DEVELOPMENT OF NOVEL VACCINE FORMULATIONS AGAINST PERTUSSIS FOR EARLY LIFE VACCINATION IN THE PRESENCE OF MATERNAL ANTIBODIES

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By

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ABSTRACT

Whooping cough caused by an infection with *Bordetella pertussis* or *Bordetella parapertussis* is a highly contagious respiratory disease. Globally, pertussis is the most prevalent vaccine-preventable disease. Even though the introduction of whole-cell (wP) and acellular pertussis (aP) vaccines has greatly reduced the burden of the disease, whooping cough still remains a problem in neonates and adolescents. *B. pertussis* is responsible for about 30 million cases of the disease each year, 90% of which are found in developing countries. About 300,000 of those infected, mostly infants, die from the infection. Various countries, especially developing nations, have reported an increase in infant morbidity due to pertussis. Recently, a rise in pertussis cases has also been observed in developed nations such as United States and Canada. Thus, novel vaccines against pertussis are urgently needed that would provide early and life-long protection.

Neonatal vaccination is challenging due to the presence of maternal antibodies (MatAbs) and the bias towards mounting Th2-type immune responses following early life vaccination. Our objective was to generate a novel vaccine against whooping cough that would offer protection in infancy in the presence of vaccine-neutralizing MatAbs. In order to first establish the model of interference, we vaccinated neonatal mouse pups and piglets in the presence and absence of passive immunity. Our experiments revealed that MatAbs interfered with active immunization using pertussis toxoid (PTd) and the level of passively transferred antibodies directly correlated with the level of interference that was observed. Nevertheless, we showed that this phenomenon could be overcome by using a second booster immunization or by co-formulating the toxoid with innate stimuli such as CpG ODN. Moreover, we also demonstrated that vaccination in the presence of MatAbs does not prevent responses to booster doses given later in life.

In order to improve the vaccine efficacy and immunogenicity we co-formulated the antigen with a novel adjuvant combination composed of CpG ODN, innate defense regulator peptide (IDRP) and polyphosphazene (PP). The model antigen ovalbumin (OVA) and adjuvants were formulated into PP microparticle and soluble formulations. These formulations were titrated and delivered to neonatal mice via parenteral and mucosal routes. Our experiments revealed that co-formulation of the antigen with the
novel adjuvant platform resulted in a higher antibody production as compared to vaccinating with antigen alone. In addition, both the soluble and microparticle formulations composed of the adjuvant combination induced elevated anti-OVA IgG2a titers thus indicating a Th1-type response shift in neonatal mice. Intranasal route of vaccination was shown to be superior to parenteral vaccination as it resulted in the production of high concentrations of systemic IgG2a and IgA antibodies.

Lastly, we co-formulated PTd and filamentous hemagglutinin (FHA) with the novel adjuvant formulation and tested them in the presence and absence of passive immunity in the murine and porcine models of pertussis. Vaccines composed of the new adjuvant formulations induced an earlier onset of immunity, superior anti-pertussis IgG2a and IgA titers, and a balanced Th1/Th2-type responses when compared to immunization with Quadracel®, one of the commercially available pediatric vaccines for pertussis. Most importantly, despite having half of the antigens of the Quadracel®, the novel vaccine formulations offered protection against challenge infection in the presence of passively transferred MatAbs. Taken together our results demonstrate this novel vaccine formulation and delivery to be an excellent candidate for neonatal vaccination.
ACKNOWLEDGEMENTS

It is my pleasure to thank the many people who have made my research a success and this thesis possible.

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Volker Gerdts for his mentorship, guidance, helpful advice and discussion that has made me into a better scientist. I truly appreciate Dr. Gerdts’ support of my interest in science and research. His knowledge and logical way of thinking have been a great value to me throughout my research and thesis writing process. I would also like to thank the members of my advisory committee, Dr. Lorne Babiuk, Dr. Harry Deneer, Dr. Hugh Townsend and Dr. Vikram Misra, for their commitment, support and supervision throughout my years of study.

During this work, I collaborated with many researchers. I wish to extend my thanks to Dr. Scott Halperin and Dr. Robert E.W. Hancock for their valuable help throughout this project. I am very thankful to Dr. Don Wilson, Dr. Stewart Walker and the VIDO animal care services for their assistance throughout the many animal experiments. I would like to thank all past and current laboratory members for their valuable help and discussion throughout this project. I would also like to express my sincere thanks to Ms. Joyce Sander and Ms. Michelle Balaski for their administrative and secretarial help. Additionally, I would like to thank my fellow graduate students in particular Jay Booth, Marina Facci and Sasha Gracia for the many stimulating discussions.

Finally, I am deeply grateful to my parents Hieronim and Maria Polewicz and my sister Dorota for their unconditional support and encouragement.

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DEDICATION

I dedicate this work to my parents Hieronim and Maria Polewicz, who from an early age expressed the importance of education and unconditionally supported me throughout the way.
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<th>Description</th>
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<tbody>
<tr>
<td>ACT</td>
<td>Adenylate cyclase toxin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>aP</td>
<td>cellular pertussis</td>
</tr>
<tr>
<td>B. avium</td>
<td>Borderella avium</td>
</tr>
<tr>
<td>B. hinzii</td>
<td>Bordetella hinzii</td>
</tr>
<tr>
<td>B. holmensii</td>
<td>Bordetella holmensii</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td><strong>Bordetella bronchiseptica</strong></td>
</tr>
<tr>
<td>B. parapertussis</td>
<td><strong>Bordetella parapertussis</strong></td>
</tr>
<tr>
<td>B. pertussis</td>
<td>Bordetella pertussis</td>
</tr>
<tr>
<td>Bac2a</td>
<td>Bactenecin 2a</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>bDC</td>
<td>Blood-derived dendritic cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming units</td>
</tr>
<tr>
<td>CpG ODN</td>
<td>Cytosine-phosphate-guanosine oligodeoxynucleotides</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell-mediated immunity</td>
</tr>
<tr>
<td>CRAMP</td>
<td>Cathelin-related antimicrobial peptide</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DTaP</td>
<td>Diphtheria, Tetanus, acellular pertussis vaccine</td>
</tr>
<tr>
<td>DTwP</td>
<td>Diphtheria, Tetanus, whole-cell pertussis vaccine</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>FcRn</td>
<td>Neonatal Fc receptor</td>
</tr>
<tr>
<td>FHA</td>
<td>Filamentous hemagglutinin</td>
</tr>
<tr>
<td>FIM</td>
<td>Fimbriae</td>
</tr>
</tbody>
</table>
FITC

*H. influenzae*

hBD-1

hBD-2

HBs-Ag

HDP

HEL

Hib

HIV

HSV

i.c.

IDRP

IFN-α

IFN-γ

IgA

IgE

IgG

IgG1

IgG2a

IgM

IL-1

IL-1β

IL-4

IL-5

IL-6

IL-8

IL-10
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IL-17</td>
<td>Interleukin-17</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin-18</td>
</tr>
<tr>
<td>IL-23</td>
<td>Interleukin-23</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>IPV</td>
<td>Inactivated polio vaccine</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major histocompatibility complex II</td>
</tr>
<tr>
<td>moDC</td>
<td>Monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MYd88</td>
<td>Myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer (cells)</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PAMPSs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PCEP</td>
<td>Poly[d(sodium carboxylatoethylphenoxy)phosphazene]</td>
</tr>
<tr>
<td>PCPP</td>
<td>Poly[d(sodium carboxylatophenoxy)phosphazene]</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>plgR</td>
<td>Polymeric immunoglobulin receptor</td>
</tr>
<tr>
<td>PLG</td>
<td>Poly-lactide-co-glycolide</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly-D,L-lactic-co-glycolic acid</td>
</tr>
<tr>
<td>PP</td>
<td>Polyphosphazene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PRN</td>
<td>Pertactin</td>
</tr>
<tr>
<td>PT</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>PTd</td>
<td>Pertussis toxoid</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncitial virus</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline with tween</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper lymphocyte type-1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper lymphocyte type-2</td>
</tr>
<tr>
<td>Th17</td>
<td>T-helper lymphocyte 17</td>
</tr>
<tr>
<td>Thp</td>
<td>T-helper cell precursor</td>
</tr>
<tr>
<td>TLR2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TLR9</td>
<td>Toll-like receptor 9</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-beta</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>wP</td>
<td>Whole-cell pertussis</td>
</tr>
</tbody>
</table>
1. LITERATURE REVIEW

1.1 WHOOPING COUGH

1.1.1 Disease

Whooping cough or pertussis is a respiratory disease caused by infection with *Bordetella pertussis* or *Bordetella parapertussis*. *B. pertussis* is transmitted via airborne aerosol droplets from an infected, coughing, sneezing person [1]. Whooping cough can be categorized into three stages [2]. The first catarrhal stage consists of a bacterial incubation period of 5-7 days prior to the development of symptoms. Persons are most infectious throughout this period. During this stage, the infected individual experiences symptoms similar to those of the common cold such as rhinorrhea, light cough and fever. The second stage of whooping cough, the paroxysmal stage, is characterized by a dry, nonproductive cough. During that time, the coughing attacks may be so severe can cause vomiting [3]. The coughing reflex attempts to clear the breathing passage and produces a typical whoop, from which the name “whooping cough” derives [4]. Typically, an infected individual experiences many intense coughing attacks throughout the day. This stage of the infection lasts for two to six weeks. The paroxysmal stage is followed by the last stage of pertussis, the convalescent phase, during which coughing spasms decrease in both severity and frequency. Complications arising from whooping cough typically occur in infants and young children. Most common complications include seizures, encephalopathy and secondary bacterial infections such as pneumonia [5-8].

Symptoms of the disease are most severe in infants and young children. Most deaths associated with pertussis take place in non-immunized neonates less than six months of age or infants who had not yet received complete series of vaccinations [9-12]. The symptoms are milder in older children and adults. Severity of the disease depends on the age of the patient as well as the remaining immune status from previous vaccination(s) or infection [13].

1.1.2 Infection

Whooping cough infection requires exposure to *B. pertussis* or *B. parapertussis*, followed by its attachment to the ciliated respiratory epithelial cells. Replicating bacteria causes localized tissue damage and systemic toxicity (Figure 1.1). Bacterial adhesins
including filamentous hemagglutinin (FHA), pertactin (PRN) and fimbriae (FIM) are responsible for attaching the organism to target respiratory cells. Other toxins such as pertussis toxin (PT) and adenylate cyclase toxin (ACT) allow the pathogen to damage the epithelial lining [14]. In combination, the virulence factors create both localized and systemic manifestations of the disease. Bacterial toxins released into the local environment result in damage of the respiratory epithelium leading to the cough during the catarrhal stage of the disease. ACT aids the bacteria in evading the innate immune response and subsequent clearance of the organism [15]. Upon entry into the cell, the toxin inhibits the cytotoxic functions of neutrophils, monocytes and natural killer (NK) cells [16].
Figure 1.1 Pathogenesis of pertussis. *B. pertussis* virulence factors contribute to the severity of whooping cough by facilitating adherence to the ciliated respiratory epithelial cells and are believed to responsible for the clinical disease process.
1.1.3 Epidemiology

According to the WHO (World Health Organization), infections with *B. pertussis* and *B. parapertussis* cause 20-40 million cases of whooping cough with about 100,000-200,000 fatalities annually, mainly in unvaccinated individuals [17]. In 2009, WHO estimated 106,207 reported cases of pertussis and 82% coverage with at least three primary doses of either whole-cell (wP) or acellular (aP) vaccines (Figure 1.2). The organization also estimated that pertussis was responsible for 195,000 deaths globally in 2008. WHO recognizes whooping cough as one of the ten most common causes of infection and death from an infectious agent. 95% of all pertussis cases are seen in developing countries. Pertussis is also one of the most common vaccine preventable diseases in industrialized nations [11]. In 1993, whooping cough was found to be the most common vaccine preventable disease among children five years of age or younger. The resurgence of the disease was due to the increasing population of susceptible adults [18]. Many European countries including England [19], the Netherlands [20] and Germany [21] also reported increased incidence of pertussis in the 1990’s.
Figure 1.2 Immunization coverage with pertussis vaccines in infants, 2009. Vaccination with inactivated whole-cell or acellular pertussis combined with diphtheria and tetanus toxoid vaccines, at least 3 primary doses.

Crowcroft et al. [22] developed a method, which allowed estimation of the global burden of pertussis. The authors assessed the proportion of vulnerable children becoming infected in areas with suboptimal vaccination coverage (<70%) in 1999 at 30% by 1 year, 80% by 5 years, and 100% by 15 years of age and for areas with excellent vaccine coverage (> or =70%) at 10% by 1 year, 60% by 5 years, and 100% by 15 years.

In the past, whooping cough mainly affected infants and young children. Recently, however, countries such as Canada and United States which have good pertussis control through vaccination, have shown an increase in the occurrence of the disease in older age groups (adolescents and adults) [23, 24]. Even though those countries have well-developed pertussis immunization programs, whooping cough still remains a problem in neonates and adolescents [25, 26]. Neonates suffer from pertussis due to the fact that they are either too young to have received their vaccination or have not yet
received a full series of vaccinations. Infants contract pertussis from their infected parents or members of the household and they cannot be protected by current vaccination schedule. An increase in the incidence of pertussis in older children and adults has been associated with waning of immunity in those who were vaccinated [27]. It has been postulated that increased susceptibility to pertussis infection in adolescents is marked by waning immunity from early life vaccination followed by reduced boosting strategies [28].

Since most infants with pertussis contract the infection from household contact, maternal and household vaccination (cocooning strategy) has been proposed in order to reduce the incidence of the disease in the neonates [29, 30]. Such cocooning strategy has been suggested in some industrialized countries such as Australia, Germany and France [14]. In fact, due to an increase of infant pertussis this strategy was recently introduced in the Province of Saskatchewan, Canada. The Saskatoon Health Region recommends vaccinating mothers and fathers of newborn infants. The cocooning strategy as well as vaccinating children and adolescents may indirectly protect newborn infants, however, information on the effect of this approach at the population level is quite limited.

1.2 BORDETELLA PERTUSSIS

1.2.1 General introduction

*Bordetellae* are small, non-motile, Gram-negative coccobacilli initially isolated by Bordet and Gengou in 1906. The bacteria infect the ciliated epithelium of the human respiratory tract [31]. The genus *Bordetella* is composed of multiple species including *B. pertussis, parapertussis and bronchiseptica* [32]. *B. pertussis* is a strictly human pathogen and a causative agent of whooping cough. *B. parapertussis* is responsible for causing milder pertussis-like disease in humans. *B. bronchiseptica* colonizes other mammals such as dogs, pigs, rabbits and guinea pigs. The pathogen is responsible for causing atrophic rhinitis in pigs [33] and kennel cough in dogs [34]. *B. avium* infects birds and can be associated with turkey coryza. *B. hinzii* and *B. holmensii* may occasionally cause human infections [4].
Members of the genus *Bordetella* have been demonstrated to enter and survive within phagocytic and nonphagocytic eukaryotic cells [35, 36]. For example, *B. pertussis* has been shown to survive in human macrophages [36] and has been shown to be present in the alveolar macrophages of children infected with human immunodeficiency virus (HIV) [37]. This intracellular phase of infection has also been demonstrated in mice [38]. Intracellular infection and survival by *B. pertussis* has a great implication for the mechanisms of vaccine action since a successful vaccine against whooping cough must induce both humoral and cellular immunity.

*B. pertussis* is difficult to culture and requires enriched media such as Bordet-Gengou medium or charcoal blood agar. Charcoal blood agar, which contains 10% sheep blood is a nonselective medium and must include an antibiotic such as cephalexin to reduce the growth of other bacteria [4]. *B. pertussis* grows best at 37°C in a humid environment. The agar plates might be incubated up to seven days before colonies can be seen.

1.2.2 Virulence factors

1.2.2.1 Pertussis toxin

PT is a virulence factor responsible for most systemic symptoms of disease in individuals infected by *B. pertussis* [39]. PT is a 105 kDa protein composed of six subunits. PT is a classical A-B toxin consisting of two parts: an enzymatically active A (S1) subunit and a B oligomer (Figure 1.3), which is responsible for attaching to receptors on target cells. The B oligomer binds surface glycoproteins on various mammalian cells such as ciliated respiratory epithelial cells [40], macrophages and lymphocytes [41, 42]. This nontoxic part of the toxin is made of subunits S2-S5. PT’s A subunit is an ADP ribosyl transferase, which increases levels of intracellular cyclic adenosine monophosphate (cAMP), which in turn affects signaling pathways of many cells. This function has an influence on the cells of the immune system. It disrupts the cellular function of phagocytes such as macrophages by decreasing their phagocytic activities, chemotaxis, engulfment and bactericidal killing [43]. Increased levels of cAMP
result in increased insulin production as well as increased sensitivity to histamine [44, 45]. In turn, these increased levels of insulin lead to hypoglycemia. Altered levels of histamine release are associated with hypotension and can lead to shock. PT has also been associated with inhibition of neutrophil and lymphocyte chemotaxis [46].

![Crystal structure of pertussis toxin.](image)

**Figure 1.3 Crystal structure of pertussis toxin.** The S1 subunit is responsible for the enzymatic activity of the toxin. The remaining subunits (S2-S5) make the B oligomer, which attaches the receptor to target cells.

1.2.2.2 Filamentous hemagglutinin

FHA is a large molecular weight (220 kDa) surface protein and a component of the cell envelope. This nontoxic pertussis antigen is involved in the initial colonization of bacteria. FHA mediates attachment to ciliated epithelial cells of the upper respiratory tract [47]. The protein has also been shown to exhibit immunosuppressive and immunomodulatory activities [48]. Following *B. pertussis* infection, infected mice generate FHA-specific T regulatory lymphocytes and antibodies. The T cells produced secrete interleukin-10 (IL-10) and suppress Th1 (T helper lymphocyte type-1) responses against *B. pertussis*, which mediates protective immunity against the pathogen [49]. It has
also been demonstrated the contact of FHA with macrophage receptors results in repression of interleukin-12 (IL-12) through an IL-10 dependent mechanism [50, 51]. It has been suggested that FHA might also act as a bridge adhesin allowing the attachment of other micro-organisms. Such property may explain the additional infections, which typically occur during pertussis infections [52].

FHA is a virulence factor included in acellular pertussis vaccines. FHA is quite immunogenic as individuals who receive FHA-containing pertussis vaccines mount a strong antibody reaction to the protein [53, 54]. Acellular whooping cough vaccines, which contain both PT and FHA, have slightly superior effectiveness as compared to monocomponent PT vaccines [55].

1.2.2.3. Pertactin

PRN, a 69-kDa outer membrane protein plays a function in the bacterial adherence to monocytes [56]. The protein, however, does not adhere to epithelial cells [57]. Based on vaccine trials, it has been suggested that PRN might be the most vital of all B. pertussis adhesins [58]. Cherry et al. [58] and Storsaeter et al. [59] performed a study identifying protective antibodies to B. pertussis antigens following both whole-cell and acellular vaccination. The studies concluded that high antibody levels to PRN, FIM and PT can be correlated with lowest risk of infection. Data from the trials concluded that antibody to PRN was the most significant in inducing protection. Protection against infection may be achieved by blocking PRN-mediated attachment of the pathogen to target cells [16]. Hellwig et al. have shown that antibodies against this immunoprotective antigen are needed for effective phagocytosis of B. pertussis by the host immune cells [60].

1.2.2.4 Fimbriae

FIM proteins are involved in colonization of the respiratory tract [61]. The proteins allow for bacterial adherence to epithelial cells and monocytes [57]. FIM has also been shown to induce nitric oxide (NO) production by macrophages [62]. Using in vivo studies in mice, it was shown that Fim B. pertussis strains with a deletion of the Fim genes were impaired in their ability to multiply in the trachea and nasopharynx [63].
Using both mouse and rat models, it was shown that FIM was absolutely necessary for *B. bronchiseptica* persistence in the trachea [64]. FIM has immunomodulatory and anti-inflammatory functions and in mice can inhibit bacterial killing by lung macrophages [65]. This virulence factor also exhibits an immunomodulatory activity. FIM can act as a T-dependent antigen for an early immunoglobulin M (IgM) response and inducing a T-helper lymphocyte type 2 (Th-2) host response following *Bordetella* infection [16]. Data from vaccine trials in children showed that FIM contributes to protection against infection [58]. A vaccine containing FIM as well as PT, FHA and PRN showed superior effectiveness than a vaccine composed of PT, FHA and PRN [66].

1.2.3 Vaccination against pertussis

1.2.3.1 Whole-cell pertussis vaccine

Inactivated wP vaccines have been a part of childhood vaccination for many decades. The vaccines introduced in the 1940’s dramatically reduced the public health impact of pertussis [67]. Before the introduction of pertussis vaccines, average annual incidence rates of whooping cough in industrialized countries were 150-200/100,000. Introduction of an extensive pertussis vaccination during the 1950s-1960s dramatically reduced (> 90%) whooping cough incidence and mortality [31]. Similarly, the introduction of the wP vaccine rapidly reduced the number of whooping cough cases in Canada (Figure 1.4) Pertussis vaccines have been part of the WHO Expanded Programme on Immunization since its launch in 1974. According to the WHO, since the end of the 1980’s, around 80% of infants worldwide have received at least the three primary doses of the pertussis vaccines [31]. Most wP vaccines exist in combination with tetanus and diphtheria toxins (DTwP). DTwP vaccines contain aluminum salt as the adjuvant. wP vaccines are thought to be ≥ 80% efficacious in eliminating symptomatic pertussis [3].
Immunization of infants with the wP vaccine is frequently (1 in 2-10 injections) related with slight adverse reactions including swelling, local redness, pain, anorexia, irritability and fever [31, 68]. In rare cases, the vaccine is associated with hypotonic-hyporesponse episodes (<1 in 1000-2000) [31, 69]. Lipopolysaccharide (LPS) is the main contributor of reactogenicity to the wP vaccines. The lipid A region of LPS, which contains most of the endotoxin activity, is the most likely source of reactogenicity of whole-cell vaccines [70]. Because of the reaction and concerns regarding vaccine safety, aP vaccines were developed in 1970. These vaccines are now being used in many countries including Canada.

Combination vaccines composed of wP vaccine combined with tetanus and diphtheria toxoids were the fundamentals of all other infant combination vaccines. DTwP vaccines have been combined with the following antigens: *H. influenzae* type b polysaccharide (Hib), Hepatitis B surface antigen (HBs-antigen), and inactivated polio vaccines (IPV).
1.2.3.2 Acellular pertussis vaccine

Concerns regarding adverse side effects of wP vaccines led to development of aP vaccines. These vaccines include virulence factors such as PT, FHA, PRN and FIM. aP vaccines displayed less side effects and their safety profile was the main driver that led to the replacement of wP vaccines. Another advantage of aP vaccines is their reproducible manufacturing process. The vaccine is composed of purified antigens, with removal of non-protective, toxic components such as LPS and other unwanted parts of the bacterial cell wall. When manufacturing wP vaccines the process and strains differ from manufacturer to manufacturer. Significant differences may be found in the amount of PT and FHA in different wP vaccines. On the other hand, the dose of antigens is more easily controlled during the manufacture of aP.

The first aP vaccines developed in Japan in 1981 were prepared using a co-purification process. The first vaccine, also called Takeda-type vaccine was produced by Takeda Chemical Industries in Osaka. This vaccine contained a higher dose of FHA as compared to PT and agglutinins [71]. The vaccine was created as a trivalent DTaP vaccine by Wyeth and Lederle Laboratories. The vaccine was extensively studied in the United States [72] and Japan [71]. The second type of aP vaccine used in Japan was produced by Biken (Research Foundation for Microbial Diseases of Osaka University). The antigens used in this vaccine were purified separately and contained the same concentrations of both PT and FHA. In a study performed by Aoyama at al. vaccine efficacy estimates were 88%, 77% and 78% for the Takeda-type vaccine, Biken-type vaccine and a wP vaccine, respectively [73].

Most aP vaccines contain four to five separately purified pertussis antigens (PT, FHA, PRN and FIM type 2 and type 3). The vaccines also differ in the bacterial clone used for antigen manufacturing methods, purification and detoxification of PT, and type of adjuvant/preservative used [14]. The standard dose of pertussis vaccine used to vaccinate infants and young children is 0.5 ml of volume. One such vaccine is the Quadracel® vaccine produced by Sanofi Pasteur Limited, which contains 15 Lf Diphtheria toxoid, 5 Lf Tetanus toxoid, acellular pertussis [20 µg chemically detoxified PTd, 20 µg FHA, 3 µg PRN, 5 µg FIM types 2 and 3], 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 used as the adjuvant. The vaccine is administered intramuscularly in the anterolateral thigh of young children aged less than one year. In older age group and adults, the vaccine is delivered in the deltoid muscle [14]. The adult vaccines, such as the Adacel® vaccine, contain lesser amounts of detoxified PT (2.5 µg) and FHA (5 µg). Administration and schedule for pertussis vaccines vary considerably between counties. The WHO recommends a three-dose primary series for infants. The first dose should be administered at six weeks with subsequent doses at four-eight weeks apart, at age 10-14 weeks, and 14-18 weeks. The dose of the advised primary series should be concluded by the age of six months. A booster dose is recommended for children around the second year of life [14]. In Canada, routine series of immunization of infants occur at two, four and six months of life. The children are then boosted at 18 months and between four-six years of life (Public Health Agency of Canada).

As previously mentioned, the switch from wP to aP vaccines was mainly driven by safety concerns. Many studies have demonstrated the increased safety following immunization with aP vaccines [66, 74]. Although aP vaccines are associated with lower reactogenicity, some cases of local reactions such as leg swelling and redness have been reported, especially following multiple booster doses of the vaccine [75, 76]. Evidence suggests that the leg swelling is a product of immunoglobulin-E (IgE)-mediated angioedema as opposed to an Arthus reaction [75]. It was shown that booster immunization of children with aP vaccine was associated with enhanced Th-2 type cytokine production [77]. The increased production of interleukin-4 (IL-4) and interleukin-5 (IL-5) increase IgE production and leading to IgE-dependent mast cell degranulation. These cells, in turn, release mediators such as Th2-type cytokines and chemokines that boost leukocyte recruitment to the site of injection. The local reaction induced by a booster shot of aP vaccine could be a type of an anaphylactic reaction produced by increased levels of Th2 cytokines and IgE [77]. A decrease in the number and frequency of immunization doses may lessen the severity of local reactions. This, however, could intensify the risk of acquiring whooping cough due to waning immunity.
Like wP vaccines, aP vaccines were primarily co-delivered with diphtheria and tetanus toxoids. Similarly, the vaccines were as well formulated with other antigens such as Hib polysaccharide, HBs-antigen and IPV vaccines. These combination vaccines are used as primary immunization for infants. Combination vaccines used as booster vaccines have reduced concentrations of antigens to further decrease the reactogenicity of booster doses. Recently, a study concluded that reduced dose combination vaccines with pertussis components are as effective as their equal non-combined vaccines [78].

The elimination of most non-protective, toxic components such as LPS throughout the purification process became the main advantage of the aP vaccine as compared to the wP vaccine. With the development of novel pertussis vaccines, quickly came a general agreement for the need of a safer pertussis toxin. This key virulence factor was then properly treated to obliterate its toxicity but maintaining its immunogenicity. It soon became clear that detoxified pertussis toxin (transformed into PTd) and FHA should be part of all aP vaccines (WHO).

In order for PT to be a part of aP vaccines it must be completely detoxified without losing its immunogenicity. Detoxification of the toxin is performed by genetic or chemical means. Chemically detoxified toxins are treated with either hydrogen peroxide or formaldehyde [79]. The S1 peptide of the toxin does not contain lysine making it unable to be detoxified with low doses of formaldehyde. In order to achieve total detoxification, the protein must be extensively treated with formaldehyde [80]. Usage of chemically detoxified toxins is associated with possible side effects. The procedure of chemical detoxification comes with a risk of reversion to toxicity or incomplete detoxification [81]. These reasons lead to the replacement of chemically inactivated to genetically inactivated toxoids for the use in pertussis vaccines. Most licensed multivalent aP vaccines, however, contain a chemically detoxified pertussis toxin.

Genetically detoxified PTd contains two amino acid replacements (Arg$^9$ → Lys and Glu$^{129}$ → Gly) in the active site of the enzyme [82]. This replacement of amino acids inactivated the catalytic part of the enzyme making it non-toxic. Multiple in vivo and in vitro studies have been performed to compare the immunogenicity of chemically and
genetically detoxified pertussis toxins. The studies concluded that genetically inactivated recombinant toxoid was superior immunogenically with an increased protective efficacy [82]. The PT9K/129G molecule increased the quality of the antibody response following vaccination. It was demonstrated that the genetic detoxification of PT maintains B and T cell epitopes of naïve PT, unlike chemical means of detoxifying which alter protein immunogenicity [83, 84].

A study published by Per Ibsen in 1996 assessed the effect of chemical and genetic detoxification of PT on epitope detection by a large collection of murine monoclonal pertussis toxin antibodies [79]. Ibsen concluded that toxin-neutralizing epitopes were conserved in the genetically detoxified PT, however, they were destroyed in the chemically detoxified PT. A study performed by the National Institute of Allergy and Infectious Diseases (NIAID) compared 13 aP vaccines [54]. This phase II clinical trial carried out in infants demonstrated the increased magnitude of the immune response following vaccination with aP vaccines composed of the recombinant PT. The study concluded that the genetically detoxified PTd induced 10-20 times greater anti-PT response as compared to vaccines composed of chemically inactivated toxoid. Another vaccine efficacy trial was performed in Italy and involved a large number of infants [82, 85]. The study demonstrated the vaccine composed of the recombinant PT was highly efficacious. During the first 30 months of the study lesser cases of pertussis were observed in children vaccinated with genetically detoxified toxin than children vaccinated with an aP vaccine composed of chemically inactivated toxin.

As previously mentioned, wP vaccines produced enhanced cellular immune responses, which were associated with increased vaccine efficacy [86]. WP vaccines, however, were associated with side effects, which led to their replacement with aP vaccines. WP vaccines induced Th1-type cells, while aP vaccines induce T cells with a Th2-type bias [86]. Cellular immune responses to pertussis are longer lasting than antibodies and it seems that T cells have an effect in long-term whooping cough protection [87]. wP vaccines were associated with interferon-gamma (IFN-γ) production, which was demonstrated to play a significant role in innate and adaptive immunity to *B. pertussis* [88]. Vaccination with aP vaccines does not induce IFN-γ and is mediated by
the production of immunoglobulin-G1 (IgG1) antibodies in mice [89]. Protective Th1-type responses induced via wP vaccines or previous infection are associated with IL-12 production by antigen presenting cells such as macrophages and dendritic cells. Vaccine components responsible for these responses are LPS and active PT [90].

1.2.3 Immune response to infection with B. pertussis

Estimates of the human duration of immunity against pertussis following natural infection range from 7-10 years [91, 92] to 20 years [28]. Estimates of the extend of the immunity gained after wP vaccination range from 4 [93, 94] to 12 years [19, 95]. A study performed by Simondon et al. demonstrated a difference in vaccine efficacy between the aP and wP vaccines [96]. The trial concluded that vaccination of 18 month-4 year old children with aP resulted in a higher incidence of pertussis as children vaccinated with wP vaccine. There was no difference, however, in the incidence rate in children younger than 18 months. The data suggested a longer duration of protective immunity against infection following vaccination with wP than by aP vaccine. Natural infection of humans with B. pertussis results in the production of cellular immune responses [97]. A Th1-type immune responses to PT, FHA and PRN are produced shortly following infection. Vaccination with wP vaccine also resulted with a Th1-type response production. Unlike natural infection and wP immunization, vaccination with aP vaccine is recognized by superior stimulation of Th2 cells.

Recovery from natural B. pertussis infection or immunization of mice with wP vaccine induces a Th1-type response. Immunization of mice using aP vaccines elicits Th2-type responses [98]. Native infection and vaccination with wP but not aP vaccines results in the induction of IL-12 [90]. Using the murine respiratory challenge model, Mills and colleagues have demonstrated the model to be a good correlate for protection following wP and aP vaccination in humans. The studies also concluded that both humoral and cell-mediated immunity (CMI) are required to obtain protection against infection following vaccination [89]. Antibody-mediated immune responses limit the degree of infection and reduce the destruction of the respiratory epithelia and immune cells. However, in order to achieve complete bacterial clearance, CMI responses must be
induced [99]. Using the murine respiratory challenge model, it was shown that immunization with an aP vaccine resulted in production of Th2-type responses, which were associated with delayed bacterial clearance. In contrast, natural infection and vaccination with a wP vaccine induced Th1-type responses, which were associated with rapid bacterial clearance [99]. Studies in mice primed either by a prior infection or by immunization with a wP vaccine (encourage strong Th1-type responses) have shown a prompt influx of neutrophils and lymphocytes with a lesser significant influx of macrophages. Priming of mice with an aP vaccine (producing Th2-type response) was associated with a less dramatic influx of neutrophils or T cells into the lungs following a challenge [86, 100].

As depicted in Figure 1.5 both vaccine types produce different types of immunity using distinct mechanisms. wP vaccines contain inactivated cell extracts and remaining bacterial toxins like LPS. The endotoxin activates macrophages to produce interlukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interlukin-18 (IL-18) and IL-12. These pro-inflammatory cytokines control the formation of Th1 cells from the precursor T cell (Thp). In addition, LPS contributes to the adverse side effects following immunization with wP vaccines. The cytokines produced allow B cells to secrete immunoglobulin G2a (IgG2a). Cytokines produced by Th1 cells such as IFN-γ, allow for the production of opsonizing antibodies and triggering neutrophils and macrophages to engulf intracellular bacteria. aP vaccines do not have residual bacterial toxins, but included antigens including FHA that encourage IL-10 production, which induces Th2 cells. The Th2 cells produced, in turn, allow murine B cells to produce IgE and IgG1, to facilitate the neutralization of toxins [86].
Figure 1.5 Mechanisms of pertussis immunity induced wP and aP vaccines Whole-cell vaccine containing residual toxins such as LPS stimulate Th1 responses via the production of IL-12, TNF-α and IL-1β. In contrast, acellular vaccines, which contain virulence factors like FHA, encourage IL-10 induction thus resulting in Th2 responses.

It has been demonstrated that Toll-like receptor 4 (TLR4) is required for successful clearance of primary infections with *B. pertussis* [101]. The receptor mediates IL-10 production, which triggers antigen-specific regulatory T cells that leads to the resistance to *B. pertussis* by inhibiting inflammatory pathology. It was also shown that TLR4 plays a vital role in protective cellular immunity following vaccination with wP vaccines. The immunity involves interleukin-23 (IL-23) and IL-1-driven interleukin-17 (IL-17) that improves the bactericidal activity of macrophages [102]. Genetically detoxified PTd (PT9K/129G) is the superior aP vaccine antigen candidate as it has been shown to induce Th1/Th17 immune responses [103]. The study also demonstrated the mutant’s utilization of TLR4/TLR2 (Toll-like receptor 2) engagement. Through IL-10, PTd favors
the expansion of Th1/Th17 lineages, which as mentioned above are involved in mediating host immunity to \textit{B. pertussis} infection.

Natural infection and vaccination with wP vaccines induce cellular immune responses mainly mediated by IFN-\(\gamma\), which are required to inhibit the spread of the disease. Ironically, the components of the vaccine that contribute to its efficacy are also responsible for its reactogenicity. aP vaccines do not contain the toxic components and are much safer to use. These vaccines result in the induction of Th2-type responses with immunity enduring for about 6-10 years. Thus, new types of pertussis vaccines are required which are safe to use, modify the immune response into a Th1-type and offer long-lasting immunity. Because most cases of whooping cough are observed in young infants, better vaccines are needed for early life vaccination. As the majority of the disease occurs in developing countries, more affordable vaccines with fewer doses would be ideal to stop the spread of pertussis in those regions of the world.

### 1.2.4 Animal models

#### 1.2.4.1 Murine model

Even though humans are the biological host for \textit{B. pertussis}, a considerable attempt has been made to reproduce the disease in mice, rats, rabbits and non-human primates. Mice are the most commonly used species to study pertussis infections. Murine models have greatly contributed to the existing knowledge of immunity to \textit{B. pertussis}. In addition, mice have been utilized to study the safety and efficacy of both wP and aP vaccines. The intracerebral (i.c.) Kendrick test was used to analyze the effectiveness of wP vaccines [104]. The Kendrick model was originally developed in 1950’s. Since then, both an intranasal (i.n.) and an aerosol models have been developed.

The response to infection depends on the age of mice as well as the dose of the challenge infection. Adult mice typically do not develop severe clinical symptoms, neonatal mice, however, depending on their age can get reasonably sick and exhibit weight loss, hypoglycemia and leukocytosis [105]. Because mice are unable to cough, they do not show the paroxysmal coughing upon \textit{B. pertussis} challenge. Mice also cannot
spread the infection to other mice. The murine model bears resemblance to the human infection as neonatal, sucking mice have a higher susceptibility to infection than adult mice. Following a respiratory challenge by an intranasal administration or aerosol, mice resemble similar systemic symptoms as human infants caused by PT such as hypoglycemia, leukocytosis and histamine sensitization [105-107]. Upon respiratory pertussis infection, immunocompetent adult mice can recover from the disease. Immunodeficient [88] and neonatal/infant mice, however, succumb to the infection with mostly fatal outcomes [108].

Mills and colleagues demonstrated that adult mice immunized with wP and aP vaccines are able to clear the bacterial infection following challenge [89]. The results suggest that the mouse respiratory challenge corresponds with vaccine efficacy in children [89, 109]. It was also shown that the murine intranasal challenge model could distinguish between divalent and trivalent aP vaccines from the same producer having varying effectiveness [110]. Taken together, the studies propose the murine respiratory challenge models are useful in the development of new pertussis vaccines, correlates of protection and mechanisms of immunity against *B. pertussis*.

1.2.4.2 Porcine model

Our laboratory developed a new pertussis disease model in newborn piglets [111]. Piglets were intrapulmonary infected with 5 x 10⁹ colony-forming units (CFU) of *B. pertussis* strain Tohama I. After a few days, nasal discharge, breathing complications, coughing and loss of weight were observed in the piglets. Post mortem examinations revealed severe lung lesions associated with pertussis as well as bronchopneumonia. Immunohistochemical analysis demonstrated an influx of *B. pertussis* bacteria within the airways. These cells were adhered to the respiratory epithelia lining or phagocytosed by immune cells including macrophages and neutrophils. Systemic effects included hypoglycemia and lymphocytosis. In a separate study Elahi *et al.* described that *B. parapertussis* can infect older piglets and cause pertussis-like illness when delivered intrapulmonarily [112]. Elahi *et al.* also demonstrated the presence of protective maternal antibodies (MatAbs) in piglets born of vaccinated sows [113] (Fig. 1.6). The study revealed pertussis specific IgG and secretory immunoglobulin-A(slIgA) in the serum and
bronchoalveolar lavage fluid (BAL) in offspring born of vaccinated mothers. The development of this new model will allow a better understanding of the disease and assist in the development of more successful vaccines against whooping cough. Moreover, these findings enable the study of pertussis vaccine efficacy in the neonate in the presence of maternal antibodies (MatAbs).

Figure 1.6 Lungs of piglets born to primed and non-vaccinated sows. Piglets were infected with $5 \times 10^9$ CFUs *B. pertussis* strain Tohama 1. Piglets were born either from PBS-treated control sows (A) and (C) or sows vaccinated with heat-inactivated *B. pertussis* (B) and (D). Piglets were euthanized two (A and B) and four (C and D) days post infection.

The pig model might be the superior model for studying pertussis as pigs and humans share numerous physiological characteristics [114] including many functions of the innate and adaptive immune responses. Additionally, the porcine model allows access to various immune compartments and samples including serum, colostrum, milk and bronchoalveolar lavage fluid. High concentration of immunoglobulin G (IgG),

immunoglobulin A (IgA) and IgM antibodies are transferred through porcine colostrum and milk, which allows the study of the effects of MatAbs on neonatal vaccination. In both humans and rodents, maternal immunoglobulin-G (IgG) is transferred through the placenta [115]. Since immunoglobulins cannot cross the placenta in pregnant sows [116] [117], piglets born of naïve and vaccinated sows can be exchanged between litters shortly after birth. This effort allows to eliminate the sow effect when studying the influence of MatAbs.

1.2.5 Maternal immunization

The discovery of the effective placental transfer of maternal anti-pertussis IgG and the fact that vaccine-specific antibodies in infants reflected levels of their mothers [118, 119], maternal immunization was identified as another means of preventing neonatal pertussis. It was shown that vaccinating pregnant women during pregnancy with wP vaccine resulted in the increase of agglutinin antibodies [120]. A study performed by Cohen et al. investigated the occurrence of pertussis in 100 infants born of mothers vaccinated during pregnancy with wP vaccine. The study identified zero cases of pertussis following documented exposures in infants born of vaccinated mothers. Three of the six infants exposed to pertussis and born of non-vaccinated mothers developed whooping cough [121]. A recent study demonstrated that immunization with TDaP vaccine during pregnancy had a profound effect on maternal and neonatal serum antibodies [122]. The study revealed newborns born of mothers vaccinated during pregnancy had significantly higher antibody concentrations to all antigens. However, even though it is possible to measure passively acquired antibodies in infants, the amount of antibodies needed to offer protection following pertussis infection is unknown [120].

Healy et al. [123] and Gall [124] encourage the importance of immunizing pregnant women with the acellular pertussis vaccine, which would offer better passive immunity to their infant. Kathryn Edwards found that elevated concentrations of maternally transferred antibodies to PT were related with a lesser pertussis toxin antibody responses to wP. This trend, however, was not associated following vaccination with aP vaccine [125]. Edwards suggested that maternal vaccination is a valid method for protecting the newborn from pertussis until active immunity is present. Pre-existing
pertussis antibody titers seem not to interfere with infant immune responses to the aP vaccine [126]. These findings provide a rationale for administering aP vaccines to pregnant women in order to decrease the pertussis burden in neonates.

The Canadian Center for Vaccinology at Dalhousie University in Halifax is currently performing a clinical trial in order to evaluate to potential for immunological protection of the neonate via maternal immunization. The trial involves immunization of pregnant women with DTaP (Adacel®) vaccine during the mid third trimester of pregnancy to evaluate the possibility of offering immunological protection to the newborn by supplying passive placentally transported serum, colostrum and breast milk antibodies against pertussis. The study will determine the rate of MatAb decay from the time of immunization until a year postpartum. The trial will also establish the concentrations of antibody passed to the neonate. Additionally, the objectives include establishing whether maternal immunization obstructs the active antibody induction following DTaP-IPV-Hib vaccination of infants born of mothers boosted at some stage of the third trimester of pregnancy.

Protection of neonates induced by passive immunity was also demonstrated using animal models of pertussis. Suckling mice born of vaccinated mothers showed protection against infection with B. pertussis [127]. Our group has also shown a potential benefit in vaccinating mothers in order to reduce neonatal whooping cough. Sows were vaccinated with heat-inactivated B. pertussis during pregnancy. Neonatal piglets were encouraged to suckle colostrum and milk after birth before they were challenged with B. pertussis. Considerable concentrations of specific IgG and sIgA were identified in the serum and BAL of piglets born of primed sows. After infection with B. pertussis, clinical symptoms, pathological alterations, and bacterial shedding were substantially decreased in piglets that had obtained passively acquired immunity. These findings provide more evidence that maternal immunization could provide an option to offer protection against pertussis in neonates [113]. Identifying maternal immunization as a method to prevent neonatal pertussis, poses another need for neonatal vaccines, which will be able to elicit protection in the presence of high levels of MatAbs.
1.3 INFANT IMMUNE SYSTEM

1.3.1 Neonatal immune system

Infants are highly susceptible to infectious diseases due to their “immature” immune system. An important challenge of neonatal vaccination includes the immaturity of the infant’s immune system, particularly its predisposition to mounting Th2-type responses [128, 129]. Neonatal dendritic cells (DCs) seem to be skewed against mounting Th1-type immune responses [130]. It has been postulated that the absence of sufficient co-stimulatory pathways may play a role in this polarization of T cells by antigen presenting cells thus resulting in a selective activation of the Th2 lineage [130]. Studies of neonatal mice established a higher necessity for accessory T cell signals in contrast to adult mice [131]. Newborn lymphocytes have also shown a reduced presence of the CD40 ligand [132]. Limiting the CD40/CD40L interaction in vivo reduces the priming of Th1 cells by reduction of IL-12 production [133]. The low level of CD40/CD40L interaction could result in deficient neonatal IL-12 production consequently leading to the development of interleukin-4 (IL-4) secreting Th2 cells. Thus, successful neonatal vaccines would need to overcome the innate neonatal polarization towards mounting Th2-like responses. This bias might be modulated by the immunological environment at the time of priming through the use of Th-1 response stimulating adjuvants in early life vaccines.

Kollmann et. al assessed TRL mediated cytokine response of neonatal and adult human blood monocuclear cells [134]. The study found differences among blood samples from neonates and adults. As compared to adults, neonatal blood monocytes produced less interferon-α (IFN-α), IFN-γ and IL-12 subunit p70. Infant cells also showed a superior capacity to produce IL-10 and IL-17 producing helper T cells (Th17). Additionally, infant cells showed an inability to produce multiple cytokines simultaneously in response to Toll-like receptors (TLR) agonists [134]. The production of Th17 cells and IL-10 may be a factor in enhanced Th2 responses. Neonatal human DCs have been shown to be incapable of expressing the IL-12(p35) gene following stimulation with TLR agonists such as LPS and poly(I:C) [135]. Similarly, neonatal human DCs are
also incompetent at producing the cytokine following stimulation with pertussis toxin or with genetically detoxified PTds [136].

This neonatal Th2-type response skewing may be a result of Th2 response predominance during pregnancy. Th1-type responses are inhibited during pregnancy as they could lead to a miscarriage. Th1-derived cytotoxic immune responses could distinguish the developing fetus as an allograft and try to eliminate it by executing an attack. Factors generated in the placenta during pregnancy skew maternal immune regulation towards a Th2-type phenotype in order to protect the fetus [137]. The factors which skew the maternal immune system to guard the developing fetus in utero in addition imprint the immune system of the neonate towards mounting Th2-like responses following vaccination. These immunological alterations in the mother during pregnancy have a profound effect on how neonatal immune cells respond to antigens. For instance, human monocytes present in the umbilical cord blood are to a lesser amount responsive upon stimulation with bacterial products such as lipopeptides and lipopolysaccharide than cells obtained from adult blood [138]. Neonates have low levels of Th1 cytokines (TNF, IL-12p70, IFN-γ) and human neonatal plasma includes increased concentrations of Th-2 cytokine interleukin-6 (IL-6) [139]. In addition, neonatal cells have impaired T-cell and antigen presenting cell interface, particularly the ligation of CD40 and CD40L [140]. Therefore, the mixture consisting of the immaturity of the neonatal immune system as well as presence of vaccine neutralizing maternal antibodies become clear obstacles to effective vaccination during the neonatal period. One means of overcoming such obstacles is by incorporating Th1-type skewing vaccine adjuvants. A successful neonatal vaccine should contain adjuvant systems which can redirect the Th2 bias and induce balanced or Th1-type responses. Such vaccines should also contain specialized vaccine delivery vehicles that protect the antigen from being neutralized by MatAbs and safely deliver it to neonatal APCs.

1.3.2 Neonatal vaccines

Even though neonatal vaccination is challenging, vaccines delivered to neonates have been proven safe and effective [141]. Currently approved vaccines administered at
birth include Hepatitis B vaccine (HBV), Bacille Calmette-Guerin (BCG) and the oral polio vaccine (OPV). HBV vaccine made using a recombinant DNA technology to produce the hepatitis B surface antigen (HBsAg) is the only vaccine given throughout the first month of life in the United States [141].

BCG, which signals via Toll-like receptor-2 (TRL-2) and TLR-4 [142] and induces Th1-type immune responses at birth [143] is the most extensively used vaccine globally [144]. Bourin Trunz et al. determined that during 2002 the 100.5 million BCG vaccines administered at birth inhibited about 30,000 cases of tuberculous meningitis and around 11,500 cases of miliary disease [145]. The efficacy of the neonatal BCG vaccination is a result of its capability to produce Th1-type immune responses [146]. Interestingly, BCG vaccination had an effect on antibody and cytokine responses to unrelated antigens (HBV and oral polio) in early life [147]. Early BCG vaccination encouraged the induction of Th1 and Th2-type cytokines following immunization with unrelated antigens. BCG boosts both Th1 and Th2-type responses to unrelated antigens most likely via its manipulation of DC maturation.

OPV vaccine given at birth has an ability to induce both mucosal and humoral antibody responses [141]. The vaccine administered at birth provides protection in parts of the world where poliomyelitis has not been controlled [148]. A study performed by Halsey et al. tested the effectiveness of the trivalent oral polio and DTP vaccines given to human neonates early in life [148]. It was concluded that even though MatAbs may alter the immune responses during early life, the priming dose of DTP could be given in the first month of life. A study by Knuf et al. demonstrated that vaccination with aP vaccine within the first week of life was safe and induced antibody responses to pertussis by three months of life [149]. Currently, the first dose of pertussis vaccine is administered at two months of life. The authors suggest that administering the vaccine at birth would further reduce the risks associated with pertussis during the window when infants are most vulnerable to the disease.

The increasing evidence suggesting that neonates can successfully respond to early life vaccination is leading to the development of novel vaccine strategies. One of such vaccines is the attenuated Listeria monocytogenes vaccine, which has shown
promise in neonatal mice [150]. A single immunization during early life induced strong primary and secondary CD4 and CD8 Th1 responses without the need for boosting. The vaccine protected young mice from a lethal infection with a wild-type pathogen.

### 1.3.3 Interference with maternal antibodies

In humans, MatAbs are transferred via placenta or through the colostrum and milk. The most effectively transferred immunoglobulin subclass is IgG1 [151], with little IgM, IgA and IgE transferred. Transfer of antibodies is facilitated by active transport using Fc receptors (FcγRs) [152]. Placental transfer of antibodies is mediated by binding of maternal IgG to Fc receptors in the placenta, which contains many FcRs, including the most important to antibody transfer, the neonatal FcR (FcRn). It is believed that by regulating the IgG transport, the FcRn greatly contributes to the control of serum IgG concentration [153]. In humans, transfer of antibodies via the placenta starts at 28 weeks gestation and the concentration of MatAbs in fetal circulation amplifies until birth [152]. At 33 weeks of gestation maternal and fetal antibody levels are of similar levels [154]. In fact, it was demonstrated that at birth fetal and maternal antibody concentrations are similar, and sometimes, the antibody levels in neonates exceed the mother’s titer [155]. Some of the factors contributing to the transport of vaccine-specific MatAbs include placental abnormalities, concentration of specific antibodies in maternal circulation, the time between maternal vaccination and delivery as well as the gestational age of the fetus [154].

Human breast milk is abundant in antibodies, especially sIgA, which have been transferred into breast milk via the polymeric immunoglobulin receptor (pIgR) [156]. sIgA transferred via breast milk is specific for a number of common respiratory and intestinal infectious agents [157]. These secretory antibodies target pathogens present in the mother’s environment, which the neonate will most probably encounter throughout its infancy. Breast-feeding is a perfect example of the exchange of mucosal immunity among the mother and her infant. During breast-feeding primed B cells migrate from the mother’s Peyer’s patch through the lymph and peripheral blood to the lactating mammary gland [158].
Presence of MatAbs is the most significant challenge for infant vaccination [159][160]. MatAbs are produced due to the mother’s previous exposure to the pathogen or vaccination. This passive immunity is transferred from mother to infant transplacentally or through the colostrum and breast milk. MatAbs offer short-lived protection against disease, but also hinder early vaccination of neonates [161]. Ironically, passive immunity protects the young from disease, but also interferes with immunization of the infant. Many examples have demonstrated important inhibitory effects on infant antibody responses. MatAb interference has been shown following vaccination with vaccines against measles [152, 162], Haemophilus influenzae type b conjugate vaccine [163], hepatitis A [164], influenza A [165], tetanus [166], and varicella-zoster [160]. Many studies have demonstrated the issue of MatAb interference following whooping cough vaccination [167-170]. Transplacental and transcolostral MatAbs to pertussis antigens can obstruct the infant’s ability to mount antibody responses following immunization with either the wP or aP pediatric vaccines [128, 168, 169, 171, 172].

MatAbs’ inhibitory effects are thought to be a result of formation of immune complexes between antibodies and antigen and include the neutralization of antigen, phagocytosis of MatAb-coated antigen, inhibition of B cell activation by Fcγ-receptor mediated signals and epitope masking by MatAbs and as a result inhibiting antigen binding to infant B cells [129, 171] (Figure 1.7). The most important determinant of infant antibody responses is found in the MatAb: vaccine antigen ratio. Neonatal antibody responses may only be initiated once MatAbs have declined beneath a particular threshold, and secondary responses may be obtained once this threshold is reached [129, 152, 171]. MatAbs have an effect on infant B cell responses but leave T cell responses unaffected [128, 171, 173]. In the presence of MatAbs, the neonatal antibody response to the specific antigen is repressed. Consequently, successful vaccines against pertussis must be able to overcome the interference of MatAbs.
Figure 1.7 Influence of maternal antibodies on early life vaccination. High levels of MatAbs could neutralize viral vaccine concentration thus inhibiting viral replication and decreasing the effectiveness of the vaccine. MatAbs also mask immunodominant specific B-cell vaccine epitopes, inhibiting contact with neonate’s B cells. Despite the inhibition of B cell responses, CD4/CD8 priming is unaffected by MatAbs.

A few strategies have been used in order to overcome maternal antibody inhibition of responses following early life vaccination. One such strategy is to use repeated immunization doses. Such an approach has been used with DTP vaccines. Another method that reduces the inhibitory effects of passive immunity takes advantage...
of the progressive decline of MatAbs. Delaying the age of first immunization until MatAbs have waned has been used in measles vaccination. Neither method permits for successful early initiation of protection of neonates [174]. Thus, novel vaccine formulations and approaches able of inducing protection in the presence of MatAbs are needed. Future vaccine strategies and formulations for infant immunization must take full advantage of the concept of vaccine adjuvanticity. Vaccine enhancing adjuvants can be divided into two categories: immunomodulators and antigen delivery systems. Immunomodulatory adjuvants directly activate APC or lymphocytes to enhance and modulate immune responses following vaccination. This class of adjuvants would allow the induction of desired, protective immunity. Antigen delivery systems integrate the antigens thus promoting their uptake, processing and presentation by APCs to B and T lymphocytes. Microspheres of biodegradable polymers are examples of such vaccine delivery adjuvants. Another exciting feature of micoparticles is their incorporation of the antigen, which could allow to protect the vaccine from being recognized and consequently neutralized by MatAbs.

1.4 STRATEGIES TO OVERCOME INTERFERNCE / FUTURE VACCINES

1.4.1 Adjuvants

1.4.1.1. CpG ODN

Originally, adjuvants were used to increase the magnitude of antibody production. Most recent adjuvants are designed to tailor the immune response, which is needed for protection. Adjuvants can be divided into two classes: delivery of antigens and immunostimulatory. Vaccine delivery adjuvants act by targeting adjuvants to antigen presenting cells. These adjuvants may also protect the antigen from being degraded as well as encourage the depot effect, which is thought to enhance antigen uptake. Thus, adjuvants are used to improve the quality and quantity of immune responses elicited by vaccines. Adjuvants are incorporated into vaccines in order to induce favorable type of immunity, for example: to induce Th-1 or Th-2-type immunity. Adjuvants not only allow
for generation of T cell memory, but also modify the specificity and affinity of the response [175].

Pathogen recognition relies mainly on the innate system. Microbes contain pathogen-associated molecular patterns (PAMPs), which are recognized and activated by pattern recognition receptors (PRRs) expressed on immune cells such as DCs [176]. Immunostimulatory adjuvants are mostly PAMPS, which are recognized by PRRs including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). These TLRs and NLRs are the most studied members of the PRR family. TLRs recognize unique patterns that are not present in the host, which allows them to sense danger and activate the innate immune system. TLRs recognize lipids, nucleic acids and proteins. Intracellular NLRs detect pathogens and stress signals such as flagellin, toxins and degradation products of peptidoglycans [177]. Most PRRs trigger the transcription factors such as nuclear factor-κB (NF-κB) and interferon regulatory factor (IRF) via pathways composed of adaptor molecules such as myeloid differentiation factor 88 (MYd88) and Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-beta (TRIF). These, in turn, are responsible for induction of genes encoding cytokines and chemokines, which shape the adaptive responses.

Cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN) motifs are an example of PAMPs that function to alert the innate immune system of possible infections. Vertebrates have evolved PRRs in order to recognize pathogen structures such as unmethylated DNA [176]. Akira et al. have shown that in mice Toll-like receptor 9 (TLR9) recognizes CpG motifs in immune cells [178]. It was also demonstrated that TLR9 is responsible for recognizing bacterial DNA in humans [179]. The primary human cells expressing TLR9 are B cells and plasmacytoid DCs (pDCs) [178]. Most members of the TLR family are expressed on the cell surface, TLR9, however, is expressed within the cell. CpG DNA is internalized by antigen presenting cells where it is recognized by TLR9 present in endocytic vesicles [178]. CpG-mediated signalling involves the MyD88 pathway, whose elements activate transcription factor NF-κB responsible for cytokine and chemokine gene expression [180].
CpG ODNs are an example of immunomodulatory adjuvants. CpGs are synthetic motifs similar to unmethylated CpG dinucleotides present in bacterial DNA. Unlike vertebrate DNA, bacterial DNA is composed of a higher concentration of CpG dinucleotides that are unmethylated, which makes it highly immunomodulatory [181]. Bacterial DNA has a positive effect on B cell proliferation and antibody secretion. Unlike vertebrate DNA, bacterial DNA acts on NK cells to lyse tumor cells and secrete IFN-γ [182]. Bacterial DNA and synthetic oligodeoxynucleotides composed of unmethylated CpG motifs trigger an immunostimulatory cascade responsible for the maturation, differentiation as well as proliferation of B and T lymphocytes, DCs, macrophages, monocytes and NK cells [183]. The cells stimulated by CpG motifs produce cytokines and chemokines which establish a pro-inflammatory (IL-1, IL-6, IL-18, TNF-α) and Th1 biased (IFN-γ, IL-12) immune environment [184].

CpG ODNs can be divided into three classes. Type A ODNs contain a mixed phosphodiester/phosphorothioate backbone with a single CpG motif [185]. The immunomodulatory activity of Type A CpG ODNs include APC maturation and stimulation of pDCs to secrete IFN-α [185]. Class B CpG ODNs are constructed using a phosphorothioate backbone and include multiple CpG motifs. This class of CpG ODNs are responsible for pDC maturation and favourably increases the induction of TNF-α and IL-6. These ODNs are also involved in B cell activation, as well as the induction of IgM [183]. The third class of CpG resembles the B type of CpG ODNs as they are completely composed of phosphorothioate nucleotides containing multiple CpG motifs. This class of ODNs combines the stimulatory and immunomodulatory properties of A and B-class ODNs. C type of CpG ODNs directly stimulate B cells and pDCs, which results in the production of IL-6 and TNF-α [183, 185].

Synthetic 18-25 base ODNs composed of CpG motifs have been tested for their adjuvant properties, either soluble or formulated into nanoparticles [186]. CpG ODNs have been shown to improve antibody responses and strongly augment Th1 responses [187]. Incorporation of CpG ODN into numerous experimental vaccines showed improved protection against pathogens such as Cryptococcus neoformans [188] and intracellular bacteria such as Francisella tularensis and Listeria monocytogenes [189].
CpG ODN has also been tested in clinical trials as an adjuvant for human vaccines [190, 191]. CpG has been tested and shown to induce IFN-γ and circumvent Th2 polarization in vaccines against Hepatitis C Virus (HCV) [192], Haemophilus influenzae type b vaccine [193], measles virus [194], anthrax [195, 196] and smallpox [197]. CpG ODNs are optimal adjuvants for neonatal vaccines. These synthetic motifs can improve antibody responses following vaccination and more importantly overcome the obstacle of the Th2-type bias associated with neonatal immunization.

1.4.1.2 Innate defense regulator peptides (IDRPs)

Synthetic cationic IDRPs are similar to host defense peptides (HDPs), which are antimicrobial innate host defense molecules found in animals, insects and plants [198]. These antimicrobial peptides are normally short (12-100 amino acids) and have a net positive charge (+2 to +9) [199]. The peptides have been isolated from a variety of sources including unicellular microorganisms, plants, insects, amphibians, birds, fish and mammals [200]. Besides displaying antibacterial and antifungal properties, the peptides can exhibit antiviral and anticancer activities. Moreover, IDRPs are involved in inflammation, healing of wounds, cytokine release and chemotaxis [201]. These peptides are also able to improve phagocytosis, neutralize the septic effects produced by LPS as well as support the recruitment of a number of immune cells to the site of inflammation [202, 203]. Mammalian peptides have been shown to be present during the induction of adaptive immunity by exhibiting their chemotactic properties for human monocytes [204] and T cells [205].

Positively charged IDRPs display immunomodulatory functions such as recruitment and activation of APCs [206]. The human cathelicidin peptide LL-37 and the murine cathelin-related antimicrobial peptide (CRAMP) induce chemokine production for human neutrophils, macrophages, monocytes and T cells [207]. LL-37 has a profound effect on adaptive immune responses. The peptide allows precursor DCs to go through phenotypic and functional changes that enhance their antigen sampling activity [208]. As these cells mature, they display an enhanced ability to promote Th1 responses. LL-37-primed DCs produce Th1 stimulatory cytokines and significantly enhanced T cell IFN-γ responses [208]. Unlike LL-37 and CRAMP cathelicidins, the 12-mer bactenecin 2a
(Bac2a) and indolicidin (13 amino acids) are smaller peptides found in the cytoplasmic granules of bovine neutrophils [209, 210]. Indolicidin has anti-endotoxin activity displayed by its inhibition of LPS-induced TNF-α secretion. The peptide also displays a capacity to produce chemokine interleukin-8 (IL-8) in a human bronchial cell line [211]. The Bac2a derivative named HH2 [212] combined with C-class CpG ODN links innate and adaptive immunity by its production of cytokines and chemokines in human mononuclear cells as well as upregulation of surface marker expression in human DCs [213]. Most interestingly, co-formulation of HH2 and CpG with PTd antigens, considerably improved toxoid-specific antibody titers in contrast to vaccination with toxoid alone [213].

Defensins are the second group of mammalian antimicrobial peptides [214, 215]. These cyclic peptides are divided into three groups based on their disulfide bond distribution (α-, β- and θ-defensins) [199]. The α and β-defensins are extensively distributed throughout the vertebrate species. θ-defensins, however, have much more limited distribution [216]. α and β-defensins are the only two classes of defensins found in humans [217]. These two classes of defensins are present in various cells including: neutrophils, macrophages, NK cells, intestinal cells, epithelial tissues, skin, mucosa, respiratory and urinogenital tracts [199]. Four α-defensins have been isolated from human neutrophils (HNP-1 to 4) [217]. Human β-defensin 1 (hBD-1) is expressed constitutively in most tissues including lung, mammary gland, salivary gland, kidney, pancreas and prostate [218]. Expression of human β-defensin 2 (hBD-2) is inducible following exposure to bacteria, LPS or pro-inflammatory cytokines including TNF-α or IL-1β [219]. In addition to their antimicrobial activity, defensins also show immunomodulatory functions. The peptides act as chemoattractants of immune cells such as monocytes [204] and stimulate cytokine and adhesion molecule expression [220].

Due to their immunostimulatory functions, short cationic peptides are promising candidates as vaccine adjuvants. In fact, IDRPs coupled with CpG ODN have been tested experimentally by Kovacs-Nolan et al. Indolicidin and CpG ODN co-administered with ovalbumin (OVA) or hen egg lysozyme (HEL) demonstrated synergistic activity between the adjuvants [221, 222]. In mice, incorporation of the two adjuvants enhanced humoral
OVA-specific immunity, but not CMI [223]. In cattle, when formulated with HEL, the adjuvants increased cell-mediated but not humeral immune responses following vaccination [222].

1.4.1.3 Polyphosphazenes (PPs)

Polyphosphazenes (PPs) are a class of synthetic, water-soluble, biodegradable polymers composed of a backbone with alternating phosphorus and nitrogen atoms with organic side groups connected to each phosphorus [224]. The most studied polyphosphazene polyacid is poly[di(sodium carboxylatophenoxy)phosphazene] (PCPP) [225]. Recently, a new polyphosphazene polyelectrolyte has been developed poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) (Figure 1.8). Due to their physio-chemical properties, nontoxic degradation products, simplicity of manufacture and matrix permeability, polyphosphazenes are an exceptional choice for a platform used for controlled vaccine/drug release [226].

![Figure 1.8 Structure of the polyphosphazene PCEP and PCPP polyelectrolytes](image)

One of the most significant potential applications of these synthetic polyelectrolytes is that when delivered with vaccine antigens they act as powerful
immunostimulants. For example, PCPP was demonstrated to have an adjuvant property to a variety of vaccine antigens including influenza [227], rotavirus [228] and cholera [229]. Mutwiri et al. demonstrated that in comparison to PCPP, PCEP was shown to be a more powerful vaccine adjuvant in trials with influenza virus X:31 [230] and HBs-antigen [231]. Vaccination of mice with influenza X:31 antigen and PCPP produced Th2-type immunity as evident by IL-4 production. In contrast, mice immunized with the same antigen and PCEP more balanced Th1/Th2-type antigen-specific responses as indicated by the production of IFN-γ and IL-4 cytokines [230].

A mechanism explaining polyphosphazene immunostimulatory activity has been suggested [232]. It is believed that the polymer-antigen combination targets the cell surface receptor of the B lymphocyte. Polyelectrolyte is able to cluster membrane proteins, which consequently results in the enhanced immune responses [232]. Multivalent receptor clustering can activate signaling pathways having an important role in biological activity and induction of immune responses [233]. Biological properties of PCPP are a result of its capacity to form water-soluble noncovalent complexes with vaccine antigens thus permitting their presentation to immune cells [232]. These water-soluble complexes of polyphosphazene and protein allow the polymer to play a role in antigen transporting. This action can also stabilize and protect protein ligands.

Polyphosphazenes have been combined with other adjuvants such as CpG ODN to increase their immunostimulatory properties. In mice, co-administration of PCPP or PCEP with CpG ODN and HBsAg resulted in a substantial increase of HBsAg-specific antibody responses. More significantly, it was observed the formulation of adjuvants changed the type of the immune response (Th1 vs. Th2) [231]. Administration of CpG ODN alone induced largely Th1-type immunity as seen by the superior IgG2a and lower IgG1 antigen-specific antibody production. A vaccine containing PCEP as the only adjuvant induced mixed Th1/Th2-type immune responses suggested by a mixed IgG2a and IgG1 antibody induction. Lastly, vaccination with PCPP alone resulted in the induction of Th2-type immunity, which was associated with production of IgG1 antibodies [231]. Mutwiri et al. confirmed the responses induced by the combination of adjuvants was at least 100-fold higher than responses induced by individual antigens,
which might lead to the conclusion that CpG ODN and PPs might act through separate signalling pathways [234].

In order to increase vaccine efficacy by improving antibody and cell-mediated immunity induction, a novel adjuvant formulation was developed, which is composed of CpG ODN, cationic host defense peptides and polyphosphazenes. The novel adjuvant platform was co-formulated with various vaccine antigens and studied experimentally. For example, when co-formulated with OVA, the mixture of CpG ODN, indolicidin and PP resulted in improved antibody and CMI in mice [235]. The study also indicated the novel adjuvant’s ability to prolong antigen preservation at injection site. Similarly mice immunized with bovine respiratory syncytial virus fusion protein and the combination of adjuvants resulted in development of enhanced humoral and cellular immune responses [222]. Vaccination with OVA co-formulated with CpG, indolicidin and PP increased the production of TNF-α, IL-12, and IL-6 by bone marrow-derived DCs [223]. This vaccine adjuvant formulation co-formulated with HEL was also tested in cattle. The experiments concluded enhanced humoral and cell-mediated immunity. In addition, it was mentioned that the adjuvant combination improved the production of IFN-α, TNF-a and IFN-γ in vitro thus suggesting the ability of the three adjuvants to work synergistically [221]. Most importantly, as described by Gracia et al. this adjuvant combination co-formulated with PTd was tested in neonatal and adult mice. This novel adjuvant induced a superior anti-PTd IgG2a response in both adult and neonatal mice. Moreover, this response was initiated early and, with a long duration [236].

1.4.2 Microparticulate vaccine delivery

Particulate delivery of vaccine antigens is a highly efficacious method for antigen delivery to APC. Microparticles containing antigens can safely deliver the vaccine by being phagocytosed by cells such as macrophages and DCs. An example of such microparticles are poly (D,L-lactic-co-glycolic acid) (PLGA) nanospheres, which were shown to be phagocytosed by human DCs and macrophages in vitro [237]. When inside an APCs, antigen is freed from the microparticles followed by loading onto major histocompatibility complex II (MHC II presentation). Poly-lactide-co-glycolides (PLG) microparticles were demonstrated to be successful adjuvants. Biodegradable PLG
microparticles increased staphylococcal enterotoxin B toxoid levels of neutralizing antibodies following vaccination [238]. Another great characteristic of PLG microparticles as vaccine delivery systems is their controlled release of antigens [239, 240]. Such controlled release of antigen may continue to stimulate the immune system over time thus allowing for fewer doses of the vaccine.

Microparticle-based vaccination has advantages over vaccination with soluble formulations. Vaccine antigen and adjuvant could be more efficiently taken up by APCs and the microparticles can amplify the retention of the vaccine at the site of its administration or the nearby draining lymph node [234]. As a result B and T cell responses may be enhanced because of the persistence of the antigen. Microparticles are also ideal for mucosal vaccination. Antigens delivered via mucosal routes may be cleared by non-specific mechanisms, be degraded by enzymes or perhaps be affected by extreme pH before successfully reaching target cells [241]. Encapsulating the antigen was demonstrated to protect it from being degraded before reaching APCs. Most importantly, hiding the antigen within the particles may decrease the recognition and neutralization of antigen by MatAbs. Microparticles have been co-formulated with adjuvants such as CpG ODN to increase vaccine efficacy [242-244]. This method resulted in the involvement of endosomal TLR-9 followed by cytokine secretion by APCs and antigen cross-presentation [245]. Another well known vaccine adjuvant muramyl dipeptide (MPD), which is a component of bacterial peptidoglycan, when encapsulated into gelatin microspheres was successfully able to activate macrophages [246]. Formulating MDP into microparticles also resulted in an enhanced immune response.

Polyphosphozenes can also be formulated into microparticles via ionic crosslinking in the presence of divalent cations [247]. In fact, as described by Eng at al. the prospective of polyphosphazene usage to deliver vaccine antigens was demonstrated when encapsulation of CpG ODN into these microspheres increased the immune responses when compared to the aqueous formulation [248]. Furthermore, Garlapati et al. showed that encapsulating OVA and CpG ODN into PCPP microparticles resulted in a potent Th1 immune response [249]. These formulations more efficiently enhanced the immune system responses as compared to the soluble formulations. These novel
formulations were also superior to the soluble vaccines at delivering the antigen to the local draining lymph nodes.

Soluble polyphosphazene microparticles can be made into microparticles by coacervation with monovalent salt solutions such as NaCl followed by stabilization by divalent cations such CaCl$_2$ [247]. Using this method, Garlapati et al. encapsulated bovine serum albumin (BSA) and OVA. The technique resulted in spherical microparticles in the range of 0.7 -3 µm in diameter [250]. Furthermore, using fluorescein isothiocyanate (FITC) labelled OVA and Alexafluor-546 labelled CpG ODN (Figure 1.9) we determined the incorporation ranged from 70% for OVA and > 90% for CpG. When lyophilized, the particles can retain their activity for a long time. When re-suspended after storage at room temperature for two months, the particles seemed to preserve their integrity.

(modified from Garlapati et al., Vaccine Dec 6;28(52):8306-14.)
Figure 1.9 Confocal laser scanning microscope images of FITC-OVA/ALEXAFLUOR-546-CpG PCPP microspheres. Top left panel shows green fluorescent FITC-OVA and the top right panel represents red fluorescent CpG-ALEXAFLUOR. The bottom image shows superimposed images.
2. HYPOTHESIS AND OBJECTIVES

2.1 OVERALL GOALS AND RATIONALE

Although vaccination against *B. pertussis* has significantly reduced the incidence of whooping cough, some 20-40 million cases of pertussis still occur worldwide each year, with 90% occurring in the developing parts of the world. Mortality is highest in infants and young children resulting in more than 200,000 deaths every year [17, 22, 251]. Rise in whooping cough cases have recently been observed in developed countries such as Canada and United States [23, 25, 252, 253]. A dramatic increase in disease has been detected in older children and adults, who can transmit the infection to young infants who are most susceptible to the disease. This trend is due in part to waning immunity over time in vaccinated individuals. In Canada a booster immunization is recommended for all adults to limit transmission to their newborns. Thus, more effective vaccines are urgently needed that provide protection at an early age of life and that can overcome the challenges of infant immunization including interference of maternal antibodies (MatAbs) and the infants’ bias towards mounting Th-2-type immune responses following early life vaccination.

The challenges for neonatal vaccines include the “immaturity” of the immune system in early life, particularly its bias for mounting Th2-type [254] responses and most importantly the interference with MatAbs [159-161]. These MatAbs come from previous exposure or vaccination of the mother. MatAbs interfere with infant immunization by neutralizing the vaccines in the case of live-attenuated vaccines and formation of immune complexes between antibodies and vaccine [129, 171]. A number of mechanisms have been suggested to describe the interference between MatAbs and active vaccination of newborns including the neutralization of viral vaccines, phagocytosis of MatAb-coated antigen, reduction of B cell activation by Fcγ-receptor mediated signals and epitope concealment by MatAbs, therefore inhibiting antigen recognition by infant B cells [171]. However, the major determinant of infant antibody responses is found in the maternal antibody: vaccine antigen ratio. Antibody responses may only be obtained when MatAbs have dropped below a particular threshold (ratio of MatAbs to vaccine antigen), and secondary responses may be acquired when this threshold is reached. During pregnancy,
the fetus and the mother show reduced Th1 responses to avoid a fetal-maternal immune attack which could potentially result in a miscarriage [254]. Due to this phenomenon neonates are programmed to mount Th2-like immune responses following early life vaccination. Novel neonatal vaccines against whooping cough which can overcome these challenges are urgently needed.

Maternal immunization has recently been identified as another means of preventing neonatal pertussis [120]. It is believed that it would be beneficial to immunize pregnant women with the acellular pertussis vaccine, which would offer greater levels of passive immunity to their newborns [122]. Identifying maternal immunization as a method to prevent neonatal pertussis poses another need for neonatal vaccines, which will be able to elicit protection in the presence of high levels of MatAbs. Novel neonatal vaccines against whooping cough are urgently needed. Such vaccines would offer long time protection, work in the presence of MatAbs and be able to induce mixed Th1/Th2 immune responses needed for protection against whooping cough. We are developing such novel vaccine formulations containing PTd, FHA and novel immunomodulators such as CpG ODN, IDRP and PP.

Our overall goal of this research project was to develop novel vaccine formulations against whooping cough, which can induce long lasting immune responses in the presence or absence of MatAbs in newborn infants following early life vaccination. The rationale of this project was to assess novel pertussis vaccine formulations based on a combination of PTd and FHA and a novel adjuvant platform in the presence of MatAbs.

2.2 HYPOTHESIS

Vaccine containing PP delivery vehicles with PTd and FHA and a combination of CpG ODN and IDRP will elicit long-lasting protection in neonates when administered in the presence of B. pertussis-specific MatAbs.

2.3 OBJECTIVES

Our main objective was to develop novel vaccine formulation against whooping cough and assess these formulations in the presence of MatAbs.
The following four aims were designed to achieve this goal:

**Aim 1. Assess maternal antibody interference when vaccinating with vaccine antigen alone.**
To test and prove that MatAb interference occurs when vaccinating both mothers and offspring with the vaccine antigen alone.

**Aim 2. Evaluate different strategies of maternal immunization and assess the levels of MatAbs in mice and piglets.**
To titrate the levels of MatAbs and study the effects of high and low concentrations of MatAbs on infant immune responses.

**Aim 3. Test novel vaccine formulations in the presence of MatAbs in mice and piglets.**
To test various novel vaccine formulations in the absence and presence of various amounts of MatAbs.

**Aim 4. Assess protection against challenge infection with *B. pertussis*.**
To test the efficacy of the potential whooping cough vaccine candidate against bacterial challenge infection in the presence and absence of MatAbs.
3. INFLUENCE OF MATERNAL ANTIBODIES ON ACTIVE PERTUSSIS TOXOID IMMUNIZATION OF NEONATAL MICE AND PIGLETS

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3.1 INTRODUCTION

_B. pertussis_, the causative agent of pertussis (whooping cough), is a Gram-negative bacillus. This serious respiratory infection is easily transmitted by close contact, mainly through droplets [16]. Although vaccination with whole cell and acellular vaccines has significantly reduced the incidence of the disease, some 20–40 million cases of pertussis occur globally each year, 90% of which are found in developing countries. About 200 000–400 000 of those infected, mostly infants, die from the disease [17] [251]. Numerous countries, especially developing nations, have reported an increase in infant morbidity due to pertussis [5]. A rise in pertussis cases has also been observed in developed countries such as the United States [253] [25] and Canada [23]. Recently there has been a dramatic increase in disease in older children and adults [25] [123] [255] [256], who can transmit the infection to young infants who are most susceptible to the disease [257]. This trend is due, in part, to waning immunity over time in vaccinated individuals [258] [12] [259].

Whooping cough is most severe in neonates who are too young to have been immunized and in infants who have not been completely immunized [260] [11]. Most deaths occur in the first 3 months of life [151], before administration of the first dose of the pertussis vaccine [151], which is given at two months of age. In most European and North American countries, pertussis vaccines are currently administered at two, four and six months of age, at a time when MatAbs (MatAbs) are declining [123] [125] [261].

Multiple doses of the vaccine are needed for protection as MatAbs pose an important challenge for infant vaccination [159] [160]. MatAbs result from the mother’s previous exposure to disease or vaccination and are transferred from mother to infant transplacentally or through thecolostrum and breast milk. The most efficiently transferred immunoglobulin subclass is IgG1 [151]. Passive immunity provides short-lived protection against disease, but also interferes with vaccination of neonates [161].
Thus, passive immunity is a double-edged sword; it protects the young from disease, but also interferes with immunization of the infant. For example, important inhibitory effects on infant antibody responses have been observed following immunization with vaccines against measles [162] [152], *Haemophilus influenzae* type b conjugate vaccine [163], hepatitis A [164], influenza A [165], tetanus [166], and varicella-zoster [160].

Numerous studies have addressed the issue of MatAbs following whooping cough vaccination [167] [168] [169] [170]. Transplacental MatAbs to pertussis antigens can interfere with the infant’s ability to mount antibody responses following immunization with either the whole cell (DTwP) or acellular (DTaP) pertussis pediatric vaccines [169] [168] [171] [128] [172]. MatAbs that possess such inhibitory effects are thought to be a result of the formation of immune complexes between antibodies and antigen and include the neutralization of antigen, phagocytosis of MatAb-coated antigen, inhibition of B cell activation by Fcγ-receptor mediated signals, and epitope masking by MatAbs thus preventing antigen binding to infant B cells [171] [129]. However, the major determinant of infant antibody responses is found in the ratio of the antigen concentration to the MatAb concentration. Antibody responses may only be obtained when MatAbs have fallen below a specific threshold, and secondary responses may be elicited as soon as this threshold is reached [171] [129] [152]. MatAbs influence infant B cell responses but leave T cell responses unaffected [171] [173] [128]. In the presence of MatAbs, the neonate’s antibody response to the specific antigen is repressed. Consequently, successful vaccines against pertussis must be able to overcome the interference of MatAbs.

Mice are the most commonly used animal model for studying pertussis [106] [104, 109]. Murine immunization followed by a respiratory challenge has been used to evaluate efficacy of diphtheria-tetanus-pertussis acellular (DTaP) and whole cell (DTwP) vaccines [86, 110]. When infected with *B. pertussis*, neonatal mice show symptoms such as weight loss and hypoglycemia [105]. Furthermore, the mouse model is not ideal as it is limited in which samples can be collected. We developed a new pertussis disease model in newborn piglets [111, 113]. Pigs and humans share many physiological characteristics [114], including specific functions of the innate and adaptive immune response. The model also provides access to various immune compartments and samples.
such as serum, colostrum, milk and bronchoalveolar lavage (BAL) fluid. Large amounts of IgG and IgA antibodies are transferred through porcine colostrum and milk, which allows us to study the effects of MatAbs. In humans and rodents, maternal IgG is transferred through the placenta [115]. Since immunoglobulins cannot cross the placenta in pregnant sows [116] [117], we are able to exchange piglets between sows to eliminate the mother effect.

In the present study two animal species were used to examine the effect of MatAb on infant pertussis toxoid (PTd) vaccination. We investigated the interference of MatAbs on PTd vaccination as well as the role of a booster dose and adjuvants as a means of trying to overcome such interference. Pregnant mice and sows were vaccinated with PTd and the offspring were immunized within the first week of life either in the presence or absence of MatAbs. While interference with vaccination was observed after a single immunization with PTd, co-formulation with adjuvants and a booster immunization was able to overcome this interference and resulted in the successful immunization of both neonatal mice and pigs. The level of interference was dependent on the immunization regimen for the mother and directly correlated with the amount of MatAbs present at the time of neonatal vaccination.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Male and female BALB/c mice were purchased from Charles River (Montreal, Quebec, Canada). All female mice were housed in separate cages. Breeding cages were checked on a daily basis and births were recorded. Offspring were kept with their mothers until weaned at about four weeks. At that time, the pups were separated according to sex.

Sows were pre-screened for cross-reactive antibodies to *B. bronchiseptica*. Pregnant Laundrace sows were purchased from the Saskatoon Prairie Swine Centre, University of Saskatchewan. The animals were stimulated to farrow by intramuscular (i.m) injection of 2 ml prostaglandin (Planate; Schering-Plough Canada Inc., Pointe-Claire, Quebec, Canada) at 113th day of gestation and another 1 ml at 114th day. Piglets
were born on day 114 and 115 of gestation. Nursing piglets were kept in the same isolation rooms in separate pens. All experiments were performed according to the guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

3.2.2 Vaccination of mice and pigs

Both mice and sows and their offspring were immunized with genetically detoxified pertussis toxoid (PTd) kindly provided by Novartis Vaccines and Diagnostics (Siena, Italy). This antigen was shown to be completely safe with an antigenic profile comparable to wild-type PT, and an immunogenicity that is greater than chemically detoxified PTd [82]. Dams were immunized one week prior to becoming pregnant with a subcutaneous injection of 1 µg PTd in Phosphate Buffered Saline (PBS, pH=7.2, 1.54 mM KH₂PO₄, 155.17 mM NaCl, 2.71 mM Na₂HPO₄·7 H₂O) (Gibco). One week into the pregnancy (two weeks before delivery) dams were boosted in the same manner. The control animals were subcutaneously treated with 100 µl PBS prior to pregnancy. Pregnant sows were vaccinated intramuscularly on each side of the neck (trapezius muscle) behind the ear with 5 µg of PTd in 1 ml of PBS and 30 % Emulsigen (MVP Laboratories, Ralston, NE; oil-in-water emulsion) resulting in a total dose of 10 µg/per sow. The control sow received the same volume of PBS and Emulsigen in each side of the neck. Neonatal mice were randomly assigned and vaccinated at 7 days of age. At that time, the development of the immune system is comparable to that of a newborn human [262, 263]. Pups were either vaccinated with 1 µg of PTd diluted in PBS or were injected with PBS. All vaccinations were 50 µl in volume and injected subcutaneously. Neonatal piglets were randomly assigned and immunized at 3-5 days of age with 10 µg PTd plus 150 µg CpG ODN 10101 (Pfizer Canada, Kirkland, Quebec) in 1 ml of PBS or treated with 1 ml of PBS. All piglets were vaccinated i.m. in the side of the neck.

3.2.3. Sample collection

Blood samples were collected from mouse dams before vaccination and at the time of vaccination of their pups. In order to evaluate immunity in the offspring following vaccination, serum samples of neonatal pups were collected 2, 4, 6 and 8
weeks post vaccination. All blood samples were centrifuged (4547 x g) and serum stored at –20 °C.

Sows were bled before priming, boosting and after farrowing. Sow colostrum and milk samples were collected. Rennet tablets (Sigma-Aldrich) were added to samples and incubated overnight at 37 °C. Once clots were formed, the samples were centrifuged at 1349 g for 20 minutes. Centrifugation resulted in the formation of three layers. The middle layer (whey) was removed and stored in –20 °C. Newborn piglets were bled before vaccination as well as 1, 2, 3 and 4 weeks past vaccination.

3.2.4. ELISA

Polystyrene microtiter plates were coated with 0.25 µg/ml (100 µl per well) PTd for analysis of murine serum samples. The antigen was diluted in coating buffer (sodium carbonate buffer, (15 mM Na₂CO₃ and 35 mM NaHCO₃, pH 9.6). The plates were incubated overnight at 4 °C. Sera were diluted four-fold, starting with 1:100 dilution. Biotin-conjugated goat anti-mouse immunoglobulin G (IgG; 1:10,000 dilution; Invitrogen, Camarillo, CA) was used for the detection of PTd specific IgG. Detection was carried out by addition of streptavidin peroxidase (1:500 dilution; Jackson Laboratories). Serum samples and antibodies were diluted in TBST gelatin (Tris Buffered Saline pH 7.3) containing 0.5% Tween and 0.5 % gelatin (Sigma). The reaction was visualized by p-nitrophenylphosphate. No anti-PTd IgA was detected in pup serum.

Polystyrene microtiter plates were coated with 0.5 µg/ml (100 µl per well) PTd for analysis of porcine serum and colostrum samples. The antigen was diluted in coating buffer. The plates were incubated overnight at 4 °C. The plates were washed and blocked with TBST gelatin. Sera, colostrum and milk were diluted four-fold, starting with 1:40 dilution. Alkaline-phosphatase-conjugated goat anti-pig immunoglobulin G (IgG; 1:5,000 dilution; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used for the detection of PTd specific IgG. The reaction was visualized by p-nitrophenylphosphate (Sigma-Aldrich). The plates were read at 450 nm with an iMark Microplate Reader (Bio-Rad Laboratories). PTd-specific antibody titers were calculated by Microplate Manager 6.0
(Bio-Rad Laboratories). To assess *Bordetella bronchiseptica* specific antibodies, polystyrene microtiter plates (Immulon 2HB; Dynex Technologies, Chantilly, VA) were coated with 2 µg/ml (100 µl per well) sonicated, heat-inactivated *B. bronchiseptica*. The ELISA was performed as described above.

3.2.5. Statistical analysis

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

3.3 RESULTS

3.3.1 Interference with MatAbs in mice

In order to establish the model and ensure that high and low levels of MatAbs could be induced, dams were divided into three groups. Six dams were primed and boosted during pregnancy with 100 µl of PTd (1µg/animal). Another set of dams (n=6) were vaccinated subcutaneously only once during pregnancy. Six control dams were injected subcutaneously with 100 µl PBS four weeks before delivery. Serum was collected prior to priming, at the time pups were born, and when pups were weaned at four weeks of age. At four weeks post immunization, primed and boosted dams developed 6-8 fold higher anti-PTd IgG titers compared to primed only dams. Control dams did not generate any anti-PTd IgG antibodies (Fig. 3.1). To confirm that maternal anti-PTd antibodies from vaccinated mouse dams were transferred to their offspring, control pups were bled and evaluated for the levels of antibodies against PTd. Throughout the experiment, the levels of anti-PTd IgG antibodies were significantly
higher in pups born of primed and boosted mothers (Fig. 3.2A) compared to offspring born of only primed mothers (Fig. 3.2B). No antibodies were detected in pups born of PBS vaccinated dams (Fig. 3.2C). Pups of control dams responded well to PTd vaccination and showed a ten-fold increase in anti-PTd IgG levels between 4 and 8 weeks post vaccination (Fig. 3.2C). In contrast, anti-PTd IgG antibody concentrations remained at the same or lower level in vaccinated pups born of high and low titer dams, indicating an impairment of the immune response in those pups (Fig. 3.2A and 3.2B). No significant differences in the level of antibodies were observed between vaccinated and non-vaccinated pups. Thirteen out of 16 pups born of low titer dams (Fig. 3.2B) and all of the pups born of high titer dams did not respond to a single PTd vaccination between four and eight weeks post immunization (Fig. 3.2A). Anti-PTd IgA antibodies were not detected in dam or pup serum.
Figure 3.1. Induction of IgG antibodies in the serum of dams. Dams were subcutaneously primed and boosted with 1 µg PTd (●), primed with 1 µg PTd (★) or PBS (▲). Dams (six per group) were primed four weeks prior to delivery and one group (●) boosted two weeks later. Serum samples were collected prior to prime (week 0), at the time pups were born (week 4) as well as at the time the pups were weaned (week 8). Serum samples were analysed using an ELISA assay. Data sets with differing subscripts indicate statistical difference.(p < 0.05).
Figure 3.2 Induction of IgG antibodies in the serum of mouse pups. Neonatal pups were subcutaneously primed with 1 µg PTd (●) or PBS (★). Pups were born to three groups of mothers: primed and boosted with 1 µg PTd (A), primed with 1 µg PTd (B) or PBS vaccinated (C). Pups were primed at seven days of age. Serum samples were collected 2, 4, 6 and 8 weeks post vaccination and analysed using an ELISA assay (p < 0.05).

3.3.2 High and low MatAb titers interfere with PTd vaccination of neonatal mice

To determine the effect of the total level of MatAbs on infant vaccination, dams were primed and boosted with either 0.05 or 1 µg of PTd or PBS. At seven days of age, half of the pups from each mother were vaccinated with 1 µg of PTd and the other half treated with PBS. Immunization with 0.05 and 1 µg PTd resulted in induction of anti-PTd antibodies in dams (Fig. 3.3). Anti-PTd IgG titers in pups born to 0.05 µg PTd vaccinated dams (Fig. 3.3B) were about twenty-fold lower than PTd-specific antibodies in pups born
of 1 µg PTd vaccinated dams (Fig. 3.3A). There were no anti-PTd antibodies transferred to pups born of PBS vaccinated dams (Fig. 3.3C). However, in the presence of lower titers of MatAbs, three out of seven pups seem to respond to PTd vaccination where none of the pups born of high titer dams respond. The results indicate that the level of interference directly correlated with the level of MatAbs.

Figure 3.3. Induction of IgG antibodies in the serum of mouse pups. Neonatal pups were subcutaneously primed with 1 µg PTd (●) or PBS (★). Pups were born to three groups of mothers: primed and boosted with 1 µg PTd (A), primed and boosted with 0.05 µg PTd (B) and PBS vaccinated (C). Pups were primed at seven days of age. Serum samples were collected 3, 4, 6 and 8 weeks post vaccination and analysed using an ELISA assay (p < 0.05).
3.3.3 Single vaccination with high and low amounts of PTd cannot overcome interference of MatAbs

To test whether vaccination of the neonate with different concentrations of PTd can overcome the interference with MatAbs, pups were vaccinated with various amounts of PTd. Dams were immunized with PTd or PBS. Half of the pups from each dam were subcutaneously vaccinated with 50 µl of either: 0.05 µg PTd, 0.1 µg PTd or 0.5 µg of PTd and other half with PBS (50 µl) at seven days of age. The pups were bled at four different times: 2, 4, 6 and 8 weeks post vaccination for ELISA analysis. PTd-specific antibodies were detected in serum of vaccinated and control mouse pups. There were no anti-PTd antibodies (IgG) detected in pups vaccinated with 0.05 and 0.1 µg of PTd (data not shown). Vaccination of pups with 0.5 of PTd resulted in production of anti-PTd IgG antibodies four weeks following immunization (Fig. 3.4B). Using 1 µg of PTd for vaccination of dams and 0.5 µg for pups (Fig. 3.4A) there were no differences in anti-PTd titers in vaccinated pups as compared to non-vaccinated pups (both groups born of vaccinated mothers).

Figure 3.4 Induction of IgG antibodies in the serum of mouse pups. Neonatal pups were subcutaneously primed with PTd (●) or PBS (★). Pups were primed with 0.5 µg PTd (A and B). Pups were born to two groups of mothers: PBS vaccinated (B) and primed with 1 µg PTd (A). Pups were primed at seven days of age. Serum samples were collected 2, 4, 6 and 8 weeks post vaccination and analysed using an ELISA assay. (p < 0.05).
3.3.4 A booster immunization can overcome MatAb interference in the presence of low titers of MatAbs but not in the presence of high titers of MatAbs

Previous experiments showed that vaccination of neonatal mice with 1 µg of PTd without adjuvants was not able to overcome the interference of MatAbs. We introduced a booster shot of PTd to see if this could overcome this interference. Neonatal pups born of high MatAb titer dams and low MatAb titer dams were either immunized once or twice. Control pups were injected with PBS. Pups were bled 3, 4, 6 and 8 weeks post vaccination.

Passive transfer of antibodies to pups born to vaccinated dams (Fig. 3.5B) was about 10-fold lower than the passive transfer of PTd specific antibodies to pups born of vaccinated and boosted dams (Fig. 3.5A). There were no anti-PTd antibodies transferred to pups born of PBS vaccinated dams (Fig. 3.5C). Waning of MatAbs was evident in both groups at eight weeks at which time the levels of maternal anti-PTd antibodies were still high in offspring born to high titer dams. Vaccinating and boosting pups born to naïve mothers resulted in superior antibody production one week after the boost. All of the six animals had anti-PTd antibodies following the boost (Fig. 3.5C). It was evident that a boost immunization of pups was not able to overcome interference of high titers of MatAbs. Twice immunized pups had similar anti-PTd IgG serum concentration as compared to PBS vaccinated pups (Fig. 3.5A). Booster immunization of pups with PTd was able to overcome interference in the presence of lower concentrations of MatAbs (single vaccinated dams). Following the boost, anti-PTd antibody titers were about 100-fold higher than in single vaccinated pups or control pups (Fig. 3.5B).
Figure 3.5 Induction of IgG antibodies in the serum of mouse pups. Neonatal pups were subcutaneously primed and boosted with 1 µg PTd (●), primed with 1 µg PTd (★) or PBS (▲). Pups were born to three groups of mothers: primed and boosted with 1 µg PTd (A), primed with 1 µg PTd (B) and PBS vaccinated (C). Pups were primed at seven days and boosted at thirty one days of age. Serum samples were collected 3, 4, 6 and 8 weeks post vaccination and analysed using an ELISA assay. (p < 0.05).

3.3.5 Priming occurred in the presence of MatAbs

To test if it would be possible to prime an immune response in the presence of MatAbs, neonatal pups were vaccinated in the presence of MatAbs and boosted once MatAbs waned. Dams were vaccinated once or twice with PTd or PBS during pregnancy. At seven days of age, a third of the pups of each dam were subcutaneously vaccinated with 1 µg of PTd and a third of all pups with PBS. Throughout the experiment pup serum was analysed for the presence of maternal anti-PTd antibodies. Once MatAbs were no longer detectable in pup serum (16 weeks), animals were boosted. Pups not primed as neonates were vaccinated for the first time once MatAbs were no longer detectable. Pups
born to naïve dams had high anti-PTd IgG titers following a single vaccination. Antibody titers increased significantly following the boost (Fig. 3.6C), as expected for a secondary response. Similarly, titers in pups born of vaccinated dams and boosted at 16 weeks of age significantly increased following the boost (Fig. 3.6A and Fig. 3.6B). Primed and boosted pups (Fig. 3.6A) had much higher antibody titers at 18 and 20 weeks of age than singly vaccinated pups (Fig. 3.6B), demonstrating that this was a result of a secondary response.

Figure 3.6 Induction of IgG antibodies in the serum of mouse pups. Neonatal pups were subcutaneously primed as neonates and boosted with 1 µg PTd once MatAbs waned (●), primed with 1 µg PTd once MatAbs waned (★) or PBS (▲). Pups were born to three groups of mothers: primed and boosted with 1 µg PTd (A), primed with 1 µg PTd (B) and PBS vaccinated (C). Pups were primed at seven days and boosted at thirty one days of age. Serum samples were collected every two weeks. Pre boost, 16, 18 and 20 weeks post boost bleeds were analysed using an ELISA assay (p < 0.05).
3.3.6 Porcine model of MatAb interference

To study MatAb interference in pigs, sows were vaccinated intramuscularly with PTd (10 µg) and emulsigen four weeks prior to farrowing, and boosted after two weeks. The control sow was treated at the same time points with the same volume of PBS. Sow serum samples were taken before each vaccination and before farrowing. After farrowing, sow colostrum and milk samples were collected and analysed for anti-PTd IgG antibodies. PTd-specific antibodies were detected in serum and colostrum of vaccinated sows (Fig. 3.7). These MatAbs were passively transferred to the offspring (Fig. 3.8A).

![Figure 3.7 Anti-PTd IgG serum and colostrum levels in PTd vaccinated and control sow.](image)

Serum was collected at day -28, which represents pre bleed/day of priming and day -14, which represents pre boost. Colostrum samples were collected at day zero, which is the day piglets were born and day four at which time the piglets were vaccinated.
To assess interference with transferred MatAbs half of the piglets of each sow were intramuscularly vaccinated at three days of age with 10 µg of PTd and 150 µg CpG 10101. Control pigs received PBS. All vaccinated animals were boosted with 10 µg of PTd and 150 µg CpG 10101 at two weeks of age. As shown in figure 8, immunization and boosting with PTd and CpG ODN induced anti-PTd specific antibodies in the presence of MatAbs (Fig. 3.8A). Thus, use of an adjuvant and a second booster immunization was able to overcome interference with MatAbs. Primed and boosted piglets had about twelve-fold higher anti-PTd serum antibody titers than their PBS vaccinated littermates.

**Figure 3.8 Induction of IgG antibodies in the serum of piglets.** Neonatal piglets were intramuscularly primed and boosted with 10 µg PTd plus 150 µg CpG 10101 (●) or PBS (★). Piglets were born to two groups of sows: primed and boosted with 10 µg PTd (A) and PBS vaccinated (B). Piglets were primed at four days of age and boosted two weeks after. Serum samples were collected pre vaccination, 1, 2, 3 and 4 weeks post vaccination and analysed using an ELISA assay (p < 0.05).

### 3.4 DISCUSSION

The overall goal of our research program is to develop novel vaccine formulations against pertussis that can induce protection in newborn infants following early life vaccination. The vaccine should elicit protection even when administered in the presence
of MatAbs [128, 168, 169, 171]. Thus, it was necessary to dissect the role of MatAbs in interference with the neonatal response to active immunization.

Pertussis vaccines have been tested previously in the presence of MatAbs. In these studies, it was shown that the aP vaccines performed better than the wP vaccine [126, 167, 264]. These findings were confirmed by studies in which infants with high levels of MatAbs mounted lower levels of anti-PT IgG antibodies after whole cell vaccination than infants with lower levels of MatAbs [126, 167-169, 172]. Thus, it was speculated that aP vaccines might be less affected by MatAbs than wP vaccines following early life vaccination [126, 167, 172, 264]. This might be because of the higher concentration of pertussis-specific antigens in the pediatric aP vaccine than in the wP vaccine relative to the concentration of MatAbs [171, 261]. It was also postulated that MatAbs induced by wP vaccination of the mother might have less specificity for the five recombinant antigens in the aP vaccine [79, 82, 120, 171, 265]. This was confirmed by the observation that most new mothers would have received aP vaccine as a series of their childhood vaccinations. Here, we demonstrated that MatAbs produced by a monovalent aP vaccine interfere with neonatal aP pertussis vaccination. We also showed the importance of the ratio of MatAb to the vaccine antigen. Responses in the neonate may only be elicited when MatAbs have fallen below a specific threshold, which is defined by MatAb: vaccine antigen ratio.

Most pertussis MatAb studies in mice have been performed with multiple aP and wP antigens [127, 266]. Here, we assessed MatAb interference with a single antigen only. Dams were immunized during gestation with varying amounts of the antigen, resulting in high or low levels of MatAbs. Mothers were either immunized once or twice with PTd to increase the levels of passively transferred antibodies, which directly correlated to the amount of antibodies in dam serum. Vaccination of pups confirmed that the induction of specific antibodies in the offspring directly correlated to the level of passively transferred MatAbs. Interference was observed with doses as low as 0.05 and 1 µg of PTd for vaccination of mothers and 1 µg for pups. Single PTd vaccination did not provide enough antigen to skew the MatAb: vaccine antigen ratio [129] [171]. Similarly, immunization with 0.5 and 1 µg PTd for pups resulted in anti-PTd titers similar to those seen in non-
vaccinated pups. We next tested whether booster vaccination in pups can overcome the interference with MatAbs in the presence of high and low levels of MatAbs. Priming and boosting pups with 1 µg of PTd did not overcome interference in the presence of high titers of MatAbs. However, this strategy was able to overcome interference in the presence of lower concentrations of MatAbs (single vaccinated dams). Our results are not surprising as the major determinant of infant antibody responses is controlled by the ratio of MatAb levels to the concentration of vaccine antigen [129, 171]. Importantly, we showed that it is possible to prime in the presence of MatAbs. Mice primed as neonates had higher anti-PTd IgG titers following a second immunization at 16 weeks of age than their littermates who were immunized once as adults. We did not detect any anti-PTd IgA antibodies in the dam and pup serum. This might be due to the systemic route of vaccination of dams.

In order to enhance anti-PTd antibody induction in piglets, we included an adjuvant in our vaccine formulation and added a booster shot. At three days of age, half of the piglets per sow were vaccinated and boosted with 10 µg of PTd and 150 µg CpG 10101 and another half with PBS. Use of 10 µg PTd and CpG 10101 as an adjuvant for priming and boosting piglets was able to overcome interference by MatAbs.

The current study was performed to examine the interaction between PTd and MatAbs. We established the negative impact of MatAbs and their interference on neonatal murine and porcine vaccination. We determined that including an adjuvant in our vaccine formulations or adding a booster vaccine can overcome the negative effects of passive immunity. Our next goal is to use novel adjuvants such as CpG ODN, innate defense regulator peptides and PTd in our vaccine formulations as well as new delivery mechanisms that could potentially protect the vaccine antigen from neutralizing MatAbs. In subsequent experiments we plan to measure the priming of the immune response in neonates in the presence of MatAbs and the degree of protection against challenge infection.
4. NOVEL ADJUVANT COMBINATION CO-FORMULATED WITH OVA INTO MICROPARTICLE AND SOLUBLE VACCINES INDUCES EARLIER AND TH1/TH2 BALANCED IMMUNITY IN NEONATAL MICE

4.1. INTRODUCTION

Adjuvants are incorporated into vaccines in order to increase vaccine immunogenicity. They were originally used to increase the magnitude of antibody production. Most recent adjuvants are designed to tailor the immune response, which is needed for protection. Adjuvants can be divided in two classes: antigen delivery and immunostimulators. Vaccine delivery adjuvants act by targeting adjuvants to antigen presenting cells. These adjuvants may also protect the antigen from being degraded as well as encourage the depot effect, which is thought to enhance antigen uptake. Thus, adjuvant usage provides the link between innate and adaptive immune systems.

Originally, adjuvants were used in order to increase the extent of the immune response to vaccines, which was measured by antibody production or protection against infection. As the understanding of the immune system and vaccination became more complete, adjuvants were starting to be designed in order to provide better adaptive responses ie. to produce a good quality immune response. For example: to induce Th1-type or Th2-type immunity. Adjuvants do not only allow for generation of T cell memory, but also modify the specificity and affinity of the response [175]. Thus, adjuvants are used to improve both the quality and quantity of immune responses elicited by vaccines. Adjuvants allow for seroconversion in populations with decreased ability to respond to vaccines such as neonates and elderly [175]. Recently, a novel approach to vaccination has been based on the usage of combination of adjuvants. Combining various TLR (Toll like receptor) agonists or delivery systems with immunostimulatory adjuvants have shown a synergistic effect in vaccination [234].

The major focus of our research is the development of novel vaccine formulations and delivery systems for vaccinating neonates. Vaccination of young infants is complicated due to two factors: interference of MatAbs (MatAbs) and the “immaturity” of the infant’s immune system, mainly its bias towards mounting T helper 2 (Th2)-type
responses [129]. We took advantage of the immunostimulatory and the antigen delivery properties of adjuvants and developed a novel adjuvant platform to help overcome the difficulties associated with neonatal vaccination. The adjuvant combination is composed of novel adjuvants including CpG ODN, IDRP and PP.

CpG ODNs are TLR-9 agonists. CpG ODN motifs are synthetic unmethylated peptides containing repeating sequences of cytosine and guanosine. CpG ODN mimics microbial DNA resulting in the induction of Th-1 responses and upregulation of molecules involved in antigen presentation and co-stimulation [267, 268]. The second component of the adjuvant platform, IDRPs are synthetic host defense peptides, which are involved in inflammation, proliferation, healing of wounds, cytokine release and chemotaxis [201]. These cationic peptides (net positive charge +2 to +9) are also able to increase phagocytosis, neutralize the septic effects produced by LPS as well as sustain the recruitment of a number of immune cells to the site of inflammation [202, 203].

The final adjuvant component, PPs are a class of synthetic, water-soluble, biodegradable polymers composed of a backbone with alternating phosphorus and nitrogen atoms with organic side groups attached to each phosphorus [224]. PTd are powerful immunostimulants and demonstrated to have a strong adjuvant property to a variety of viral and bacterial antigens including influenza [227], rotavirus [228] and cholera [229]. One of the greatest features of this adjuvant, however, is the fact that it can be formulated into microparticles using easy and inexpensive methods [269]. Encapsulating the vaccine antigen and adjuvants may hide the components from vaccine neutralizing MatAbs and safely deliver it to neonatal antigen presenting cells. The novel adjuvant formulation might also enhance the Th1-type response shift following administration.

In the current study, we formulated OVA with CpG ODN, IDRP and poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) PP into soluble and microparticle vaccines. We titrated the adjuvant dose in neonatal mice and studied the effect of various routes of delivery such as subcutaneous (s.c.), intramuscular (i.m) and intranasal (i.n.) on neonatal immune responses. Following vaccination, the pup sera were analyzed for the concentration and kinetics of OVA-specific antibodies produced.
4.2. MATERIALS AND METHODS

4.2.1 Animals

Male and female BALB/c mice were purchased from Charles River (Montreal, Quebec, Canada). Animals were bred, and taken care of by the Animal Care staff at the Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan (Saskatoon, SK). Breeding cages were set up and each dam was housed in a separate cage. The cages were checked daily for new births. Offspring were kept with their mothers until weaned at about four weeks of life at which time the pups were separated according to sex. Animals were maintained in compliance with the ethical guidelines if the University of Saskatchewan as well as the Canadian Council for Animal Care.

4.2.2 Vaccine components

OVA Grade V (Sigma, USA) was used as a model antigen. CpG-ODN 10101 (TCGTCGTTTTCGCGCGCGCGCCG) was obtained from Pfizer (Ottawa, ON). IDRP 1002 (VQRWLIVWRIRK) was manufactured by Genscript (Picataway, NJ). The polyphosphazene PCEP was synthesized by the Idaho National Laboratory (Idaho Falls, ID) by a method previously described [230] including minor modifications. Lyophilized PTd were dissolved in Dulbecco’s PBS (pH 7.4; Sigma-Aldrich, MO) to a concentration of 3 mg/ml and stored in the dark at room temperature.

4.2.3 Vaccine formulation

All adjuvant formulations consisted of PCPP, IDRP 1002 and CpG ODN at a ratio of 1:2:1. Standard formulations consisted of 2.37 µg CpG ODN 10101, 4.74 µg IDRP 1002 and 2.37 µg of PCEP (EP3). In an adjuvant dose titration experiment the standard dose was referred to as the High dose. The Medium dose consisted of half of the normal dose: 1.19 µg CpG ODN 10101, 2.37 µg IDRP 1002 and 1.19 µg of PCEP. The low dose of the adjuvant combination was composed of: 0.6 µg CpG ODN 10101, 1.19 µg IDRP 1002 and 0.6 µg of PCEP.

The negatively charged CpG ODN was first complexed with cationic IDRP in a ratio of 1:2 (w/w) for 30 minutes at 37°C. Following incubation the rest of the components were added such as OVA and PCPP. This procedure yielded the soluble
(SOL) vaccine formulation. Microparticle (MP) formulations were obtained by addition of PCPP and 6.2% NaCl to the complexed CpG ODN and IDRP. The mixture was incubated at room temperature for 20 minutes after which 8.8% CaCl$_2$ was added and incubated for 20 minutes on a nutator. Microparticles were then centrifuged at 1340 g for 10 minutes at room temperature. Before collection, the particles were washed once with ddH$_2$O and centrifuged once again.

### 4.2.4 Immunization of mice

Neonatal BALB/c mice were primed at two weeks of life and boosted four weeks later. Various routes of immunization were used to prime and boost the animals. Routes of immunization included: s.c., which resulted in an 50 µl injection between the shoulder blades, i.m. 12.5 µl into each quadriiceps muscle and i.n., which delivered 12.5 µl into each nostril. Blood samples were taken from neonatal mice at 2, 4, 6 and 8 weeks post primary vaccination. All blood samples were centrifuged (4547 x g) and serum stored at –20 °C.

### 4.2.5 OVA-specific antibody detection by an ELISA

Polystyrene microtiter plates (Immulon 2 HB; Thermo, Milford, MA) were coated overnight at 4 °C with 10 µg/ml (100 µl per well) OVA for analysis of murine serum samples. The antigen was diluted in coating buffer (15 mM Na$_2$CO$_3$ and 35 mM NaHCO$_3$ pH 9.6). The plates were washed between each step with Tris-Buffered Saline (pH 7.3) containing 0.5% Tween (TBST). Sera and detection antibodies were diluted in TBST containing 0.5% gelatin (TBSTg). Sera were diluted four-fold, starting with 1:100 dilution. For the detection of antigen-specific IgA, sera were diluted starting with 1:4 dilution. Biotin-conjugated goat anti-mouse immunoglobulin G, G1, G2a and A (1:10,000 dilution; Invitrogen, Camarillo, CA) was used for the detection of OVA-specific antibodies. The plates were incubated for one hour at 37°C. Following the incubation, the plates were washed and alkaline phosphatase conjugated with streptavidin (Jackson ImmunoResearch; West Grove, PA) diluted 1:5000 was added and incubated for another hour. The plates were then washed five times and p-nitrophenylphosphate (PNPP) (Sigma-Aldrich, St. Louis, MO; dilution 1 mg/ml) added to each well. The
reaction was allowed to develop until yellow color appeared. The plates were read using the microplate reader (Bio-Rad iMark Microplate Reader; Philadelphia, PA) at 405 nm with a reference of 490 nm.

4.2.6 Statistical analysis

Because data was not normally distributed it was rank transformed followed by analysis by one-way analysis of variance (ANOVA). In 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. Statistical analysis was carried out using GraphPad Prism software, version 5.0b.

4.3 RESULTS

4.3.1 Novel adjuvant combination microparticle vaccine significantly increases antibody titers compared to vaccinating with antigen alone

In order to assess the adjuvanticity of the novel adjuvant combination, neonatal mice were vaccinated with antigen alone as well as three microparticle vaccines composed of OVA and various concentrations of adjuvants. Neonatal mice were primed at two weeks of life and boosted four weeks later. The vaccines were delivered by two routes: intramuscular and intranasal. The mice were bled throughout the experiment and their serum analysed for various antibody isotypes using an ELISA assay.

The results revealed that both OVA alone and OVA adjuvanted with the novel CpG ODN and IDRP formulated into polyphosphazene microparticle vaccines induced high anti-OVA IgG (Fig. 4.1A) and IgG1 (Fig. 4.2A) antibody titers following intramuscular vaccination. The adjuvanted vaccines delivered intramuscularly induced an earlier onset of immunity and resulted in all animals responding to the immunization after a single dose of the vaccine unlike vaccinating with antigen alone (Fig. 4.1A and Fig. 4.2A). Mice vaccinated intranasally were able to induce earlier and higher IgG (Fig. 4.1B) and IgG1 (Fig. 4.2B) antibody concentrations than mice vaccinated with antigen alone. The adjuvanted vaccines induced higher antigen specific IgG2a titers in intramuscularly (Fig. 4.3A) and intranasally (Fig.4.3B) vaccinated mice as compared to
vaccinating with OVA alone. In fact, half of the intramuscularly vaccinated with antigen alone mice did not induce anti-IgG2a antibodies at the time the final bleed (Fig. 4.3A). Two out of nine intranasally vaccinated mice did not produce anti-IgG2a following vaccination with OVA alone (Fig. 4.3B). Parenteral route of vaccination did not result in significant anti-OVA serum IgA production following vaccination with any of the vaccines. Mucosal vaccination, however, resulted in much higher anti-OVA IgA (Fig. 4.4B) antibody production compared to vaccination with antigen alone.
Figure 4.1 Induction of anti-OVA specific IgG serum antibodies in pups. Antibody induction following intramuscular (A) and intranasal (B) vaccination. Neonatal mice were primed and boosted with 10 µg OVA (●), 10 µg OVA plus high adjuvant concentration (2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3) microparticle vaccine (★), 10 µg OVA plus medium adjuvant concentration (1.19 µg CpG 10101, 2.37 µg IDRP 1002, 1.19 µg EP3) microparticle vaccine (▲) or 10 µg OVA plus low adjuvant concentration (0.6 µg CpG 10101, 1.19 µg IDRP 1002, 0.6 µg EP3) microparticle vaccine (■). Pups were primed at two weeks of life and boosted four weeks later. Serum samples were collected throughout the experiment and analysed using an ELISA assay. Data sets with different subscripts are significantly different (p < 0.05).
Figure 4.2 Induction of anti-OVA specific IgG1 serum antibodies in pups. Antibody induction following intramuscular (A) and intranasal (B) vaccination. Neonatal mice were primed and boosted with 10 µg OVA (●), 10 µg OVA plus high adjuvant concentration (2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3) microparticle vaccine (★), 10 µg OVA plus medium adjuvant concentration (1.19 µg CpG 10101, 2.37 µg IDRP 1002, 1.19 µg EP3) microparticle vaccine (▲) or 10 µg OVA plus low adjuvant concentration (0.6 µg CpG 10101, 1.19 µg IDRP 1002, 0.6 µg EP3) microparticle vaccine (■). Pups were primed at two weeks of life and boosted four weeks later. Serum samples were collected throughout the experiment and analysed using an ELISA assay (p < 0.05).
Figure 4.3 Induction of anti-OVA specific IgG2a serum antibodies in pups. Antibody induction following intramuscular (A) and intranasal (B) vaccination. Neonatal mice were primed and boosted with 10 µg OVA (●), 10 µg OVA plus high adjuvant concentration (2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3) microparticle vaccine (★), 10 µg OVA plus medium adjuvant concentration (1.19 µg CpG 10101, 2.37 µg IDRP 1002, 1.19 µg EP3) microparticle vaccine (▲) or 10 µg OVA plus low adjuvant concentration (0.6 µg CpG 10101, 1.19 µg IDRP 1002, 0.6 µg EP3) microparticle vaccine (■). Pups were primed at two weeks of life and boosted four weeks later. Serum samples were collected throughout the experiment and analysed using an ELISA assay (p < 0.05).
Figure 4.4 Induction of anti-OVA specific IgA serum antibodies in pups. Antibody induction following intramuscular (A) and intranasal (B) vaccination. Neonatal mice were primed and boosted with 10 µg OVA (●), 10 µg OVA plus high adjuvant concentration (2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3) microparticle vaccine (★), 10 µg OVA plus medium adjuvant concentration (1.19 µg CpG 10101, 2.37 µg IDRP 1002, 1.19 µg EP3) microparticle vaccine (▲) or 10 µg OVA plus low adjuvant concentration (0.6 µg CpG 10101, 1.19 µg IDRP 1002, 0.6 µg EP3) microparticle vaccine (■). Pups were primed at two weeks of life and boosted four weeks later. Serum samples were collected throughout the experiment and analysed using an ELISA assay (p < 0.05).
### 4.3.2 Intranasal route of immunization is superior to parenteral vaccination

In a separate experiment the novel microparticle and soluble adjuvant formulations were titrated and various routes of immunization compared. Soluble and microparticle vaccine formulations composed of OVA and various concentrations of novel adjuvants were used to vaccinate two-week old mice. The vaccines were delivered by parenteral (subcutaneous and intramuscular) and mucosal (intranasal) routes.

Both microparticle and soluble vaccine formulations induced similar levels of anti-OVA IgG (Fig. 4.5) and IgG1 (Fig. 4.6) antibodies following parenteral and mucosal vaccination routes. Decreasing the concentration of adjuvants did not significantly effect the levels of anti-OVA IgG and IgG1 produced. Even the lowest concentration of adjuvants allowed the neonatal system to respond to vaccination. All vaccines and routes of immunization were successful at inducing anti-OVA IgG2a (Fig. 4.7) antibodies and all three concentrations of adjuvants produced similar titers. Intramuscular (Fig.4.7B) and intranasal (Fig. 4.7C) vaccination with microparticle vaccine formulations produced the most tight data points. Only microparticle vaccines delivered intranasally induced significant anti-OVA IgA serum titers (Fig. 4.8C). In this case, the concentration of the adjuvant platform had an effect on the levels of antibodies produced. The highest concentration of the adjuvants resulted in the highest levels of anti-OVA IgA produced.
Figure 4.5 Induction of anti-OVA specific serum IgG antibodies in pups. Antibody induction following subcutaneous (A), intramuscular (B) and intranasal (C) vaccination with microparticle vaccine formulations as well as subcutaneous vaccination with soluble vaccine formulations (D). Mouse pups were primed and boosted with 10 µg OVA plus high adjuvant concentration (2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3) vaccine (●), 10 µg OVA plus medium adjuvant concentration (1.19 µg CpG 10101, 2.37 µg IDRP 1002, 1.19 µg EP3) vaccine (■) or 10 µg OVA plus low adjuvant concentration (0.6 µg CpG 10101, 1.19 µg IDRP 1002, 0.6 µg EP3) vaccine (▲). Pups were primed at two weeks of life and boosted four weeks later. Serum samples were collected throughout the experiment and analysed using an ELISA assay (p < 0.05).
Figure 4.6 Induction of anti-OVA specific serum IgG1 antibodies in pups. Antibody induction following subcutaneous (A), intramuscular (B) and intranasal (C) vaccination with microparticle vaccine formulations as well as subcutaneous vaccination with soluble vaccine formulations (D). Mouse pups were primed and boosted with 10 µg OVA plus high adjuvant concentration (2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3) vaccine (●), 10 µg OVA plus medium adjuvant concentration (1.19 µg CpG 10101, 2.37 µg IDRP 1002, 1.19 µg EP3) vaccine (■) or 10 µg OVA plus low adjuvant concentration (0.6 µg CpG 10101, 1.19 µg IDRP 1002, 0.6 µg EP3) vaccine (▲). Pups were primed at two weeks of life and boosted four weeks later. Serum samples were collected throughout the experiment and analysed using an ELISA assay (p < 0.05).
Figure 4.7 Induction of anti-OVA specific serum IgG2a antibodies in pups. Antibody induction following subcutaneous (A), intramuscular (B) and intranasal (C) vaccination with microparticle vaccine formulations as well as subcutaneous vaccination with soluble vaccine formulations (D). Mouse pups were primed and boosted with 10 µg OVA plus high adjuvant concentration (2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3) vaccine (●), 10 µg OVA plus medium adjuvant concentration (1.19 µg CpG 10101, 2.37 µg IDRP 1002, 1.19 µg EP3) vaccine (■) or 10 µg OVA plus low adjuvant concentration (0.6 µg CpG 10101, 1.19 µg IDRP 1002, 0.6 µg EP3) vaccine (▲). Pups were primed at two weeks of life and boosted four weeks later. Serum samples were collected throughout the experiment and analysed using an ELISA assay (p < 0.05).
Figure 4.8 Induction of anti-OVA specific serum IgA antibodies in pups. Induction of titers following subcutaneous (A), intramuscular (B) and intranasal (C) vaccination with microparticle vaccine formulations as well as subcutaneous vaccination with soluble vaccine formulations (D). Mouse pups were primed and boosted with 10 µg OVA plus high adjuvant concentration (2.37 µg CpG 10101, 4.74 µg IDRP 1002, 2.37 µg EP3) vaccine (●), 10 µg OVA plus medium adjuvant concentration (1.19 µg CpG 10101, 2.37 µg IDRP 1002, 1.19 µg EP3) vaccine (■) or 10 µg OVA plus low adjuvant concentration (0.6 µg CpG 10101, 1.19 µg IDRP 1002, 0.6 µg EP3) vaccine (▲). Pups were primed at two weeks of life and boosted four weeks later. Serum samples were collected throughout the experiment and analysed using an ELISA assay (p < 0.05).
4.4 DISCUSSION

In the present study we compared parenteral and mucosal delivery of novel vaccine formulations in neonatal mice. Both soluble and microparticle formulations containing OVA co-administered with a novel adjuvant formulation composed of CpG ODN, IDRP and PCPP were assessed for their induction of systemic immunity. The adjuvant formulation was titrated to test the effectiveness of lower doses on the adjuvanticity of the novel formulation. Our results demonstrate that the route of immunization and formulation into microparticles had a substantial impact on the degree and quality of the immune response.

Neonatal vaccination is complicated due to the presence of vaccine neutralizing MatAbs and the neonate’s innate bias towards mounting Th2-like responses [129, 171]. Use of immunostimulating adjuvants such as CpG ODN, IDRPs and PP may shift the bias towards Th1 type responses, which was the basis why those adjuvants were chosen for our studies. Additionally, PTd can be formulated into microparticle vaccine delivery vehicles [247], which made them an attractive candidate as an adjuvant for neonatal vaccines. Such microparticles could not only hide the vaccine antigen from MatAbs but also safely deliver the vaccine to neonatal antigen presenting cells. Antigens encapsulated in microparticle delivery systems are more efficiently taken up by antigen presenting cells, which migrate to the nearby lymphoid organs leading to a sustained release of antigen over time [249, 270]. Most importantly, co-encapsulation of both antigen and adjuvants in microparticles allows for their co-delivery to the same antigen presenting cells. This in turn, allows for processing and presentation of antigen in the presence of co-stimulatory signals, which is responsible for more powerful immune responses.
The current study demonstrated that the microparticle and soluble vaccine formulations composed of a novel adjuvant platform induced high concentrations of antigen-specific antibodies. The vaccines induced high anti-OVA IgG (Fig. 4.1 and Fig. 4.5) and IgG1 (Fig. 4.2 and Fig. 4.6) titers despite the route of their administration to neonatal mice. Moreover, antigen co-formulated with the vaccine platform outperformed vaccinating with antigen alone in the production of serum IgG2a antibodies (Fig. 4.3). The presence of IgG2a isotype of antibodies is indicative of a Th1 shift desired for successful neonatal vaccination. Decreasing the concentration of adjuvants did not affect the levels of IgG2a antibodies produced, which can make a vaccine more cost effective, especially when designing vaccines for use in developing countries. Finally, the microparticle vaccines delivered intanasally induced antigen-specific serum IgA (Fig. 4.4B), while vaccinating with antigen alone did not produce any significant concentrations of that particular isotype of antibody.

This research suggests that vaccinating with a microparticle vaccine formulation containing novel adjuvants has a great potential as a vaccine platform for protecting during early infancy. Most promising is intranasal (mucosal) delivery as it produces both IgG2a and IgA antibodies. Even though most pathogens enter the host via mucosal surfaces, most vaccinations are administered systemically. Systemic vaccination might be less than optimal as it does not successfully induce local mucosal responses. Mucosal immunity is necessary to prevent the early stages of infection where the pathogen is blocked right at its entry point into the host. Mucosal vaccination is believed to be the most successful in preventing infections caused by pathogens that enter the host via the mucosa. There are several advantages of mucosal vaccination. The vaccination is needle-
free, which may increase vaccine compliance. Mucosal vaccination can induce strong sIgA and cytotoxic lymphocyte responses against pathogens entering via mucosa surfaces and also systemic IgG responses [271]. Moreover, the mucosal route of vaccination has been hypothesized to circumvent the challenges associated with MatAbs on active neonatal vaccination. It is believed that targeting neonatal vaccine delivery to the mucosal surfaces such as the respiratory (intranasal) tract or the intestine (oral) may overcome the interference of MatAbs as these are the sites where there is a restricted transport of MatAbs [152, 272].

Due to the fact that mucosal vaccination is noninvasive and needle-free, induces mucosal and systemic immunity and has a potential to overcome the interference of MatAbs, it is an attractive candidate for neonatal vaccines. Because the mucosal immune system can operate independently of the systemic immune system, it might allow successful neonatal vaccination in the presence of vaccine-neutralizing MatAbs. The optimal (neonatal) mucosal vaccine would most likely be composed of a vaccine delivery adjuvant such as microparticles. Antigens delivered via mucosal routes may be cleared by non-specific mechanisms, be degraded by enzyme or perhaps be affected by extreme pH before successfully reaching target cells [241]. Encapsulating the antigen would protect it from being degraded before reaching antigen presenting cells. It might also retain the antigen longer within the cells thus continuing to stimulate the system. Thus, the microparticle vaccine composed of the novel adjuvant combination might be an excellent candidate for neonatal vaccination.

Our research showed that co-formulating OVA with a combination of CpG ODN, IDRP and PCEP allows for the generation of mixed Th1/Th2-type responses in neonatal
mice. Intranasal vaccination with the adjuvant platform formulated into microparticles induced both IgG2a and IgA tiers. The work presented is done with OVA as the model antigen. We are currently using the same adjuvant platform and delivery system to develop a novel vaccine against whooping cough. We are testing the formulations in the presence and absence of vaccine-neutralizing MatAbs. We believe that encapsulating *Bordetella pertussis* antigens co-formulated with our novel adjuvant formulation and delivered by a mucosal route will generate protective immune responses in the whooping cough challenge model. This research will lead to the development of novel vaccine formulations for neonates in both developed and developing nations.
5. NOVEL VACCINE FORMULATIONS AGAINST PERTUSSIS OFFER EARLIER ONSET OF IMMUNITY AND PROVIDE PROTECTION IN THE PRESENCE OF MATERNAL ANTIBODIES

5.1 INTRODUCTION

Whooping cough is a serious respiratory disease caused by infection with *Bordetella pertussis*. The disease is responsible for severe respiratory symptoms in infants and young children. In rare cases complications can include seizures, pneumonia, encephalopathy and death [16]. 20–40 million cases of pertussis occur globally each year, most of which are found in developing countries. 200,000–400,000 of these cases, mostly infants, die from the disease each year [17, 22, 251]. Pertussis can affect all age groups, but 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 [31, 262]. Even though the incidence of disease has dramatically decreased with the introduction of wP (inactivated bacteria) and aP (purified proteins) vaccines, many cases are still reported even in developed nations [23, 25, 252, 253]. In 2010, United States and Canada experienced more than 20,000 cases with more than 15 deaths [273-275]. Therefore, more effective neonatal pertussis vaccines are needed to reduce the incidence of whooping cough and provide long lasting protection.

The 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 potentially lead to a miscarriage [254]. Thus, neonatal immune responses are skewed towards a Th2-type bias following vaccination [128, 129]. These passively derived antibodies are a result from previous exposure or vaccination. Ironically, while these MatAbs provide short-lived protection against disease they can also interfere with active vaccination of neonates [159-161].
MatAbs have been demonstrated to form immune complexes with antigen, which leads to the neutralization of vaccines, phagocytosis of MatAb-coated antigen and inhibition of B cell activation by Fcγ-receptor mediated signals [171]. MatAbs can also mask immunodominant vaccine epitopes thus preventing antigen binding to infant B cells [129, 171]. In this context, the ratio of the MatAb: vaccine antigen seems to be critical. Antibody responses to vaccine antigen may only be produced once MatAbs have fallen below a specific threshold [129, 171]. MatAb inhibitory effects on infant antibody responses have been observed following immunization with vaccines against measles [152, 162], HIB conjugate [163], hepatitis A [164], influenza A [165], tetanus [166], and varicella-zoster [160]. MatAb interference has also been observed following neonatal vaccination with either wP or aP vaccines [128, 167-172]. Antibodies against *B. pertussis* are transferred from mother to infant transplacentally or through the colostrum and breast milk. The IgG1 immunoglobulin subclass is the most efficiently transferred antibody [151].

The goal of the present study was to evaluate novel vaccine platforms that when combined with pertussis antigens would efficiently protect neonates in the presence of MatAbs. The adjuvant platform consists of three components that effectively induce both innate and adaptive immunity in the neonate. The first component of the novel adjuvant formulations are cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN), which operate via signaling through TLR-9 ligands [276]. In humans, CpG motifs bind to Toll-like receptor-9 (TLR-9) on dendritic cells (DCs) and B cells, which results in the production of interleukin-12 (IL-12) and interferon-α (IFN-α). These CpG ODNs stimulate human B cells and plasmacytoid dendritic cells (pDCs), promoting the production of Th1 responses and pro-inflammatory cytokines [86, 277]. The second component of the platform is a synthetic cationic IDR peptide. These cationic peptides are derivatives of host defense peptides, which induce chemokine production and/or act as chemokines [278], promote wound healing, cell trafficking and modulate the responses of DCs and cells of the adaptive immune response [279-281]. Most recently, it was shown that IDRs can act as adjuvants vaccines [282-284].
The third component of our vaccine platform are PPs. These synthetic polymers are water soluble and biodegradable. PPs are composed of an inorganic backbone of alternating phosphorus (P) and nitrogen (N) atoms and two side groups attached to each P [226]. PPs are inexpensive to produce, they can be lyophilized and be stored for a long time at room temperature. PPs have also been shown to be potent immunomodulators [224, 228, 285]. For example, poly[di(sodium carboxylatoethylphenoxy) phosphazene] (PCEP) was able to induce balanced or Th1-type immune responses in mice when immunized with influenza antigens [230]. Another feature of this adjuvant is the ability to form microspheres [247, 269, 286] for vaccine delivery. Coacervation of polyphosphazenes with NaCl followed by cross-linking with Ca$$^{++}$$ ions results in the creation of small particles sized between 1-5 µm [247]. Microparticles protect the vaccine antigen from degradation or from being recognized and neutralized by MatAbs. Encapsulated antigens may be delivered to infant antigen presenting cells without being recognized by MatAbs.

We previously showed that combining all three adjuvants has a synergistic effect on immune responses [221, 223, 234, 236, 250]. The objective of the current study was to test whether a vaccine composed of a combination of adjuvants with pertussis antigens would be able to overcome interference produced by MatAbs. Novel adjuvants such as CpG ODN, IDR peptide and PP delivery systems were tested in combination with PTd and FHA pertussis antigens for their ability to induce immunity in neonates despite the presence of MatAbs. We tested these vaccines using our novel pertussis disease model in newborn piglets [111, 113]. In addition to the porcine model, we also used the murine model, which is the most common model for studying pertussis vaccination [104, 106, 109].

5.2 MATERIALS AND METHODS

5.2.1 Animals

Male and female BALB/c mice were obtained from Charles River (Montreal, Quebec, Canada). Animals were kept and cared for by the Animal Care staff at the
Every dam was housed in a separate cage. Offspring were kept with their mothers until weaned at around the fourth week of life at which time the pups were separated according to sex.

All sows were pre-screened for cross-reactive antibodies to *Bordetella bronchiseptica*. Pregnant Landrace sows were obtained from the Saskatoon Prairie Swine Centre, University of Saskatchewan. The animals were encouraged to farrow by intramuscular (i.m.) injection of 2 ml prostaglandin (Planate; Schering-Plough Canada Inc., Pointe-Claire, Quebec, Canada) on the 113th day and another 1 ml on the 114th day of gestation. Consequently, piglets were born on day 114 and 115 of gestation. Nursing piglets were kept in same isolation rooms in separate enclosures. Shortly after birth, half of the piglets of each litter were exchanged between sows to decrease the mother effect. All experiments were performed in agreement with the guidelines proposed by the University of Saskatchewan and the Canadian Council for Animal Care.

### 5.2.2 Vaccine components

Recombinant genetically detoxified PTd was kindly provided by Dr. Rino Rappuoli, Novartis. FHA antigen was purchased from List Biological Laboratories, Inc. (Campbell, California). CpG-ODN 10101 (TCGTCGTTTTCGCGCGCGCGCCG) was acquired from Pfizer (Ottawa, ON). IDR peptide 1002 (VQRWLIVWRIRK) was synthesized by Genscript (Picataway, NJ). The PP PCEP (VIDO-EP#3) was synthesized by the Idaho National Laboratory (Idaho Falls, ID) by a method previously described [230] with minor modifications. Lyophilized PP were dissolved in Dulbecco’s PBS (pH 7.4; Sigma-Aldrich, MO) and stored in the dark at room temperature.

The commercial pediatric pertussis vaccine Quadracel® was purchased from Sanofi Pasteur (Toronto, ON). 0.5 ml human dose of the vaccine 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
used as the adjuvant in the vaccine. In order to compare the novel vaccine formulations to the commercial vaccine, antigen dose was calculated to contain a matching dose to that in the Quadracel® vaccine.

5.2.3 Vaccination of mice

Dams were subcutaneously (s.c.) immunized between the shoulder blades or intranasally (i.n.) with 1 µg (100 µl) of genetically detoxified PTd, PBS (pH= 7.2; 1.54 mM KH$_2$PO$_4$, 155.17 mM NaCl, 2.71 mM Na$_2$HPO$_4$-7 H$_2$O; Gibco, Invitrogen; Carlsbad, CA) or the Quadracel® (Sanofi Pasteur Limited, Toronto, Ontario, Canada) vaccine. A number of dams were boosted in the same manner two weeks following the primary vaccination. Seven or 14 day old neonatal mice were vaccinated via numerous routes including: s.c (50 µl between the shoulder blades), i.m. (12.5 µl into each quadricep 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. Both components were placed in a 1.5 ml Eppendorf tube (VWR; West Chester, PA) and co-incubated for 30 minutes in a block heater at 37°C. Neonatal mice were vaccinated with 1 µg of both PTd and FHA. CpG-ODN, IDR and PCEP were delivered at a ratio of 1:2:1. 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 phosphate buffered saline (PBS, pH=7, 1.54 mM KH$_2$PO$_4$, 155.17 mM NaCl, 2.71 mM Na$_2$HPO$_4$ 7 H$_2$O) (Gibco).

5.2.4 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-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).
5.2.5 Microparticle vaccine preparation

CpG-ODN and IDR peptide were complexed in a ratio of 1:2 at 37°C for 30 min. PCEP and the antigen(s) were added, following the incubation. Then 6.2% NaCl was added into the mixture and incubated in the dark at room temperature for 20 minutes. When the incubation was over, the mixture was added into 8.8% CaCl$_2$ and mixed on a nutator for 20 minutes in the dark at room temperature. The microparticles were then collected by centrifugation at 1340 g for 10 minutes at room temperature and washed once with ddH$_2$O. The vaccine was re-suspended in PBS (pH 7.2) to the desired volume.

5.2.6 Sample collection

Mouse dams were bled pre and post vaccination. Neonatal mice were bled 2, 4, 6 and 8 weeks (unless otherwise stated) post primary vaccination. All blood samples were centrifuged (4547 x g) and serum stored at –20 °C. Nasal washes were collected four and seven days post infection. Nasal lavages were collected 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 minutes. Neonatal piglets were bled pre-vaccination as well as 1, 2, 3 and 4 weeks following the primary vaccination (unless otherwise stated). Piglet serum was stored in serum blocks at –20 °C.

5.2.7 B. pertussis challenge

B. pertussis Tohama I strain was stored at −80°C in Casamino Acids plus 10% glycerol. Bacteria were grown on charcoal agar plates containing 10% (vol/vol) sheep blood and 40 µg/ml cephalexin (Sigma-Aldrich) at 37°C. Bacteria were harvested into Stainer-Scholte (SS) media and inoculated to make puddle plates. Following a 48-hour incubation, bacteria were collected by scraping off and re-suspending in SS medium. Bacteria were collected by centrifugation at 4,500 $\times$ g for 10 min. The pellets were re-suspended in PBS and their optical density (OD) at 600 nm was determined using a spectrophotometer. The challenge dose viable count was determined by plating serial
dilutions of the bacterial suspension onto charcoal blood agar plates and incubation at 37°C for five to seven days.

At 9-10 weeks of age the pups were intranasally challenged with 4 x 10^6 CFU *B. pertussis*. Half of the pups were sacrificed four 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 and Company, Sparks, MD) plates to determine the number of bacteria present in the lung. The plates were incubated at 37 °C for one week before the colonies were counted.

5.2.8 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 Na₂CO₃ and 35 mM NaHCO₃, pH 9.6). Sera were diluted four-fold, starting with 1:100 dilution. TBST gelatin (Tris Buffered Saline pH 7.3 containing 0.5% Tween and 0.5 % gelatin (Sigma) was used to dilute the samples and antibodies. For the detection of nasal wash IgA, ELISA plates were coated with 0.50 μg/ml of antigen. The samples were not diluted and added at neat concentration. Biotin-conjugated goat anti-mouse immunoglobulin G, G2a and A (1:10,000 dilution; Invitrogen, Camarillo, CA) was used for the detection of PTd and FHA specific IgG, IgG2a and IgA antibodies. Amplification of the signal was carried out by addition of streptavidin alkaline phosphatase (1:5000 dilution; Jackson ImmunoResearch; West Grove, PA). The reaction was visualized by p-nitrophenylphosphate (Sigma-Aldrich, St. Louis, MO; dilution 1 mg/ml). Samples were analysed using a microplate reader (Bio-Rad iMark Microplate Reader; Philadelphia, PA) at 405 nm with a reference of 490 nm.

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. The plates were washed followed by blocking with TBST gelatin for one hour at room temperature. Sera were diluted four-fold, starting with 1:40 dilution. Alkaline phosphatase-conjugated goat
anti-pig immunoglobulin G (IgG; 1:5,000 dilution; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was used for the detection of PTd and FHA specific IgG. The reaction was visualized by p-nitrophenylphosphate. Samples were analysed using a microplate reader at 405 nm with a reference of 490 nm.

5.2.9 Statistical analysis

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

5.3. RESULTS

It was previously reported that vaccines against pertussis can be affected by maternal antibodies[128, 167-172]. We previously described that vaccination with PTd antigen alone was not able to overcome this interference. The goal of the experiments presented here was to test the effect of co-formulating pertussis antigens with novel adjuvants for their ability to induce protection against challenge infection in neonates in the presence of MatAbs. A series of experiments were performed in which mothers were vaccinated during gestation and their offspring shortly after birth. Antibody responses were compared to neonates born of naïve mothers.

5.3.1 Role of novel vaccine adjuvants

To study MatAb interference with neonatal vaccination, mouse dams were divided into two groups. Ten dams were immunized and boosted with PTd at four weeks and two weeks prior to birth. Seven control dams were treated subcutaneously with PBS
four weeks before delivery. Pups were bled three weeks following vaccination to assess the levels of PTd-specific serum antibodies. Throughout the experiment, levels of maternal anti-PTd IgG antibodies were only present in pups born to 2x vaccinated mothers. No antibodies were detected in the animals of the control group born of PBS vaccinated dams. Newborn mice responded well to pertussis vaccination in the absence of MatAbs as demonstrated by an increase in anti-PTd serum IgG. Vaccinating pups with PTd and combination of CpG, IDR peptide and PP resulted in earlier onset of immunity and higher antibody titer as compared to vaccination with the Quadracel® vaccine and PTd alone (Fig. 5.1A). Anti-PTd IgG titers were around 500-fold higher following a single vaccination with the novel adjuvant combination as compared to the titers produced by vaccination with the Quadracel® vaccine (Fig. 5.1A).
Figure 5.1 Induction of anti-PTd IgG antibodies in the serum of mouse pups. Neonatal pups were 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 vaccinated at seven days and boosted at thirty-one days of age. 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) (p < 0.05).
Analysis of antibody titers in serum of 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. 5.1B). In contrast, immunization with PTd co-formulated with CpG, IDR peptide and PP resulted in enhanced immune responses (Fig. 5.1B).

These results were confirmed in our recently developed pig model. To this end, MatAbs were induced by vaccinating with PTd two and four weeks prior to delivery. At three days of age, neonatal piglets were intramuscularly vaccinated with PTd co-formulated with CpG, IDR peptide and PP soluble vaccine or the Quadracel® vaccine and boosted after two weeks. ELISA results revealed that in the presence of MatAbs, the novel vaccine formulation induced significantly higher anti-PTd IgG antibodies compared to vaccination with the Quadracel® vaccine (Fig. 5.2).
Figure 5.2 Induction of anti-PTd IgG antibodies in the serum of piglets. Neonatal piglets were intramuscularly primed and boosted with 10 µg PTd plus 150 µg CpG 10101, 300 µg IDR peptide 1002 and 150 µg EP3 soluble vaccine (●) or the Quadracel® vaccine (■). Piglets were born to intramuscularly PTd vaccinated and boosted sow. Piglets were vaccinated at three days of age and boosted two weeks post priming. Serum samples were analysed using an ELISA assay (*p < 0.05).

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

To improve vaccine efficacy, a second antigen, FHA, was added into the formulation. In an effort 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 the pups were boosted in the same manner and challenged at ten weeks with *B. pertussis* to assess the efficacy of each vaccine. ELISA results revealed that this microparticle vaccine
produced higher anti-PTd (Fig. 5.3A) and anti-FHA (Fig. 5.3B) IgG titers, both pre and post boosting, when compared to vaccination with the Quadracel® vaccine. The novel vaccine, incorporated into a microparticle delivery vehicle, also induced higher anti-PTd (Fig. 5.3C) and anti-FHA (Fig. 5.3D) IgG2a antibodies as compared to the commercial vaccine. Both pertussis vaccines protected the pups from a challenge of $4 \times 10^6$ CFU of *B. pertussis* as demonstrated by the significant reduction of bacteria in mouse lungs four and seven days post infection (Fig. 5.3E).
Figure 5.3 Induction of anti-PTd and FHA IgG antibodies, anti-PTd and FHA IgG2a antibodies and bacterial load (CFU/lung) following challenge infection in pups. Neonatal pups were 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 x 10⁶ 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) (p < 0.01).
Since mucosal vaccination induced both systemic and mucosal immunity and could avoid the interference with MatAbs the bivalent vaccine formulations were also tested in the presence of passive immunity using an intranasal route of administration. Dams were intranasally vaccinated (and boosted) with PTd and PBS during gestation. At two weeks of life neonatal pups were intranasally vaccinated with PTd and FHA co-formulated with CpG, IDR peptide 1002 into PP microparticle or soluble vaccine, Quadracel® vaccine or PBS. The microparticle and soluble vaccine formulations induced high anti-PTd IgG titers, both pre and post boost, in pups born of naïve dams (Fig. 5.4A), as well as those born of low (Fig. 5.4C) and high titer dams (Fig. 5.4E). Both soluble and microparticle vaccines also induced significant levels of anti-FHA IgG antibodies in the absence (Fig. 5.4B) and presence of passive immunity (Fig. 5.4 D, F). When compared to the Quadracel® vaccine, these novel vaccine formulations also induced higher anti-PTd IgG2a serum titers in the absence (Fig. 5.5 A) and presence (Fig. 5.5 C, E) of passive immunity. Interestingly, early life vaccination with the novel microparticle and soluble vaccine formulation induced around a 1000 fold higher concentration of anti-PTd IgG2a in the presence of high titers of MatAbs (Fig. 5.5E) than vaccination with the commercially available vaccine. A similar trend was noticed in the production of anti-FHA IgG2a antibodies. Novel vaccine formulations resulted in superior anti-FHA IgG2a titers in the absence (Fig. 5.5B) and presence (Fig. 5.5 D, F) of passive immunity. PTd and FHA co-formulated with novel adjuvants protected the pups from *B. pertussis* infection in the presence and absence of MatAbs (Fig. 5.5G).
Figure 5.4 Induction of anti-PTd and FHA IgG antibodies in the serum of mouse pups.

Neonatal pups were 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. 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) (p < 0.05).
Figure 5.5 Induction of anti-PTd and FHA IgG2a antibodies in the serum of mouse pups.

Neonatal pups were 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. Serum samples were analysed using an ELISA assay. Pups were challenged with 4 x 10⁶ CFU B. pertussis at ten weeks of life and sacrificed four days later. Different subscripts indicate significant differences between groups. Within each time point (a) is significantly different than (b), (c) and (d) (p < 0.05).
An additional test in mice included vaccinating (and boosting) dams, during pregnancy, with the Quadracel® vaccine. Unlike previous experiments, 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 vaccinating with the commercial vaccine. Both the Quadracel® vaccine and the novel vaccine formulations induced high anti-PTd IgG titers in the absence (Fig. 5.6 A) and presence (Fig. 5.6 C, E) of MatAbs. Additionally, all pertussis vaccines induced high concentrations of anti-FHA titres in the absence (Fig. 5.6 B) and presence (Fig. 5.6 D, F) of passive immunity. The vaccines composed of the novel adjuvant platform however, induced much higher anti-PTd and FHA IgG2a antibody titers (Fig. 5.7) as compared to vaccination with the commercially available vaccine. Vaccination with novel vaccines resulted in at least 1000-fold higher production of anti-PTd IgG2a in the presence of high titers of MatAbs than vaccination with the Quadracel® vaccine (Fig. 5.7E). Similarly, the vaccines induced superior anti-FHA IgG2a titers in the presence of passive immunity (Fig. 5.7F). Moreover, the soluble vaccine formulation produced the highest nasal lavage anti-PTd (Fig. 5.8 A, B) and FHA (Fig. 5.8 C, D) IgA antibody concentration. Both the microparticle and soluble pertussis vaccine formulations protected the pups following infection (Fig. 5.8 E, F).
Figure 5.6 Induction of anti-PTd and FHA IgG antibodies in the serum of mouse pups.

Neonatal pups were 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. 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) (p < 0.05).
Figure 5.7 Induction of anti-PTd and FHA IgG2a antibodies in the serum of mouse pups.

Neonatal pups were 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. 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) (p < 0.05).
Figure 5.8 Induction of anti-PTd and FHA IgA antibodies in the nasal washes of mouse pups. Neonatal mice were 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 analyzed using an ELISA assay. Pups were challenged with 4 x 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) (p < 0.05).
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 the boost our microparticle-formulated vaccine induced higher anti-PTd (Fig. 5.9A) and anti-FHA IgG (Fig. 5.9B) antibodies compared to the commercial vaccine.
Figure 5.9 Induction of anti-PTd and FHA IgG antibodies in the serum of piglets. Neonatal piglets were intramuscularly vaccinated and boosted with 10 µg PTd and 10 µg FHA co-formulated with 150 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) (p < 0.05).
5.4 DISCUSSION

Whooping cough is one of the most prevalent vaccine preventable disease [287]. Globally pertussis is thought to be one of the top ten causes of death in children [22]. The goal of our research is to develop novel vaccine candidates which could improve the effectiveness of immunization and reduce the burden of pertussis. In the present study, various routes of immunization were tested for the induction of *B. pertussis* immune responses in the neonates using novel soluble and microparticle formulations containing a combination of adjuvants including CpG-ODN, IDR peptides and PCEP polyphosphazene. These studies led to the conclusion that the novel pertussis vaccine formulations had a major impact on the magnitude and quality of the immune response in neonatal mice and piglets.

Use of optimized adjuvants can result in the stimulation of both innate and adaptive immunity, leading to stronger and longer lasting immune responses to neonatal vaccination. Combining adjuvants often results in stronger immune responses than using a single adjuvant [234]. It was previously shown that co-formulating polyphosphazenes with CpG-ODN enhanced adjuvanticity and resulted in an enhanced efficacy of vaccines [231, 234, 288]. Similarly, co-formulating CpG-ODN with IDRs and PP can greatly enhance vaccine immunogenicity [221-223]. In the present study using the murine model, we showed that co-formulating PTd with the novel combination of adjuvants resulted in higher anti-PTd IgG titers and earlier onset of immunity as compared to vaccination with PTd alone or the commercial pediatric pertussis vaccine Quadracel® composed of PT, FHA, PRN and Fim 2 and 3. These enhanced immune responses were induced in the presence of MatAbs. Similar results were also observed in the porcine model. PTd co-formulated with CpG, IDR and PCEP resulted in superior anti-PTd IgG responses in neonatal piglets in the presence of MatAbs.

Polyphosphazenes were used here since they are not only strong immune modulators [230] but can also be formulated into microspheres and act as a vaccine delivery system [247]. Formulating a vaccine into microparticles protects it from degradation while increasing its uptake by antigen presenting cells [249, 250, 270]. Microparticles such as polyphosphazenes, liposomes, PLGA particles and ISCOMs are
designed to protect the antigen and be phagocytosed by antigen presenting cells [289]. Microparticles are intended to deliver the antigen into the phago-endosome of antigen presenting cells where it is processed and loaded into MHC molecules. This would be greatly assisted by the cationic IDR peptides that act as cell penetrating peptides to translocate themselves and cargo into cells [290]. In addition these peptides have a profound role in recruiting immune cells through stimulation of local chemokine production [291]. Pathogen recognition receptors like TLR9 are present in the phago-endosome. 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. Thus, we formulated the pertussis antigens and adjuvants into microparticles to protect them from degradation as well as recognition of the antigen by MatAbs.

To further increase the efficacy of these vaccines, another pertussis antigen, FHA, was added into the formulation. The efficacy of this bivalent soluble and microparticle vaccines was compared to the commercially available pertussis vaccine. Neonatal mice intramuscularly vaccinated with PTd and FHA co-formulated with the novel adjuvant platform microparticle vaccine showed full protection following *B. pertussis* challenge. As shown in the results, the vaccine also induced higher anti-PTd and FHA IgG antibodies as compared to vaccinating with the Quadracel® vaccine. Most interestingly, the microparticle vaccine formulation induced much 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 mucosally delivering the vaccines in the presence of low and high titers of MatAbs. The mucosal route of vaccination has been hypothesized to circumvent the challenges associated with MatAbs on active neonatal vaccination. It is believed that targeting neonatal vaccine delivery to the mucosal surfaces such as the respiratory (intranasal) tract or the intestine (oral) may overcome the interference of MatAbs as these are the sites where there is a restricted transport of MatAb [272]. 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 [89, 292]. The efficacy of microparticle and soluble vaccines was proven by *B. pertussis* clearance following the infection. The vaccines protected neonatal pups in the presence and absence of MatAbs. The vaccines induced a similar level of 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 formulated vaccines. Vaccination resulted in the induction of superior anti-PTd and FHA IgG2a serum titers as compared to pups vaccinated with the commercially available vaccine. Interestingly, the novel soluble vaccine produced the highest concentrations of anti-PTd and FHA IgA found in the nasal lavages. All three vaccines significantly reduced the bacterial load following vaccination. In this instance, however, we observed a higher concentration of bacteria in vaccinated pups born of high and low titer dams. This could be explained by a higher concentration of pertussis specific MatAbs at the time of challenge as this time dams were vaccinated with the commercial vaccine containing multiple antigens. In other studies the dams were vaccinated with a single antigen, which resulted in a total clearance of bacteria in vaccinated pups. The increased lung bacterial counts in pups might be a result of a higher concentration of anti-pertussis vaccine-neutralizing MatAbs. Furthermore, the microparticle vaccine formulation was also successful in the porcine model. Formulating PTd, FHA and adjuvant combination into polyphosphazene 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 triple 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 in Quadracel® vaccine. The novel vaccine formulations also induced high anti-PTd IgG2a antibodies, which is indicative of a Th-1 shift, an outcome much desired of newborn vaccines. Formulating the vaccine into microparticles, protected it from degradation and delivered it to antigen presenting cells. Another excellent characteristic of encapsulated vaccines are beneficial antigen release kinetics. Antigen is released slowly over time thus providing continuous stimulation of the immune system. Such a vaccine would be an ideal vaccine candidate especially in developing countries where most cases of pertussis occur and vaccine coverage is low. Encapsulating a vaccine 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.
6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION

According to the WHO, infectious diseases globally account for ~ 2 million deaths per year in newborns and infants less than six months of age. These diseases are mostly caused by infections with Gram-positive bacteria (Group B Streptococcus, *S. pneumoniae*), Gram-negative bacteria (*B. pertussis, E. coli*) and viral infections such as herpes simplex virus (HSV), respiratory syncitial virus (RSV) and rotavirus [293]. The increased vulnerability to infections emphasizes the need for more successful vaccines for early life vaccination of neonates. Vaccination at birth offers numerous benefits, predominantly for non-industrialized nations where birth might be the only connection with the healthcare system. Early life vaccination is also correlated with a considerably enhanced degree of vaccination coverage compared to immunization administered at later time points [294]. Currently, only three vaccines are approved which can be given at birth: hepatitis B, tuberculosis and oral polio [141], thus indicating the unmet need for improved neonatal adjuvants and vaccines that are capable of requiring fewer doses and improving the intensity, rate and longevity of protection.

The goal of our study was to develop novel vaccine formulations against whooping cough, which would provide protection early in life in the presence of MatAbs. Early life vaccination must overcome the polarization of fetal and neonatal immune responses towards Th2-type immunity [137, 295] and the presence of vaccine neutralizing MatAbs [129, 171]. In this context, it was suggested that recovery from a natural *B. pertussis* infection is dependent on the presence of Th1-type responses. Current aP vaccines are adjuvanted with alum, which result in a Th2-type immune responses following immunization [98]. These suboptimal immune responses might be the reason for an early waning of vaccine-induced immunity and the resurgence of the disease in countries with vast vaccine coverage [23, 24]. We developed a novel adjuvant platform to overcome the challenges associated with neonatal vaccination. The adjuvant formulation was based on CpG ODN, IDRP and PP vaccine delivery vehicles. When co-formulated with pertussis antigens, the adjuvant platform resulted in the production of both cell-
mediated and humoral immunity. In addition, when delivered intranasally the vaccine produced a mucosal antibody response.

The study began with the establishment of the murine and porcine models for studying maternal antibody interference. We titrated both the levels of MatAbs and the concentration of PTd for neonatal vaccination. Our results concluded that vaccinating with PTd alone was not able to overcome the interference of MatAbs, even in the presence of low concentrations of passive immunity. A booster dose of the antigen, however, was able to overcome this interference in the presence of low levels of passive immunity but not in the presence of high levels of MatAbs. Using two animal models, we showed that MatAbs interfere with the generation of antibodies during the primary immune response in infancy. We also demonstrated that this phenomenon could be overcome by the addition of innate stimuli such as CpG ODN. Interestingly, we also showed that priming in the presence of MatAbs does not prevent responses to booster doses given later in life. Taken together, the experiments revealed the possibility to successfully immunize at birth even in the face of MatAb inhibition.

In order to achieve a balanced Th1/Th2 immunity following early life vaccination, we combined a model antigen OVA with the novel combination of adjuvants. CpG ODN, IDRP and PP co-formulated with OVA into soluble and microparticle vaccines were used to vaccinate neonatal mice. We titrated the adjuvant formulations and delivered them via parenteral and mucosal routes. The vaccines containing the adjuvants induced greater concentrations of serum antibodies shortly following the vaccination. Both soluble and microparticle vaccines composed of the novel adjuvant platform and delivered intranasally resulted in superior IgG2a and IgA serum antibody production as compared to vaccinating with antigen alone. Interestingly, decreasing the adjuvant concentration did not change the magnitude of IgG2a production. These results indicate that a mucosal administration of the novel vaccine formulations has great potential for intranasal early life vaccination.

Our last set of experiments involved co-formulating pertussis antigens such as PTd and FHA with the novel adjuvant platform into microparticle and soluble vaccine formulations. The basis for formulating the vaccine into PP microparticle delivery
vehicles was the fact that it might protect the vaccine antigens from being recognized and consequently neutralized by MatAbs. We tested the vaccine efficacy by a challenge infection in the presence and absence of passive immunity. Co-formulation of PTd with the combination of novel adjuvants resulted in a higher anti-PTd IgG concentration and earlier onset of immunity as compared to vaccination with PTd alone or the commercial aP pediatric vaccine. The vaccines based on PTd and FHA as well as the adjuvant formulation induced superior levels of IgG2a when compared to vaccination with the commercially available vaccine. Furthermore, following intranasal administration the soluble vaccine formulation produced high levels of IgA in the nasal wash. The efficacy of the novel microparticle and soluble vaccine formulations was confirmed by the total *B. pertussis* clearance following infection. Our studies confirm the possibility of using innate adjuvants and delivery vehicles to induce protection following early life vaccination in the presence of MatAbs.

The goal of numerous studies around the globe is to identify appropriate adjuvants for early life vaccination. Adjuvants that induce Th1-polarizing cell-mediated immunity are particularly sought after for vaccination shortly after birth. Novel adjuvants could enable the production of improved vaccines for the use in neonates thus reducing the morbidity and mortality of infections. Our results indicate the possibility of using innate adjuvants to enhance immune responses and manipulate the neonatal immune system and for driving Th1-type responses. In this context, we showed that neonatal vaccination is feasible even in the presence of MatAbs. This approach is expected to expand in the future, especially against infections that require early protection.

The need for improved neonatal vaccines will entail the development of new adjuvants able to activate particular PRRs. The current knowledge of PRRs in the neonate offers new prospects for developing novel vaccine delivery systems and adjuvants Proper use of novel adjuvants might also overcome the challenges associated with the presence of passive immunity at the time of vaccination. Our research demonstrated the possibility of using microparticles as a strategy to induce protection in early life. Such vaccine delivery would not only hide the antigens from being neutralized by MatAbs but also protect from the harsh environment at mucosal surfaces. As always with novel vaccines,
safety concerns will be a top priority for vaccinating this vulnerable population. As with all new drugs, novel vaccine formulations and adjuvants will be required to undergo a rigorous safety analysis. Safety and efficacy of early life vaccination in animal models must be considered during the development of novel neonatal vaccines.

With the need for new methods to improve early life vaccination the applicability of neonatal animal vaccination models to humans must be discussed. An important aspect to think about understanding animal models is the comparatively large deviation of the innate immune systems between species. For instance, the innate immune system of mice is different from that of humans [296]. Timing of vaccine administration is a crucial part of neonatal animal vaccination models. For example, most studies have concentrated on mice that are around one week of age to model neonatal responses [297]. Knowing the necessity of creating vaccines active around the time of birth, novel animals models might have to be implemented to study vaccination of animals on their first day of life. Even though murine models are crucial for immunologic research, results in mice do not always convert exactly to that of humans. In addition to the murine model, our research showed the porcine model as a great alternative to study neonatal vaccination. Pigs are more closely related to humans and their offspring are of similar size of that of neonatal humans at birth.

All together, our data showed the negative impact of MatAbs on early life vaccination. These inhibitory effects, however, can be overcome by co-formulating the vaccine antigen with proper Th-1-type response redirecting adjuvants. The results indicate that neonatal vaccination can be safe, effective and can become the main approach in protecting the vulnerable newborns and infants. Since most vaccine formulations to be administered in early life have been adjuvanted with alum, novel adjuvants which are more effective at birth might be the solution for developing novel and more successful neonatal vaccines [141, 293]. Further research in neonatal and infant immunology and ongoing safety trials are needed for the development of novel vaccine formulations to meet the challenges of global infections.

Early life vaccination against hepatitis B, polio and tuberculosis showed that neonatal vaccination is effective, well tolerated and an excellent preventative strategy to
The focus of future research should aim at better understanding of how neonatal vaccines responses are obtained and sustained. This can be accomplished by identification of novel adjuvants and vaccine formulations for the use in early life. Such strategy could reduce the incidence of current vaccine preventable diseases such as pertussis, rotavirus and influenza.

6. 2 GENERAL CONCLUSIONS

After summarizing and discussing all results shown in all chapters general conclusions would be:

- We confirmed in two animal models (murine, porcine) that MatAbs interfere with the generation of antibodies during the primary immune response in early life.

- Priming in the presence of passive immunity does not prevent responses to booster doses given later in life.

- Co-formulation of a model antigen OVA with CpG ODN, IDRP and polyphosphazene into microparticle and soluble vaccine formulations induced superior antibody responses in neonatal mice compared to vaccination with antigen alone.

- By assessing various routes for early life vaccination we concluded that mucosal (intranasal) vaccination is superior to parenteral vaccination as it results in the production of both systemic and mucosal immunity.

- In neonates, co-formulating PTd and FHA with the adjuvant platform into microparticle and soluble formulations resulted in balanced Th1/Th2 response as compared to vaccination with the commercially available vaccine Quadracel®, which produced Th2-type responses.
- The novel vaccine formulations composed of the adjuvant platform and pertussis antigens provided similar level of protection as the currently available vaccine against challenge infection even in the presence of high levels of MatAbs.
7. APPENDIX

7.1 CONTRIBUTIONS TO PEER REVIEWED MANUSCRIPTS

7.1.1 “Strategies to link innate and adaptive immunity when designing vaccine adjuvants.


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Dr. Garlapati and myself established the methods to generate polyphosphazene microparticles. Together we tested various methods for preparing microparticles and assessed their size. We labeled both the antigen and adjuvant and studied their distribution within the microparticle.

7.1.1.1 ADJUVANTS FOR VACCINES

Adjuvants constitute important components of human and animal vaccines. They can be grouped into particle-based delivery systems, such as liposomes, micro- or nanoparticles, and molecules that either directly or indirectly induce the expression of cytokines and chemokines thereby modulating the local microenvironment for activation and stimulation of immune cells. Most of today’s adjuvants have been developed empirically and include a wide variety of formulations including cell-wall components, alum, QuilA, carbomers, and oil-in water emulsions to name a few. With the recognition of pathogen recognition receptors (PRRs) such as Toll-like, mannose and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), it has become clear that many of these adjuvants signal through highly specific pathways resulting in increased NF-κB and/or type I interferon (IFN) production, which subsequently leads to an up-regulation of chemokines and cytokines needed for maturation of dendritic cells (DCs) and improved presentation of the antigen. Since invading microorganisms are likely to simultaneously interact with many PRRs, we hypothesize that effective vaccine formulations need to stimulate multiple PRRs to both enhance the magnitude and the
quality of immune responses to the vaccine antigens. Here, we highlight some of our strategies to enhance immune responses against *Bordetella pertussis*, an important human pathogen responsible for more than 300,000 deaths and 50 million cases in infants and young children worldwide [22]. We recently demonstrated that newborn piglets are highly susceptible to infection with *B. pertussis* and show severe signs of respiratory distress, weight loss and moderate to mild fever. The pathology following infection is similar to that seen in human infants including a thickening of the alveolar wall, severe influx of macrophages and neutrophils and complete tissue destruction of underlying interstitial tissues [111]. Using this model our research is focused on utilizing innate immune modulators such as CpG ODN, host defence peptides (HDPs) and polyphosphazenes (PPs) to activate and imprint neonatal DCs towards a Th1 type of response, which ultimately will help to enhance neonatal immunity against infectious diseases such as pertussis. Here, we highlight the potential of some of these immune modulators for use as vaccine adjuvants for neonatal vaccines.

7.1.1.2 HOST DEFENSE PEPTIDES

HDPs, also called cationic antimicrobial peptides, are innate immune molecules found in almost every life form. Their wide spectrum of functions includes direct antimicrobial activities, immunostimulatory functions of both innate and acquired immunity, as well as involvement in wound healing, cell trafficking, vascular growth and both the induction and inhibition of apoptosis [208, 280, 281, 299-301]. For example, HDPs have been shown to recruit immature DCs and T-cells, enhance glucocorticoid production, macrophage phagocytosis, mast cell degranulation, complement activation, and IL-8 production by epithelial cells [207, 279, 302, 303]. Other HDPs have been demonstrated to neutralize pro-inflammatory cytokine induction and lethality in response to LPS/endotoxin [207, 280, 299, 301, 304-309]. For example, the innate defense-regulator peptide (IDR-1), which targets monocytes and macrophages, provided protection against infection with multi-resistant bacteria in mice, and induced a more balanced or controlled immune response by decreasing pro-inflammatory cytokines such as TNF-α and IL-6 at the site of infection [290].
HDPs can be largely grouped structurally into defensins and cathelicidins based on the respective presence of β-sheets and α-helices [310]. They are expressed by a wide range of cells including epithelial cells, neutrophils, macrophages and DCs [281]. Expression is often regulated by the presence of microorganisms [311] and/or stimulation with TLR ligands, such as LPS. HDP may also act as TLR ligands. For example, TLR4 can directly stimulate the expression of murine β-defensin 1 in immature DCs and lead to maturation of these cells [312]. Interestingly, some HDPs such as LL-37 were able to modulate the effects of TLR agonists in the presence of LPS by decreasing the amount of NF-κB translocation into the nucleus consequently altering patterns of gene expression [307]. Furthermore, HDPs have been demonstrated to also enhance adaptive immune responses, and thus are considered an important link between innate and acquired immunity. For example, the human neutrophil peptides (HNP) 1 to 3, human β-defensins (HBD) 1 and 2, as well as murine β-defensins were shown to chemoattract immature DCs, lymphocytes, monocytes and macrophages [204, 313-315]. Recruitment of immature DCs occurred through signaling via the chemokine receptor 6 [302, 313] and other not yet identified receptors [314]. Maturation of DCs was demonstrated following co-culture of immature DCs with HDPs [305]. Moreover, fusion of the murine β-defensin 2 with the gene encoding the human immunodeficiency virus-1 glycoprotein 120 (HIV gp120) resulted in specific mucosal, systemic, and CTL immune responses after immunization [312, 313]. Ovalbumin-specific immune responses were enhanced after intranasal co-administration of ovalbumin and HNP1-3 in C57/Bl mice [282] and intraperitoneal injection of HNP1-3 and KLH of B-cell lymphoma idiotype Ag into mice enhanced the resistance to subsequent tumor challenge [283]. Fusion of β-defensins mBD2 or mBD3 to a B-cell lymphoma epitope sFv38 induced stronger anti-tumor immune responses in mice [312, 313]. Thus, these examples provide evidence that HDPs have been successfully used as adjuvants to enhance vaccine-specific immunity.

To investigate the potential of HDPs for enhancing the immune response in neonates, we are currently using murine, human and porcine DCs. Screening of HDPs is based on the ability to induce expression of chemokine and cytokines in these cells, as well as the up-regulation of co-stimulatory markers and MHC class II. For example, two
subsets of porcine DCs, namely monocyte-derived DCs (moDC) and blood-derived DCs (bDC) are being used which include both myeloid and lymphoid DCs. MoDC were generated by isolation of CD14$^{+}$ cells (monocytes) and subsequent culturing in the presence of IL-4 and GM-CSF [316, 317], whereas bDC were isolated based on their expression of CD172$^{+}$[317]. Figure 7.1 shows an example of the expression of pro-inflammatory cytokines in moDC and bDC following stimulation with HDP. MoDC were stimulated at day 6 of culture with 133 µg/ml of the 12 amino acid peptide HH2 (VQLRIRVAVIRA-NH$_2$). BDC were isolated and rested for 16 hours, after which time they were stimulated in the same manner. Twenty-four hours after stimulation, supernatants were collected from both moDC and BDC for interleukin (IL)-8 analysis by ELISAs. Following an eight-hour stimulation of moDC, cells were collected for qPCR analysis. Figure 1a shows that stimulation with HH2 resulted in enhanced expression of interleukin IL-8 in moDC but not in bDC. Furthermore, 8 hour stimulation by peptide HH2 resulted in a 6- and 8-fold respective increase in the expression of IL-12p40 and IL-17 in moDC (Figure 7.1b). IL-17 plays a role in the activation of macrophages to kill $B$. pertussis [102], recruitment of neutrophils and in an increase of IL-8 production [318]. Thus, this example shows that HDPs can induce the expression of cytokines involved in the recruitment and activation of immune cells. Current research is focused on assessing potential synergies between CpG ODN and HDPs to further enhance specific immune responses against $B$. pertussis in newborn pigs.
Figure 7.1 The effect of peptide stimulation on porcine bDC and moDC. After 24-hour stimulation with peptide HH2 (133 µg/ml) IL-8 levels were examined by ELISAs in moDC and BDC (A). Following an 8-hour stimulation with HH2, the gene expression of IL-12p40 and IL-17 was examined by qPCR in moDC (B). Results are demonstrated as mean ± SEM, (n=4). The following primers were used: IL17F:ACGTACGTGCTACGT; IL17R:AGCTGTAACCGTT; IL12p40-F: GAAATT CAGTGTCAAAAAGCAGCAG; IL12p40-R: TCCACTCTGTACTTCTTACTCCC. The IL-8 was detected by ELISA using the anti-IL8 antibodies (R&D MAB5531 at 2 ug/ml; R& D BAF 535 at 25 ng/ml), and recombinant cytokine standards (R&D 533-IN, concentration of highest standard 40ng/ml).

7.1.1.3 CpG ODN

Bacterial DNA, as well as short oligonucleotides containing ‘CpG motifs’ (CpG ODN), are potent immune modulators in both human and animal species. CpG ODN signal through TLR9, and their immunomodulatory activity, either as ‘stand alone’-innate
immune treatments or as vaccine adjuvants, has been shown by numerous investigators in a variety of species. Excellent reviews are available to summarize the activity of CpG ODN [190, 276, 319-321]. When used as vaccine adjuvant, CpG ODN promote predominantly Th-1 type immune responses in adults, a quality needed for optimal protection against pertussis [86, 102, 292, 322-324].

The strong ability to skew vaccine-induced immune responses towards a Th-1 type response make CpG ODN a logical choice to stimulate balanced or Th1-type immune responses in the neonate. To date immunomodulatory activities of CpG ODN that enhance neonatal immune responses have been demonstrated in a variety of species including mice, humans and pigs [139, 325-334]. In the case of a hepatitis B vaccine co-formulated with CpG ODN, these responses were enhanced even in the presence of MatAbs [335].

To assess the ability of neonates to respond to stimulation with CpG ODN in vitro several studies were performed using either neonatal PBMC or DCs, which were isolated from either human cord blood or the blood of animals. For example, comparable amounts of IFN-α were found in whole blood from adults and neonates following stimulation with CpG both neonatal and adult DCs can elicit Th1 responses [336, 337]. However, in this study the response in DCs was down-regulated by IL-10 secretion from CD5+ B cells in response to systemic inflammation following TLR9 triggering [337]. It has also been demonstrated that stimulation with CpG ODN induced secretion of IgM, up-regulation of expression of HLA-DR and CD86, induction of MIP-1 α, and proliferation of adult and cord blood B cells [338]. Furthermore, similar amounts of IgM were produced by adult and umbilical cord B cells following stimulation with CpG ODN [339]. In contrast, the production of IFN-α in response to CpG ODN was dramatically impaired in cord blood plasmacytoid DCs [340] whilst it was also demonstrated that immune responses to tetanus toxoid, co-formulated with CpG ODN, were higher in adults than in newborns [341]. Similarly, evidence exists that neonatal immune responses to CpG ODN differ from those seen in adults and indeed Th2-responses to allergens were increased following addition of CpG ODN to house dust mite allergens [342]. This contradictory evidence
highlights the need for further research to understand CpG ODN activity in the neonate and to also assess the long-term consequences of treating neonates with CpG ODN.

More recent evidence to support the use of CpG ODN in the neonate comes from recent observations demonstrating that CpG ODN can stimulate the expression of the BAFF-receptor TACI, a factor needed for survival of activated B cells and plasmablasts [343]. CpG ODN, therefore, might help to extend the lifespan of neonatal plasma cells and induce the earlier development of germinal centres [128]. Stimulation of B2 and B1 cells with LPS or CpG ODN not only induced MyD88-dependent plasma cell differentiation and intracellular expression of BAFF and APRIL [344] but also strongly up-regulated the expression of the BAFF-receptor TACI [345, 346] needed for survival of activated B cells and plasmablasts. Thus, in addition to skewing the immune response towards a Th1 type immune response in the neonate, CpG ODN may help to elicit effective cell priming and long term responses in the neonate.

7.1.1.4 POLYPHOSPHAZENES

PPs are synthetic, water-soluble and biodegradable polymers that can function both as vaccine adjuvants as well as delivery-vehicles for vaccines when formulated into microspheres. Polyphosphazene polymers have long chain backbones of alternating nitrogen and phosphorous atoms with two side groups attached to each phosphorous [347]. Different side groups can be substituted at these positions to synthesize polymers with different physiochemical properties, such as water solubility and biodegradability, which make them amenable for use as biomedical polymers, membranes, hydrogels, bioactive and biodegradable polymers (Allcock, 1990). PPs have been used extensively for drug and vaccine delivery. For example, poly[di(sodium carboxylatophenoxy)phosphazene] (PCPP) displayed strong adjuvant activity in mice with a variety of viral and bacterial antigens [227-229] and poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) not only enhanced the magnitude but also modulated the quality of immune responses to influenza X:31 antigen towards a Th1 type immune responses, resulting in more balanced immunity [230]. PCEP similarly induced a balanced Th1/ Th2-type immune response with Hepatitis B surface antigen, and the magnitude of antibody responses was much higher than with the conventional
adjuvant alum, which induced a predominantly Th2-type response (Mutwiri 2006; Figure 7.2). Furthermore, PPs are very safe to use. Their water soluble nature reduces the risk of injection site reactions, which is often seen when using conventional adjuvants like mineral oil and Alum (Payne 1998). Thus, the combined effects of their potent adjuvanticity and negligible toxicity make them potential components for commercial vaccine formulations. We are currently assessing a panel of modified PPs for their ability to enhance specific immune responses against *B. pertussis*. Indeed, preliminary experiments already indicate that the co-formulation of PPs with pertussis toxoid (PTd) and CpG ODN leads to higher antibody responses and secretion of PTd-specific SIgA into BAL and nasal fluids in mice (data not shown). We expect that these responses can be further enhanced by using PP-based microparticles, which contain antigen, CpG ODN and HDPs (Figure 7.3).

![Figure 7.2 Adjuvanticity of PCEP.](image)

**Figure 7.2 Adjuvanticity of PCEP.** Balb/c mice (n=6) were given a single immunization with 10 μg HBsAg alone or in combination with alum or PCEP. IgG1 and IgG2a serum antibody responses were assessed by ELISA at 12 weeks after immunization.
7.1.1.5 MICROPARTICLE-BASED DELIVERY

Particle-based delivery of antigens has proven to be highly efficacious for antigen delivery, especially when compared to the delivery of soluble proteins. Microparticles are phagocytosed by a variety of cells including macrophages and DCs [237, 348]. Once taken up by these cells, antigen is released and subsequently selected for presentation via MHC II. Interestingly, this process occurs in a phagosome-autonomous manner and is controlled by the presence of TLR ligands [349]. As a result, DCs can distinguish between self and non-self antigens allowing for self/non-self discrimination [349]. Furthermore, by being present in either early or late endosomes, various TLRs can be stimulated, therefore enhancing the overall response to the antigen [350].

Particulate delivery systems, such as microparticles and nanoparticles, are typically less than 10 µm in size and consist of hydrophobic polymers or polysaccharides with the protein of interest incorporated at incorporation efficiencies of between 70 and 90%. Concerns regarding the use of particle-based delivery systems include inefficient incorporation, stability and integrity of the antigen during the formulation process or storage [351]. By creating a depot effect, microparticles help to increase the persistence of antigens for a longer time, which is important for the induction of efficient protective T-cell responses [350, 352]. Furthermore, by masking the antigen inside the particles, microparticles help overcome interference with MatAbs, which is a major challenge for vaccinating the neonate. Microparticles are typically co-formulated to deliver both the antigen and adjuvant to the target cell. Indeed, microparticles and liposomes have been successfully used for delivery of a wide range of antigens and adjuvants including CpG ODN using models for cancer, allergies and infectious diseases [242, 243, 353-361]. In primary human plasmacytoid DCs, CpG ODN was delivered by cationized gelatin nanoparticles and this resulted in IFN-α production [362]. Poly(lactic-co-glycolic) microspheres have also been used for both the delivery of antigen and CpG ODN to APC, and their delivery resulted in the activation of endosomal TLR [245]. Maturation and cytokine secretion as well as antigen-cross-presentation was observed. Furthermore, in the same study immunization with these microspheres triggered clonal expansion of primary and secondary antigen-specific CD4+ and CD8+ T cells in vivo.
Many of the currently used microparticles, however, have the disadvantage of exposing antigen during the assembly process to harsh conditions such as high temperature, organic solvents or low pH levels [363]. The ability of PPs PCPP and PCEP to form microspheres under mild conditions either by using spray drying of polymer-protein mixtures onto CaCl$_2$ solution (Allcock, 1990), coacervation with NaCl and subsequent stabilization of microparticle sized coacervates by cross-linking with Ca$^{++}$ ions (Andrianov et al 1998), or by ionic complexation of PP with spermine [269], makes them attractive encapsulation agents. This is particularly useful for encapsulation of biologically labile entities, such as proteins, CpG ODN and/or HDPs. Using the coacervation technique with bovine serum albumin (BSA) and chicken ovalbumin (OVA), we observed spherical microparticles in the range of 0.7 – 3.0 µm in diameter,(Figures 7.3 and 7.4). Using FITC labeled OVA and Alexafluor-546 labeled CpG ODN we showed that the incorporation ranged from 70% to >90% respectively. The integrity of the particles after lyophilization and resuspension appeared to be normal even after storage at room temperature for 2 months. Uptake studies using porcine moDC at a ratio of 5 microparticles per DC confirmed that the particle uptake was apparent at 30 minutes after addition of microparticles (Figure 7.5). Current research in our lab is focused on further improvement of these microparticles using layer-by-layer (LbL) microparticles, which consist of colloid sized core particles onto which oppositely charged molecules are added [364]. The generation of these particles has several advantages including the potential of adding multiple adjuvants onto the outside layers.
Figure 7.3 Formation of PCPP-ovalbumin microparticles. Scanned electron microscopy (SEM) of PCPP-Ovalbumin microparticles prepared by coacervation method (1,000 X magnification). The scale corresponds to 5 µm.
Figure 7.4 Formation of a PCPP-microparticle. SEM of the PCPP-Ovalbumin microparticles at 10,000 X magnification, showing a spherical structure with smooth surface with frequent blebs on the surface.
Figure 7.5 Uptake of PCPP-ovalbumin microspheres by MoDC. Monocyte-derived porcine DCs were overlaid with PCPP-Ovalbumin-CpG microparticles in 5:1 Mps:DCs. The ovalbumin was labeled with FITC and the CpG ODN labeled with Alexa-Fluor 546 Dye. The photomicrographs (40X magnification) were taken using a Zeiss Fluorescent microscope under transmitted light (A), TRITC (B) and FITC (C) filters after 30 min of MP addition. The above results were also confirmed by FACS (results not shown).

7.1.1. 6 CONCLUSION

Adjuvants are important components of vaccines, both for humans and animals. Here, we have highlighted the potential of CpG ODN, HDPs and PPs as adjuvants for neonatal vaccines. CpG ODN, HDPs and PPs act via different pathogen recognition receptors and signaling pathways, each of them resulting therefore in slightly different activation of the innate immune system. By combining these immune modulators and thereby providing multiple signals for stimulation of the immune system, we may be able to develop highly effective vaccine formulations for both adults and neonates.
7.1.2 “Antibody responses in adult and neonatal BALB/c mice to immunization with novel *Bordetella pertussis* vaccine formulations”

Gracia A., Polewicz M., Halperin SA., Hancock RE., Potter AA., Babiuk LA., Gerdts V.

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Aleksandra Gracia and myself worked on the formulation of the vaccine platform. Together we established the proper doses of the antigens and adjuvants to result in optimal induction of immunity. Jointly, we established a proper dose and protocol for *B. pertussis* challenge infections.

7.1.2.1 SYNOPSIS OF THE MANUSCRIPT

The manuscript describes the creation and testing of the novel adjuvant formulation composed of CpG ODN, IDRP and PP. The adjuvant platform combined with PTd into soluble formulations was tested in adult and neonatal mice. Co-formulation of the adjuvants with PTd increased the serum IgG2a and IgG1 antibody titers in adult mice as compared to vaccination with the antigen alone. These results indicate the improvement of the overall, as well as support of a Th1-type immunity. Similar trend was observed in neonatal mice. Moreover, the novel vaccine formulation induced superior IgG2a response when compared to immunization with the commercially available pertussis vaccine. Interestingly, the novel vaccine formulation produced elevated IgG2a response even after a single immunization, which is substantially fewer than the three to five doses presently needed for commercial pertussis vaccines. The response produced following vaccination with the new platform was not only initiated earlier but also persisted over a period greater than 22 months. The results indicated that this adjuvant technology can be potentially used as a platform for future neonatal vaccines.
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