA) were coated overnight at 4 °C with 0.25 μg/ml of PTd antigen in coating buffer (15.0 mM Na_2CO_3 and 34.88 mM $NaHCO_3$). The plates were then incubated for 2 h with serially diluted sera. Goat anti-mouse biotin conjugated detection antibodies specific for mouse IgG1 and IgG2a (Caltag Laboratories, Burlingame, CA; 1/10,000 dilution) were used to examine the PTd-specific antibody titres within the sera. Streptavidin Alkaline Phosphatase (Jackson ImmunoResearch; West Grove, PA; 1/5000 dilution, starting concentration 500 μg/ml) was used to amplify the reaction, and p-nitrophenyl phosphate (Sigma–Aldrich, St. Louis, MO; dilution 1 mg/ml) was used as a substrate. Samples were analyzed using a spectrophotometer (Bio-Rad iMark Microplate Reader; Philadelphia, PA) at λ405 nm with a reference of λ490 nm.

2.5. Statistics

Data was analyzed for normal distribution and significance using the STATA program version 10 for Windows (StataCorp LP, College Station, TX). One-way ANOVA was used to determine the presence of a significant difference between groups, followed by Scheffe's test to identify which of the groups were significantly different. A p-value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Selection of adjuvant candidates

Class-A (CpG 8954), Class-B (10103), and a Class-C (10101) CpG ODN were tested to determine which was best able to stimulate a Th-1 type immune response in combination with PTd. Adult mice were immunized at time zero and 4 weeks with 0.05 μg of PTd plus each of these CpG ODNs, and at 2-week intervals, the IgG1 and IgG2a

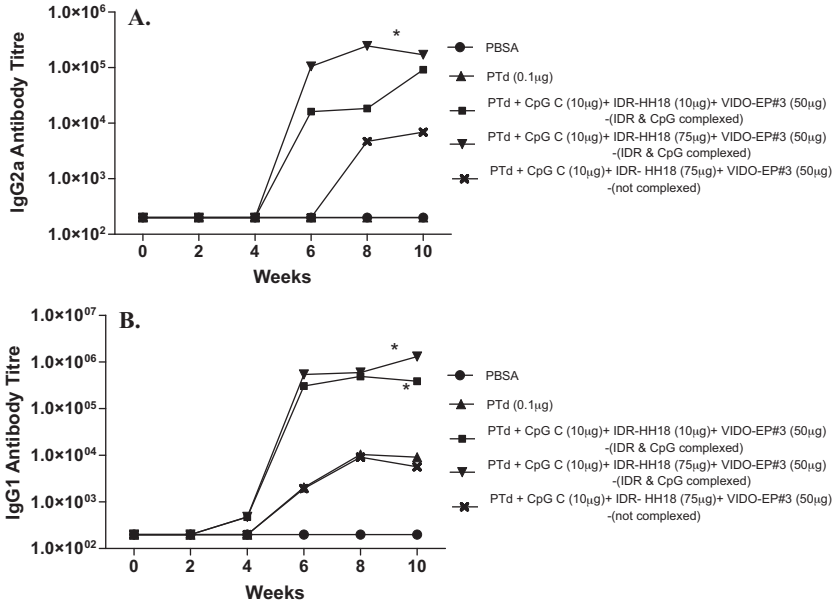

Fig. 2. PTd-specific serum Ab responses to immunization with various adjuvant platform combinations. Adult BALB/c mice ($n = 10$ mice per group) were immunized with either PTd antigen alone, PBS, or antigen combined with various formulations of CpG ODN, IDR, and PP combined at 8 weeks of age. An identical secondary immunization was given at 4 weeks. Sera were collected immediately prior to the initial immunization and every 2 weeks thereafter for 10 weeks. (**A**) Statistically significant IgG2a antibody differences occurred between the adjuvant platform combination containing 75 μg IDR-HH18 (complexed with CpG ODN) relative to PTd ($p < 0.05$) treated mice. (**B**) Statistically significant IgG1 antibody differences occurred between the adjuvant platform combination containing 75 μg IDR-HH18 (complexed with CpG ODN) relative to PTd ($p < 0.01$) treated mice and the non-complexed adjuvant formulation ($p < 0.01$).

serum titres were compared. Immunization with 10 μg of each class of CpG ODN resulted in a statistically significant increase in IgG2a titres (indicative of a Th1 response) as compared to either PTd only or 10 μg of corresponding GC 2137 or GC 2243 backbone control ODN (Fig. 1A), but between the CpG groups no significant difference was observed in the IgG1 titres (indicative of a Th2 response) (data not shown). Class-C CpG 10101 ODN was selected for inclusion in the vaccine adjuvants described here, as its mechanism of action is reported to combine the qualities of both Class-A and Class-B CpG ODN [32].

The polyphosphazenes PCEP (VIDO-EP#3) and PCPP (VIDO-PP#4), were selected for testing. Adult mice were immunized with 0.10 μg of PTd and either 50 μg or 100 μg of each polyphosphazene, with and without 10 μg of the selected CpG ODN, and the serum IgG1 and IgG2a titres were compared. Both the 50 μg/mouse and 100 μg/mouse doses of VIDO-EP#3, in combination with CpG C ODN and PTd, were found to induce higher IgG2a titres than VIDO-PP#4 and CpG C, however, these increases were not statistically significant. We observed a 500-fold increase in IgG2a titre, as compared to PTd antigen alone, after boosting with VIDO-EP#3 at four weeks. A statistically significant difference in IgG2a titres was observed in mice immunized with PTd and 50 μg VIDO-EP#3 compared with mice immunized with PTd and 50 μg VIDO-PP#4 (Fig. 1B). No statistically significant differences in the IgG2a titres occurred after immunization with either dose of VIDO-PP#4. The IgG1 titres showed no significant differences between the two doses of the two polyphosphazenes. Mice vaccinated with PTd antigen alone failed to produce statistically significant PTd-specific IgG2a titres as compared to unvaccinated mice over the entire course of the experiment. However, these mice did display a significant increase in IgG1 response. When CpG was included in the formulation, we observed a statistically significant increase in IgG2a titres; however, there were other differences noted between the two PP-adjuvanted groups. None of the mice vaccinated with 50 μg/mouse VIDO-PP#4, PTd and CpG ODN produced a significant IgG2a response. When the dose was increased to 100 μg/mouse VIDO-PP#4 (still including CpG), three mice failed to respond post-vaccination, and four mice responded after 8 weeks or longer, instead of the usual four to six weeks. In contrast, all except one mouse vaccinated with 50 μg/mouse VIDO-EP#3, PTd, and CpG ODN produced an IgG2a response, and only two mice vaccinated with 100 μg VIDO-EP#3 failed to produce detectable IgG2a. Thus VIDO-EP#3 as a vaccine adjuvant appeared to be a more consistent stimulator of the IgG2a response, and was selected for further formulations.

Two IDRs, IDR-HH2 (VQLRIRVAVIRA-NH_2) and IDR-HH18 (IWVIWRR-NH_2) were synthesized and tested. Adult mice were immunized with 0.1 μg of PTd together with 75 μg or 150 μg (3.75 mg/kg or 7.50 mg/kg, respectively) of IDR-HH2 or IDR-HH18, were boosted in the same manner after 4 weeks, and the IgG1 and IgG2a serum responses were analyzed. Vaccination with each IDR and PTd at 75 μg/mouse or 150 μg/mouse doses resulted in no detectable IgG2a response. When the IDRs were combined with a 10 μg/mouse dose of CpG C ODN and PTd, a statistically significant IgG2a response was observed in mice immunized with 75 μg/mouse of IDR-HH18 as compared to mice immunized with PTd only (Fig. 1C).

Vaccination with 150 μg/mouse of this IDR resulted in the ablation of the IgG2a response. The lower dose of IDR-HH18 combined with CpG ODN was the only IDR group tested that was statistically different from the PTd antigen alone. Thus, there appeared to be an inverse dose effect, with lower doses of IDR able to increase the IgG2a titres better than higher doses. Vaccines formulated with IDR-HH2 resulted in no significant IgG2a response, and addition of CpG C ODN did not improve the results. Upon measuring the IgG1 titres, we observed that either IDR alone resulted in a greater increase in IgG1 titres as compared to either IDR in combination with CpG C (data not shown). However, once again lower doses appeared to perform better in the IDR and CpG C combination groups. Due to the IgG2a results, as well its potential effects on cellular recruitment and cytokine levels, IDR-HH18 was selected as the IDR for continued testing.

Table 1
Individual IgG2a antibody responses and antibody kinetics post-vaccination for Fig. 2a.

Vaccine group[a]	Non-R[b]	Late-R[c]	Avg-R[d]	Mouse total
PTd (0.10 μg)	7	2	1	10
PTd (0.10 μg) + CpG C (10 μg) + IDR-HH18 (10 μg) + VIDO-EP#3 (50 μg)-complexed	3	1[e]	6	10
PTd (0.10 μg) + CpG C (10 μg) + IDR-HH18 (75 μg) + VIDO-EP#3 (50 μg)-complexed	–	–	10	10
PTd (0.10 μg) + CpG C (10 μg) + IDR-HH18 (75 μg) + VIDO-EP#3 (50 μg)-not complexed	2	3	5	10

[a] Vaccine components of group, antibody titre results shown in Fig. 2a.
[b] Non-R: number of "non-responding mice", a mouse that has non detectable antibody titre over the course of the experiment.
[c] Late-R: number of "late-responding mice", a mouse that has no detectable antibody titre until 8 weeks post-immunization or later.
[d] Avg-R: number of "average-responding mice", a mouse that has detectable antibody titre prior to 8 weeks post-immunization.
[e] 1 mouse was found dead in this group.

3.2. Testing the combined adjuvant platform in adult mice

After the selection of each of the components, a vaccine combination using the adjuvant platform was developed. It had been found *in vitro* that when CpG ODN was added to IDRs, a precipitate complex was formed that was able to enhance chemokine production *in vitro*, as well as to increase mucosal and systemic responses *in vivo* [33]. Thus, vaccine groups were included to confirm the ability of this complexation to increase immune responses *in vivo*. Vaccine formulations consisted of 0.1 μg PTd, 10 μg CpG ODN, 50 μg VIDO-EP#3, and either 10 μg or 75 μg of IDR-HH18. To create the complexed precipitate, CpG C and IDR-HH18 were precombined and heated at 37 °C for 30 min. Non-complexed groups had CpG C and IDR-HH18 kept separate until immediately prior to injection. Adult mice were immunized and boosted with variations of the adjuvant platform combination and the serum IgG2a and IgG1 titres were compared (Fig. 2A and B).

A large increase in serum IgG2a titres was observed in mice immunized and boosted with the adjuvant platform formulations as compared to PTd antigen alone (Fig. 2A). An earlier, stronger IgG2a response was observed when the adjuvants were complexed prior to vaccination and this difference was statistically significant ($p < 0.05$) between the groups containing a 75 μg dose of IDR. Even when the IDR dose was lowered to 10 μg/mouse, antibody titres rose earlier and higher in the complexed adjuvant platform group as compared to the non-complexed group.

The IgG1 serum response after vaccination showed a similar trend to the IgG2a (Fig. 2B). There was a significantly earlier and greater IgG1 response when the adjuvants were included in the vaccine, as compared to antigen alone. There was also a significant difference in IgG1 titres seen between the complexed and non-complexed adjuvant platform groups; however, there was no difference seen between the complexed groups containing the higher 75 μg/mouse dose of IDR, and the lower 10 μg/mouse dose.

When comparing the IgG2a responses, we observed several non-responders in the PTd only group (Table 1). Three mice in this PTd group that did not produce detectable IgG2a also failed to produce an IgG1 response to the antigen. In contrast, mice from the higher dose 75 μg/mouse IDR group complexed with CpG ODN all showed a measurable IgG2a response to vaccination, with no late responders. All of these mice also had an IgG1 response. In the non-complexed adjuvant platform group containing 75 μg/mouse of IDR, there were two IgG2a non-responder mice, and three late-responders. The adjuvant platform group containing 10 μg of IDR complexed with CpG ODN contained three IgG2a non-responders. These three IgG2a non-responders were also IgG1 late-responders.

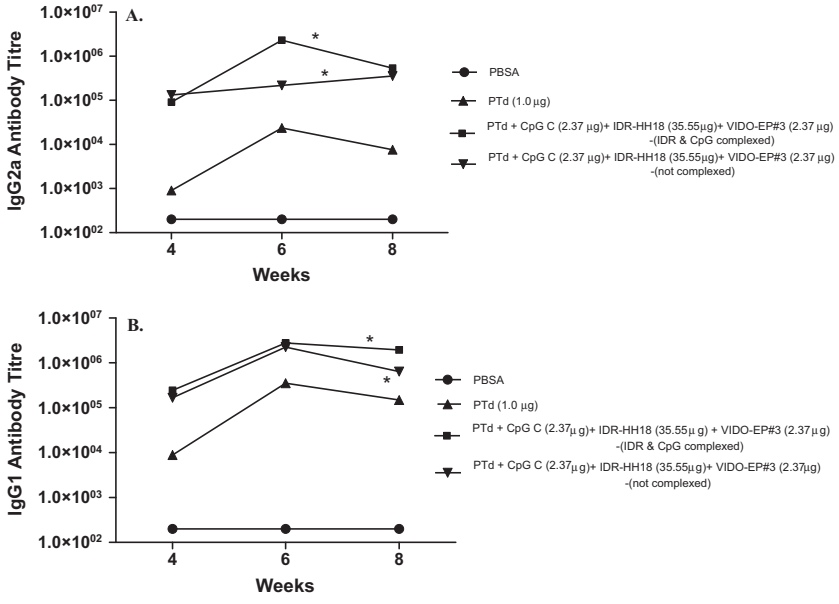

Fig. 3. PTd-specific neonatal serum Ab responses to immunization with adjuvant platform combinations. Neonatal BALB/c mice were immunized with either PTd antigen alone, antigen combined with various formulations of CpG ODN, IDR, and PP combined ($n = 8$ mice per group), or PBS ($n = 5$ mice per group) at 7 days of age. An identical secondary immunization was given at 4 weeks. Sera were collected at 4 weeks after the initial immunization prior to the boost, and every 2 weeks thereafter until 8 weeks post-initial immunization. (**A**) At week 6–8, statistically significant differences in IgG2a antibody titres occurred between the complexed adjuvant platform combination and PTd ($p < 0.001$) treated mice. There was also a statistically significant difference in IgG2a antibody titres ($p < 0.05$) seen between the complexed and non-complexed adjuvant platform combinations. (**B**) At week 6–8, statistically significant differences occurred between the complexed or non-complexed adjuvant platform combination relative to PTd treated mice ($p < 0.05$). There was no statistically significant difference seen between the complexed and non-complexed adjuvant combinations.

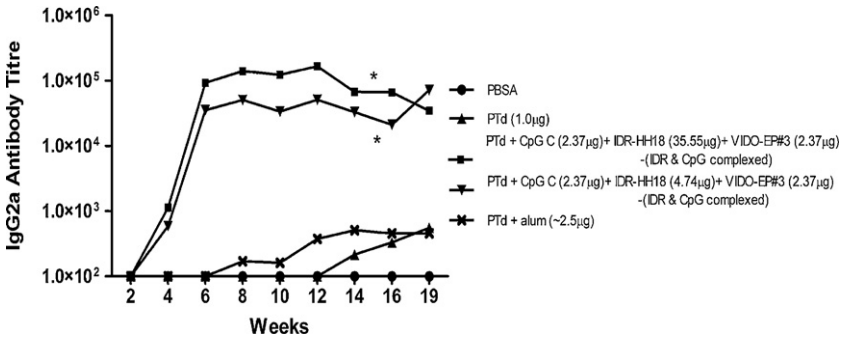

Fig. 4. PTd-specific neonatal serum IgG2a Ab responses to immunization with various adjuvant platform combinations or alum. Neonatal BALB/c mice were immunized with either PTd antigen alone, antigen combined with various adjuvant platform combinations, antigen with alum ($n = 10$ mice per group), or PBS ($n = 9$ mice per group), at 7 days of age. An identical secondary immunization was given at 4 weeks. Sera were collected at 2 weeks after the initial immunization prior to the boost, and every 2 weeks thereafter. Statistically significant differences occurred between the adjuvant platform combination containing 35.55 μg IDR and the combination containing 4.74 μg relative to PTd treated mice ($p < 0.05$, and $p < 0.01$, respectively) at selected time points. There was also a statistically significant difference found between the adjuvant platform combination containing 35.55 μg of IDR and the PTd and alum group ($p < 0.01$). There were no differences between the two adjuvant platform combination formulations.

3.3. Triple adjuvant combination in BALB/c neonatal mice

Seven-day old mice were used in our neonatal experiments. This age has been shown to correlate best with the human neonate's immune system [11,12]. BALB/c pups were immunized, and the serum IgG2a and IgG1 titres monitored over time (Fig. 3A and B). A higher dose of PTd was used for vaccination in the neonatal model to compensate for the reduced ability of the immature immune system to respond to the antigen, and the dosages of other vaccine components used were adjusted for the smaller weight of the pups in comparison to adult mice. Vaccine formulations consisted of 1.0 μg PTd, 2.37 μg CpG ODN, 2.37 μg VIDO-EP#3 and 35.55 μg of IDR-HH18, both complexed and non-complexed.

Large increases in the serum IgG2a response were observed in mice immunized and boosted with each adjuvant platform combination tested. The differences between the responses to the complexed adjuvant formulation, or the non-complexed formulation, and PTd alone were statistically significant ($p < 0.001$, and $p < 0.05$, respectively). Immunization with the complexed adjuvant platform vaccine also resulted in significantly ($p < 0.05$) higher IgG2a titres as compared to the non-complexed combination.

The IgG1 titres in this experiment followed a very similar pattern to the IgG2a response, with adjuvant combinations inducing approximately 10 times higher titres than the antigen alone (Fig. 3B). There was no significant difference seen in the IgG1 titres between the complexed and non-complexed vaccine groups, however, the mouse to mouse variability in antibody titre was larger in the non-complexed vaccine groups.

3.4. Variations of the adjuvant ratio in neonatal mice

In vitro evidence indicated that an adjuvant ratio of 1:2:1 (CpG ODN:IDR:PP) was most effective at increasing chemokine production *in vitro* [33]. In our previous experiments, a ratio of 1:15:1 was being used, thus, a comparison of this ratio to the *in vitro* suggested adjuvant ratio was performed. Neonatal 7-day old mice were immunized with vaccine formulations either containing 1.0 μg PTd alone, or consisting of the 1:15:1 ratio containing 1.0 μg PTd, 2.37 μg CpG, 35.55 μg of IDR-HH18, and 2.37 μg VIDO-EP#3 (complexed); or the 1:2:1 adjuvant ratio containing 1.0 μg PTd, 2.37 μg CpG, 4.74 μg of IDR-HH18 and 2.37 μg VIDO-EP#3 (complexed). A control group received 1.0 μg PTd combined with 2.5 μg alum (aluminum phosphate), the commercially used adjuvant that induces characteristically strong Th-2 type immune responses.

A large increase in the IgG2a titres already occurred after the first immunization with either ratio of the adjuvant platform formulation, as compared to antigen alone (Fig. 4). No significant difference was seen between the adjuvant platform combination containing a dose of 35.55 μg of IDR-HH18 (1:15:1 ratio) and the combination with the lower 4.74 μg IDR dose (1:2:1 ratio). Very low IgG2a titres were found in the PTd + alum immunized group. Within this alum group, there were five non-responder mice and one late-responder out of a total of 10 mice (Table 2). In the adjuvant combination group with 35.55 μg of IDR-HH18, all mice showed an IgG2a response and there were no late responders. In the adjuvant combination group containing 4.74 μg of IDR, there was one mouse that was a late responder, however all mice eventually showed an IgG2a response post-vaccination.

3.5. Duration of immunity

The antibody levels of the group containing an adjuvant ratio of 1:2:1 were followed over a longer period of time to examine when they would decrease (Fig. 5). Very high IgG2a titres were induced after immunization and boosting, and these titres remained elevated at the highest level for approximately 52 weeks. Past this point, the antibody titres decreased slightly, however, the IgG2a antibody response has remained highly elevated for more than 22 months, with the experiment still ongoing.

Table 2
Individual IgG2a antibody responses and antibody kinetics post-vaccination for Fig. 4.

Vaccine group[a]	Non-R[b]	Late-R[c]	Avg-R[d]	Mouse total
PTd (1.0 μg)	1	6	3	10
PTd (1.0 μg) + CpG C (2.37 μg) + IDR-HH18 (35.55 μg) + VIDO-EP#3 (2.37 μg)-complexed	–	–	10	10
PTd (1.0 μg) + CpG C (2.37 μg) + IDR-HH18 (4.74 μg) + VIDO-EP#3 (2.37 μg)-complexed	–	1	9	10
PTd (1.0 μg) + alum in PBSA (2.5 μg)	5	1	4	10

[a] Vaccine components of group, antibody titre results shown in Fig. 4.
[b] Non-R: number of "non-responding mice", a mouse that has non detectable antibody titre over the course of the experiment.
[c] Late-R: number of "late-responding mice", a mouse that has no detectable antibody titre until 8 weeks post-immunization or later.
[d] Avg-R: number of "average-responding mice", a mouse that has detectable antibody titre prior to 8 weeks post-immunization.

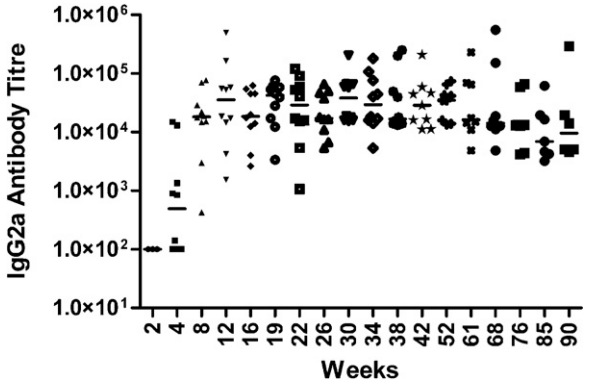

Fig. 5. PTd-specific neonatal serum IgG2a Ab responses to immunization with a 1:2:1 ratio of CpG:IDR:PP adjuvant combinations over an extended time period. Neonatal BALB/c mice were immunized as in Fig. 4, with an adjuvant platform combination, (n = 10 mice per group) at 7 days of age. An identical secondary immunization was given at 4 weeks. Sera were collected at 2 weeks after the initial immunization prior to the boost, and every 2 weeks thereafter, until week 10, after which sera were collected approximately every 4 weeks.

3.6. Comparison of single and double immunizations with a commercial vaccine

The ability of these adjuvants to induce a response comparable with, or ideally superior, to that seen after immunization using a commercial vaccine and the response to one or two doses were examined.

Vaccine formulations consisted of 1.0 μg PTd, 2.37 μg CpG ODN, 4.74 μg IDR-1002, 2.37 μg VIDO-EP#3, as well as the commercial vaccine Quadracel™. The IDR-1002 and CpG ODN components were complexed. Single and double immunization groups for the adjuvant platform combination and Quadracel™ were included. IDR-HH18 was replaced by a third generation synthetic IDR, IDR-1002 (VQRWLIVWRIRK-NH_2) because of its ability to induce superior chemokine responses *in vitro* and *in vivo* [29]. Neonatal mice were immunized subcutaneously with the formulations and the IgG2a serum responses were compared (Table 3) (Fig. 6).

A large increase in IgG2a titre was seen after both a single and a double immunization with the adjuvant platform formulations, as compared to PTd antigen alone; however there was a noticeable difference between the single dose and the boosted groups, with the boosted group developing much higher titres. Immunization with the commercial Quadracel™ vaccine, currently used for infant vaccination, resulted in very low to negligible IgG2a titres after a single dose, with an increase in titre occurring when boosted. However, even within the boosted Quadracel™ group, the resulting IgG2a titres were still lower than or similar to those that occurred in the single dose adjuvant platform group. The IgG2a antibody response in the adjuvant platform and Quadracel™ groups remained elevated for over four months.

Thus it appears that through using the adjuvant platform formulation, a greater Th-1 immune response can be induced, even after a single immunization, than that seen with a commercial vaccine.

4. Discussion

Whooping cough remains a major threat to infants and children in the developing world, with serious complications resulting from the disease including encephalopathy, convulsions, brain damage, and death (reviewed in [34]). Current pertussis vaccines cannot be administered until 6–8 weeks of age, as prior to this time several factors, including the presence of maternal antibodies and the innate Th-2 bias of the infant immune system, can modulate response to vaccination. Since neonates are known to be the most severely affected by whooping cough, a protection gap exists prior to the possibility of immunization, during which infants are at serious risk. To circumvent this gap a novel neonatal vaccine formulation, able to successfully overcome infants' Th-2 bias as well as the interference of maternal antibodies, is required.

Combinations of adjuvants may be used to induce superior immune responses than achieved by single adjuvants alone. It has been found that the simultaneous stimulation of several TLRs using multiple adjuvants in mice can improve the affinity maturation and neutralizing antibody production in vaccinated animals [35]. This positive modulation effect is not seen with alum, the currently used adjuvant in most human vaccines, including pertussis vaccine. Using mixed TLR ligands in vaccine formulations, the immune response can be increased until it reaches a threshold of activation necessary for antibody maturation [35]. Co-administration of TLR-9 agonists has also recently been tested in humans and found to enhance affinity maturation of specific anti-vaccine antibodies [36]. Because of this, we have sought to formulate effective, multi-component-adjuvanted vaccines with a combination of a known TLR ligand and other adjuvants to create a more balanced immune response and protect neonates against pertussis.

Three classes of adjuvants were selected. These act via complementary mechanisms and are thus speculated to simultaneously activate the immune system in distinct ways. Class C CpG ODN is a well characterized TLR 9 agonist, combining the mechanistic abilities of the other CpG classes by inducing both the secretion of IFN-α from pDC and the proliferation of B and NK cells [32]. Although the precise mechanism of action of the polyphosphazene PCEP is unknown, its adjuvant effects are suspected to result at least partially from a depot effect. Polyphosphazenes have been shown to stimulated increased cytokine production, and it is also speculated that they may stabilize antigens, allowing more efficient presentation to immune cells [37,38]. IDRs have a plethora of immunomodulatory effects; however, their ability to recruit cells to the site of injection and activate DCs, resulting in increased APC function is speculated to be important for increasing the cellular immune response necessary to clear *B. pertussis* infection.

Evidence from this report indicates that the combination of PTd antigen plus CpG C, IDR-HH18 or IDR-1002, and VIDO-EP#3 has advantages over the use of each adjuvant individually or as a doubly

Table 3
Individual IgG2a antibody responses and antibody kinetics post-vaccination for Fig. 6.

Vaccine group[a]	Non-R[b]	Late-R[c]	Avg-R[d]	Mouse total
PTd (1.0 μg) + CpG C (2.37 μg) + IDR-1002 (4.74 μg) + VIDO-EP#3 (2.37 μg)-complexed and single dose	2	–	8	10
PTd (1.0 μg) + CpG C (2.37 μg) + IDR-1002 (4.74 μg) + VIDO-EP#3 (2.37 μg)-complexed	–	1	8	9
Quadracel™ (dose includes 1.0 μg chemically detoxified PTd)-single dose	6	1	3	10
Quadracel™ (dose includes 1.0 μg chemically detoxified PTd)	2	3	5	10

[a] Vaccine components of group, antibody titre results shown in Fig. 6.
[b] Non-R: number of "non-responding mice", a mouse that has non detectable antibody titre over the course of the experiment.
[c] Late-R: number of "late-responding mice", a mouse that has no detectable antibody titre until 8 weeks post-immunization or later.
[d] Avg-R: number of "average-responding mice", a mouse that has detectable antibody titre prior to 8 weeks post-immunization.

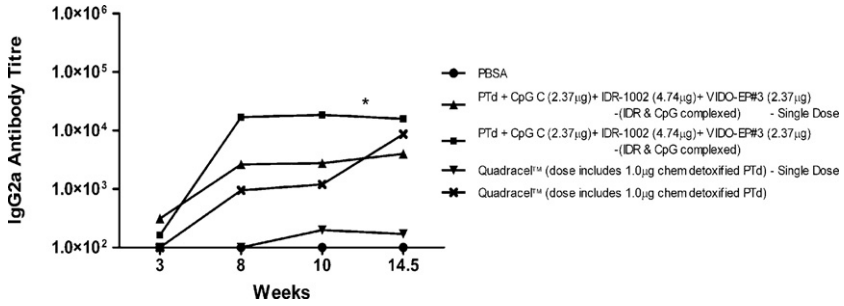

Fig. 6. PTd-specific neonatal serum Ab responses to single or double immunization with PTd formulated with adjuvant platform combinations or the commercial vaccine Quadracel™. Neonatal BALB/c mice were immunized with either PTd antigen combined with the adjuvant platform, or Quadracel™ ($n = 10$ mice per group), or PBS ($n = 9$ mice per group) at 7 days of age. An identical secondary immunization was given at 4 weeks to some groups. Sera were collected at 3 weeks after the initial immunization, and also at 8, 10 and 14.5 weeks post-initial immunization. Statistically significant differences occurred between 2 doses of the adjuvant platform relative to a single dose of Quadracel™ ($p < 0.05$) treated mice.

adjuvanted vaccine formulation. Vaccination using the developed complexed adjuvant platform was found to result in the earlier onset of the IgG2a antibody response in both adults and neonates. This was a distinct asset when compared to the responses to vaccination with PTd antigen alone, antigen adjuvanted with alum, a single-dose of licensed Quadracel™ vaccine, and using the non-complexed adjuvant platform.

When the immune response to vaccination with PTd antigen was examined with each adjuvant individually in adults, it was found that both CpG ODN and PP were able to significantly increase IgG2a titres, indicating an enhanced Th-1 immune response, as compared to PTd only. Class C was selected due to its unique ability to combine the effects of the other classes. The selection of the PP VIDO-EP#3 was based on its ability to induce higher IgG2a titres, and thus a stronger Th-1 immune response than VIDO-PP#4 in the absence of CpG ODN after a primary injection. Also, overall, a higher proportion of immunized mice were able to produce detectable IgG2a titres when this VIDO-EP#3 was used. Immunization with IDRs alone as an adjuvant failed to increase IgG2a titres. A possible explanation is that the IDRs are working through a different mechanism than the other adjuvants, for example it is well understood that IDRs do not interact with TLRs but do modulate signal transduction downstream of TLRs and demonstrate synergy with TLR agonists including CpG ODN [23,39]. However, since the likely effect of this component is enhancement of the recruitment of immune cells, the apparent inability of IDRs to affect the IgG2a response by themselves may be irrelevant. IDRs are known to have chemotactic effects on immune effector cells, and our synthetic IDRs have been shown to induce increased production of chemokines *in vitro* and *in vivo* [33]. Their cell recruitment effect may possibly be achieved through changing the microenvironment at the vaccination site, resulting in increased APC recruitment and subsequent increases in cytokine production that results in an increased cellular immune response.

When we combined CpG ODN and IDR together in a complexed precipitate form, a significantly stronger and earlier IgG2a response occurred in both adult and neonatal mice over that seen when the components were added separately. A higher dose of IDR also resulted in a higher proportion of mice responding with detectable IgG2a to vaccination. The indication is that IDRs have an effect on the ability of the mice to respond to the vaccine antigen by producing antibodies, and thus IDRs may in fact result in an increase of the Th-1 immune response. This would indicate that the addition of a certain amount of the IDR component allows the required threshold of activation needed for antibody maturation to be reached. The absence of a detectable antibody response in some mice within a vaccine group, while the other mice develop a very good response, supports the supposition that there is a stimulation threshold necessary to achieve activation of the humoral response.

After selection of each single adjuvant, these were combined together with PTd antigen to make an adjuvant platform combination vaccine. In adult mice vaccinated with the selected adjuvant platform, the IgG2a and IgG1 serum antibody titres were significantly increased as compared to immunization with antigen alone. This indicates an enhancement of the overall, as well as support of a Th-1 immune response. Immunization with the adjuvant platform vaccine in adult mice was able to induce a higher and longer-lasting immune response than single adjuvants alone. Complexation was found to cause a large increase in antibody production, with the greatest enhancement of IgG2a titres resulting after a 75 μg IDR dose, with a lower 10 μg dose of IDR appearing to have a lesser effect, although the difference was not statistically significant. There are several other known functions of IDRs, such as activation and maturation of dendritic cells and chemokine induction, which were not assessed in using the readouts of IgG1/IgG2a titres. These functions may be very important in inducing a strong overall immune response to our vaccine formulations. PTd alone at the dosages used was found to be very poor at inducing an IgG2a response, and somewhat better at inducing an IgG1 response, which was commensurate with previous experiments performed. In particular, there were several IgG2a non-responder mice seen in the PTd only group, with some mice in this group also failing to produce an IgG1 response to the antigen. It is notable that mice in the 75 μg/mouse IDR complexed group all had a measurable IgG2a and IgG1 response to vaccination, with no late responders, indicating that complexation not only increased the overall immune response in these mice, it enabled us to ensure that mice that might not otherwise respond to vaccination would do so.

Immunization in the neonates with the adjuvant platform combination was found to induce significantly higher IgG2a responses than PTd antigen alone, confirming the ability of the formulation to increase the Th-1 response, even within a model less likely to respond in a Th-1 type fashion. The results of complexation of the IDR and PP components mirrored those seen in the adult mouse model, with significantly higher IgG2a titres occurring after complexation. This further indicates that complexation has an important effect on the ability of the vaccine combinations to induce a Th-1 response, although we do not know if this involves co-localization of components or genuine synergy between associated components. The neonatal mice were found to respond with detectable IgG2a titres to both the antigen alone as well as to the adjuvanted groups. A possible explanation for this is the higher dose of antigen being used to vaccinate in the neonatal model. When neonatal mice were immunized using alum as an adjuvant, the IgG2a response was almost non-existent, and was significantly lower than the response induced by the adjuvant platform combinations. This was expected, as alum is an adjuvant that typically induces a Th-2 type immune response [40]. Within the alum group,

there was a 50% IgG2a non-response rate among the mice, indicating a poor Th-1 response being induced when using this adjuvant. Since alum is currently uses to adjuvant human acellular pertussis vaccines, the ability of our adjuvant platform to induce superior IgG2a titres is promising.

When neonatal mice vaccinated with the adjuvant platform were followed long term over nearly two years, the IgG2a antibody titres were shown to remain elevated over the entire period. This indicates that the Th-1 immune response being induced by the platform formulation is not only superior in magnitude to that achieved after immunization with PTd only, but that it is also a long-lasting response.

When the ability of the adjuvant platform formulation to induce an immune response after a single immunization was examined, it was found that, although the IgG2a titres were superior following a second immunization, the titres seen after a single dose were still greater than those induced by a single dose of the commercial Quadracel™ vaccine, and appeared in fact to be superior to two doses of that vaccine. Thus it appears that through using the current adjuvant platform, a greater Th-1 immune response could be induced than that seen with a commercial vaccine, and a good IgG2a Ab response might be induced even after a single immunization.

In conclusion, we have shown that through use of our adjuvant platform, a greater Th-1 immune response could be induced than that seen with a commercial vaccine, and a good IgG2a Ab response appeared to be induced even after a single immunization, which is substantially fewer than the three to five doses currently required for commercial acellular vaccines. Overall, the adjuvant platform formulation has the potential to improve the balance of the neonatal immune response to pertussis, allowing an earlier, stronger, and more effective response to be mounted to deal with infection. Further characterization of the mechanisms involved in the change of the immune response is needed to optimize these formulations.

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