Development of a Novel Adjuvant Platform for Neonatal Vaccines Against Pertussis

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By

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ABSTRACT

Whooping cough is a common childhood disease caused by infection with the bacterium *Bordetella pertussis* or *Bordetella parapertussis*. Although there are both whole-cell and acellular vaccines commercially available, whooping cough has been called the worst-controlled vaccine-preventable disease in existence today. Recent data indicates that there were approximately 16 million cases of whooping cough worldwide in 2008, resulting in 195,000 deaths**.** The majority of these deaths were in developing countries, in infants that were too young to have been vaccinated, or who had not received their full course of vaccinations. Although previously considered under control within most developed countries due to widespread vaccination, a resurgence of whooping cough has been occurring in many developed countries including Canada, the United States, and the United Kingdom. Recent outbreaks in North America have underscored the need for new and more effective vaccines that can be given during the neonatal period, to protect infants in the crucial first few months of life, when they are most susceptible to the disease, and that will provide long-lasting protection against pertussis.

The neonatal immune system has several functional differences from the adult system, and there are specific difficulties that must be overcome when attempting to create a vaccine that can be effective in the neonate. The inherent Th2 bias of the infant immune system is a key difference, with the induction of Th1 type humoral and cell-mediated immune responses being more difficult to achieve due to this state, although not impossible. Another important issue is the presence of maternal antibodies in infants, which can also interfere with vaccination and achieving a sufficient primary immune response.

We tested several adjuvants for immunogenicity, including various dosages and forms of cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN), innate defense regulator peptides (IDRP), and polyphosphazenes (PP), in order to design a novel adjuvant platform that could be combined with our pertussis vaccine antigen, pertussis toxoid (PTd). After selection of the appropriate form and dose of each adjuvant component, we tested the ability of various formulations of the adjuvants in adult and neonatal mice to induce mixed Th1 and Th2 humoral responses, in order to further refine our adjuvant platform. We found that a ratio of 1:2:1 of CpG ODN: IDRP: PP was ideal, and demonstrated that the pre-complexing of the IDRP and CpG ODN components was able to induce significantly higher Th1 (IgG2a) antibody titres than when

the vaccines were not complexed. Our vaccine platform was able to induce strong Th1 and Th2 antibody titres in both adult and neonatal BALB/c mice, with the immune response being of a mixed Th1/Th2 type. However, there was an especially significant increase in the Th1 type humoral response using our novel adjuvant platform when compared to immunization with either current commercial vaccines such as Quadracel®, or when using the standard vaccine adjuvant alum. The Th1 antibody response was extremely long-lasting, with strong IgG2a titres being found up to 2 years post-vaccination.

When an examination of the cell-mediated immune response was performed in adult and neonatal BALB/c mice, it was found that a strong secretory IFN-γ (Th1) response was present post-vaccination in the splenocytes of mice immunized with our novel vaccine. This corresponded with a large number of IFN-γ secreting cells present as visualized through ELISPOTs. The IL-5 (Th2) response, however, was found to be decreased in mice that received our novel vaccine as compared to mice vaccinated with Quadracel®, with no detectable cytokine secreted by stimulated splenocytes *in vitro*, and few to no IL-5 secreting cells visible through ELISPOT.

In order to further improve our vaccine, a second antigen, pertactin (PRN), was added to the formulation, and neonatal mice were immunized and subsequently challenged with live *B. pertussis*. The mice that received the two-antigen vaccine were completely protected against the bacterial infection, and showed strong humoral Th1 and Th2 responses. Several animals that received our two-antigen vaccine showed complete bacterial clearance. This full protection and clearance was superior to the results seen using the commercial vaccine Quadracel®.

Finally, the adjuvant platform was further examined to test its variability and cell recruitment functions. The novel adjuvant platform was able to induce a strong mixed Th1 and Th2 humoral response when combined with a different vaccine antigen, hepatitis B surface antigen (HBsAg), and showed similar results to those seen using PTd, with a significant increase in the Th1 (IgG2a) antibody response when compared to immunization of mice with alum as an adjuvant. Replacing the CpG ODN component of the adjuvant platform with Poly I:C induced lower Th1 antibody responses when the vaccine was delivered subcutaneously or intranasally, however when given intramuscularly it was able to induce IgG2a titres equal to that of the adjuvant platform containing CpG ODN.

When the ability of adjuvant platform to recruit cells to the site of injection was examined, it was determined through histological analysis that the reaction seen was localized and not systemic, and required the presence of both the adjuvant platform and the vaccine antigen together at the same site for induction of the inflammatory response. The response seen at the site of injection was characterized by an influx of neutrophils to the site, with increased vasculitis and little to no necrosis. There was little to no inflammatory response seen over a 48 hour period in mice injected with the adjuvant platform alone, with the PTd antigen alone, or with the adjuvant platform and PTd given in different legs, indicating that a site-specific response is occurring that requires both the presence of the adjuvants and the antigen to be induced.

In summary, we have developed a novel vaccine formulation against *B. pertussis*, which is able to induce strong humoral and cell-mediated immune responses, especially Th1 responses, in both adult and neonatal mice, with the immune responses induced being long-lasting and protective against infection. We have also demonstrated that our novel adjuvant platform itself is adaptable for use with other vaccine antigens and routes of administration, and it may be possible to adjust its components while maintaining efficacy.

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LIST OF ABBREVIATIONS

a.a	Amino acid
Ab	Antibody
ACT	Adenylate cyclase toxin
Ag	Antigen
ANOVA	Analysis of variance
APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
ASC	Antibody-secreting cell
AS02	Adjuvant system 02
AS03	Adjuvant system 03
AS04	Adjuvant system 04
BCR	B cell receptor
BMSC	Bone marrow stromal cell
C1q	Complement component 1, q subcomponent
C3d	Complement component 3d
C5aR	Complement component 5a receptor
CCR6	CC chemokine receptor type 6
CD	Cluster of differentiation
CpG ODN	Cytosine-phosphate-guanosine oligodeoxynucleotide
Con A	Concanavilin A
CRAMP	Cathelin-related antimicrobial peptide
CpG	Cytosine-phosphate-guanosine
CR3	Complement receptor 3
CTL	Cytotoxic T-lymphocyte

DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot assay
Fc	Fragment, crystallizable region
Fcγδ R	Fragment crystallizable region–δ receptor
FDC	Follicular dendritic cell
FHA	Filamentous haemagglutinin
FIM	Fimbriae
FPRL-1	Formyl peptide receptor like-1
GC	Germinal Center
HBD	Human β-defensin
HBsAg	Hepatitis B surface antigen
HLT	Heat-labile toxin
HNP	Human neutrophil peptide
IDRP	Innate defense regulator peptide
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6

IL-10	Interleukin-10
IL-12	Interleukin-12
IL-12p40	Interleukin-12 subunit p40
IL-12p70	Interleukin-12 subunit p70
IL-23	Interleukin-23
IRF-3	Interferon regulatory factor-3
IRF-7	Interferon regulatory factor-7
IRIV	Immunopotentiating reconstituted influenza virosomes
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LN	Lymph node
MAb	Maternal antibody
Mac-1	Macrophage-1 antigen
mDC	Myeloid dendritic cell
MEM	Minimum essential medium
MHC	Major histocompatibility complex
mM	Millimolar
Mϕ	Macrophage
MPL	Monophosphoryl Lipid A
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK	Natural killer cell
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain

ODN	Oligodeoxynucleotide
OVA	Ovalbumin
Pa	Acellular pertussis vaccine
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBSA	Phosphate-buffered saline calcium and magnesium-free
PCEP	Poly[di(sodiumcarboxylatoethylphenoxy)phosphazene]
PCPP	Poly[di(carboxylatophenoxy)phosphazene]
pDC	Plasmacytoid dendritic cell
PMN	Polymorphonuclear cell
PNPP	P-nitrophenyl phosphate
Poly I:C	Polyinosinic polycytidylic acid
PP	Polyphosphazene
PRN	Pertactin
PRR	Pattern recognition receptor
PTd	Genetically detoxified pertussis toxoid
PT	Pertussis toxin
Pw	Whole-cell pertussis vaccine
RNA	Ribonucleic acid
sIgA	Secretory IgA
TBST	Tris buffered-saline/0.01% Tween 20
TBSTg	TBST/0.5% gelatin
TCR	T cell receptor
TCT	Tracheal cytotoxin

TGFβ	Transforming growth factor beta
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
T_{reg}	Regulatory T cell
VCAM-1	Vascular cell adhesion molecule 1
WHO	World Health Organization
WWW	World wide web

CHAPTER 1: LITERATURE REVIEW

1.1 *Bordetella pertussis*

1.1.1 Overview

The disease whooping cough, resulting from infection with the bacteria *Bordetella pertussis* and *B.parapertussis* is endemic worldwide. While remaining an important cause of infant and child illness in developing nations, it is also undergoing a resurgence within the developed world. The disease is most dangerous to infants and young children, in particular those who have not received their full course of vaccinations. As current pertussis vaccines are not administered prior to two months of age, there is a significant window of vulnerability present at infancy, the very time when the human immune system is least capable of dealing with the type of infection causing the disease. Thus, it is no surprise that the majority of the mortality from pertussis is seen in very young infants. There is evidence that infants who recover from the disease suffer from reduced weight gain during early childhood as compared to those that were not infected, and may also have an increased risk of asthma symptoms and respiratory infections later in life [1]. Thus, a vaccine is needed that can address the need to immunize earlier in life, and can meet the specific requirements of stimulating the infant immune system.

1.1.2 B. pertussis

The *Bordetella* genus encompasses six species including *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. avium*, *B. hinzii*, and *B. holmezii* [2].The two species associated with whooping cough, *B. pertussis* and *B. parapertussis*, are closely related with similar morphology and cause similar disease in humans [3, 4]. *Bordetella bronchiseptica* is usually found as a common commensal animal pathogen, however it has been found in humans causing zoonotic disease, and it has been suggested that this is the species from which both *B. parapertussis* and *B. pertussis* evolved [4, 5]. *B. pertussis* is a Gram-negative aerobic coccobacillus which attaches itself to the ciliated epithelial cells of the mucosa in the mammalian upper respiratory tract [3]. It most commonly acts as an extracellular pathogen, however it is also capable of invading human alveolar macrophages, and thus functioning in an intracellular manner [6].The bacterium is approximately 0.8 μm by 0.4 μm in size, non-encapsulated, non-motile, and non-spore

producing [3]. *B. pertussis* is slow-growing, taking at least three to five days to produce pinpoint colonies, and is most commonly grown on blood-containing medium, as it is nutritionally fastidious and has a requirement for nicotinamide [5]. It expresses several virulence factors, the most important being pertussis toxin (PT), a 105kDa protein unique to *B. pertussis*, which is an A-B toxin similar to cholera toxin [2, 7]. There are two main subunits, with the A (S1) subunit being enzymatically active, and the B (S2-S5) oligomer having no enzymatic activity and only responsible for the binding of the toxin to the cell [7]. The S1 subunit is released into the cell, and functions as an ADP-ribosyltransferase which ribosylates the Gi subclass of G proteins, resulting in a disruption of intracellular signalling [7, 8]. Mac-1 (macrophage-1 antigen, also known as CR3 or CD11b/CD18) has been implicated as the cell surface receptor being bound by the B oligomer of PT [9]. Adenylat cyclase toxin (ACT) is a hemolysin that is secreted extracytoplasmically in large quantities by the bacteria. It enters target cells through contact and after activation by calmodulin within the host cell, functions by enzymatically inhibiting the natural phagocytic and cytotoxic functions of cells responsible for immune defense including neutrophils, monocytes, and natural killer (NK) cells [3, 7]. It has also been shown to modulate host dendritic cell (DC) function through blocking IL-12p70 production, resulting in Th17 cell promotion and Th1 limitation [10]. Lipopolysaccharide (LPS), also known as endotoxin, a characteristic component of Gram-negative bacterial cell walls, is also produced by *Bordetella* species, and is the pathogen associated molecular pattern (PAMP) that functions as the agonist of the innate immune pattern recognition reception (PRR) toll-like receptor 4(TLR4). In *B. pertussis*, the lipopolysaccharide (LPS) is missing a long O-antigenic chain, thus it is referred to as lipooligosaccharide (LOS) [7]. LPS and LOS are made up of a saccharide chain and a lipid A portion, which results in toxic effects. There are also other toxins produced by the bacterium, including heat-labile toxin (HLT) and tracheal cytotoxin (TCT), the first of which is protoplasm localized causing tissue damage through dermonecrosis and vasoconstriction, and the second which is a fragment of the cell wall peptidoglycan and destroys the ciliated cells within the trachea [2, 3]. Filamentous haemaggultinin (FHA) is a large 220 kDa surface protein of the cell envelope that is partially responsible for mediating adhesion to the ciliated host cells in the respiratory tract [2, 7]. Pertactin (PRN) is an autotransporter protein of 68-70 kDa that is also involved in the binding of the bacterium to the cell [2, 3]. Further involved in mediating host cell

adhesion are the fimbriae (FIM)-2 and 3 proteins, serotype-specific agglutinins which are found on the surface of the bacterium [7].

1.1.3 Disease and Clinical Symptoms

Whooping cough is acute respiratory infection, with a characteristic paroxysmal cough, which causes a "whoop" sound upon inhaling that gives the disease its name. It is transmitted person to person through airborne droplets, and symptoms begin to appear after an incubation period of 7-10 days [11]. The catarrhal phase of the disease, where patients are most infective, lasts from one to two weeks, and is characterized by coughing, fever and rhinorrhea [3, 11]. The next phase, lasting two to four weeks, is called the paroxysmal phase. This is where the characteristic "whoop" sounding cough usually appears, most severely at night, with other attending complications such as vomiting being common [11]. Finally, there is the convalescent phase of one to three weeks, during which the cough gradually declines. Patients remain infectious to others for a period of approximately five weeks [3]. Complications arising from whooping cough are most likely to be seen in young infants, and can include bronchopneumonia and acute encephalopathy, which is particularly dangerous, with often lethal results [3, 11]. Treatment of the disease involves administration of macrolide antibiotics, most commonly erythromycin. If given in the early catarrhal phase, antibiotic therapy may be able to reduce or prevent clinical symptoms, however when administered later in the course of disease, antibiotics can only reduce transmissibility, and have little effect on symptomology [3, 11].

1.1.4 Epidemiology

There were an estimated 18.4 million cases of whooping cough worldwide in 2004, resulting in 254,000 deaths [12]. Of these cases, 12.7 million are found in Africa or South-East Asia [12]. The disease causes high morbidity in unvaccinated children, and may still be underestimated or misdiagnosed in both developed and developing nations [13]. There is a much higher case-fatality rate in developing nations (approximately 1-3%) as compared to developed countries (0.04%), which makes the unbalanced distribution of disease cases even more devastating [14].

Recently in developed nations such as Canada and the United States, there has been an increase in the number of people infected with pertussis, despite relatively high rates of vaccination coverage [14-17]. Particularly noticeable is the increase in infections seen among adolescents and young adults, who can then proceed to infect those too young to be immunized [15]. A major source of primary exposure for infants has been found to be contact with an infected household or family member, often an adolescent child who was partially or even fully immunized [15, 16]. Current measures being proposed to deal with the issue include booster vaccination specifically of immediate family members of young infants (the "cocoon strategy"), a pre-school booster, as well as vaccination of adolescents in general [15, 18, 19]. Some suggested causes of this disease resurgence are age-cohort effects, a lack of vaccine coverage, and possible issues with vaccine effectiveness or waning post-vaccination immunity [17, 20, 21].

1.1.5 Immune Response to Infection

Infection with *B. pertussis* in infants as young as 4 months old results in induction of T cells specific for several of the bacterial antigens, such as PT, FHA or PRN, which show the characteristics of Th1 cells, and are able to secrete the Th-1 type cytokine interferon-γ (IFN-γ) and interleukin-2 (IL-2), but not the Th2 cytokines IL-4 or IL-5, in response to antigen restimulation [22, 23]. It appears that natural infection in humans tends to polarize responding T cells in the Th1 direction, an initial indication of the importance of the Th1 or cell-mediated immune response in combating pertussis infection. This was confirmed by studies in mice showing that T cell-deficient mice were unable to clear pertussis infection, and adoptive transfer of CD4+ T cells from immune animals was able to mediate protection against bacterial challenge, even when there was a lack of detectable serum antibody [22, 24]. The subset of T cells conferring the protective effects were shown to be Th1-like cells that secreted IFN-γ and not IL-4 [24]. Although the importance of IFNγ produced by Th1 cells has thus been established in cell-mediated immunity to pertussis, other cells have also been implicated in protection. Stimulation of NK cells by DC through production of IL-12 and IL-23 results in IFN-γ early on in pertussis infection, with a lack of NK cells resulting in an enhanced Th2 response [25]. It has also recently been shown that the ACT is able to modulate the actions of human DC through reducing the production of the Th1 promoting cytokine IL-12p70, which results in promotion of

a Th17 type immune response and an inhibition of the Th1 immune response to infection [10]. IL-12 is also produced by macrophages in response to *B. pertussis* infection in mice [26].The Th1 cells induced by infection are detectable in the spleen and lymph nodes of mice within two to three weeks after infection [23]. When naive mice are infected with pertussis, an acute influx of immune cells including macrophages, neutrophils and lymphocytes into the lung occurs. This cell infiltration also occurs in immune mice, however it is less dramatic and more transient by comparison [23]. There is also a strong induction of IgG antibodies against PT, FHA, PRN, FIM, ACT, LPS, and other *B. pertussis* antigens seen after natural infection, and evidence that humoral immunity does play a role in protection, with both humoral and cell-mediated immune responses being required for optimal protection [23]. This was shown through the targeted disruption of immunoglobulin (Ig) heavy-chain genes, with Ig knockout mice developing chronic infection after *B. pertussis* infection [27].The antibodies produced were thought to function by inhibition of bacterial adhesion and uptake, toxin neutralization or opsonizing the bacterium in order to facilitate phagocytosis [23].

Recent evidence indicates that natural immunity after recovery from infection with pertussis is substantial, and longer-lasting (possibly up to 30 years) than whole-cell pertussis (Pw) vaccine induced immunity (6-12 years), which it was originally thought to be similar in duration to [11, 20]. However, the duration of immunity is inherently variable from person to person.

1.1.6 Immune Response to Vaccination

Although there are both whole-cell (Pw) and acellular pertussis (Pa) vaccines available which have served to decrease the incidence of disease, the clinical correlates of immunity to pertussis remain unknown. In developing countries, Pw vaccines remain the most commonly used, due to their lower cost, while many developed nations now exclusively use Pa vaccine [11]. Pw vaccines are usually inactivated by formalin, and contain whole cell bacteria including PT, ACT, FHA, and LPS, while Pa vaccines have the LPS and various other cell components removed and the PT treated to remove its toxicity [7]. Both types of vaccine include an aluminum salt as an adjuvant, and have been shown to induce strong antibody titres against the included antigens, in proportion to the number of doses received, and both can result in immunity against infection, with Pw being suggested to have higher efficacy, but Pa resulting in lower reactogenicity and

reduced frequency of systemic and local reactions [7]. There are no clear correlations between serum antibody levels and protection [28]. Immunization with Pw is similar to natural infection in its ability to stimulate macrophage production of IL-12 and induce antigen-specific Th1 cells in mice, resulting in protection against bacterial challenge [26]. In contrast, immunization of mice with Pa resulted in no detectable production of IL-12 by restimulated macrophages with the main antigenic components of the vaccine. The addition of IL-12 to Pa is able to enhance its efficacy through polarization of the T cell response in the Th1 direction, suggesting that the induction of IL-12 by Pw may be involved in its higher efficacy [26]. There is also a demonstrated role of IFN-γ in immunity resulting from Pw vaccination, similar to that seen after natural infection [27]. This once again contrasts with Pa, which mostly mediates protection through strong induction of antibody against the included antigens [27]. TLR4 has also been shown to be important in the DC response to immunization with Pw, with DC activation through TLR4 resulting in production of IL-23, IL-12, IL-1β and tumor necrosis factor (TNF)-α [29].Thus it appears that Pw tend to induce Th1 type and cellular responses while Pa induce humoral and Th2 biased responses.

1.2 Overview of the Neonatal Immune System

1.2.1. Characteristics of the Neonatal Immune System

The neonatal immune system, defined as the first 28 days of life in humans, is no longer seen as immature in nature, but as shifted in its tendencies. During pregnancy, an increase in hormones such as progesterone assists in the down-regulation of Th1 and pro-inflammatory responses in the mother, resulting in a Th2 bias [30, 31]. This helps prevent an alloimmune response to the fetus [32]. This Th2 bias is reflected in the immune system of both the fetus, and the neonate, and results in a limited capacity to clear intracellular infections. There are also high levels of functional T-regulatory (T_{reg}) cells ($CD4^{+}/CD25^{high}$) found in both fetal lymphoid tissue and neonatal cord blood, which function to suppress the Th1 response in order to prevent autoimmune reactions to self-antigens [33]. There are high levels of the anti-inflammatory cytokine IL-10 produced in the neonate, which results in the impairment of neutrophil responses

[32]. In prematurely born neonates, the IL-10 response to TLR stimulation remains, however there is a profound deficiency in the IL-12 and IL-23 subunit p40, resulting in an even weaker Th1 response and greater susceptibility to infection [34]. Recent data has indicated that human neonatal myeloid dendritic cell (mDC) levels are notably low at birth, and increase to adult levels within the first year of life, however the levels of plasmacytoid dendritic cells (pDC) in neonates remains lower than adults until approximately 2 years of age [35].

Newborn infants passively receive antibodies from their mothers. IgG antibodies against several *B. pertussis* antigens have been detected in cord blood and serum of infants prior to their first dose of vaccine, which were likely transferred through transplacental passage of antibody from the mother [7]. In contrast, no cord blood samples have been found to contain IgM antibody, and only 5% of infants tested had IgA antibody against pertussis in their serum samples [7].

Antibodies induced in response to bacterial or viral antigens tend to be short-lived and weak in the neonate, and remain so until about one year of age [36]. A possible reason for the short-lived antibody responses in the neonate is the impaired homing ability of long-lived plasmacytes to the bone marrow [36]. This often results in a complete lack of antibody response or a very weak response to a vaccine given at birth. However, antigen-specific memory B cells can be induced in early infancy [37]. Infants are also less able to produce IgG2a antibodies in particular. The infant antibody response is characterized by a predominance of IgM, although the isotype switch to IgG is possible, it is dominated by IgG1 and IgG3 antibodies [36]. Neonates are also very limited in their response to T-independent, such as bacterial capsular polysaccharides and other carbohydrates, as well as T-dependent antigens [38]. The follicular dendritic cells (FDC) of neonatal mice are unresponsive, which results in a limited number of germinal centre (GC) induced in response to antigen stimulation [39]. The immature FDC and resulting delay in GC induction causes limitations in antibody secreting cell (ASC) differentiation, resulting in limited primary IgG responses, even when strong immunostimulants such as cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN) are used. In humans, the appearance of fully mature FDC is thought to occur around two months of age, with GC occurring at approximately four months [39]. Stromal cells of the bone marrow (including endothelial cells), provide migration signals, such as vascular cell adhesion molecule (VCAM)-1 and E-selectin, able to

induce leukocytes to travel into and out of the bone marrow compartment [40]. Plasmablasts generated in the spleen of neonates successfully migrate to the bone marrow however, due to the inability of neonatal bone marrow stromal cells to support their survival (due to insufficient bone marrow stromal cell (BMSC) expression of a proliferation inducing ligand (APRIL)), the plasmablasts are unable to survive as ASC [41, 42]. This could explain the lack of antibody response to very immunogenic antigens in neonates as compared to adults.

Maternal antibodies (MAb) passively transferred to and present in the neonate can interfere with the humoral response to vaccination in various titre-dependent ways. They can bind to B cell epitopes of the vaccine antigen, blocking the infant B cells from recognizing their specific epitopes, or they can bind to and neutralize live vaccines [36]. The presence of MAb tends to not affect T cell responses, as antigen:MAb immune complexes can be taken up by antigen presenting cells (APC) and presented to $CD4^+$ and $CD8^+$ T cells successfully through the fragment crystallizable region-γ receptor (FcγR) [43]. Strategies to avoid MAb interference with vaccination include increasing the antigen dose, using mucosal routes of immunization (since the MAb concentration at the mucosa is much lower than in the serum), microencapsulation of antigen, and developing novel vaccine formulations able to result in prolonged immune stimulation, so that the MAb levels have time to wane [36, 43].

The decreased $CD4^+$ and $CD8^+$ T cell function in the neonate is due to a suboptimal ability of APC to interact with the T cells, as well as a low number of APC overall [36, 44]. The mDC of human neonates express lower levels of major histocompatibility complex II (MHC II) and cluster of differentiation (CD)80/86, and are impaired in their IL-12p70 and IFN-α (resulting in less IFN-γ induction) production, resulting in a decreased capacity to mount Th1 type responses as compared to adult mDC [44, 45]. Human neonatal pDC also have a reduced ability to respond to stimulation with TLR-7 and TLR-9 agonists with IFN-α and IFN-β production, even though they express similar levels of the PRR as adult pDC [37]. This reduced response disappears by one year of age [35]. However, neonatal mDC and pDC are able to produce equal or greater amounts of the cytokines IL-1β, IL-6, IL-23 and IL-10 in response to TLR ligands as adult cells, further demonstrating the neonatal bias in the Th-17 and Th-2 direction [45]. It has been suggested that these results are due to neonatal deficiencies in interferon regulatory factor 3 (IRF-3) activation in mDC, and impaired IRF-7 translocation in human pDC [44].

Soon after birth, the neonatal gut begins to be colonized by commensal bacteria. Since a strong inflammatory response to the LPS of Gram-negative bacteria could be dangerous, the intestinal cells of the neonate temporarily lose their responsiveness to TLR-4 [32]. The initial post-birth exposure of intestinal epithelial cells to LPS stimulates the upregulation of the microRNA miR-146a, which represses the TLR-signaling molecule IL-1 receptor-associated kinase 1 (IRAK1) [46]. This down-regulates the production of the IRAK1 protein in the cells, and protects the mucosa from damage.

In mice, the neonatal period is less clearly defined than in humans. Although many previous studies defined one to three day old mice as neonates, the immune maturation of mice at this age poorly correlates to that of humans. The newborn mouse is less developed than the human, with a lack of terminal deoxynucleotidyl transferase expression, which is found even in the human fetus [36]. This relative immaturity can be demonstrated by neonatal thymectomy, which does not significantly affect future human responses, but drastically affects those seen in mice [36]. However, at the age of one week, mice immunized with weak vaccine antigens (such as diphtheria toxoid) that do not show primary antibody responses, are able to show clear antibody responses and induction of memory B cells after subsequent immunizations, indicating that seven day old mice are a better correlate to the human neonate [36].

1.2.2. Comparison with the Adult Immune System

Although the neonatal immune system is able to successfully produce Th1 type immune responses, these responses tend to be lower in comparison to those seen in similarly treated adults. Neonates also have reduced levels of complement components such as complement component 3d (C3d) in their blood as compared to adults, which may be partially responsible for their susceptibility to encapsulated bacteria [32, 36]. Vaccines which are able to induce a strong IgG2a response in adults are often unable to do so in infants [36]. A major difference between the neonatal and adult immune systems is seen within the DC. Although the cord blood of neonates indicates that they express similar amounts of TLRs on their monocytes, the response of neonatal cells to TLR stimulation is different, with less capacity for IL-12p70, IFN-α and

IFN-γ production, similar or greater for IL-6, IL-23 and IL-1β, and far greater ability with respect to IL-10 production [33]. When LPS is used to stimulate human neonatal DC through TLR4 activation, there is IL-23 production, however, adult DC produce IL-12, with the resulting CD4 T cell differentiation favouring Th17 cells in the neonate and Th1 cells in the adult [37]. However, it is possible to induce adult-like T cell responses in the neonate using specific adjuvants or vaccines, for example the Bacille Calmette-Guérin vaccine [37]. Another important difference between neonates and adults is the pDC to mDC ratio, which is approximately 3:1 as compared to 1:3, respectively [44]. This means that there are relatively more DC that can promote an inflammatory response through IFN-α production in the neonate, and less that are able to induce T cell differentiation in the Th1 direction through IL-12. Although the ability of neonatal mDC to produce IL-12p70 (contributing to the Th1 response) starts increasing from birth, the capacity of the mDC to produce the cytokine in response to TLR stimulation remains less than half of that seen in adult cells, even at 2 years of age [35]. IFN-γ production by infant mononuclear cells in response to stimulation with TLR-7 or TLR-8 also remains below adult levels up to 2 years of age, while the increased neonatal Th17 response, as indicated by higher than adult levels of IL-23 production at birth, decreases to adult levels within the first year of life [35]. Finally, there is an increased IL-10 response seen in cord blood from neonates at birth, with the production of this cytokine decreasing during infancy and dropping to adult levels between one to two years of age [35].

1.3 Linking the Innate and Adaptive Immune Systems

The innate immune system is phylogenetically conserved, and encoded in fixed germline receptors such as the TLR [47, 48]. It functions by recognizing damage associated molecular patterns (DAMP) and PAMP and mounting subsequent immediate non-specific pro-inflammatory (such as IL-1β, IL-6 and IL-8) immune responses [49]. The key difference between the innate and adaptive immune responses is the specificity and delayed reaction of the adaptive immune response, which uses gene rearrangement, somatic hypermutation, and Ig-class

switching, to generate antigen-specific effector cells that can recognize particular epitopes on a pathogen and results in immune memory [49]. Since the adaptive immune response is improved through exposure to particular pathogens (with subsequent exposures to a pathogen resulting in a faster and stronger specific immune response), while the innate immune response does not depend on prior exposure, the innate immune response is particularly key in the initial few months of life, when the adaptive response is still being formed. It should be noted that defects in innate immunity although more rare, are more likely to be lethal than those in adaptive immunity, however dysfunctional adaptive immunity is responsible for many common problems such as allergy, tissue graft rejection, and most autoimmune disorders [49]. Successful activation of both the innate and adaptive immune responses, often through induction of appropriate cytokines, is key to designing an effective vaccine. There are many linkages between the innate and adaptive immune system, a key one being in the effector cells used to trigger the immune responses. Innate responses are immediate and mostly triggered through the detection of pathogens using PRR on macrophages (Mϕ), DC, neutrophils, eosinophils, NK cells, and mast cells [49]. Some of these same immune cells when activated, subsequently act as APC and initiate the response of the antigen-specific effector B and $CD4^+$ or $CD8^+$ T cells of the adaptive immune system. This response requires clonal expansion and differentiation of the effector lymphocytes, usually requiring three to five days to occur [49]. This combined effect is referred to as the instruction of the adaptive immune system by the innate immune system. One aspect of this instruction is the upregulation, through activation of the NF-κB (Nuclear factor κ-light-chain-enhancer of activated B cells) pathway, of the co-stimulatory molecules CD80 and CD86 on APC such as the DC [47]. PRR and other molecules, such as C-reactive protein, function through opsonization, activation of complement and pro-inflammatory pathways, as well as through induction of apoptosis [49]. PRR can be expressed extracellularly as in TLR-4, or intracellularly as in nucleotide-binding oligomerization domain (NOD) proteins (as well as TLR-9), both of which respond to LPS. When bound by their respective ligand, TLR induce expression of various host defense mechanisms, such as the production of pro-inflammatory cytokines, upregulation of co-stimulatory molecules, and production of antimicrobial peptides [49].TLR can function through pathways that are dependent or independent of the adaptor protein myeloid differentiation primary response gene 88 (MyD88), with most TLR using the MyD88-dependent pathway, resulting in the release of NF-κB, a conserved transcription factor

involved in DC development and Ig light chain transcription, which promotes the induction of pro-inflammatory responses [47, 49]. The MyD88-independent pathway, used in TLR3 and TLR4 responses, activates the transcription factor IRF-3, which can induce IFNα/β production [47].

One of the key molecules involved in the linking of the innate and adaptive immune systems is the PRR TLR-9, which recognizes unmethylated bacterial or viral DNA containing the CpG motif, as well as the synthetic CpG ODN. The cell types that express TLR9 vary between species. Many murine cells, including B cells, monocytes and both cDC and pDC express TLR9, however human expression is far more limited, and mostly seen in pDC and B cells [50]. TLR-9 is located within the endoplasmic reticulum of cells and upon CpG binding, it is translocated to the endosome, dimerized, and proceeds to recruit MyD88 and trigger a cascade that results in production of NF-κB and the upregulation of pro-inflammatory cytokines TNF-α, IL-6, IL-12, and co-stimulatory molecules such as CD80, CD86, and MHC II [50].

The DC, in its function as a professional APC, is key to the linkage of innate and adaptive immunity through TLR, and are the most powerful stimulators of naive T cells [51]. DC are commonly divided into two categories, myeloid (mDC) and plasmacytoid (pDC), based on their hematopoietic origin, with mDC being derived from a myeloid progenitor and pDC from lymphoid progenitor cells. The mDC characteristically produce IL-12, and express TLR-2 and TLR-4, while the pDC are able to produce high levels of IFNα, and express TLR-7 and TLR-9. A third category, follicular DC (FDC), are not thought to be of hematopoietic origin, but resemble DC in their long dendrite-like processes. The mDC and pDC originate within the bone marrow, and subsequently circulate within the blood before migrating to peripheral tissues such as the spleen, liver and lung [52]. It has been shown that circulating DCs have strong bone marrow tropism, and can also migrate back to the bone marrow in a manner dependent on VCAM-1 and E-selectin, where they are better retained than in the peripheral tissues [52]. Those DCs that take up antigen (Ag) in the peripheral tissues and then return to the bone marrow are able to strongly induce proliferation of Ag-specific CD8+ T memory cells (while these cell types

rarely interact when Ag is not present), presenting a pathway by which DC can link the innate and adaptive immune responses [52].

The immature DC circulating in the periphery constantly sample the environment, and, due to their high capacity for endocytosis, take up pathogens [49]. When peripheral immature DC endocytose a microbial component such as LPS or CpG ODN, their maturation is induced through the upregulation of MHC class I and II molecules, as well as the expression of several TLR (such as TLR-1, 2, 4, 5) [47, 52]. This maturation induces upregulation of co-stimulatory molecules and production of the pro-inflammatory cytokines, and is followed by the migration of the DC to the draining lymph nodes (LN) where they present the processed antigen to naive T and B cells and induce the specific adaptive immune response [49]. The maturation of DC by response to a "danger signal", such as LPS, is a crucial step as immature DC are poor stimulators of T cells [53].The particular cytokines produced by the DC have an effect on the type of immune response generated, with IL-12 production by mDC after TLR4 or TLR9 stimulation able to promote cell differentiation towards the Th1 type [47]. The activation of TLR on pDC induces the production of large amounts of IFNα/β, which promotes the differentiation of human peripheral blood mononuclear cells (PBMC) into DC, and the production of Th1 chemokines as well as antibodies for protection against viral infection [53]. The Th1 immune response is MyD88 dependent, while the Th2 type immune response can be generated through the MyD88-independent pathway [47]. It should be noted that although the various DC subsets express different TLR, there are some, such TLR7 that are expressed in both types. However the response to TLR7 activation is different in mDC, which produce IL-12, as compared to pDC, which produce IFNα, indicating that it is not only the type of TLR engaged that directs the response generated, but also the type of DC being activated [47]. It has also been shown that small numbers of DCs located in peripheral tissues actually migrate back into the blood, through which they can travel to non-lymphoid tissues and present Ag to Ag-experienced T cells already disseminated in the tissue, thus functioning to "boost" the memory response [52]. Due to their crucial role in inducing Ag-specific T cell responses, DC have frequently been examined in clinical trials as possible cellular adjuvants, especially for various types of cancer [54, 55]. Other vaccines use adjuvants that target the DC through intracellular signaling, such as CpG-B ODN, which signals through TLR-9 to decrease IFNα and IFNβ production in murine mDC and pDC,

as well as human PBMC [56]. Finally, some adjuvants have been designed to specifically bind to and act upon the DC itself, through the pre-complexing of Ag to DC-specific antibody (Ab), and subsequent Ab-mediated targeting of cell surface proteins [57].

Another APC which can function as a bridge between the innate and adaptive immune response is the Mϕ. There are two Mϕ phenotypes, M-1 and M-2. It was discovered that in typically Th1-biased mouse strains such as C57BL/6, Mϕ (M-1) stimulated with LPS or IFN-γ were more sensitive, and easily activated to produce nitric oxide (NO) (which inhibits cell division), while in Th2-biased strains such as BALB/c, LPS stimulated Mϕ (M-2) to increase the metabolism of arginine to produce ornithine (which stimulates cell division) [58, 59]. The balance between the M-1 and M-2 phenotypes is regulated by Mϕ TGF-β1, which inhibits the induction of NO synthase and stimulates arginase. This balance is not dependent on T or B cells [58]. The dominant phenotype can influence whether a Th1 or Th2 response will occur. When activated through TLR or scavenger receptors, Mϕ also produce IFNα/β and upregulate co-stimulatory molecules, and thus the ability of the cell to function as an APC is increased [48]. Mϕ can also be activated by the innate immune response through recognition of complement components, antibodies (through Fc receptors), or in response to IFN-γ, all of which results in an increased secretion of cytokines which then affect the direction of the adaptive immune response [48].

B cells also link the innate and adaptive immune systems as they express both PRR such as TLR and antigen-specific B cell receptors (BCR) [60]. B cells are also capable of acting as APC. TLR pathways are known to affect B-cell activation, proliferation and class-switch recombination, however the exact factors required remain unknown. The B cells in the marginal zone of the spleen are considered more "innate-like" and show greater IgM secretion and plasma cell differentiation in response to TLR3 and TLR4 ligands than follicular B cells in mice [60]. It has also been demonstrated that there are higher levels of TLRs on memory B cells as opposed to naïve cells, and the memory cells are better able to differentiate into plasma cells after further TLR stimulation, indicating that the activation signals provided to immature B cells by TLR ligands during the innate immune response may lead to their maturation and increased ability to function as part of the adaptive immune response [60].

Finally, the complement system is a main element of the innate immune system, and consists of over 30 soluble plasma and cell-mediated proteins [61, 62]. It functions to discriminate between self and non-self, as well as to recognize danger signals, and has several methods to deal with these issues, including opsonization of pathogens, chemoattraction of inflammatory cells, and direct lysis of pathogens using the membrane attack complex [61]. It is now thought that not only does complement function as an innate immune response, it also links the innate and adaptive responses. Recently it has been shown that the complement component 1 q subcomponent (C1q) component of complement, which binds the Fc receptor on immune complexes and opsonized pathogens in order to activate the classical complement pathway, is not only produced by Mϕ, but also by DC [61, 62]. It has been shown in mice that C1q is produced by immature DC, with this production being downregulated after DC maturation, and C1q knockout mice produce reduced antibody and IFN-γ T cell responses [61]. Human monocyte derived DC were activated and matured when exposed to C1q, indicating that this complement component may function as a link between the immature DC that function within the innate immune system and the mature DC that activate the adaptive specific immune response [61]. The C3 component of complement is responsible for opsonizing foreign pathogens and marking them for destruction by the membrane attack complex. B lypmhocytes are able to bind C3, and the depletion of this component results in an impaired humoral immune response [62]. It has now been shown that when the BCR encounters C3 bound antigens, there is a large reduction in the threshold needed for B cell activation, allowing a stronger humoral immune response [62]. There is evidence indicating a link between complement activation and the enhancement of T cell immune responses, with mice treated with a complement component 5a receptor (C5aR) antiagonist producing fewer antigen specific CD8 T cells after infection, and those that are deficient in C5aR having reduced responses to infection [62]. The C5aR complement component has also been shown to have a synergistic effect in combination with TLR4, which further indicates the likelihood that complement can have a similar function as the PRR in activating the adaptive immune response [62]. An analogue of the C5aR C-terminal region, called EP67, has been examined as an adjuvant in both young and elderly mice. It was shown to successfully function as a complement agonist, engaging and activating APC and enhancing the induction of Ag-specific humoral and cell-mediated immune responses [63]. Other adjuvants have been found to function by binding complement proteins, such as CTA1-DD (a mixture of the ADP-

ribosylating CTA1 subunit of cholera toxin with a d-dimer, derived from *Staphylococcus aureus* protein A), which binds to complement receptors on FDC, and subsequently enhances GC reactions and serum Ab titres [64].

1.4 Adjuvants

1.4.1 Innate Defense Regulator Peptides

These peptides have been known by several names, such as 'antimicrobial peptides', 'cationic host defense peptides', or, most recently 'innate defense regulator peptides'. IDR peptides are a key part of the innate immune system, and are produced by the PMN and epithelial cells of most vertebrate species [49]. There are two major types of antimicrobial peptides, defensins and cathelicidins [65]. All IDRP have a cationic charge resulting from the presence of positive a.a residues such as lysine, tryptophan and arginine, as well as an amphipathic structure and a large proportion (50% or greater) of hydrophobic residues [66]. Defensins are classified into two major subfamilies, α and β, which are structurally similar, and there is also a third θ subfamily [65]. Defensins all have direct antimicrobial effects, and are able to kill both Gram-negative and positive bacteria *in vitro* as well as fungi and some parasites [65]. Defensins kill pathogens by inserting into and disrupting the cell membrane, resulting in pores which cause membrane permeability and leakage [65]. α-defensins also have anti-viral activity, while β-defensins function by interacting with CC chemokine receptor 6 (CCR6), with both types also causing mast cell degranulation [65]. Defensins have also been shown to enhance the adaptive immune response through acting as chemoattractants for immature human DC [65]. Since the release of defensins is often an innate immune response to infection, the recruitment of immature DC to the site will allow the adaptive immune response to be effectively generated. The defensin mBD2 has also been shown to promote the maturation of DC in mice, activating the DC through a TLR4-dependent pathway [65, 67]. Human α and β-defensins are able to bind microbial antigens with high affinity in both a defensin and antigen-specific manner, which may attenuate

the production of pro-inflammatory cytokines through alteration of the interaction of the APC with the antigen [67]. Defensins may also function as adjuvants and facilitate the delivery of bound antigen to APC while enhancing the immunogenicity of the antigen [67]. The α-defensins known as human neutrophil peptides (HNPs) were shown to enhance ovalbumin (OVA)-specific humoral serum responses after intra-nasal vaccination, as well as increasing the production of OVA-specific IFN-γ, IL-5, IL-6, and IL-10 by $CD4^+$ T cells [68, 69]. For β-defensins, human beta defensin (HBD)-1 and HBD-2 were able to induce significantly higher OVA-specific Ab in mice after intra-nasal vaccination [69].

Cathelicidins are also found in various species and consist of an N-terminal preregion, a conserved cathelicidin-like proregion, and a C-terminal microbicidal domain [65]. The only known cathelicidins generated in humans and mice are called LL-37 (also known as hCAP18) and cathelin-related antimicrobial peptide (CRAMP), respectively. Cathelicidins function by acting upon formyl peptide receptor-like 1 (FPRL1)-expressing cells, such as endothelial cells, and have been shown to be chemotactic for leukocytes such as monocytes, neutrophils and mast cells [65]. The best characterized cathelicidin is LL-37, which has been shown to have antimicrobial effects, angiogenic effects in wound healing, as well as enhancing the expression of chemokines CXCR2 and CCR2 in Mϕ, which indirectly assists in immune cell recruitment [65]. LL-37 is produced by phagocytic leukocytes and epithelial cells, and is found at high levels at sites of inflammation or infection [70]. It is able to modulate the responses of DC through enhancement of Th1 type cytokine secretion and upregulation of the DCs endocytic capacity [71, 72]. This immunomodulatory property of the peptide was shown to be independent of its antimicrobial effects [71]. LL-37 has also been tested in combination with CpG ODN as a cancer therapy, and was shown to significantly increase the anti-tumour effects of CpG ODN treatment in a mouse model of ovarian cancer, and increase the proliferation and activation of NK cells [73]. CRAMP has also been studied as an adjuvant in mice, and was found to induce dose-dependent humoral and cellular Ag-specific immune responses to OVA [67]. Other IDRP such as the small bovine derived Bac2A and Indolicidin, have been shown to have similar abilities to inhibit LPS-induced TNF-α secretion *in vitro* in human monocytes, however the peptides varied in some of their chemotactic abilities, indicating the diversity of effects that are possible with these compounds [74]. The immunomodulatory abilities of various IDRP result in their being

examined for use as adjuvants or anti-infective therapeutics. The peptide IDR-1, which is not directly antimicrobial, was shown to be protective against infection with both Gram-negative and positive bacteria when administered from 48h prior to 6h after infection, with this protection being mediated by monocytes and Mϕ [66, 75]. The synthethic peptide IDR-1002, a derivative of Bac2a, shows promise as a possible adjuvant due to its ability to induce chemokines in human PBMC [76]. This peptide has also been shown to be effective at leukocyte recruitment and was protective against bacterial infection due to the combination of chemokine induction and neutrophil and monocyte recruitment to the infection site [76]. Another example of a cathelicidin being considered as an adjuvant is salmon cathelicidin (asCATH), which has been shown to transiently and specifically stimulate IL-8 production in order to recruit immune cells to the site of infection, and has been suggested as a possible adjuvant for aquaculture [77].

There is evidence that TLR are involved in IDR peptide secretion and thus in the antimicrobial effect of the peptides [47]. LL-37 has also been shown to modulate the inflammatory response to TLR agonists [78]. The fact that new high-throughput methods of IDRP generation have been developed further adds to their attractiveness as adjuvants, as the cost of production has been significantly lowered [79]. Other examples of antimicrobial peptides and proteins being studied for their adjuvant capabilities include lactoferrin and melittin [67, 80].

1.4.2 CpG Oligodeoxynucleotides

CpG ODN are synthetic mimics of unmethylated bacterial DNA sequences, and have very well characterized and strong adjuvant properties. They are recognized as a PAMP by the PRR TLR9, which is expressed intracellularly in B cells and pDC in humans, and in mDC and Mϕ in mice [47, 81]. CpG ODN are internalized through non-specific uptake into the cell, and subsequently colocalize with TLR9 in the endosome, resulting in the activation of a MyD88-dependent pathway that releases NF-κB [47, 81]. They can be a strong activator of DC production of IL-12, which polarizes T cells in the Th1 direction, and thus CpG ODN are well-known as Th1 promoting adjuvants [47]. There are three classes of CpG ODN, referred to as A, B, and C-Class, which vary in the effects produced. The A-Class function through activation of pDC cells in humans and result in strong IFNα production, B-Class stimulates the direct

activation of B and NK cells, and C-Class combines the effects of both of the previous classes [81, 82]. A recent comparison of several motifs from each class *in vivo* in mice indicated that, when used as adjuvants, B and C-Class CpG ODN were able to induce strong Th-1 biased antibody, $CD4^+$ and $CD8^+$ responses, while the A-Class CpG ODN were unable to affect the IgG1/IgG2a antibody ratio and did not elicit Ag-specific IFN-γ secreting $CD4^+$ and $CD8^+$ T cells [83]. All three classes function through TLR9 activation, and all stimulate the secretion of IL-6 in human PBMCs [81]. CpG have been tested and shown to be strong immunostimulants in an enormous variety of species, including but not limited to; mice, cattle, sheep, nonhuman primates, humans, dogs, cats and fish [84]. They are potent mucosal adjuvants, and can be used as a stand-alone treatment of respiratory infections in mice, since when given intra-nasally they quickly induce a transient cytokine response which allows DC and NK cells to be activated and recruited to the lung [85]. CpG ODN have also been tested in clinical trials in humans and are able to enhance the avidity maturation process when included with a Hepatitis B vaccine, resulting in a larger number of high avidity antibodies to the vaccine antigen [86, 87]. CpG ODN have been tested in humans in many types of vaccines, including, but not limited to, those against Hepatitis B Virus, Influenza, and *Plasmodium falciparum* (Malaria), resulting in strong immune responses and an acceptable safety profile [88-91]. There are many more vaccines containing CpG ODN that have been tested in animal models, including those against HIV, human papilloma virus, and *Mycobacterium tuberculosis* (tuberculosis) [92-94]. The method of formulation and delivery of CpG can enhance the innate and adaptive immune responses resulting after vaccination [84]. For example, the covalent linkage of CpG ODN to antigen has been shown to increase the immune responses even further, and it has been suggested that the immune response to CpG ODN is dependent on the administration route [87]. In mucosally immunized mice, spleen cells produced strong IFNγ as opposed to IL-4 upon antigen re-stimulation [82]. It has also been shown in mice that immunization with CpG ODN results in strong levels of IFN-γ production and IL-12 in the serum [84].

CpG ODN show promise as adjuvants for neonatal vaccination, and addition of CpG ODN to neonatal vaccines has been shown to successfully enhance the IFN-γ response to adult levels in some cases [36]. The use of CpG ODN as a vaccine adjuvant in neonatal mice and piglets has also been shown to promote the switch from a Th2 towards a Th1 type of response

[87]. Although CpG ODN are strongly immunostimulatory, they have a very good safety profile at therapeutic doses, although toxicity has been seen in cases of daily high dose injections [87].

1.4.3 Polyphosphazenes

Polyphosphazenes (PP) are biodegradable polymers made up of an inorganic alternating phosphorus-nitrogen backbone with additional reactive side groups. They are linear polymers with a high molecular weight and there can be two side groups attached to each phosphorus atom [95]. The versatility of PP is due to the large number of various side groups that can be attached, including hydrophilic groups such as amino acid esters, lactate and imidazole, or hydrophobic ones including aryloxy and fluoroalkoxy groups, allowing the extreme fine tuning of the polymer backbone, and the fact that when degraded, the resulting products are usually nontoxic [95-97]. PP can be designed to degrade at a biological pH, or at certain temperatures, which allows them to be used as a biodegradable matrix, with great potential for use in vaccines, controlled drug delivery, or as tissue engineering scaffolds [95, 97]. They can be designed to vary in size, stability, and ability to load protein, and this flexibility gives them a huge range of possible applications [96]. PP have been found to function as potent immunostimulants when conjugated to antigenic proteins even in a noncovalent manner [96]. It has been shown that when the polyphosphazene Poly[di(carboxylatophenoxy)phosphazene] (PCPP) was mixed with antigen, water-soluble noncovalent PP:Ag complexes were formed. It is thought that these are able to target the lymphocyte cell surface and cluster together the membrane proteins, which results in the stimulation of intracellular ionic fluxes, followed by ATPase activation and an enhancement of the immune response [96]. PCPP has also been tested for use as a mucosal adjuvant for intranasal immunization against respiratory pathogens, and was shown to be an effective inducer of sIgA and IgG antibody production in the mucosal secretions and serum of immunized mice, and was able to protect against intranasal challenge with infectious agents including *B. pertussis*

and *Streptococcus pneumoniae* [98]. The immune responses seen were of a mixed Th1 and Th2 type, with strong IgG2a and IgG1 titres along with IFN-γ and IL-4 production [98].The polyphosphazene Poly[di(sodiumcarboxylatoethylphenoxy)phosphazene] (PCEP) has also been characterized and compared to PCPP as a vaccine adjuvant in mice, and it has been found that it too is capable of inducing strong antigen specific immune responses [99]. Immunization with PCEP was shown to induce the production of IFN-γ and IL-4, as well as resulting in superior amounts of IFN-γ and IgG2a production as compared to PCPP, indicating that it may be able to promote a more Th1 type immune response than PCPP, although it does induce both Th1 and Th2 type responses [99]. When compared to the most commonly used vaccine adjuvant alum, both PCPP and PCEP were able to induce significantly superior IgG2a antibody responses, as alum resulted in a mostly IgG1 or Th2 type response, suggesting that the PP may be used to modulate the immune response towards a more balanced or mixed Th1/Th2 response [99, 100].

Lastly, the ability to formulate PP including PCPP and PCEP into microparticles is a promising strategy for use in vaccine antigen and adjuvant delivery [100]. Microparticles are able to be phagocytosed by Mϕ and DC, inside which the antigen can be released and more efficiently presented to the antigen-specific cells of the adaptive immune response [100]. The particles, usually less than 10μm in size, are also are able to create a depot effect, allowing antigen to be masked from interfering MAb, as well as to maintain the antigen presence at the site of injection for a longer period, which allows efficient T-cell responses to be induced [100].

1.4.4 Combination Adjuvants

Through combining different adjuvants, one can try to elicit the best possible immune responses to vaccination. Ideally, synergistic effects between adjuvants can improve the immune responses several fold over use of any of the components alone. There are many combination adjuvants that are currently being examined for use in vaccines. A good example of this is the adjuvant system 04(AS04) adjuvant. AS04 contains monophosphoryl lipid A (MPL) adsorbed onto alum, with MPL being the only TLR ligand currently approved for use in human vaccines. MPL signals through TLR-4, as it is a derivative of the Lipid A molecule found on the LPS of

Gram-negative bacteria, while alum has recently been proven to signal through the Nalp3 NOD-like receptor [101]. There are MPL adjuvanted vaccines currently available for Hepatitis B (Fendrix®) as well as Human Papilloma Virus (Cervarix®), and MPL has been studied in clinical trials of several cancer vaccines (both alone and in combination with other adjuvants) [102, 103]. Use of either alum or MPL alone only stimulates a mild immune response to the HPV vaccine, but in combination, strong immune responses result [104]. MPL has also been examined in mice in combination with the innate antimicrobial protein lactoferrin as an adjuvant complex, and was an efficient stimulator of both humoral, and cellular immune responses [80].

There are few adjuvants currently approved for human use, including alum, MPL, AS04, MF59, adjuvant system 03 (AS03), and immunopotentiating reconstituted influenza virosomes (IRIVs) [105]. The first adjuvant licensed for use in humans after alum was MF59™, an adjuvant consisting of a squalene in oil emulsion, that is used in the Fluad™ and Focetria™ vaccines against H1N1, and has been shown to stimulate production of $CD4^+$ memory cells [106-108]. When tested in combination with microparticles and an HIV protein, the combined microparticles in MF59 adjuvant combination was able to induce significantly greater antibody responses as compared to the separate adjuvants in mice and baboons [109]. A possible mechanism of action for MF59™ is that microdroplets of the emulsion are taken up by Mϕ, which then secrete cytokines to enhance the immune response [110]. Squalene is also a key ingredient in AS03, the combination adjuvant licensed for use in humans, which is composed of squalene, another immunostimulatory oil called DL-α-tocopherol (a vitamin E derivative), and the surfactant polysorbate 80. AS03 is also included in the human H1N1 type influenza vaccines Pandemrix™ and Arepanrix™, as well as in the H5N1 Prepandrix™ vaccine [107, 111]. The H1N1 vaccines adjuvanted with either AS03 or MF59™ were able to induce strong antibody responses in adult vaccinated populations, as determined through serum hemagglutination-inhibition assays [112, 113]. In addition, an AS03-adjuvanted H1N1 vaccine has recently been shown to be highly immunogenic in children 6-35 months of age, with an acceptable reactogenicity profile [114].

Microparticles have been studied as delivery vehicles for CpG ODN adjuvants, and are able to induce strong immune responses, especially when antigen is encapsulated into the microparticle along with the CpG ODN, with these responses being greater than those seen with

the adjuvant components delivered separately [84]. When combined with the encapsulated antigen OVA, a stronger cytotoxic T-lymphocyte (CTL) response and increased cross-priming was seen in mice after vaccination as compared to when the components were given separately [115]. Simple co-formulation of CpG ODN with PP (not in microparticle form) has also been shown to have a synergistic effect on the serum IgG titre and neutralizing antibody titre of mice immunized against bovine respiratory syncytial virus, as compared to the use of each adjuvant separately [116].

Liposomes have also been used to encapsulate CpG ODN and this has been shown to enhance the effects of the adjuvant in many ways, including protection from nuclease activity, a decrease in the rate of adjuvant distribution, and facilitation of CpG ODN delivery to the cytoplasm [84]. Microparticles have also been studied as delivery vehicles for CpG ODN, and are able to induce strong immune responses, especially when antigen is encapsulated into the microparticle along with the CpG ODN, with these responses being greater than those seen with the adjuvant components delivered separately [84]. IRIVs are proteoliposomes made of surface glycoproteins from the influenza strain H1N1, and have been used as adjuvants in the human hepatitis A vaccine Epaxal™ [110, 111]. The virosomes can be loaded with Ag, with the hemagglutinin protein of the influenza virus targeting the virosome to the APC, which subsequently endocytoses and intracellularly processes it, finally expressing the vaccine Ag through MHC I and II molecules [110, 117]. IRIVs are also currently in clinical trials for use in vaccines against malaria [118].

When used in combination, the IDRP LL-37 has been shown to promote the sensing of CpG ODN in human B cells and pDC *in vitro*, with 20 times less CpG ODN required to create a reaction in the presence of the peptide [119]. When the synthetic IDRP HH-2 was complexed with a CpG ODN and used with a pertussis toxoid antigen, the combined adjuvant was found to have a synergistic effect on induction of the chemokine MCP-1, as well as on the upregulation of the co-stimulatory molecule CD80 on monocytes and mDC [120]. Production of the IgG1, IgG2a and IgGA antibody titres was also strongly induced with the combination of adjuvants [120].

Other important adjuvants currently being studied both alone and in various combinations currently being studied include immune stimulating complexes (ISCOM, containing the saponin Quil-A), the purified plant extract QS-21**,** imiquimod, and AS02 (containing MPL^{TM} and QS-21 in an oil-in-water emulsion), as well as various combinations of TLR agonists. It has been found that simultaneously stimulating several TLR during vaccination against Respiratory Syncytial Virus was able to improve antibody affinity maturation in mice, and the resulting antibodies were better able to neutralize the pathogen [121]. There have been synergistic increases in cytokine production when multiple TLR stimulation of different pathways has been used to stimulate DC [104].

CHAPTER 2: RATIONALE, HYPOTHESIS AND OVERALL OBJECTIVES

2.1 Hypothesis

Co-formulation of PTd with CpG ODN, IDRP, and PP, promotes a long-lasting protective balanced or Th1-biased response against *Bordetella pertussis* in neonatal mice following vaccination.

2.2 Overall Objectives

1. Characterize the magnitude and isotype profile of the humoral immune response in BALB/c mice vaccinated as adults with PTd and various adjuvant formulations.

2. Characterize the magnitude and isotype profile of the humoral response of BALB/c mice vaccinated as neonates with PTd and various adjuvant formulations

3. Further characterize the quality of the immune response in vaccinated BALB/c mice through examination of the cytokine profile, the duration of immunity, protection against challenge infection, and comparison of single versus two-dose immunizations.

4. Characterize the adjuvant platform further by examining safety and cell recruitment, as well as the versatility of the adjuvant platform with other antigens.

CHAPTER 3: ANTIBODY RESPONSES IN ADULT AND NEONATAL MICE TO IMMUNIZATION WITH NOVEL *BORDETELLA PERTUSSIS* VACCINE FORMULATIONS

3.1 Introduction

Whooping cough is an acute, highly communicable infection caused by the Gram-negative bacterium *Bordetella pertussis*. It is a major childhood illness in the developing world and even in several industrialized nations [11, 14, 122]. Pertussis causes significant morbidity and mortality mostly in infants and young children, who are either too young to have been vaccinated (< two months old), or who have not received their full series of vaccinations [11, 14, 123]. Although it is reported that >80% of people in industrialized nations have been vaccinated against pertussis, there has been a resurgence of the disease within the past two decades [14, 122, 123].

B. pertussis is generally considered to be an extracellular pathogen that localizes at the epithelial cell surface and thus it was previously believed that humoral immunity and a Th2-type response would mediate protection, as is usually the case for extracellular bacteria [6]. Although antibodies can be protective against infection, as shown by passive antibody transfer, experiments in mice have shown that circulating antibodies are not necessary for maintenance of vaccine-mediated immunity [26, 27]. Since *B. pertussis* is also able to invade human and mouse lung macrophages, full clearance of this pathogen requires an additional strong cell-mediated immune response (CMI), with antibody alone being insufficient [6, 24, 26, 27]. Studies of pertussis infection in human infants and mice indicate that a strong Th-1 response, specifically mediated by IFN-γ, is required to resolve infections [22, 25, 27]. Polarizing the T-cell response after vaccination towards a Th-1 type response has been shown to improve the effectiveness of acellular pertussis vaccines [27]. Indeed, respiratory-challenge protection has been shown in the absence of a detectable antibody response, but is correlated with a strong Th-1 response [26].

The neonatal immune response is functionally skewed towards a Th-2 type bias, [36, 37], in part due to the repression of Th-1 responses in the mother and fetus during pregnancy, and

compromised antigen presentation. This immune bias can preclude successful infant immunization when a pathogen, such as pertussis, requires the induction of a balanced or Th1-type immune response through vaccination. Recent studies in mice have indicated that use of appropriate adjuvants, such as simultaneous stimulation of multiple Toll-like receptors (TLR), can augment the immune response in neonates and may compensate for a suboptimal immune system [124]. Thus, to achieve the best possible immune response to vaccination in neonates, multiple adjuvants working synergistically through multiple mechanisms may be required. In the present study we have evaluated a combination of novel adjuvants based on three distinct types of immunomodulators, which together promote a more balanced or Th-1 type response.

Cytosine-phosphate-guanosine oligodeoxynucleotides (CpG ODN) are intracellular TLR-9 ligands and potent activators of the innate and adaptive immune systems of many species (reviewed in [82]). CpG ODNs promote a Th-1 biased pro-inflammatory response with increased IL-1, IL-6, IL-18, IFN-γ and IL-12 cytokine production; these then induce the maturation of APCs (reviewed in [82, 87]). CpG ODN have been used as adjuvants to promote switching from a Th-2 response to a Th-1 response in neonatal mice[87]. Since TLR signalling and activation is necessary for DC maturation and B cell activation, marked effects on antibody production would also be expected, making CpG ODN an excellent candidate adjuvant for a vaccine aimed at shifting the immune response towards the Th-1 direction [84].

Polyphosphazenes (PPs) are synthetic polymers with an inorganic backbone of alternating phosphorus (P) and nitrogen (N) atoms and two side groups attached to each P (reviewed in [95]). PPs can form microspheres to encapsulate possible vaccine antigens [96, 97]. Two PPs in particular have been frequently examined as potential adjuvants, polyphosphazene polyelectrolyte, poly[di(sodium carboxylatoethylphenoxy)phosphazene] (PCEP) and poly[di(sodium carboxylatophenoxy)phosphazene] (PCPP). PCEP is a strong adjuvant that, when compared with PCPP in a murine model, was found to be an even better enhancer of antigen-specific Th-1 and Th-2 immune responses. PCEP modified the immune response from a Th-2 bias to a mixed Th-1/Th-2 response, with both PPs being vastly more potent adjuvants than alum [99].

Cationic innate defence regulator peptides (IDRP) are naturally-occurring innate host defence molecules found in animals, insects, and plants. Although originally studied for their (often-

weak) direct antimicrobial activities, they have a number of immunomodulatory properties. These include recruitment and selective activation of immune cells including dendritic cells (DC) through mechanisms independent of TLRs, synergy with CpG ODNs, and an ability to protect against a variety of bacteria in mouse infection models (reviewed in [125, 126])[78]. IDRPs are evolutionarily conserved and involved in the interface between innate and adaptive immunity [71, 72]. At physiological concentrations, IDRPs can induce the chemotaxis of immune effector cells, through chemokine induction or leukocyte recruitment, to the site of infection, allowing innate, then adaptive immune responses to be generated [74-76, 127]. Evidence exists that IDRPs such as cathelicidins and defensins can function as potent adjuvants and promote antigen-specific cellular and humoral immune responses in murine models [65].

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

3.2 Materials and Methods

3.2.1. Animals

Adult male and female BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). All mice were housed and cared for in the Animal Care facilities at VIDO, University of Saskatchewan (Saskatoon, SK). 8-week old females were used for all adult mouse studies, and mice were bred in the Animal Care facilities at VIDO, University of Saskatchewan, to obtain neonatal mice. Neonates were immunized at 7 days and housed with their dams until weaning at four weeks of age. Mice were selected at random for cage assignments, with each cage housing mice from at least two different groups. When sampling, mice were selected at random in equal numbers from each cage housing that group. Animal technicians were not blind to the group assignment of mice, however those performing the experiment were. All animal experiments followed the guidelines of the University of Saskatchewan and the Canadian Council for Animal Care. All animal experiments were performed in agreement with the guidelines proposed by the University of Saskatchewan and the Canadian Council for Animal Care.

3.2.2. Immunization of Mice

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

3.2.3 Vaccine Components

Genetically detoxified PTd was provided by Novartis (formerly Chiron) (Siena, Italy) [128]. Received batches were centrifuged at 1350 g for 10 min before being stored at -20°C in aliquots. Frozen aliquots were thawed at room temperature and then centrifuged at 1350 g for 10 min. The supernatents were removed and subsequently stored at 4°C for use in the vaccine formulations. Three IDRPs, IDR-HH2, IDR-HH18, and IDR-HH1002, were synthesized using a standard solid phase FMOC method [76]. De-protected IDRPs were cleaved from the resin and purified by reverse phase HPLC prior to lyophilization. Lyophilized IDRPs were resuspended in de-ionized water prior to dilution in PBS for use. CpG ODN were purchased from Coley Pharmaceuticals (now Pfizer). The polyphosphazenes PCEP (VIDO-EP#3) and PCPP (VIDO-PP#4) were synthesized as described previously [99] with minor modifications. The synthesis was performed by the Idaho National Laboratory (Idaho Falls, ID), under the supervision of Dr. John Klaehn. Polyphosphazenes were received in lyophilized form and stored at 4°C in the dark, then dissolved for use in Dulbecco's PBS (1.54 mM KH_2PO_4, 155.17 mM NaCl, 2.71 mM Na_2HPO_4-$7H_2O$, Sigma-Aldrich, MO) by gentle shaking for 36 hours at room temperature (RT). After resuspension, polyphosphazenes were stored in aliquots at RT in the dark.

Quadracel®™ was purchased from Sanofi Pasteur (Toronto, ON). The human dosage of 0.5 ml, contains 15 Lf-units of Diphtheria toxoid, 5 Lf Tetanus toxoid, acellular pertussis [20 μg chemically-detoxified pertussis toxoid (PT), 20 μg filamentous haemagglutinin (FHA), 3 μg pertactin (PRN), 5 μg fimbriae types 2 and 3 (FIM)], inactivated poliomyelitis vaccine (IPV) 40 D-antigen units type 1 (Mahoney), 8 D-antigen units type 2 (MEF-1) and 32 D-antigen units type 3 (Saukett)] with 1.5 mg aluminum phosphate as adjuvant. For immunization of mice, the volume administered was adjusted to contain a dose of chemically-detoxified PT identical to that of the genetically-detoxified PTd in the novel vaccine formulations studied here.

3.2.4. ELISA analysis of PTd-specific Abs

Blood was collected through tail bleeding at two week intervals, starting at zero weeks (at the time of the initial immunization) for adults, and four weeks post-initial immunization for

neonates. Serum was then collected by centrifugation of the blood in a microcentrifuge at 5940 g for five minutes, and this was used to perform ELISAs.

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

3.2.5. Statistics

Data were analyzed for normal distribution and significance using the STATA program version 10 for Windows (StataCorp LP, College Station, TX). Data was assessed for normality using the Shapiro-Wilk test. In order to achieve a normal distribution of data, the sums of all of the repeated measures were taken, and these sums subsequently ranked. The resulting ranked data passed the normality test ($p > 0.05$). One-way ANOVA was used on the normally distributed data to determine the presence of a significant difference between groups, followed by Scheffe's test to separate the means and identify which groups were significantly different. A p-value of $p < 0.05$ was considered statistically significant.

3.3 Results

3.3.1. Selection of adjuvant candidates

Class-A (CpG 8954), Class-B (10103), and a Class-C (10101) CpG ODN were tested to determine which was best able to stimulate a Th-1 type immune response in combination with PTd. Adult mice were immunized at time zero and 4 weeks with 0.05 μg of PTd plus each of these CpG ODNs, and at 2-week intervals, the serum IgG1 and IgG2a serum titres were compared. Immunization with 10 μg of each class of CpG ODN resulted in a statistically significant increase in IgG2a titres (indicative of a Th1 response) as compared to either PTd alone or 10 μg of the corresponding GC 2243 (for Class-A) or GC 2137 (for Class-B/C) backbone control ODN (Fig 3.1A). However, no significant difference was observed in the IgG1 titres (indicative of a Th2 response) between the CpG groups compared to each other or to PTd alone (data not shown). Class-C CpG 10101 ODN was selected for inclusion in the vaccine adjuvants described here, as its mechanism of action is reported to combine the qualities of both Class-A and Class-B CpG ODN [81].

The polyphosphazenes PCEP (VIDO-EP#3) and PCPP (VIDO-PP#4), were selected for testing. Adult mice were immunized with 0.10 μg of PTd and either 50 μg or 100 μg of each polyphosphazene, with or without 10 μg of the selected CpG ODN, and the serum IgG1 and IgG2a titres were compared. Both the 50 μg/mouse and 100 μg/mouse doses of VIDO-EP#3, in combination with CpG C ODN and PTd, were found to induce higher IgG2a titres than VIDO-PP#4 and CpG C, however, these increases were not statistically significant. We observed a 500-fold increase in IgG2a titre, as compared to PTd antigen alone, after boosting with VIDO-EP#3 at four weeks. A statistically significant difference in IgG2a titres was observed in mice immunized with PTd plus 50 μg or 100 μg VIDO-EP#3 as compared to mice immunized with PTd plus 50 μg VIDO-PP#4 (Fig 3.1B). No statistically significant differences in the IgG2a titres, as compared to PTd alone, occurred after immunization with either dose of VIDO-PP#4. The IgG1 titres showed no significant differences between the two doses of the two polyphosphazenes. Mice vaccinated with PTd antigen alone failed to produce statistically significant PTd-specific IgG2a titres as compared to unvaccinated mice over the entire course of the experiment. However, these mice did display a significant increase in IgG1 response. When

CpG was included with the polyphosphazenes in the adjuvant formulation, we observed a statistically significant increase in IgG2a titres as compared to PTd alone; however, there were other differences noted between the four PP plus CpG-adjuvanted groups. None of the mice vaccinated with 50 μg/mouse VIDO-PP#4, PTd and CpG ODN produced a significant IgG2a response. When the dose was increased to 100 μg/mouse VIDO-PP#4 (still including CpG), three mice failed to respond post-vaccination, and four mice responded after 8 weeks or longer, instead of the usual four to six weeks. In contrast, all except one mouse vaccinated with 50 μg/mouse VIDO-EP#3, PTd, plus CpG ODN produced an IgG2a response, and only two mice vaccinated with 100 μg VIDO-EP#3 (plus PTd and CpG ODN) failed to produce detectable IgG2a. Thus VIDO-EP#3 as a vaccine adjuvant appeared to be a more consistent stimulator of the IgG2a response, and was selected for further formulations.

Two IDRPs, IDR-HH2 (VQLRIRVAVIRA-NH_2) and IDR-HH18 (IWVIWRR-NH_2) were synthesized and tested. Adult mice were immunized with 0.1 μg of PTd together with 75 μg or 150 μg (3.75 mg/kg or 7.50 mg/kg respectively) of IDR-HH2 or IDR-HH18, were boosted in the same manner after 4 weeks, and the IgG1 and IgG2a serum responses were analyzed. Vaccination with each IDRP and PTd at 75 μg/mouse or 150 μg /mouse doses resulted in no detectable IgG2a response. When the IDRPs were combined with a 10 μg/mouse dose of CpG C ODN and PTd, a statistically significant IgG2a response was observed in mice immunized with 75 μg/mouse of IDR-HH18 as compared to mice immunized with PTd only (Fig 3.1C).

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

Figure 3.1 PTd-specific serum IgG2a Ab responses to immunization with various adjuvants.

Adult BALB/c mice (n = 8 or 10 mice per group) were immunized with PTd antigen alone, PBS, or antigen combined with various dosages of CpG ODN, IDRP, or PP at 8 weeks of age. An identical secondary immunization was given at 4 weeks. Sera were collected immediately prior to initial immunization and every 2 weeks thereafter for 10 weeks. Median titres are shown. **A)** Statistically significant differences occurred between each class of CpG ODN relative to PTd alone ($p<0.05$) treated mice. **B)** Statistically significant differences occurred between CpG ODN and PTd alone ($p<0.001$), 50 μg or 100 μg of VIDO-EP#3 and PTd alone ($p<0.005$), and also between 50 μg of VIDO-PP4 as compared to 50 μg or 100 μg of VIDO-EP#3 ($p<0.001$). **C)** Statistically significant differences only occurred between CpG ODN combined with 75 μg IDR-HH18 as compared to PTd alone ($p<0.05$) treated mice.

3.3.2. Testing the Combined Adjuvant Platform in Adult Mice

After the selection of each of the components, a vaccine combination using the adjuvant platform was developed. It had been found *in vitro* that when CpG ODN was added to IDRPs, a precipitate complex was formed that was able to enhance chemokine production *in vitro*, as well as to increase mucosal and systemic responses *in vivo* [120]. Thus, vaccine groups were included to confirm the ability of this complexing of components to increase immune responses *in vivo*. Vaccine formulations consisted of 0.1 μg PTd, 10 μg CpG ODN, 50 μg VIDO-EP#3, and either 10 μg or 75 μg of IDR-HH18. To create the complexed precipitate, CpG C and IDR-HH18 were pre-combined and heated at 37°C for 30 min. Non-complexed groups had CpG C and IDR-HH18 kept separate until immediately prior to injection. Adult mice were immunized and boosted with variations of the adjuvant platform combination and the serum IgG1 and IgG2a titres were compared (Fig.3.2A & B).

A large increase in serum IgG2a titres was observed in mice immunized and boosted with the adjuvant platform formulations as compared to PTd antigen alone (Fig. 3.2A). An earlier, stronger IgG2a response was observed when the adjuvants were complexed prior to vaccination. The noted difference in response was statistically significant ($p < 0.05$) between the two groups containing a 75 μg dose of IDRP. Even when the IDRP dose was lowered to 10 μg/mouse, antibody titres rose earlier and higher in the complexed adjuvant platform group as compared to the non-complexed group.

The IgG1 serum response after vaccination showed a similar trend to the IgG2a (Fig. 3.2B). There was a notably earlier and significantly greater IgG1 response when the adjuvants were included in the vaccine, as compared to antigen alone. There was also a significant difference in IgG1 titres seen between the complexed and non-complexed adjuvant platform groups; however, there was no difference seen between the complexed groups containing the higher 75 μg/mouse dose of IDRP, and the lower 10 μg/mouse dose. There was also a notable IgG1 response to mice immunized with PTd alone, similar in strength to that of the non-complexed adjuvant platform group, which is similar to that seen in other experiments, as immunization with PTd alone tends to result in an IgG1 response as opposed to IgG2a.

When comparing the IgG2a responses, we observed several non-responders in the PTd only group (Table 3.1). Three mice in this PTd group that did not produce detectable IgG2a also failed to produce an IgG1 response to the antigen. In contrast, mice from the higher dose 75 μg/mouse IDRP group complexed with CpG ODN all showed a measurable IgG2a response to vaccination, with no late responders. All of these mice also had an IgG1 response. In the non-complexed adjuvant platform group containing 75 μg/mouse of IDRP, there were two IgG2a non-responder mice, and three late-responders. The adjuvant platform group containing 10 μg of IDRP complexed with CpG ODN contained three IgG2a non-responders. These three IgG2a non-responders were also IgG1 late-responders.

Table 3.1 *Individual IgG2a antibody responses & antibody kinetics post-vaccination for Fig 3.2a*

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

[a] Vaccine components of group, antibody titre results shown in Figure 3.2a.

[b] Non-R = Number of "non-responding mice", a mouse that has non detectable antibody titre over the course of the experiment

[c] Late-R = Number of "late-responding mice", a mouse that has no detectable antibody titre until 8 weeks post-immunization or later

[d] Avg-R = Number of "average-responding mice", a mouse that has detectable antibody titre prior to 8 weeks post-immunization

[e] 1 mouse was found dead in this group

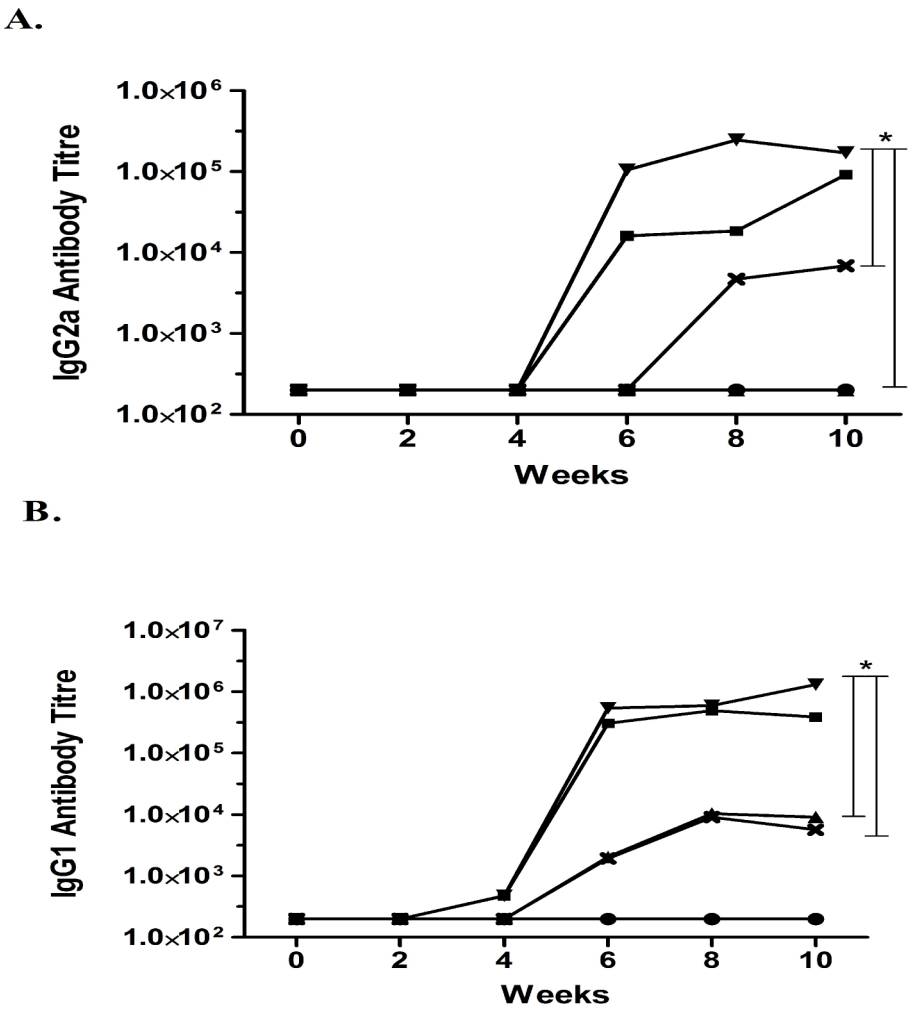


Figure 3.2 PTd-specific serum Ab responses to immunization with various adjuvant platform combinations.

Adult BALB/c mice (*n* = 10 mice per group) were immunized with either 0.1μg PTd antigen alone (▲), PBS (●), PTd (0.1μg)+ CpG C(10μg)+ IDR-HH18(10μg)+ VIDO-EP#3(50μg), with the IDRP & CpG components complexed (■), PTd (0.1μg)+ CpG C(10μg)+ IDR-HH18(75μg)+ VIDO-EP#3(50μg), with the IDRP & CpG components complexed (▼), or PTd (0.1μg)+ CpG C(10μg)+ IDR-HH18(75μg)+ VIDO-EP#3(50μg), with the IDRP & CpG components not complexed (**X**), at 8 weeks of age. An identical secondary immunization was given at 4 weeks. Sera were collected immediately prior to the initial immunization and every 2 weeks thereafter for 10 weeks. Median titres are shown. **A)** Statistically significant IgG2a differences occurred between the adjuvant platform containing 75μg IDR-HH18 (complexed with CpG ODN) relative to PTd ($p<0.005$) treated mice and between the non-complexed and complexed adjuvant platforms containing 75 IDR-HH18 ($p<0.05$). **B)** Statistically

significant IgG1 differences occurred between the adjuvant platform containing 75 μg IDR-HH18 (complexed with CpG ODN) relative to PTd ($p<0.005$) treated mice and the non-complexed adjuvant formulation ($p<0.005$).

3.3.3. Triple Adjuvant Combination in BALB/c Neonatal Mice

Seven-day old mice were used in our neonatal experiments. This age has been shown to correlate best with the human neonate's immune system [36, 37]. BALB/c pups were immunized, and the serum IgG2a and IgG1 titres monitored over time (Fig. 3.3A & B). A higher dose of PTd was used for vaccination in the neonatal model to compensate for the reduced ability of the immature immune system to respond to the antigen, and the dosages of other vaccine components used were adjusted for the smaller weight of the pups in comparison to adult mice. Vaccine formulations consisted of 1.0 μg PTd, 2.37 μg CpG ODN, 2.37 μg VIDO-EP#3 and 35.55 μg of IDR-HH18, both complexed and non-complexed.

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

The IgG1 titres in this experiment followed a very similar pattern to the IgG2a response, with adjuvant combinations inducing approximately 10 times higher titres than the antigen alone (Fig. 3.3B). There was no significant difference seen in the IgG1 titres between the complexed and non-complexed vaccine groups, however, the mouse to mouse variability in antibody titre was larger in the non-complexed vaccine groups.

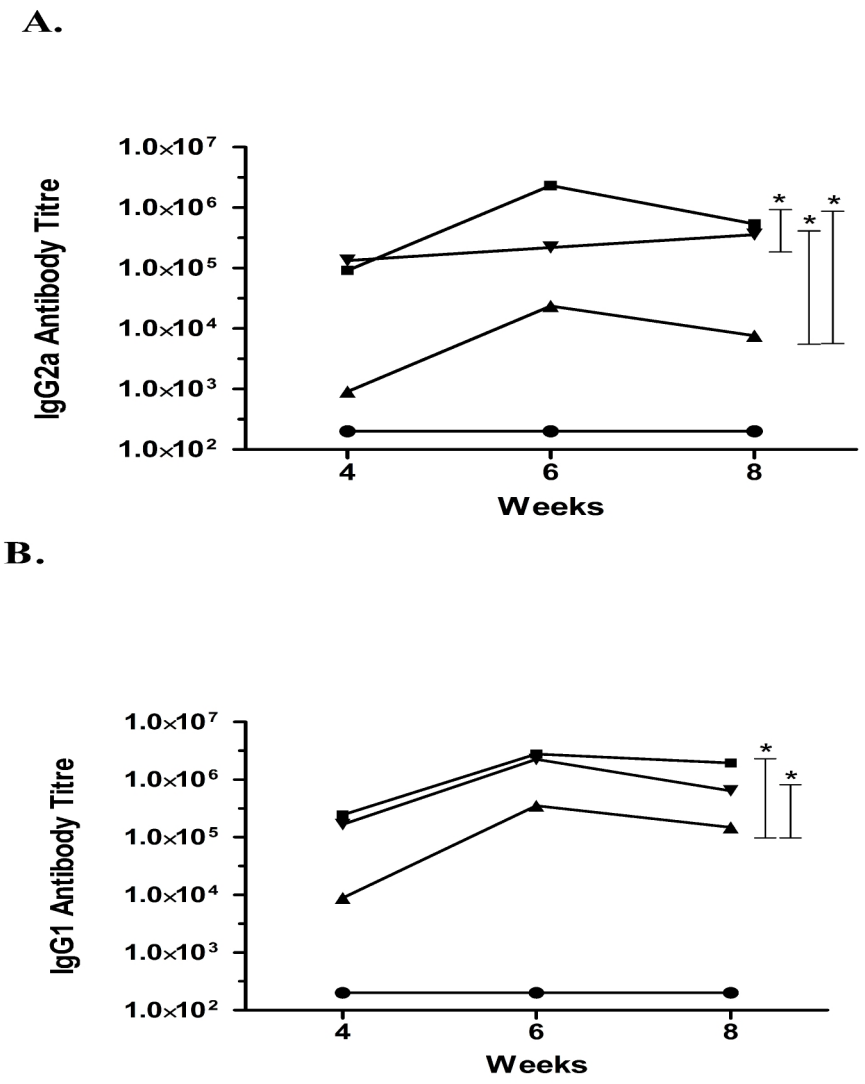


Figure 3.3 PTd-specific neonatal serum Ab responses to immunization with adjuvant platform combinations.

Neonatal BALB/c mice (n=8 mice per group) were immunized with either 1.0μg PTd antigen alone (▲), PTd (1.0μg)+ CpG C(2.37μg)+ IDR-HH18(35.55μg)+ VIDO-EP#3(2.37μg), with the IDRP & CpG components complexed (■), PTd (1.0μg)+ CpG C(2.37μg)+ IDR-HH18(35.55μg)+ VIDO-EP#3(2.37μg), with the IDRP & CpG components not complexed (▼), or PBS (●, *n* = 5 mice per group) at 7 days of age. An identical secondary immunization was given at 4 weeks. Sera were collected at 4 weeks after the initial immunization prior to the boost, and every 2 weeks thereafter until 8 weeks post-initial immunization. The median titres are shown. **A)** At week 6 to 8, statistically significant differences in IgG2a antibody titres occurred between either the complexed adjuvant platform combination ($p<0.001$) or the non-complexed platform ($p<0.05$) relative to PTd treated mice. There was also a statistically significant difference in IgG2a antibody titres ($p<0.05$) seen between the complexed and non-complexed adjuvant platform combinations. **B)** At week 6 to 8, statistically significant differences in IgG1 titres occurred between the complexed or non-complexed adjuvant platform relative to PTd treated mice ($p<0.001$).

3.3.4. Variations of the Adjuvant Ratio in Neonatal Mice

In vitro evidence indicated that an adjuvant ratio of 1:2:1 (CpG ODN: IDRP: PP) was most effective at increasing chemokine production *in vitro* [120]. In our previous experiments, a differing ratio of 1:15:1 was being used, thus, a comparison of the two adjuvant ratios was performed. Neonatal 7-day old mice were immunized with vaccine formulations either containing 1.0 μg PTd alone, or consisting of the 1:15:1 ratio containing 1.0 μg PTd, 2.37 μg CpG, 35.55 μg of IDR-HH18, and 2.37 μg VIDO-EP#3 (complexed); or the 1:2:1 adjuvant ratio containing 1.0 μg PTd, 2.37 μg CpG, 4.74 μg of IDR-HH18 and 2.37 μg VIDO-EP#3 (complexed). A control group received 1.0 μg PTd combined with 2.5 μg alum (aluminum phosphate), the commercially used adjuvant that induces characteristically strong Th-2 type immune responses.

A large increase in the IgG2a titres already occurred after the first immunization with either ratio of the adjuvant platform formulation, as compared to antigen alone (Fig 3.4). This difference was statistically significant for both adjuvant platform groups over the course of the experiment. No significant difference was seen between the adjuvant platform combination containing a dose of 35.55 μg of IDR-HH18 (1:15:1 ratio) and the combination with the lower 4.74 μg IDRP dose (1:2:1 ratio). Very low IgG2a titres were found in the PTd + alum immunized group. Within this alum group, there were five non-responder mice and one late-responder out of a total of 10 mice (Table 3.2). In the adjuvant combination group with 35.55 μg of IDR-HH18, all mice showed an IgG2a response and there were no late responders. In the adjuvant combination group containing 4.74 μg of IDRP, there was one mouse that was a late responder, however all mice eventually showed an IgG2a response post-vaccination.

Table 3.2. *Individual IgG2a antibody responses and kinetics post-vaccination for Fig 3.4.*

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

[a] Vaccine components of group, antibody titre results shown in Figure 3.4.

[b] Non-R = Number of "non-responding mice", a mouse that has non detectable antibody titre over the course of the experiment

[c] Late-R = Number of "late-responding mice", a mouse that has no detectable antibody titre until 8 weeks post-immunization or later

[d] Avg-R = Number of "average-responding mice", a mouse that has detectable antibody titre prior to 8 weeks post-immunization

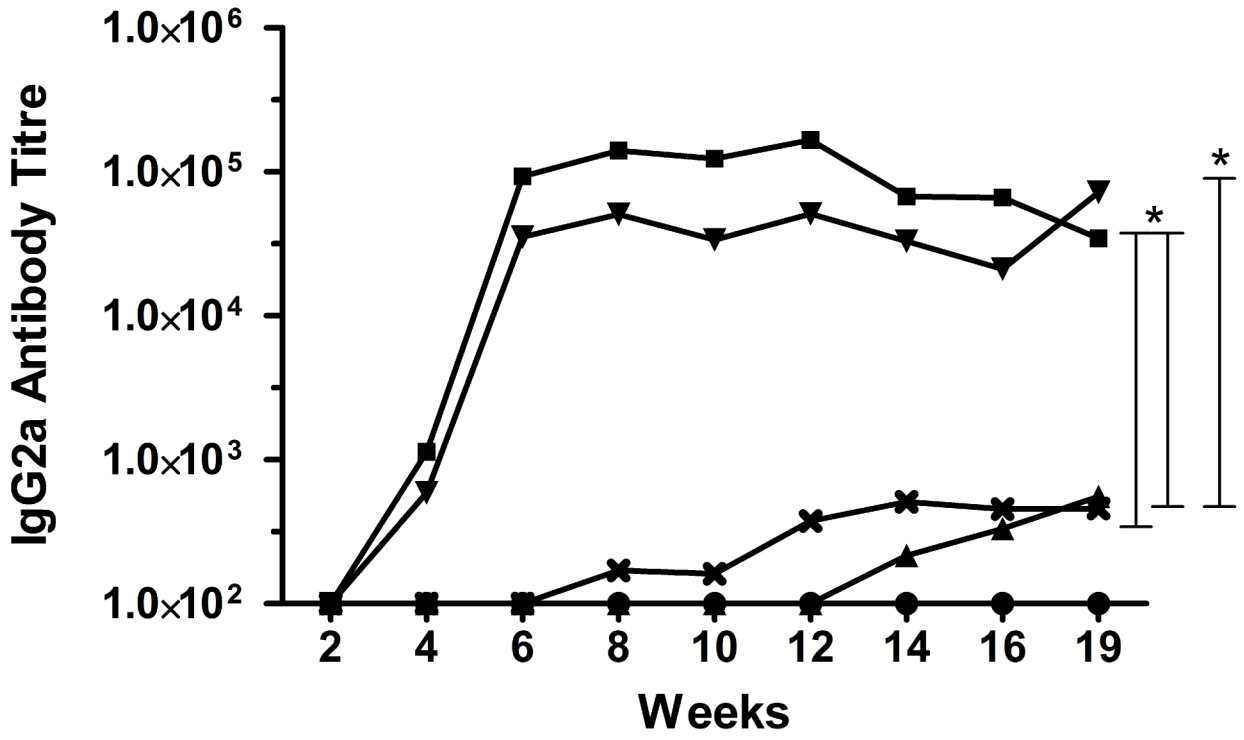


Figure 3.4 PTd-specific neonatal serum IgG2a Ab responses to immunization with various adjuvant platform combinations or alum.

Neonatal BALB/c mice were immunized with either 1.0μg PTd antigen alone (▲), PTd (1.0μg)+ CpG C(2.37μg)+ IDR-HH18(35.55μg)+ VIDO-EP#3(2.37μg), with the IDRP & CpG components complexed (■), PTd (1.0μg) +CpG C(2.37μg)+ IDR-HH18(4.74μg)+ VIDO-EP#3(2.37μg), with the IDRP & CpG components complexed (▼), PTd(1.0μg) + alum (2.5μg) (**x**, *n* = 10 mice per group), or PBS (●, *n* = 9 mice per group), at 7 days of age. An identical secondary immunization was given at 4 weeks. Sera were collected at 4 weeks after the initial immunization prior to the boost, and every 2 weeks thereafter. The median titres are shown. Statistically significant differences occurred between the adjuvant platform combination containing 35.55 μg IDRP and the combination containing 4.74 μg relative to PTd-alone treated mice ($p<0.005$, and $p<0.05$, respectively). There was also a statistically significant difference found between the adjuvant platform combination containing 35.55 μg of IDRP and the PTd and alum group ($p<0.01$). There were no differences between the two adjuvant platform combination formulations.

3.3.5. Duration of Immunity

The antibody levels of the group containing an adjuvant ratio of 1:2:1 were followed over a longer period of time to examine when they would decrease (Fig 3.5). Very high IgG2a titres were induced after immunization and boosting, and these titres remained elevated at the highest level for approximately 52 weeks. Past this point, the antibody titres decreased slightly, however, the IgG2a antibody response remained highly elevated for over 22 months.

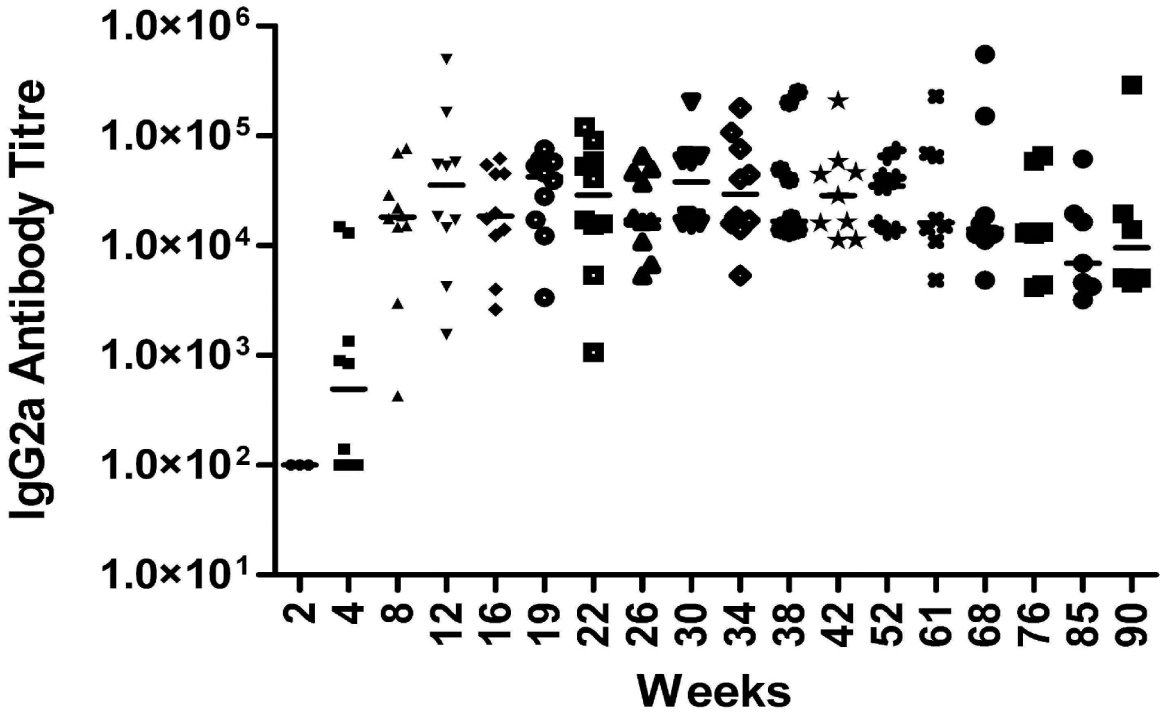


Figure 3.5 PTd-specific neonatal serum IgG2a Ab responses to immunization with a 1:2:1 ratio of CpG:IDRP:PP adjuvant combinations over an extended time period.

Neonatal BALB/c mice were immunized as in Fig 3.4, with an adjuvant platform combination, (n = 10 mice per group) at 7 days of age. An identical secondary immunization was given at 4 weeks. Sera were collected at 4 weeks after the initial immunization prior to the boost, and every 2 weeks thereafter, until week 10, after which sera were collected approximately every 4 weeks. Median titres are also indicated for each group.

3.3.6. Comparison of Single and Double Immunizations with a Commercial Vaccine

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

Vaccine formulations consisted of 1.0 μg PTd, 2.37 μg CpG ODN, 4.74 μg IDR-1002, 2.37 μg VIDO-EP#3, as well as the commercial vaccine Quadracel®. The IDR-1002 and CpG ODN components were complexed. Single and double immunization groups for the adjuvant platform combination and Quadracel® were included. IDR-HH18 was replaced by a third generation synthetic IDRP, IDR-1002 (VQRWLIVWRIRK-NH_2) because of its ability to induce superior chemokine responses *in vitro* and *in vivo* [76]. Neonatal mice were immunized subcutaneously with the formulations and the IgG2a serum responses were compared (Fig. 3.6).

A large increase in IgG2a titre was seen after both a single and a double immunization with the adjuvant platform formulations, as compared to PTd antigen alone; however there was a noticeable difference between the single dose and the boosted groups, with the boosted group developing much higher titres. Immunization with the commercial Quadracel® vaccine, currently used for infant vaccination, resulted in very low to negligible IgG2a titres after a single dose, with an increase in titre occurring when boosted. However, even within the boosted Quadracel® group, the resulting IgG2a titres were still lower than or similar to those that occurred in the single dose adjuvant platform group. The antibody titres in the boosted adjuvant platform group were significantly higher than those of the single dose commercial vaccine group. All mice in the boosted adjuvant platform group showed an antibody response, while there were two non-responding mice in the single dose group (Table 3.3). In comparison, six of the mice that received a single dose of Quadracel® did not produce a detectable IgG2a response, with the boosted group containing two non-responders. The IgG2a antibody response in the adjuvant platform and Quadracel® groups remained elevated for over four months.

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

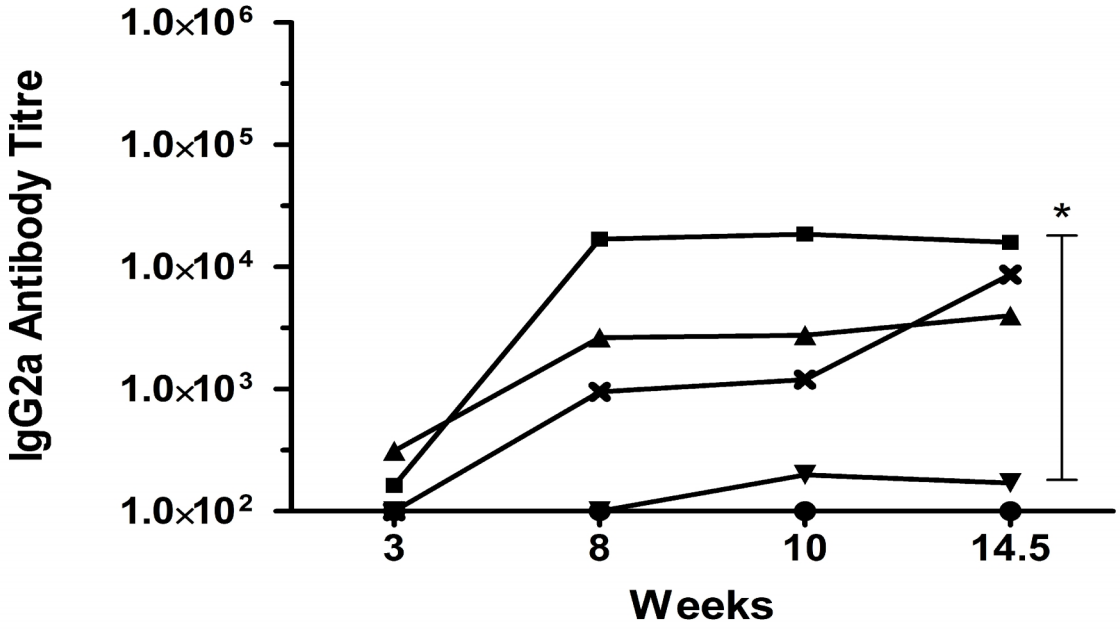


Figure 3.6 PTd-specific neonatal serum Ab responses to single or double immunization with PTd formulated with adjuvant platform combinations or the commercial vaccine Quadracel®.

Neonatal BALB/c mice were immunized with either PTd (1.0μg) +CpG C(2.37μg)+ IDR-HH18(4.74μg)+ VIDO-EP#3(2.37μg), with the IDRP & CpG components complexed (▲, single dose), PTd (1.0μg) +CpG C(2.37μg)+ IDR-HH18(4.74μg)+ VIDO-EP#3(2.37μg), with the IDRP & CpG components complexed (■, two doses), Quadracel® (*n* = 10 mice per group) with a dose including 1.0μg chemically detoxified PTd (▼, single dose), Quadracel® (**x**, two doses), or PBS (●, *n* = 9 mice per group) at 7 days of age. An identical secondary immunization was given at 4 weeks to some groups. Sera were collected at 3 weeks after the initial immunization, and also at 8, 10 and 14.5 weeks post-initial immunization. The median titres are shown. Statistically significant differences occurred between 2 doses of the adjuvant platform relative to a single dose of Quadracel® ($p<0.05$) treated mice.

Table 3.3. *Individual IgG2a antibody responses and kinetics post-vaccination for Fig 3.6.*

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

[a] Vaccine components of group, antibody titre results shown in Figure 3.6.

[b] Non-R = Number of "non-responding mice", a mouse that has non detectable antibody titre over the course of the experiment

[c] Late-R = Number of "late-responding mice", a mouse that has no detectable antibody titre until 8 weeks post-immunization or later

[d] Avg-R = Number of "average-responding mice", a mouse that has detectable antibody titre prior to 8 weeks post-immunization

3.4 Discussion

Whooping cough remains a major threat to infants and children in the developing world, with serious complications resulting from the disease including encephalopathy, convulsions, brain damage, and death (reviewed in [23]). Current pertussis vaccines cannot be administered until 6-8 weeks of age, as prior to this time several factors, including the presence of maternal antibodies and the innate Th-2 bias of the infant immune system, can modulate response to vaccination. Since neonates are known to be the most severely affected by whooping cough, a protection gap exists prior to the possibility of immunization, during which infants are at serious risk. To circumvent this gap a novel neonatal vaccine formulation, able to successfully overcome infants' Th-2 bias as well as the interference of maternal antibodies, is required.

Combinations of adjuvants may be used to induce superior immune responses than achieved by single adjuvants alone. It has been found that the simultaneous stimulation of several TLRs using multiple adjuvants in mice can improve the affinity maturation and neutralizing antibody production in vaccinated animals [121]. This positive modulation effect is not seen with alum, the currently used adjuvant in most human vaccines, including pertussis vaccine. Using mixed TLR ligands in vaccine formulations, the immune response can be increased until it reaches a threshold of activation necessary for antibody maturation [121]. Co-administration of TLR-9 agonists has also recently been tested in humans and found to enhance affinity maturation of specific anti-vaccine antibodies [86]. Because of this, we have sought to formulate effective, multi-component-adjuvanted vaccines with a combination of a known TLR ligand and other adjuvants to create a more balanced immune response and protect neonates against pertussis.

Three classes of adjuvants were selected. These act via complementary mechanisms and are thus speculated to simultaneously activate the immune system in distinct ways. Class C CpG ODN is a well characterized TLR 9 agonist, combining the mechanistic abilities of the other CpG classes by inducing both the secretion of IFN-α from pDC and the proliferation of B and NK cells [81]. Although the precise mechanism of action of the polyphosphazene PCEP is unknown, its adjuvant effects are suspected to result at least partially from a depot effect. The depot effect of polyphosphazenes has been tested and shown strong potential for use in the sustained controlled delivery of human growth hormone [129]. Polyphosphazenes have been shown to stimulated increased cytokine production, and it is also speculated that they may

stabilize antigens, allowing more efficient presentation to immune cells [98, 130]. IDRPs have a plethora of immunomodulatory effects; however, their ability to recruit cells to the site of injection and activate DCs, resulting in increased APC function is speculated to be important for increasing the cellular immune response necessary to clear *B. pertussis* infection.

Evidence from this report indicates that the combination of PTd antigen plus CpG C, IDR-HH18 or IDR-1002, and VIDO-EP#3 has advantages over the use of each adjuvant individually or as a doubly adjuvanted vaccine formulation. Vaccination using the developed complexed adjuvant platform was found to result in the earlier onset of the IgG2a antibody response in both adults and neonates. This was a distinct asset when compared to the responses to vaccination with PTd antigen alone, antigen adjuvanted with alum, a single-dose of licensed Quadracel® vaccine, and using the non-complexed adjuvant platform.

When the immune response to vaccination with PTd antigen was examined with each adjuvant individually in adults, it was found that both CpG ODN and PP were able to significantly increase IgG2a titres, indicating an enhanced Th-1 immune response, as compared to PTd only. Class C was selected due to its unique ability to combine the effects of the other classes. The selection of the PP VIDO-EP#3 was based on its ability to induce higher IgG2a titres, and thus a stronger Th-1 immune response than VIDO-PP#4 in the absence of CpG ODN after a primary injection. Also, overall, a higher proportion of immunized mice were able to produce detectable IgG2a titres when this VIDO-EP#3 was used. Immunization with IDRPs alone as an adjuvant failed to increase IgG2a titres. A possible explanation is that the IDRPs are working through a different mechanism than the other adjuvants, for example it is well understood that IDRPs do not interact with TLRs but do modulate signal transduction downstream of TLRs and demonstrate synergy with IDRPs including CpG ODN [78, 131]. However, since the likely effect of this component is enhancement of the recruitment of immune cells, the apparent inability of IDRPs to affect the IgG2a response by themselves may be irrelevant. IDRPs are known to have chemotactic effects on immune effector cells, and our synthetic IDRPs have been shown to induce increased production of chemokines *in vitro* and *in vivo* [120]. Their cell recruitment effect may possibly be achieved through changing the microenvironment at the vaccination site, resulting in increased APC recruitment and subsequent increases in cytokine production that results in an increased cellular immune response.

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

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

mice, it enabled us to ensure that mice that might not otherwise respond to vaccination would do so.

Immunization in the neonates with the adjuvant platform combination was found to induce significantly higher IgG2a responses than PTd antigen alone, confirming the ability of the formulation to increase the Th-1 response, even within a model less likely to respond in a Th-1 type fashion. The results of the complexing of the IDRP and PP components mirrored those seen in the adult mouse model, with significantly higher IgG2a titres occurring after the components were complexed. This further indicates that complexing the components has an important effect on the ability of the vaccine combinations to induce a Th-1 response, although we do not know if this involves co-localization of components or genuine synergy between associated components. The neonatal mice were found to respond with detectable IgG2a titres to both the antigen alone as well as to the adjuvanted groups. A possible explanation for this is the higher dose of antigen being used to vaccinate in the neonatal model. When neonatal mice were immunized using alum as an adjuvant, the IgG2a response was almost non-existent, and was significantly lower than the response induced by the adjuvant platform combinations. This was expected, as alum is an adjuvant that typically induces a Th-2 type immune response [132].Within the alum group, there was a 50% IgG2a non-response rate among the mice, indicating a poor Th-1 response being induced when using this adjuvant. Since alum is currently used to adjuvant human acellular pertussis vaccines, the ability of our adjuvant platform to induce superior IgG2a titres is promising.

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

When the ability of the adjuvant platform formulation to induce an immune response after a single immunization was examined, it was found that, although the IgG2a titres were superior following a second immunization, the titres seen after a single dose were still greater than those induced by a single dose of the commercial Quadracel® vaccine, and appeared in fact to be similar to two doses of that vaccine. In addition, the number of mice responding to vaccination

with a detectable IgG2a titre was superior in the groups given our adjuvant platform as compared to those that received Quadracel® vaccine. All mice that were boosted with the adjuvant platform showed an IgG2a response, however there were two mice in the Quadracel® vaccine group that did not respond. At a single dose, only two mice did not produce a detectable IgG2a response in the adjuvant platform group, as compared to six non-responders for the single Quadracel® dose. Thus it appears that through using the current adjuvant platform, a greater Th-1 immune response could be induced than that seen with a commercial vaccine, and a good IgG2a Ab response might be induced even after a single immunization.

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

CHAPTER 4: CELL MEDIATED IMMUNE RESPONSES AND PROTECTION IN ADULT AND NEONATAL MICE TO IMMUNIZATION WITH NOVEL *BORDETELLA PERTUSSIS* VACCINE FORMULATIONS

4.1 Introduction

The disease whooping cough results from infection with the Gram-negative bacterium *Bordetella pertussis* and *Bordetella parapertussis*. It is generally a childhood illness, and is seen in both developed and developing countries [11, 122, 133]. The neonate and very young children are the most susceptible to the disease, especially those who have never been immunized or have not completed their full series of immunizations, and the majority of the morbidity and mortality are seen within these groups [11, 133]. Although in several developed nations, a series of 5 doses of acellular pertussis vaccine are given to children over the period from 2 months of age until 5 years, strong herd immunity is not effectively created due to the waning immune responses to the vaccine over time [134]. In addition there are age-cohort effects where older children with incomplete immunity are able to infect younger children or infants, which has led to a resurgence of the disease in nations that were thought to not be significantly affected by this problem [17].

B. pertussis is able to function as both an extracellular and an intracellular bacterium, usually attaching to the epithelial cell surface of the respiratory tract, but also able to invade human and mouse lung macrophages [6]. Thus, both cell-mediated immune responses and humoral responses are needed to effectively protect against and clear pertussis infection [22, 25, 27]. However, the exact mechanisms of protective immunity to the disease remain unknown, and it has been shown that it is possible for vaccine protection to occur in the absence of detectable circulating antibody [26, 135]. As well, IFNγ production, $CD4^+$ Th1 cells and Th17 cells play an important role in protection against this disease [24, 25]. The whole cell pertussis vaccine (Pw) is known to induce superior levels of Th1 and Th17 cells as compared to the acellular pertussis vaccines (Pa), however due to safety concerns, their use has been discontinued in many developed nations [136, 137]. Thus it would be useful to develop a vaccine that is able to combine the effective stimulation of Th1 cell-mediated immune responses seen in the Pw vaccines, with the safety afforded by acellular vaccines.

Human infants have difficulty mounting certain immune responses, including those to T cell-independent type 2 antigens [38]. The response is seen as functionally polarized in a Th2 direction, with less antibody secretion and less potent Th1 cell-mediated immune responses able to be induced in both human and mouse neonates [36, 37, 138]. This leads to difficulties when attempting to vaccinate neonates against diseases, such as pertussis, that require strong Th1 responses for clearance. However, studies indicate that the Th2 bias can be overcome through strategies that include the efficient use of adjuvants such as multiple TLR ligands, and effective antigen dosing and route of administration, such that immunization at or soon after birth can be made effective [124, 138].

We have recently described a novel three component adjuvant platform with three components for use in immunization against *B. pertussis* [139]. The three adjuvant components are C-class CpG oligodeoxynucleotides (CpG ODN) as TLR-9 ligand and strong Th1-promoting adjuvants; Polyphosphazenes (PP), a synthetic polymer with proven adjuvant activity and antigen encapsulation abilities; and cationic innate defense regulator peptides (IDRP), representing host defense molecules that have been shown to induce cell recruitment and modulate innate immunity [72, 82, 99]. After characterizing the humoral responses in adult and neonatal mice to vaccination with various combinations of these adjuvants, the present study was undertaken in order to examine the cell-mediated immune responses to this adjuvant platform, as well as to determine whether this vaccine formulation would function to protect neonatal mice against infection with *B. pertussis*. Our results are promising, and indicate that this adjuvant platform is highly effective in the mouse model in inducing superior immunity and protection as compared to current vaccines.

4.2 Materials and Methods

4.2.1. Animals

Seven-week old male and female BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were kept at the VIDO Animal Care facility (University of Saskatchewan, Saskatoon, SK), and bred to obtain neonates. Adult mice were used for immunization experiments at 8 weeks of age. There were $n = 8$ mice per group for all cytokine experiments, and $n = 10$ mice per group for the bacterial challenge experiment. Mice were selected at random for cage assignments, with each cage housing mice from at least two different groups. For sampling, mice were selected at random in equal numbers from each cage housing that group. For the bacterial challenge experiment, five mice from each group were sampled on both days 4 and 7. Animal technicians were not blind to the group assignment of mice, however those performing the experiments were. All animal experiments followed the guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

4.2.2. Animal Immunizations and Sample Collection

Adult mice were immunized at 8 weeks of age, and given a secondary immunization after four weeks unless otherwise noted. Neonatal mice were immunized at 7 days, weaned from their dams at four weeks, and received a secondary immunization at five weeks of age unless otherwise noted. For cytokine experiments, both adult and neonatal mice were sacrificed and spleens removed at five days after the secondary immunization. For bacterial challenge experiments, animals were sacrificed and their lungs removed at three weeks, three weeks four days, and four weeks after secondary immunization. For subcutaneous immunizations, both adult and neonatal mice were immunized between the shoulder blades with 50 μL of vaccine or sterile phosphate- buffered saline (PBS; 1.54 mM KH_2PO_4, 155.17 mM NaCl, 2.71 mM Na_2HPO_4-$7H_2O$, Gibco, Invitrogen; Carlsblad, CA). For intramuscular immunizations, neonatal mice were immunized in the quadriceps muscle with 10 μL of vaccine or sterile PBS. To complex the IDRP and CpG adjuvants within the vaccines, the components were mixed together and co-incubated in a 1.5mL Eppendorf tube (VWR; West Chester, PA) for 30 minutes at 37°C.

Blood was collected from the tail veins of mice. Samples were centrifuged in a microcentrifuge at 5940 g for 5 min, and the sera removed and stored at -20° C until used for ELISA. Spleens were aseptically collected into 15 ml centrifuge tubes (VWR; West Chester, PA) containing 10 mL of RPMI media (Gibco, Invitrogen; Carlsblad, CA). To isolate splenocytes, spleens were put through a 40 μM BD Falcon™ cell strainer (BD Biosciences; Bedford, MA) and washed at 21,885 x g for 10 min in RPMI media. Red blood cells were then lysed with 1 mL of NH_4Cl lysis buffer for 30 seconds, followed by another wash using 10 mL of RPMI at 21,855 x g for 10 min. Two more identical washes were performed, after which the cells were resuspended in AIM-V media (Gibco, Invitrogen; Carlsblad, CA) at various concentrations for counting and stimulation.

4.2.3. Vaccine Components

Genetically detoxified pertussis toxoid (PTd) was received from Novartis Vaccines (formerly Chiron, Siena, Italy) [128]. Each batch of PTd was centrifuged at 1,350 x g for 10 min prior to storage in aliquots at -20 °C. Frozen aliquots were thawed at room temperature (RT) and centrifuged at 1350 x g for 10 min, after which the supernatants were used in vaccine formulations or stored at 4 °C. CpG class C ODN 10101 was purchased from Coley Pharmaceuticals (Ottawa, ON). The IDR-1002 was synthesized by the solid state phase fMOC method, after which de-protected IDRPs were cleaved from the resin [76]. Purification utilized reverse phase HPLC, followed by lyophilization. Before use in vaccine formulations, IDRPs were resuspended in de-ionized water and diluted to the appropriate concentration in sterile PBS. The polyphosphazene VIDO-EP#3 (PCEPP) was synthesized at the Idaho National Laboratory (Idaho Falls, ID) under the supervision of Dr. John Klaehn, using a previously described method [99]. Polyphosphazenes were received in the lyophilized form and stored at 4° C in the dark. Before use in vaccine formulations, stored polyphosphazenes were dissolved by shaking for 36 hours at RT in Dulbecco's PBS (1.54 mM KH_2PO_4, 155.17 mM NaCl, 2.71 mM Na_2HPO_4-$7H_2O$, Sigma-Aldrich, MO). The DTaP-IPV vaccine used was Quadracel® (Sanofi Pasteur, Toronto ON), which was purchased from the University of Saskatchewan's Western College of Veterinary Medicine (Saskatoon, SK). A human dose of 0.5 mL contains 20 μg of chemically detoxified pertussis toxoid (PT), 20 μg filamentous hemagglutinin (FHA), 5 μg fimbrial

agglutinins 2 and 3 (FIM), 3 μg pertactin (PRN), 15 Lf diphtheria toxoid, 5 Lf tetanus toxoid, 40 D-antigen units poliovirus type 1 (Mahoney), 8 D-antigen units poliovirus type 2 (MEF1), 32 D-antigen units poliovirus type 3 (Saukett), and 1.5 mg of aluminum phosphate aduvant. The dosage of vaccine used for immunization was adjusted so that the concentrations of the PTd component in the Quadracel® and the PTd component of our vaccine formulation were equal.

4.2.4. Detoxification of Pertussis Toxin

To obtain pertussis toxoid for use in ELISA and cytokine assays, aliquots of pertussis toxin glycerol were purchased from List Biological Laboratories Inc. (Campbell, CA) and chemically detoxified. For each 200μg vial, 1.4 μg of formaldehyde was added and the mixture incubated at 37°C for 2 hours, after which 25μl of 1M L-lysine was added to quench the reaction. The solution was then dialyzed using a Pierce Slide-A-Lyzer 2K MWCO dialysis cassette (Thermo Fisher Scientific; Rockford, IL) in dialysis buffer (PBS, 0.5M Urea) over 2 days at 4°C, with 3 buffer changes. The concentration of the detoxified protein was then quantified using a Micro BCA protein assay kit (Thermo Fisher Scientific; Rockford, IL).

4.2.5. In vitro Study of Splenocyte Cytokine Production

ELISA analysis of cytokine production:

Splenocytes were isolated and plated in Costar® 12 well cell culture cluster flat bottom polystyrene plates (Corning Life Sciences; Lowell, MA) in AIM-V media (Gibco, Invitrogen; Carlsblad, CA) at a concentration of 2.5 x 10^6 cells/well. Cells were then stimulated at 37°C for 72 h with either 5 μg/ml List detoxified PTd or 2.5 μg/ml Con A (Sigma-Aldrich, St. Louis, MO) as a positive control. Plates were centrifuged at 21885 g for 10 min and the supernatant was removed and stored at -80° C for later cytokine analysis. Cytokine ELISAs on the supernatants were performed in 96-well polystyrene microtitre plates (Immulon 2 HB; Thermo, Milford, MA), using DuoSet® ELISA kits (R&D Systems, Minneapolis, MN) for murine IL-5 and IFN-γ and following kit instructions. After development with Streptavidin-HRP, plates were read

using a SpectraMax® Plus^{384} Absorbance Microplate Reader (Molecular Devices; Sunnyvale, CA) at a λ of 450 nm with wavelength correction set at 540 nm.

ELISPOT analysis of cytokine production:

Ninety six well tissue culture plates (Immulon 2 HB; Thermo, Milford, MA) were coated overnight at 4° C using primary rat anti-mouse IL-5 and IFN-γ specific monoclonal antibodies in coating buffer (BD Pharmingen; San Diego, CA; 1.25 μg/mL concentration). Splenocytes were then isolated and plated in duplicate in AIM-V media (Gibco, Invitrogen; Carlsblad, CA), at a concentration of 5 x 10^5 cells/well, and stimulated at 37°C for 24 h with either 5 μg/ml detoxified LIST PTd, 2.5 μg/ml Con A, or AIM-V media as a negative control. Plates were then incubated with biotinylated rat anti-mouse secondary antibodies (BD Pharmingen; San Diego, CA; 1.25μg/mL concentration) for two hours at RT, and developed with SigmaFAST™ BCIP®/NBT chromogen (Sigma-Aldrich, St. Louis, MO; dilution: 1 tablet/10 ml PBS). The spots were counted using a Zeiss Stemi 2000 microscope. The median number of spots was also calculated and indicated by a median line within each figure.

4.2.6. Analysis of PTd-specific antibodies by ELISA

Polystyrene microtitre plates (Immulon 2 HB; Thermo, Milford, MA) were coated with 0.25 μg/mL detoxified List PTd antigen in coating buffer (15.0 mM Na_2CO_3 and 34.88 mM $NaHCO_3$) at 4 °C overnight, after which sera was added and plates incubated for two hours at RT. After washing, goat anti-mouse IgG1 and IgG2a specific antibodies (Caltag Laboratories, Burlingame, CA; dilution 1/10,000) were applied. Then streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch; West Grove, PA; dilution 1/5000, starting concentration 500 μg/mL) was added, followed by the substrate p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO; dilution 1mg/mL). The iMark Microplate Reader (Bio-Rad; Philadelphia, PA) was used to analyze the developed samples at a λ of 405 nm with a reference wavelength of λ490 nm.

4.2.7. Bacterial Challenge with Bordetella pertussis

Mice (n = 10 per group) were challenged using 5 x 10^6 cfu/mouse of *B. pertussis* Tohama I strain (obtained from Dr. Mark Peppler, University of Alberta, Edmonton, AB) in sterile PBS. The challenge inoculum was determined by scraping the bacterial growth from several Charcol Agar plates using 2mL of sterile PBS per plate. The plate growth was pooled and the resuspended bacteria were strained into a 50mL centrifuge tube (VWR; Radnor, PA) and centrifuged using an SS34 rotor in a Sorvall® Evolution RC Superspeed centrifuge (Thermo Fisher Scientific; Rockford, IL) at 3,500 x g for 10 min at 16°C. The bacteria were then rinsed with PBS and centrifuged once more at the same specifications. The OD_{600} was then measured in order to determine the challenge dose, with an OD_{600} of 1 equaling 2.5x 10^8 bacteria/mL.

The challenge was delivered intranasally to anesthetized mice, with 10μL being inserted into each nostril for a total volume of 20 μL. To determine the challenge dose actually received within the lungs, at 90 minutes post-challenge the lungs of two mice were removed into 2 mL sterilized disposable conical microtubes with silicone O-rings (VWR; West Chester, PA) containing approximately 0.5 mL of 2.3 mm Zircon/Silica beads (BioSpec Products; Bartlesville, OK) and 1 mL of SS complete media. Samples were homogenized for 30 seconds using a Mini Bead-Beater (BioSpec Products; Bartlesville, OK) set at speed level 1. Dilutions were plated onto Charcoal agar plates containing 10% sheep blood, and incubated at 37° C for 7 days after which bacterial colonies were counted. The same lung removal, homogenization, and plating procedure was followed for the rest of the mice, with half the remaining mice being sacrificed at four days post challenge, and the rest at day seven (n = 5 mice per group, per timepoint).

4.2.8. Statistics

Analysis of the data was performed using the Windows STATA 10 program (StataCorp LP, College Station, TX). Graphs and scattergrams were plotted using the GraphPad Prism® 5 program for Windows (GraphPad Software Inc., La Jolla, CA).

Data was assessed for normality using the Shapiro-Wilk test. For repeated measures data, in order to achieve a normal distribution, the sums of all of the repeated measures were taken, and

these sums subsequently ranked. The resulting ranked data passed the normality test ($p > 0.05$). For non-repeated measures data, the data was ranked, and the resulting ranks passed the normality test. One-way ANOVA was used on the normally distributed data to determine the presence of a significant difference between groups. Post-hoc tests used to separate the means and identify which groups were significantly different included Bonferroni and Scheffe. A p-value of $p < 0.05$ was considered statistically significant. For bacterial challenge experiments, antibody titres were statistically analyzed separately both prior to and after challenge, as well as together; for lung bacterial counts, each day was analyzed separately.

4.3 Results

4.3.1. Cell mediated response to the adjuvant platform in adult mice

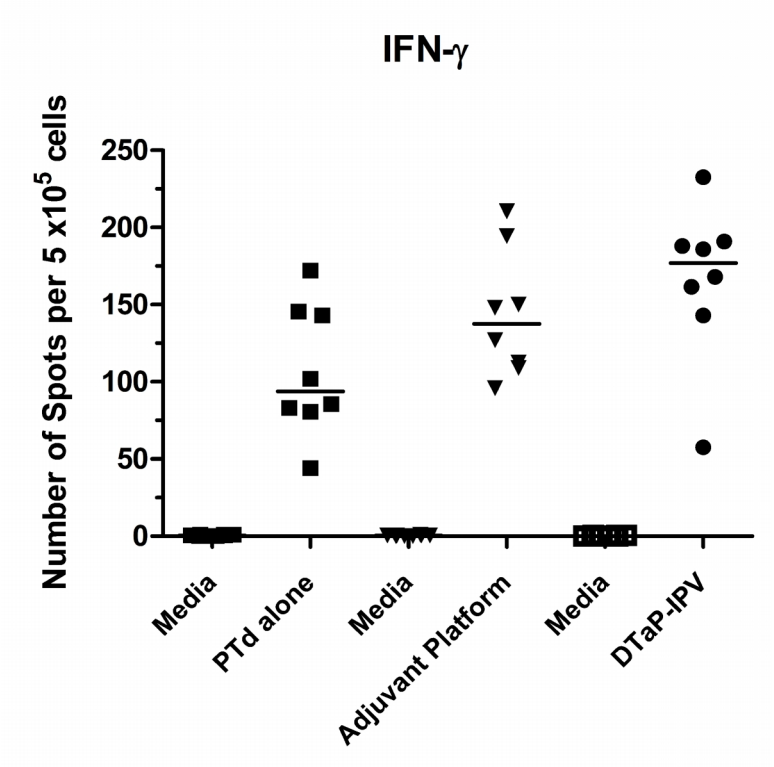
The mouse model is not an ideal correlate to the human, especially with respect to *B. pertussis*, since infection in the mouse model does not reflect the clinical symptoms or pathology of the human disease; nevertheless it is the accepted model for investigation of the pathophysiology of pertussis [140].

After immunization with the vaccines and controls, boosting at four weeks, and removal of spleens at five days post-boost, the isolated splenocytes were re-stimulated with 5 μg/mL of PTd for 24 h or 72 h (for ELISPOT and cytokine ELISA, respectively). The optimal kinetics of the cytokine response in immunized mice and the optimal antigen stimulation dose and time points for splenocyte response analysis were established in a series of pilot experiments (data not shown). The ELISPOT analysis showed that there were IFN-γ producing cells present in all the vaccine groups, ranging from a median of 94 spots up to 177 per group (Fig 4.1A). There was no statistically significant difference in the number of IFNγ–producing cells seen between mice immunized with our vaccine formulation as compared to those immunized with unformulated PTd or DTaP-IPV (hereafter referred to as DTaP). Conversely, multiple spots indicating cells producing IL-5 were seen in mice immunized with PTd alone or DTaP as compared to mice that received our vaccine formulation, which showed virtually no cytokine production (with a median number of spots of 2) (Fig 4.1B). The number of spots in these two IL-5 secreting groups ranged from a median of 32 to 27, respectively. There was a statistically significant difference seen between mice immunized with PTd alone or DTaP, as compared to those immunized with our vaccine formulation, however there was no statistically significant difference between results for the PTd alone and DTaP groups.

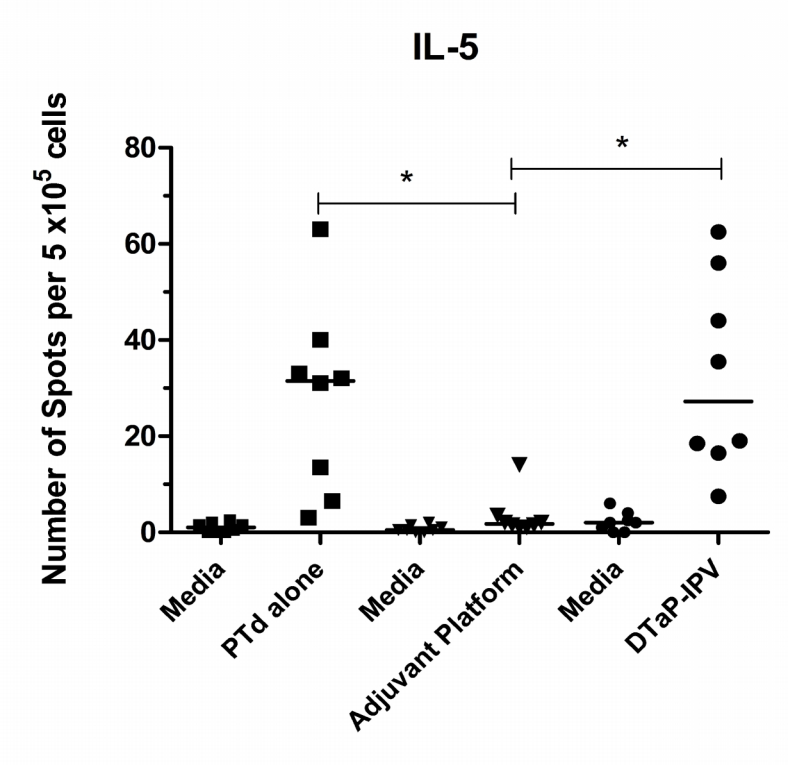
When the ratio of IFNγ : IL-5 producing cells was examined, it was shown that immunization with our vaccine formulation resulted in a large increase in the ratio, from under 10 to a median of 34, indicative of a shift in the type of immune response in favor of Th1 type immunity (Fig 4.1C).

ELISAs to examine the concentration of cytokines produced were also performed (Fig 4.2A-B). Strong production of more than 10,000 pg/mL of IFN-γ was seen after immunization with all vaccines, with no significant differences observed between the groups (Fig 4.2A). This similarity in measured cytokine production supports the ELISPOT data, which showed no significant difference in the number of cytokine-producing cells detected. There was a significantly greater amount of IL-5 produced in mice immunized with PTd alone or DTaP, as compared to those that received our vaccine (Fig 4.2B). While there was detectable IL-5 production in the PTd and DTaP groups with medians ranging from 162-56 pg/mL, respectively, there was no cytokine detected in mice that received our formulated vaccine. There were no significant differences in cytokine levels seen between mice that received PTd or DTaP, respectively. This once again mirrored the results of the IL-5 ELISPOT.

A.


IFN-γ
Number of Spots per 5 x10^5 cells
250
200
150
100
50
0
Media
PTd alone
Media
Adjuvant Platform
Media
DTaP-IPV

B.


IL-5
Number of Spots per 5 x10^5 cells
80
60
40
20
0
*
*
Media
PTd alone
Media
Adjuvant Platform
Media
DTaP-IPV

C.

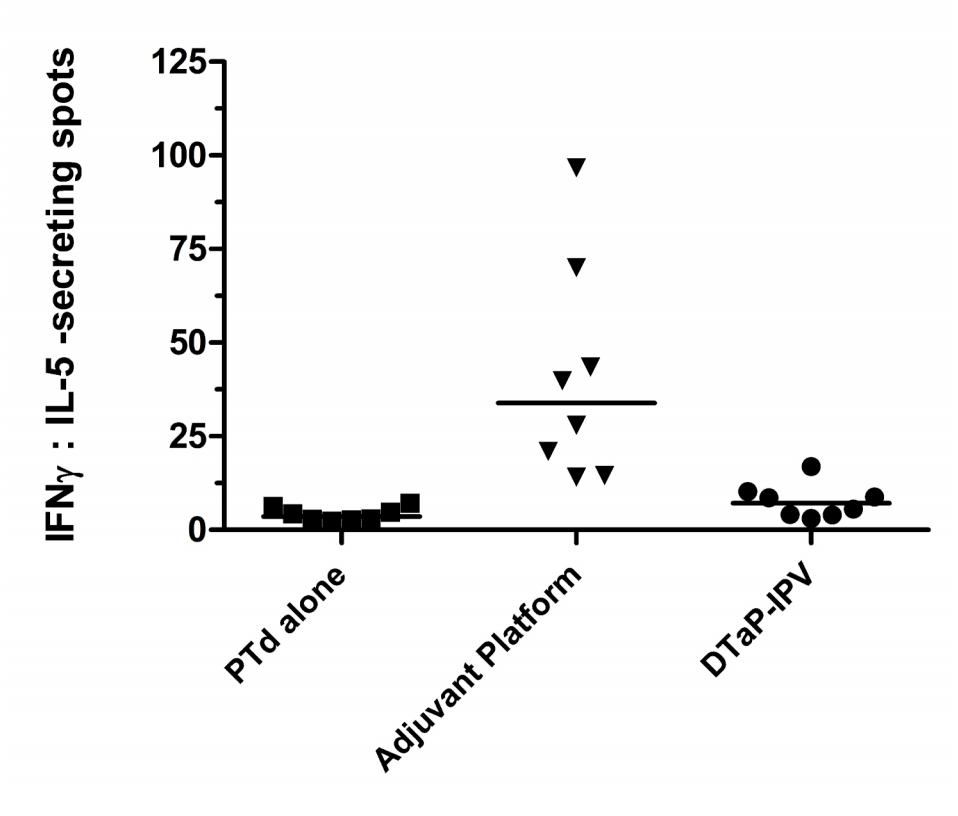
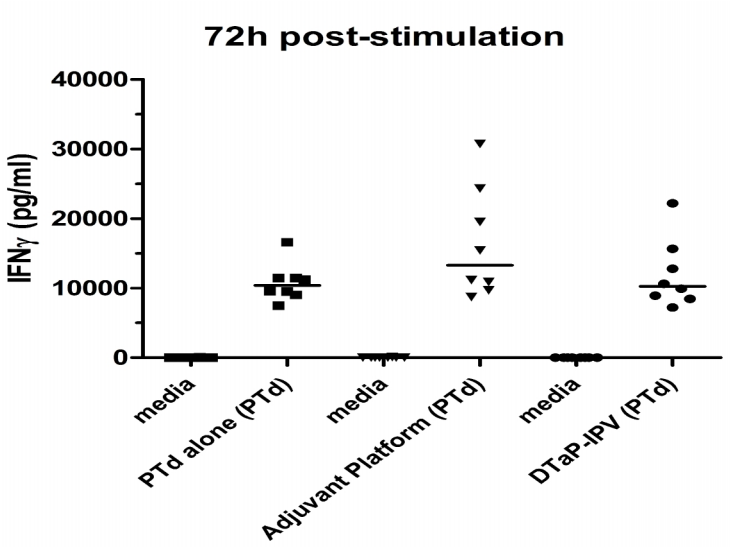


Figure 4.1 Adult IFN-γ and IL-5 ELISPOT responses to immunization with various vaccine formulations.

Adult BALB/c mice (*n* = 8 mice per group) were immunized with 0.1μg PTd antigen alone (■), PTd (0.1μg) + CpG C(10μg)+ IDR-1002(20μg) + VIDO-EP#3(10μg), with the IDRP & CpG components complexed (▼), or DTaP , with a dose including 0.1μg chemically detoxified PTd (●) at 8 weeks of age. An identical secondary immunization was given after 4 weeks. At 5 days post-boost, spleens were removed and splenocytes isolated and stimulated with either media or 5.0μg/mL of PTd for 24h. **A)** No statistically significant differences occurred in IFN-γ spot numbers between any of the vaccinated mice. **B)** There were statistically significant differences in the IL-5 results between mice treated with PTd alone or DTaP, as compared to PTd + the adjuvant platform ($p<0.001$). **C)** The ratio of IFNγ:IL-5 cells was determined by dividing the number of IFN-γ spots for each animal by the corresponding number of IL-5 spots.

A.



B.

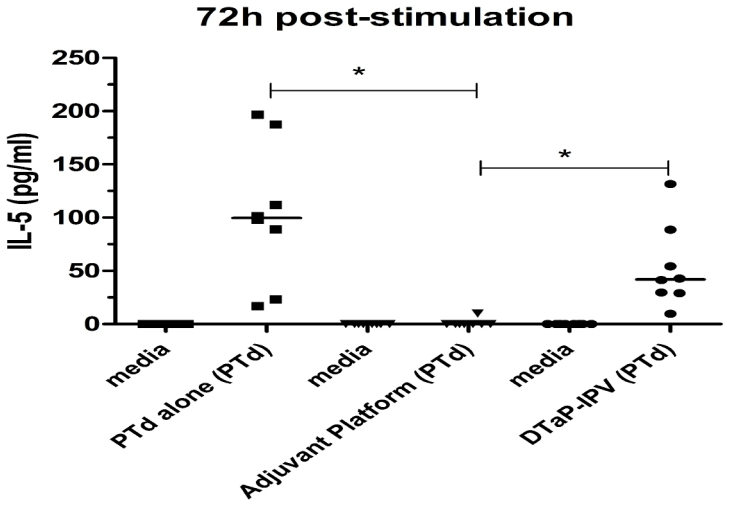


Figure 4.2 Adult IFN-γ & IL-5 cytokine ELISA responses to immunization with various vaccine formulations.

Adult BALB/c mice (*n* = 8 mice per group) were immunized with 0.1μg PTd antigen alone (■), PTd (0.1μg)+ CpG C(10μg)+ IDR-1002(20μg)+ VIDO-EP#3(10μg), with the IDRP & CpG components complexed (▼), or DTaP , with a dose including 0.1μg chemically detoxified PTd (●) at 8 weeks of age. An identical secondary immunization was given after 4 weeks. At 5 days post-boost, spleens were removed and splenocytes isolated and stimulated with either media or 5.0μg/mL of PTd for 72h. **A)** No statistically significant differences occurred in IFN-γ cytokine levels between any of the vaccinated mice. **B)** Statistically significant differences in the IL-5 cytokine levels occurred between mice treated with PTd alone or DTaP, as compared to those that received PTd + adjuvant platform ($p<0.001$).

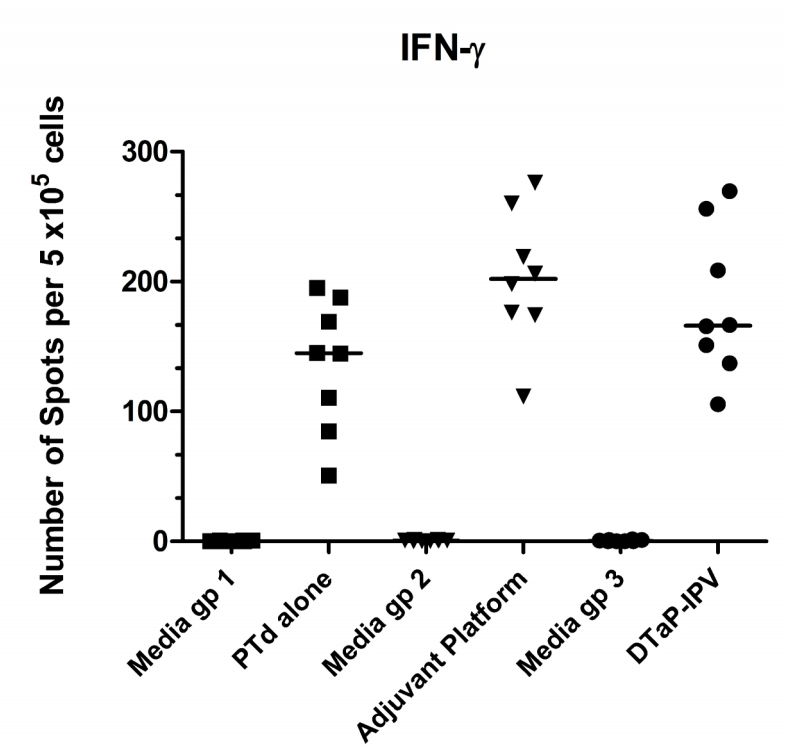
4.3.2. Cell mediated response to adjuvant platform in neonatal mice

After completion of the cytokine studies in adult mice, we began experiments in a neonatal mouse model. In order to use the best murine correlate to the human neonatal immune system, seven-day old BALB/c mice were employed [36, 37]. These mice were immunized subcutaneously with 50 μL of vaccine, the spleens removed five days after a four-week booster immunization, and the cytokine response of stimulated splenocytes determined through cytokine ELISAs and ELISPOTs. Vaccine formulations included 1.0 μg PTd, 2.37 μg CpG ODN, 4.74 μg IDR-HH1002, and 2.37 μg VIDO-EP#3, with the CpG and IDRP components pre-complexed. The adjuvant dosages were decreased by a ratio relative to the body weight of the neonatal mouse as compared to an adult, while the PTd dose was increased in order to compensate for the reduced immune response of neonates to the antigen, as based on previous studies. After immunization with the various vaccines and controls, boosting, and removal of the spleens, isolated splenocytes were stimulated with 5 μg/mL of PTd for 24 h or 72 h (for ELISPOT and cytokine ELISA, respectively).

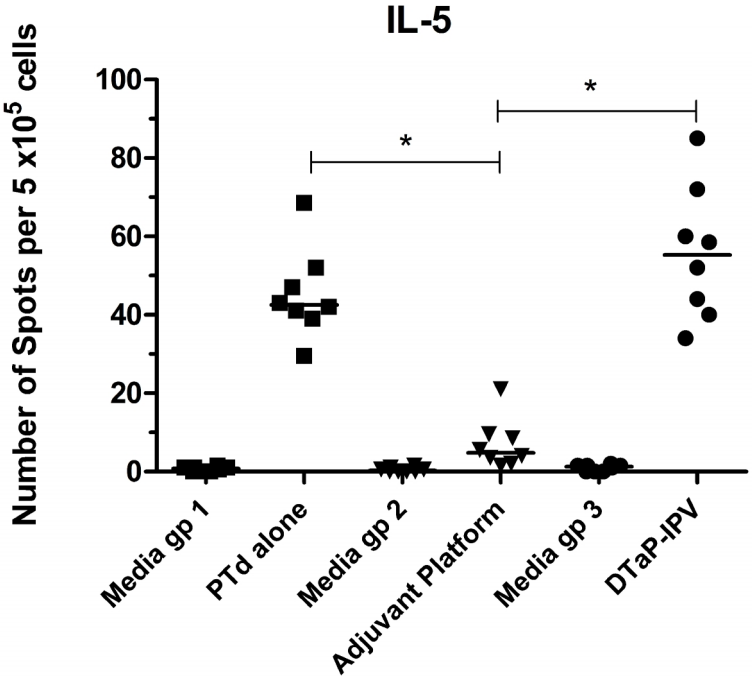
ELISPOTs showed a large number of IFNγ producing cells present in all of the vaccine groups, ranging from a median of 145 spots to 202 in each group (Fig 4.3A). There were no statistically significant differences in the number of IFNγ–producing cells seen between any of the vaccine groups. These results and spot numbers are similar in range to those seen in adult mice. For the Th2 type cytokine IL-5, spots were seen in mice immunized with PTd alone or DTaP but very few spots were found in mice that received our formulated vaccine (Fig 4.3B). The number of spots in the PTd and DTaP groups ranged from a median of 43 to 56, respectively, higher than the number of spots seen in adult mice, although the trends within the vaccine groups were the same. There was a statistically significant difference seen between mice immunized with PTd alone or DTaP, as compared to those immunized with our vaccine formulation, with no statistically significant difference noted between the PTd and DTaP controls. When these results are expressed as a ratio, the number of IFNγ : IL-5 producing cells indicated that immunization with either of our vaccine formulations resulted in a large increase in the ratio, from less than 2 to a median of 45, which indicates a shift in the type of immune response in the Th1 direction (Fig 4.3C).

ELISAs to examine the concentration of cytokines being produced were also performed on the neonatal samples (Fig 4.4A-B). Strong production ranging from 4487-6674 pg/mL of IFN-γ was seen after immunization with all vaccines (Fig 4.4A). There was no significant difference seen between the vaccine groups, indicating that our vaccine did not affect the amount of IFNγ being produced by the cells. This once again supported the ELISPOT data where there was also no significant difference seen in the number of cytokine-producing cells. With respect to IL-5 production, there was a significantly greater amount of IL-5 produced in mice immunized with PTd alone or DTaP, as compared to those that received our vaccine formulations (Fig 4.4B). While there was detectable IL-5 production in the PTd and DTaP groups, medians of 185 and 108 pg/mL, respectively, there was effectively no cytokine detected in mice given our formulated vaccine. There was no statistically significant difference seen between the PTd and DTaP mice, once again matching the IL-5 and IFN-γ ELISPOTs.

A.


IFN-γ
Number of Spots per 5 x10^5 cells
300
200
100
0
Media gp 1
PTd alone
Media gp 2
Adjuvant Platform
Media gp 3
DTaP-IPV

B.


IL-5
Number of Spots per 5 x10^5 cells
100
80
60
40
20
0
*
*
Media gp 1
PTd alone
Media gp 2
Adjuvant Platform
Media gp 3
DTaP-IPV

C.

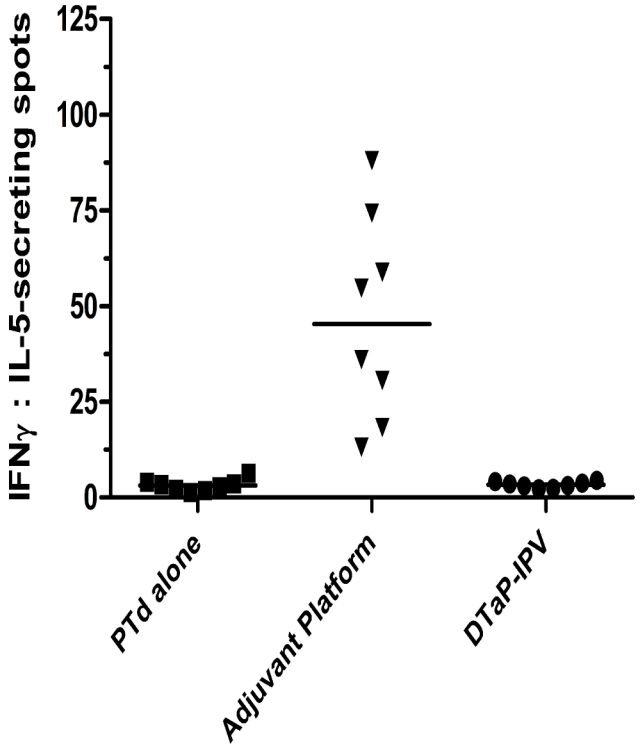
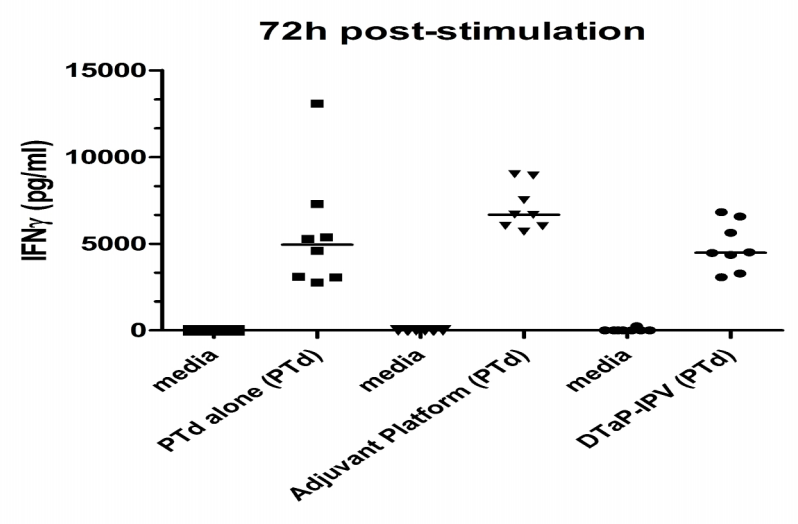


Figure 4.3. Neonatal IFN-γ and IL-5 ELISPOT responses to immunization with various vaccine formulations.

Neonatal BALB/c mice (*n* = 8 mice per group) were immunized with 1.0 μg PTd antigen alone (■), PTd(1.0μg)+ CpG C (2.37μg)+ IDR-1002 (4.74μg)+ VIDO-EP#3 (2.37μg), with the IDRP & CpG components complexed (▼), or DTaP , with a dose including 1.0 μg chemically detoxified PTd (●) at 7 days of age. An identical secondary immunization was given after 4 weeks. At 5 days post-boost, spleens were removed and splenocytes isolated and stimulated with either media or 5.0μg/mL of PTd for 24h. **A)** No statistically significant differences occurred in IFN-γ spot numbers between any of the vaccinated mice. **B)** There were statistically significant differences in the IL-5 results between mice treated with PTd alone or DTaP, as compared to PTd + the adjuvant platform ($p<0.001$). **C)** The ratio of IFNγ:IL-5 cells was determined by dividing the number of IFN-γ spots for each animal by the corresponding number of IL-5 spots.

A.



B.

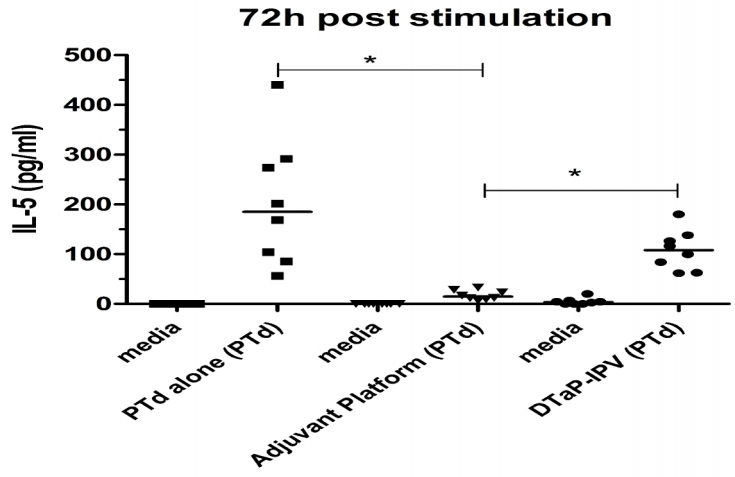


Figure 4.4. Neonatal IFN-γ and IL-5 cytokine ELISA responses to immunization with various vaccine formulations.

Neonatal BALB/c mice (*n* = 8 mice per group) were immunized with 1.0 μg PTd antigen alone (■), PTd (1.0μg)+ CpG C (2.37μg)+ IDR-1002 (4.74μg)+ VIDO-EP#3 (2.37μg), with the IDRP & CpG components complexed (▼), or DTaP , with a dose including 1.0 μg chemically detoxified PTd (●) at 7 days of age. An identical secondary immunization was given after 4 weeks. At 5 days post-boost, spleens were removed and splenocytes isolated and stimulated with either media or 5.0μg/mL of PTd for 24h. **A)** No statistically significant differences occurred in

IFN-γ cytokine levels between any vaccinated mice. **B)** Statistically significant differences in IL-5 cytokine levels occurred between mice treated with PTd alone or DTaP, as compared to PTd + the adjuvant platform ($p<0.001$).

4.3.3. Protection of immunized neonatal mice against bacterial challenge with B. pertussis

Since vaccination with our adjuvant platform appeared to shift the cytokine response towards a Th1 response, further experiments were undertaken in order to determine if our vaccine formulations were able to protect *in vivo* against infection with *B. pertussis*. To examine this, an intranasal bacterial challenge with 20 μL of $5x10^6$ cfu/mL *B. pertussis* Tohama I strain was performed. Two mice were killed on the day of challenge in order to accurately assess the inoculum dose achieved. Since the commercial DTaP vaccine contains 5 separate antigens, a second pertussis antigen, pertactin (PRN), was included in some formulations, to test if addition of a second antigen would increase the level of protection. Vaccine formulations consisted of 2.37 μg CpG ODN, 4.74 μg IDR-1002, 2.37 μg VIDO-EP#3, combined with either 1.0 μg PTd or 5.0μg PRN, or both PTd and PRN together as antigen (mixed together after complexing the CpG and IDRP components), and compared to the commercial vaccine DTaP.The bacterial load from the lungs of infected mice, as well as the PTd-specific IgG2a and IgG1 serum responses were examined.

The PTd-specific antibody ELISA results showed that there was a significant increase (more than 400 times higher) in PTd-specific IgG2a antibodies, indicative of a Th1 type immune response, when mice were immunized with either of our vaccine formulations as compared to the commercial DTaP vaccine (Fig 4.5A). It should be noted that vaccination with our vaccine formulations was able to induce a strong IgG2a as early as three weeks post-immunization, whereas mice that received DTaP did not show detectable IgG2a production until week five. A very high IgG2a antibody titre of more than $1x\ 10^5$ was reached with both of our vaccine formulations, however the titre was significantly higher in mice vaccinated with the formulation containing only the PTd antigen (reaching a maximum of 1.04×10^6), as compared to the adjuvant formulation combined with PTd and PRN (which reached a titre of $1.91x\ 10^5$). There were two mice in the DTaP group that showed no detectable IgG2a response, and two that only responded relatively late, after more than six weeks. There were no late or non-responding mice in the groups that received any of our various adjuvant platform combinations. This result

mirrored the lung bacterial loads, indicating that the inclusion of a second antigen significantly increased the immune response to the formulated vaccines.

With respect to the PTd-specific IgG1 titres, indicative of a Th2 type immune response, there were no significant differences seen between any of the vaccine groups (Fig 4.5B). All of our vaccine formulations, as well as DTaP, were able to greatly increase the IgG1 response of the mice, with the median titres ranging from 1 to 1.9x 10^6 by week six in all groups. Both the IgG2a and IgG1 results mirrored our previously published data which indicated that our vaccine formulations are superior inducers of IgG2a titres, while IgG1 titres remain approximately equal to those seen using the commercial vaccine [139].

The bacterial loads at day zero were 5.03 x 10^6 colony forming units (cfu)/ lung (Fig 4.6). At days four and seven, the bacterial load in the PBS control group had increased slightly, to a maximum of 1.32 x10^7 cfu/ lung, whereas it had decreased greatly in all other vaccinated groups, and was actually cleared completely in one mouse in the formulated dual antigen group. The bacterial load in the vaccine group containing both PTd and PRN as antigens was significantly lower than that seen in the group that received only PTd as an antigen, although both groups were significantly lower than the PBS control. When compared to the untreated (PBS) control goup, all vaccine groups showed a significant decrease in bacterial load. Nearly identically decreased bacterial loads were seen in mice that were immunized with either DTaP or our adjuvant platform using PRN as the antigen. However, it should be noted that a complete lack of bacteria at day seven was only seen in mice of the vaccine group that received our adjuvant platform combined with both PTd and PRN as antigens (there were three mice by the end of the experiment with total bacterial clearance), indicating that this formulation was best able to induce the appropriate immune responses necessary for total bacterial clearance.

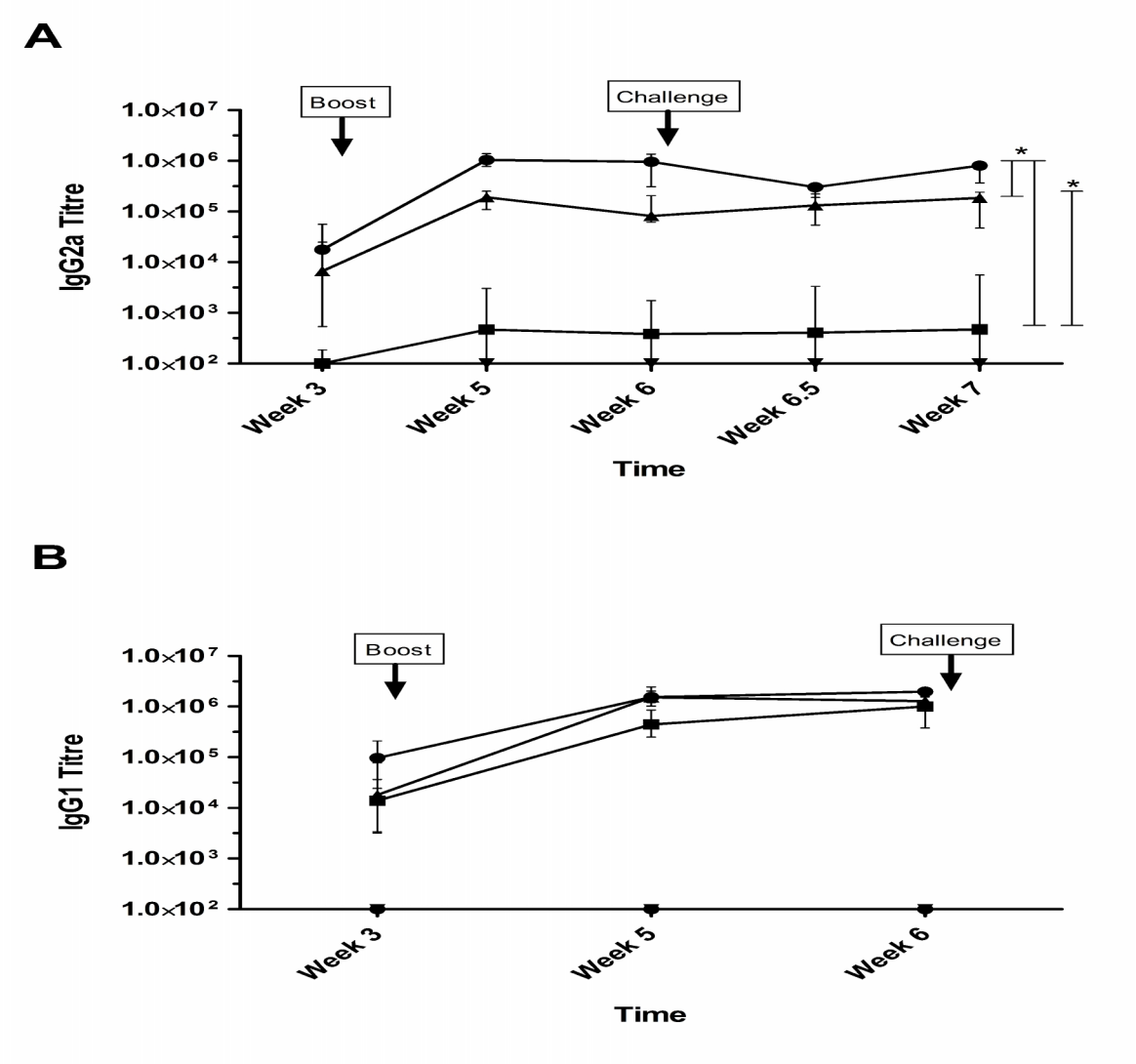


Figure 4.5. PTd-specific neonatal serum IgG2a and IgG1 Ab responses during challenge with *B. pertussis*.

Neonatal BALB/c mice (*n* = 10 mice per group) were immunized with PTd (1.0μg)+ CpG C (2.37μg)+ IDR-1002 (4.74μg)+ VIDO-EP#3 (2.37μg), with the IDRP & CpG components complexed (●), PTd (1.0μg) + PRN (5.0μg) + CpG C (2.37μg)+ IDR-1002 (4.74μg)+ VIDO-EP#3 (2.37μg), with the IDRP & CpG components complexed (▲), DTaP, with a dose including 1.0 μg chemically detoxified PTd (■), or PBSA (▼), at 7 days of age. An identical secondary immunization was given at 3 weeks. Mice were challenged with 5 x 10^6 cfu/mL of *B. pertussis* at 3 weeks post-boost. Sera were collected at 3 weeks after the initial immunization prior to the boost, and at various times thereafter. The median titres are shown. **A)** Statistically significant differences between the IgG2a titres occurred between mice treated with PTd + adjuvant platform as compared to PTd +PRN + the adjuvant platform or DTaP ($p<0.001$), as well as between the DTaP group as compared to the PTd + PRN + adjuvant platform group ($p<0.001$). **B)** No statistically significant differences in IgG1 titre occurred between any of the vaccinated mouse groups.

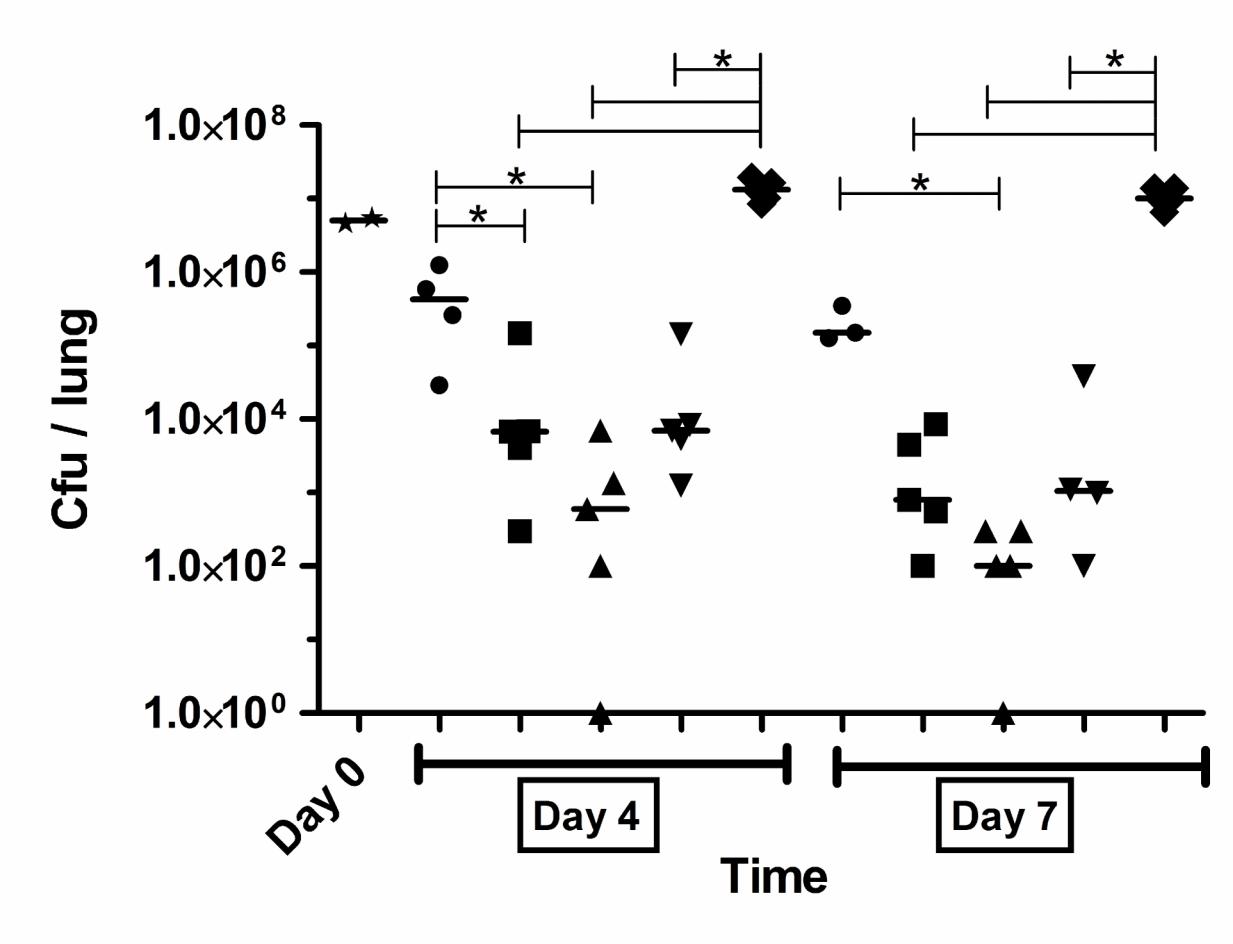


Figure 4.6. Neonatal lung bacterial counts after challenge with *B. pertussis*.

Neonatal BALB/c mice (*n* = 10 mice per group) were immunized with PTd (1.0μg)+ CpG C (2.37μg)+ IDR-1002 (4.74μg)+ VIDO-EP#3 (2.37μg), with the IDRP & CpG components complexed (●), PRN (5.0μg) + CpG C (2.37μg)+ IDR-1002 (4.74μg)+ VIDO-EP#3 (2.37μg), with the IDRP & CpG components complexed (▼), PTd (1.0μg) + PRN (5.0μg) + CpG C (2.37μg)+ IDR-1002 (4.74μg)+ VIDO-EP#3 (2.37μg), with the IDRP & CpG components complexed (▲), DTaP, with a dose including 1.0 μg chemically detoxified PTd (■), or PBSA (♦), at 7 days of age. An identical secondary immunization was given at 3 weeks. Mice were challenged as described in Figure 5. Lungs were collected at 0, 4, and 7 days after bacterial challenge. The median t counts are shown. Statistically significant differences occurred between mice immunized with PTd + adjuvant platform as compared to PTd + PRN +adjuvant platform ($p<0.005$) on both day 4 and day 7, or as compared to DTaP ($p<0.05$) on day 4 only. There were also statistically significant differences seen between mice immunized with PBSA as compared to all other vaccine groups ($p<0.01$).

4.4 Discussion

Whooping cough has re-emerged as a serious risk to infants in the developed world. Current vaccines are unable to effectively protect against the disease without several booster doses, cannot be utilized successfully prior to 6 weeks of age, and do not assure protection even if a full vaccination schedule is followed. It is clearly necessary to develop new vaccines that can be administered to and protect the most susceptible population, the neonate, and that will be fully effective in their application. In order to achieve this, a vaccine must be capable of inducing strong Th1 type humoral and cellular-mediated immunity in the neonatal system, as well being able to overcome interference from maternal antibodies. We have addressed this challenge through the combined use of the adjuvants CpG ODN, polyphosphazene, and IDR peptides. We have previously shown that our adjuvant platform induces strong long-lasting Th1 type humoral immunity, as well as the typically observed Th2 humoral immunity that is seen after immunization with currently used commercial vaccines [139]. We have also addressed the issue of maternal antibodies and found that the inclusion of a booster immunization and correct formulation of adjuvants is able to overcome this issue [141].

The cell-mediated immune response to our adjuvant platform was examined through looking at cytokine production post-vaccination. Our vaccine formulation was able to shift responses towards a more balanced response through decreasing production of Th2 type cytokines. When ELISPOTS for IL-5 and IFN-γ were performed to gauge the number of cytokine producing cells in adult BALB/c mice, the results indicated that our vaccine formulations do not induce IL-5 secreting cells, while maintaining the levels of IFN-γ secreting cells, in contrast to mice immunized with an alum adjuvanted, marketed acellular pertussis vaccine or antigen immunized mice. The results were identical to those seen in neonatal mice, indicating that our vaccine formulation is equally effective in both adults and neonates. It should be noted that the number of IL-5 secreting spots detected by ELISPOT in mice immunized with PTd alone or the licensed acellular pertussis vaccine was 26% and 52% higher in neonatal mice than the corresponding number of spots seen in adult mice.

The cytokine ELISAs results showed the same trend, with the IL-5 concentrations detected being approximately twice as high in the neonate as in adult mice, and the IFN-γ concentrations

in the neonate being from 75% to 50% of those seen in adult mice. These results indicate a tendency of the neonatal mice to produce an increased cellular Th2 response and lower, although still strong, Th1 response in comparison to the adults. This may be due to the relative difficulty of achieving a strong Th1 response in a neonatal system as compared to a mature adult one [38].

In previous experiments, we were able to demonstrate that PTd was a good model antigen with respect to the humoral response, however when mice were bacterially challenged, we did not see significantly reduced bacterial counts as compared to the licensed DTaP vaccine (data not shown). However, through improvement of our formulation by inclusion of another pertussis antigen, PRN, we have been able to surpass the alum adjuvanted marketed acellular vaccine (which contains PT, PRN, FHA, and fimbriae) not only in the induction of a IgG2a humoral response, indicative of a Th1 type response, but also in the ability to achieve bacterial clearance in the lung. Although the alum adjuvanted DTaP vaccine is effective at inducing high IgG1 titres, indicative of a Th2 type response, it is not a strong inducer of IgG2a titres, whereas our adjuvant platform, whether including a single or two antigens, is very capable of anti-PTd IgG2a induction. Not only are the IgG2a titres far superior after vaccination with our formulations as compared to DTaP (more than 400 times higher), they are detectable after only 3 weeks post-immunization, as opposed to after 5 weeks with the commercial vaccine. After challenge of mice with *Bordetella pertussis*, although DTaP was able to significantly decrease the bacterial load in the lungs of mice, total clearance was only seen in mice that were immunized with our dual-antigen adjuvant platform, indicating that we have been able to surpass the current vaccine in all examined areas of the immune response, including humoral, cell-mediated, and effective protective responses. Thus it appears that our vaccine formulations are inducing strong Th1 type cell mediated immune responses in both adult and neonatal mice which are equivalent to those seen using commercial vaccines, as well as a greater Th1 type humoral response than commercial vaccines. These vaccine formulations may also function through decreasing the Th2 type cell mediated responses. Finally, our vaccine formulations are able to successfully induce the immune response of neonatal mice in order to fully clear infection with *B. pertussis*.

In conclusion, we have demonstrated that our novel adjuvant platform is not only able to induce a shift in the adult and neonatal humoral response towards a Th1 type immune response, but that there is also a corresponding decrease in the Th2 cytokine responses, and vaccination results in protection against bacterial challenge in the neonate. This indicates that our vaccine formulation promotes an overall balancing of the immune response in the adult and neonatal mouse system by shifting responses in favor of a Th1 type response. Despite the traditional limits of the mouse model we have achieved a strong shift in the immune response, with corresponding protection against *B. pertussis* infection that is superior to currently available vaccines. The type of immune responses seen with our vaccine formulation corresponded to the results needed in order to create an effective human neonatal vaccine, and thus we believe that this adjuvant platform is very promising.

CHAPTER 5: ALTERATION OF ADJUVANT CHARACTERISTICS WITHIN A NOVEL *BORDETELLA PERTUSSIS* VACCINE FORMULATION AND ITS EFFECT ON THE IMMUNE RESPONSE IN MICE.

5.1 Introduction

Identifying adjuvants able to induce strong and lasting responses in susceptible populations such as neonates or the elderly is key for the successful development of vaccines that will be effective for these groups. In the elderly, the involution of the thymus as well the low output of naïve T and B cells makes successful stimulation and thus primary immunization difficult [142-144]. The neonatal immune system is functionally immature, and characterized by lower shorter-lasting vaccine-induced antibody responses and a Th2 type immune response that precludes the development of the strong Th1 response required for dealing with intracellular pathogens [36]. This is due to a variety of factors, including the functioning of neonatal DC as well as the immaturity of the follicular DC network and germinal centers in the neonate [39, 44]. However, adult-like cellular immune responses can be generated in the neonate through the use of appropriate adjuvants [145].

Currently, there is an interest in the use of multiple or combination adjuvants to attempt to overcome some of the difficulties faced when attempting to vaccinate specific populations including the immuno-compromised, neonates, and the elderly, with immune responses that differ from those seen in healthy adults. The ability to link innate and adaptive immunity through the use of appropriate adjuvants involving the stimulation of pattern recognition receptors such as TLRs, has been previously demonstrated in our lab and others [100, 146]. Through incorporating TLR ligands as adjuvants the immune response to several vaccines has been improved, including Hepatitis B and RSV [86, 115, 121]. TLR ligands in vaccines appear to significantly enhance the affinity maturation process and lead to the production of antibodies with higher antigen-binding affinity [86, 121]. We have previously shown that the combination of several adjuvants, including the TLR ligand CpG ODN, cationic innate defense regulator peptides, and polyphosphazenes, are able to induce strong humoral Th1-type responses in adult and neonatal mice when combined with a *B. pertussis* vaccine antigen [139]. This adjuvant

combination is not only able to induce a strong humoral response in the neonate, but is also able to overcome interference with maternal antibodies in both mice and pigs, which is another major barrier to successful infant immunization [141]. We have recently demonstrated that this adjuvant platform is also able to induce strong cell-mediated immune responses in both adult and neonatal mice, and when a second antigen is added, the vaccine developed is protective against bacterial challenge with *B. pertussis* (submitted for publication).

CpG ODN are synthetically developed unmethylated DNA sequences that mimic bacterial DNA and act as TLR 9 ligands. There are three separate classes, each with its own functional abilities [81]. A-Class CpG ODN strongly stimulate plasmacytoid DC resulting in IFNα secretion, B-Class are able to stimulate the activation of B cells and increase the production of antibodies, and C-Class combine the activities of both previous classes [81].

All CpG ODN are well-characterized as Th-1 promoting adjuvants, and CpG ODN have been tested in many animal trials as well as in clinical trials in humans for vaccines against Hepatitis B [87].

Poly I:C is a synthetic dsRNA which acts as a TLR 3 ligand. As such, it is able to induce IFN production and the maturation of DC [47]. In humans and mice, only mDC express TLR 3 (as opposed to the pDC), and TLR 3 is unique among TLRs since it is preferentially expressed in mature DC. An attractive feature of dsRNA as a potential adjuvant is its ability to signal in both a MyD88-dependent, and MyD88-independent manner. This was shown through its ability to induce activation of NF-kB in MyD88 knockout mice, although no dSRNA induced production of inflammatory cytokines was observed.

Polyphosphazenes are linear polymers composed of an inorganic phosphorus-nitrogen backbone that have great flexibility in structure through their ability to incorporate various side groups [95]. They are capable of being formed into microparticles for such varied uses as drug delivery, tissue matrix development, and vaccine antigen or adjuvant protection and delivery [95]. In addition, they have been shown to have potent immunostimulatory properties and can act as vaccine adjuvants in their own right, with different PP inducing slightly different immune responses [98, 99]. The ability of PP to act as adjuvants is not limited to their being covalently

linked to the vaccine antigen, as water-soluble non-covalent complexes are able to form when antigen and PP are merely mixed together [96]. The immune response seen with PP tends to be a mixed Th1/Th2 type response, although PCPP has been demonstrated to induce more of a Th2 type immune response, with lower IFN-γ production than PCEP, which induces strong vaccine induced antigen-specific responses that include both IFNγ and IL-4 production [99].

Cationic innate defense regulator peptides (IDRP) are naturally occurring (or derived from) molecules found in almost all species. These peptides are amphipathic, small, positively charged, and produced by a variety of host cells including phagocytes and mucosal epithelial cells [66, 126]. They are induced by the presence of pathogens and have multiple and varied functions, including direct antimicrobial activity, cytokine and chemokine induction, and wound healing [126]. The use of these peptides as vaccine adjuvants is relatively new, however they have shown promise as treatments for infection, and are able to modulate the innate immune response [75]. The synthetic peptide IDR-1002 has been shown to increase leukocyte recruitment through the increase production of the chemokine MCP-1 [76].

A vaccine that is effective through multiple methods of administration (intranasal, subcutaneous, etc.) is highly desirable, since the route of administration can be adapted to the particular circumstances being faced in the field at the time of immunization. This would be of particular use in the developing world, where access to some components of health care, such as clean needles, can be limited. Within the context of an adjuvant platform, the ability to use several routes of administration would allow various vaccines to be tailored to fit the route of infection of the particular pathogen being immunized against.

The characterization of the immune response after vaccination at multiple simultaneous injection sites allows the possibility of vaccine interference to be examined. The purpose of this study was to further characterize the developed novel adjuvant platform, and we show that this platform is able to function with different antigens, induces inflammatory cell recruitment, and is able to increase the immune response post-vaccination in a site-specific and localized manner.

5.2 Materials and Methods

5.2.1. Animals

Adult BALB/c mice were obtained at seven-weeks old from Charles River Laboratories (Wilmington, MA), and housed in the VIDO Animal Care facility (University of Saskatchewan, Saskatoon, SK). These mice were bred in-house to obtain neonates. Mice were selected at random for cage assignments, with each cage housing mice from at least two different groups. For sampling, mice were selected at random in equal numbers from each cage housing that group. Animal technicians were not blind to the group assignment of mice, however those performing the experiments were. Animal experiments were performed following the guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

5.2.2. Immunization of mice

Adult mice were immunized at 8 weeks of age and given a secondary immunization after four weeks unless otherwise noted. Neonatal mice were immunized at 7 days of age, weaned at four weeks of age, and received a secondary immunization after four weeks unless otherwise noted. For subcutaneous immunizations, adult mice were immunized between the shoulder blades with 50 μL of vaccine or sterile phosphate- buffered saline (PBS; 1.54 mM KH_2PO_4, 155.17 mM NaCl, 2.71 mM Na_2HPO_4-$7H_2O$, Gibco, Invitrogen; Carlsblad, CA). For intramuscular immunizations, adult mice were immunized in the semimembranosus muscle of the right or left leg, with 20 μl of vaccine or sterile PBS per injection site; neonatal mice were immunized in the same muscle with 10 μL of vaccine or sterile PBS. For intranasal immunizations, neonatal mice were immunized with 20 μL of vaccine or sterile PBS. Complexing of the IDRP and CpG adjuvant components within vaccines was achieved through mixing together the components and co-incubating them at 37°C for 30 minutes in a 1.5mL Eppendorf tube (VWR; West Chester, PA). Blood was collected from the tail veins of mice. Blood was centrifuged for 5 minutes in a microcentrifuge at 5940 x g in order to separate the sera, which was removed and stored at -20° C for use in ELISAs.

5.2.3. Vaccine components

Genetically detoxified pertussis toxoid (PTd) was kindly provided by Novartis Vaccines (formerly Chiron, Siena, Italy) [128]. Each batch was centrifuged at 1,350 x g for 10 min and stored in aliquots at -20 °C. Frozen aliquots were thawed at room temperature (RT) and centrifuged at 1350 x g for 10 min, after which the supernatants were used in vaccine formulations or stored at 4 °C. CpG class C ODN 10101 was purchased from Coley Pharmaceuticals (Ottawa, ON). The IDR-1002 was synthesized by the solid state phase fMOC method, after which de-protected IDRPs were cleaved from the resin [76]. Purification was performed through reverse phase HPLC, followed by lyophilization. Before use in vaccine formulations, IDRPs were resuspended in de-ionized water and diluted to the appropriate concentration in sterile PBS. The polyphosphazene VIDO-EP#3 (PCEPP) was synthesized at the Idaho National Laboratory (Idaho Falls, ID) under the supervision of Dr. John Klaehn [99]. Polyphosphazenes were received in the lyophilized form and stored at 4° C in the dark. Before use in vaccine formulations, stored polyphosphazenes were dissolved by shaking for 36 hours at RT in Dulbecco's PBS (1.54 mM KH_2PO_4, 155.17 mM NaCl, 2.71 mM Na_2HPO_4-$7H_2O$, Sigma-Aldrich, MO).

Poly I:C was purchased from Sigma-Aldrich (St.Louis, MO). Hepatitis B surface antigen (HBsAg) was purchased from Fitzgerald (North Acton, MA).

5.2.4. Preparation of samples for analysis

Following injection, sites were circled using permanent non-toxic marker, and lesion samples were removed using scissors to cut out the affected area, or through taking of a punch biopsy. For time-course experiments, the entire semimembranosus muscle was removed and sent for analysis. Samples were placed in tissue cassettes and stored in 10 % buffered formalin solution for transport to the University of Saskatchewan Prairie Diagnostic Services laboratory for histological analysis by Dr. Brendan O'Conner. Pathologist was blind to content of treatments, however group numbers were noted.

5.2.5. Histological analysis of samples

Received tissues were cut through the lesion if visible, or in half through the centre if not, and both new surfaces trimmed. Both surfaces were then placed inside a tissue cassette for further histological processing. Tissue samples were stained using hematoxylin and eosin stain (H&E stain), and stained samples were then analyzed for myopathy due to necrosis, inflammation indicated by the presence of heterophils, vasculitis, lymphadenitis, and the presence of hair shafts in the sample.

5.2.6. Detoxification of Pertussis Toxin

Pertussis toxoid used for ELISA assays was chemically detoxified from aliquots of pertussis toxin that were purchased from List Biological Laboratories Inc. (Campbell, CA). For each 200μg vial of PT in glycerol, 1.4 μg of formaldehyde was added and the mixture incubated at 37°C for 2 hours, followed by 25μl of 1M L-lysine to quench the reaction. The detoxified solution was dialyzed within a Pierce Slide-A-Lyzer 2K MWCO dialysis cassette (Thermo Fisher Scientific; Rockford, IL) in dialysis buffer (PBS, 0.5M Urea) over 2 days at 4°C, with buffer changes. Detoxification was confirmed by Chinese hamster ovary cell cytotoxicity assay (CHO assay). Increasing concentrations of detoxified protein were incubated with CHO cells and cell death analyzed. The detoxified protein concentration was determined using a Micro BCA protein assay kit (Thermo Fisher Scientific; Rockford, IL).

5.2.7. ELISA analysis of antigen-specific Abs

Polystyrene microtitre plates (Immulon 2 HB; Thermo, Milford, MA) were coated for PTd-specific ELISAs with 0.25 μg/mL detoxified List PTd antigen, and for HBsAg-specific ELISAs with 1.0 μg/mL HBsAg, each in coating buffer (15.0 mM Na_2CO_3 and 34.88 mM $NaHCO_3$) at 4 °C overnight, respectively. Sera was then added and the plates incubated at RT for two hours, followed by washing and the addition of goat anti-mouse IgG1 and IgG2a specific antibodies (Caltag Laboratories, Burlingame, CA; dilution 1/10,000). Streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch; West Grove, PA; dilution 1/5000, starting concentration

500 μg/mL) was added, and then the substrate p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO; dilution 1mg/mL). Developed samples were read using the iMark Microplate Reader (Bio-Rad; Philadelphia, PA) at a λ of 405 nm with a reference wavelength of λ490 nm.

5.2.8. Statistics

Data were statistically analyzed using the STATA 10 program for Windows (StataCorp LP, College Station, TX). Normality of data was examined by Shapiro-Wilk test. To achieve a normal distribution of data, the sums of all of the repeated measures were taken, and these sums subsequently ranked. The resulting data passed the normality test ($p > 0.05$). One-way ANOVA was used on the transformed data to determine the presence of a significant difference between treatment groups. Post-hoc tests used to determine the specific differences between groups include Bonferroni and Scheffe. A p-value of $p<0.05$ was considered statistically significant.

5.3 Results

5.3.1 Testing the adjuvant platform with a different antigen

In order to test the versatility of our adjuvant platform and its ability to affect the immune response to other antigens, HBsAg was co-formulated with the adjuvants and used for immunization. 8-week old adult BALB/c mice were immunized intramuscularly with 20 μL of vaccine, boosted after four weeks, and the sera obtained at two week intervals in order to determine the HBsAg-specific IgG2a and IgG1 serum responses through ELISAs. Vaccine formulations included 0.3 μg or a lower 0.03 μg dose of HBsAg, 10 μg CpG ODN, 20 μg IDR-HH1002, and 10 μg VIDO-EP#3, with the CpG and IDRP components pre-complexed, with Alum (25μg) used as a control adjuvant.

ELISA analysis showed that immunization with our adjuvant platform and a 0.3 μg dose of HBsAg was able to induce strong IgG2a titres, 100-fold higher than alum, reaching a median titre of 4.4×10^5 (Fig 5.1A). Mice that received the higher antigen dose with our adjuvant platform also had detectable IgG2a titres two weeks earlier than in the other vaccine groups. The titres seen after immunization with a 0.3 μg antigen dose and Alum as an adjuvant, were comparable to those seen using a 0.03 μg antigen dose and our adjuvant platform, with titres in both these groups only detectable after 4 weeks, and only reaching a median of $2.3\text{-}8.3 \times 10^3$.

Immunization with the 0.3 μg dose of HBsAg and our adjuvant platform resulted in significantly higher titres than those achieved in mice immunized with a ten-fold lower dose of antigen (0.3 μg) and the adjuvant platform or with the same antigen dose but alum as the adjuvant. This indicates a noticeable antigen dose-sparing effect seen when our adjuvant platform was used as compared to Alum.

The IgG1 responses were comparable between alum and the adjuvant platform, with the adjuvant platform and 0.3μg HBsAg inducing a strong response reaching a median titre of 4×10^5, similar to the antigen adjuvanted with alum, which reached a median titre of 1.3×10^5 (Fig 5.1B). Titres in both of these groups were significantly higher than those seen using the lower 0.03μg antigen dose combined with our adjuvant platform, which only reached a median titre of 2.5×10^3. Thus

the dose sparing effect noted above is only applicable to the IgG2a response. These results indicated that our adjuvant platform was effective in enhancing the response to HBsAg, and was able to enhance both the IgG2a and IgG1 (Th-1 and Th-2 type antibodies, respectively) responses.

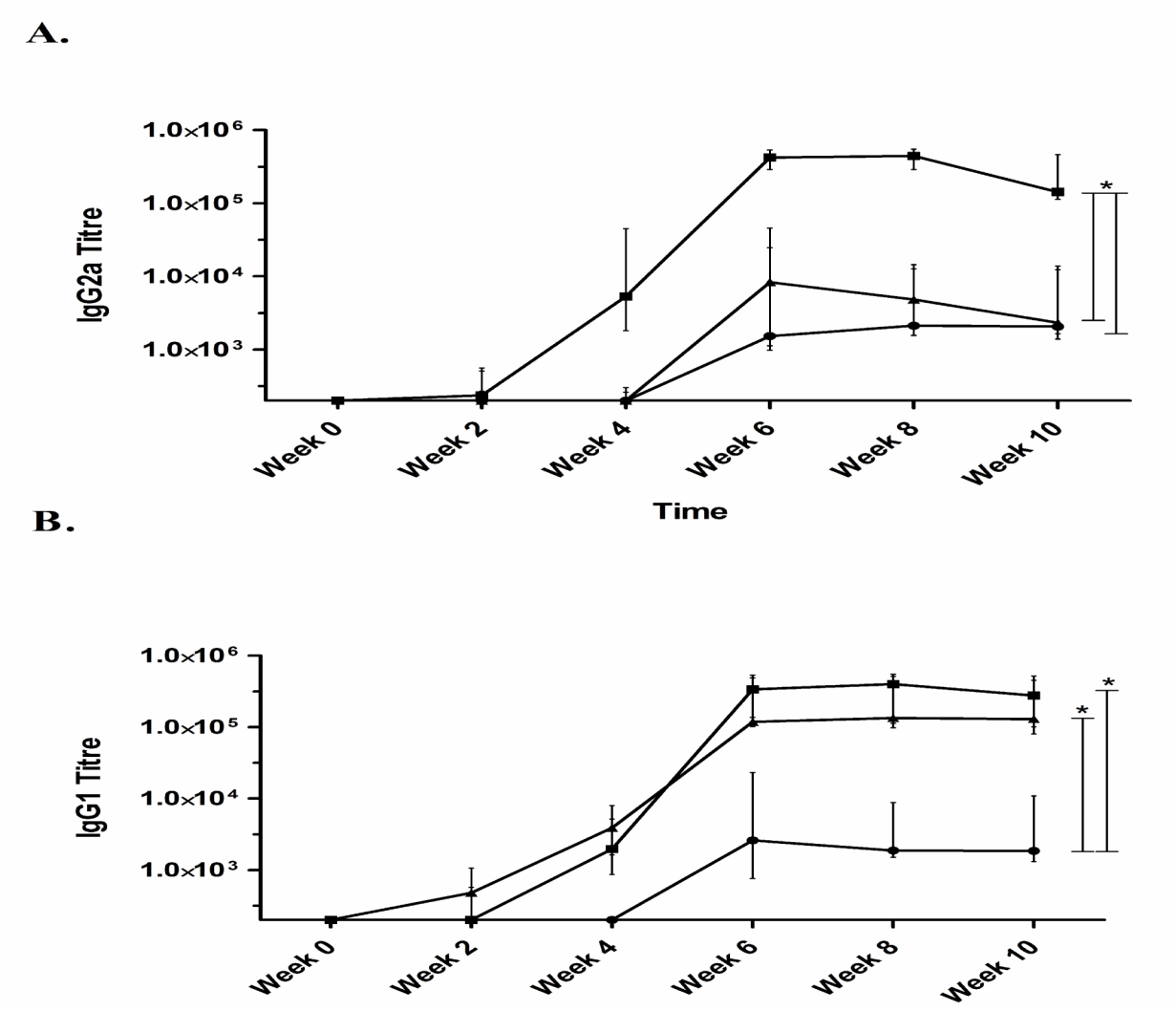


Figure 5.1 Adult IgG2a and IgG1 humoral responses to immunization with various vaccine formulations containing Hepatitis B Surface Antigen.

Adult BALB/c mice (*n* = 10 mice per group) were immunized intramuscularly with 0.03 μg HBsAg + CpG C (10μg)+ IDR-1002 (20μg)+ VIDO-EP#3 (10μg), with the IDRP & CpG components complexed (●), HBsAg (0.3μg)+ CpG C (10μg)+ IDR-1002 (20μg)+ VIDO-EP#3 (10μg), with the IDRP & CpG components complexed (■), or HBsAg (0.3μg) + Alum (25μg) (▲), at 8 weeks of age. An identical secondary immunization was given after 4 weeks. Sera were collected every 2 weeks after the initial immunization for 10 weeks. The median titres are shown. **A)** There were statistically significant differences in the IgG2a titre between mice vaccinated with 0.3 μg of HBsAg combined with the adjuvant platform, as compared to mice that received either the lower 0.03 μg antigen dose, or 0.3μg HBsAg with Alum as adjuvant ($p<0.001$). **B)** There were statistically significant differences in the IgG1 titres between mice vaccinated with either 0.3 μg of HBsAg combined with the adjuvant platform or 0.3 μg HBsAg and Alum, as compared to those that received 0.03 μg of HBsAg and the adjuvant platform ($p<0.001$).

5.3.2 Histological time-course of antigen and adjuvant platform separately and in combination

In order to examine the histological changes and cell recruitment occurring after immunization with the adjuvant platform, a time-course experiment was performed. To determine whether the adjuvant platform (or the antigen alone) was acting through the induction of a systemic inflammatory response, the vaccine components were administered separately in different legs of the animal, with a combined vaccine control group included. Adult 8 week-old BALB/c mice (n=20 mice per group) were immunized intramuscularly with 20µl of the vaccine formulation per leg (Table 5.1). Mice were boosted in the same manner after 3 weeks. The semi-membranous muscle was removed at 3h, 7h, 24h, or 48h after immunization, and the biopsy was sent to Prairie Diagnostic Services for histological examination by Dr.Brendan O'Connor. Results are summarized in Table 5.2.

Table 5.1 *Vaccine Groups for histological time-course of antigen and adjuvant platform separately and in combination*

Group	Vaccine Formulation
1	PTd (0.1µg)+ CpG C (10µg) +HH-1002 (20µg)+ EP3(10µg)- complexed and injected in Right Leg PBSA Injected in Left Leg
2	PTd (1.0µg) Injected in Right Leg CpG C (1.0µg) + HH-1002 (20µg) + EP3 (10µg)- complexed and injected in Left Leg
3	PBSA Injected in Right Leg PTd (0.1µg) alone Injected in Left Leg

A very mild inflammatory response was present by 3h in the leg of Group 1 mice following immunization with PTd and the novel adjuvant platform, with no vasculitis present. This response had increased by 7 h to a moderately localized inflammatory response with an increased presence of neutrophils (heterophils), indicating inflammation, and mild vasculitis. This response remained increased through 48h post-immunization, however the vasculitis had decreased almost completely by 24h, and was not detectable at 48h. In the leg of mice immunized with antigen alone, there was mild or no inflammation seen at 3h. At 7h, there was a very mild influx of neutrophils seen in approximately half of the mice immunized with antigen

alone, with little to no vasculitis present. There was some very mild inflammation in half of the mice at 24h and 48h, however no vasculitis was detected. In the legs of the mice immunized with adjuvants alone, there was mild inflammation seen by 3h with some influx of neutrophils, this inflammation had not dissipated by 48h. The inflammation seen in the mice immunized with both antigen and adjuvant at the same site was by far the most noticeable, with the legs receiving either adjuvant or antigen alone showing much less inflammation. The response to each component did not affect the responses occurring in the opposing leg, indicating that the vaccine is likely not creating a systemic inflammatory response. This indicates that there is a localized inflammatory response with vasculitis and a subsequent influx of neutrophils occurring when mice are immunized with antigen combined with our adjuvant platform. This response does not appear to be due to a systemic response to one of the components, since when the antigen and adjuvant platform are separated, the inflammation is decreased. Although local lymph nodes were present in approximately half of the samples examined, no lymphadenitis was noted within them. The presence of hair shafts, presumably introduced by the injection needle, may have caused some of the inflammatory response in samples where they were found.

Table 5.2 Histological results and lesion scoring of muscle samples from time-course injections.

Group 1:	PTd + Adjuvants (Right Leg)				PBS (Left Leg)			
Timepoint	Necrosis	Heterophils	Vasculitis	Hair Shafts	Necrosis	Heterophils	Vasculitis	Hair Shafts
3h	1	0	0	0	1	1	0	0
	1	1	0	0	0	1	0	0
	0	1	0	0	1	0	0	0
	0	0	0	0	0	2	1	0
	1	1	0	0	0	1	0	0
7h	2	3	1	0	0	0	0	0
	0	2	1	0	0	2	0	3
	0	2	1	0	0	2	1	1
	0	3	1	0	1	1	0	1
	1	2	1	0	0	0	0	0
24h	0	1	0	0	0	0	0	0
	1	2	0	2	0	1	1	1
	1	2	1	2	0	0	0	0
	0	1	1	0	0	1	0	0
	0	1	0	0	0	1	0	0
48h	1	3	0	0	0	1	0	0
	0	2	0	0	0	0	0	0
	0	2	0	0	0	0	0	0
	1	1	0	0	0	2	0	1
	1	2	0	0	0	0	0	0

Group 2:	PTd (Right Leg)				Adjuvant Combination (Left Leg)			
Timepoint	Necrosis	Heterophils	Vasculitis	Hair Shafts	Necrosis	Heterophils	Vasculitis	Hair Shafts
3h	0	0	0	0	0	1	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	1	1	0	0
	0	0	0	0	0	1	0	0
	0	0	0	0	1	1	1	0
7h	0	2	0	2	2	2	1	0
	0	0	0	0	1	1	1	0
	0	0	0	0	0	0	0	0
	0	0	0	0	1	1	1	1
	0	1	0	1	0	1	0	1
24h	0	0	0	0	0	2	1	0
	0	0	0	0	0	1	0	0
	1	1	0	0	0	0	0	0
	0	0	0	0	0	1	1	0
	0	1	0	0	0	0	0	0
48h	0	0	0	0	0	2	0	0
	0	0	0	0	0	2	0	0
	0	0	0	0	0	2	0	0
	0	0	0	0	0	2	0	0
	0	1	0	1	0	1	0	0

Group 3:	PBS (Right Leg)				PTd (Left Leg)			
Timepoint	Necrosis	Heterophils	Vasculitis	Hair Shafts	Necrosis	Heterophils	Vasculitis	Hair Shafts
3h	0	1	0	0	0	1	0	0
	0	1	0	0	0	0	1	0
	0	1	0	0	0	1	0	0
	0	0	0	0	0	0	0	0
	1	1	0	0	1	2	1	0
7h	0	0	0	0	0	1	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	1	0	0
	0	0	0	0	0	1	0	1
	0	0	0	0	0	1	0	1
24h	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	1	1	0	0	1	1	0	1
	0	0	0	0	0	1	0	0
	0	0	0	0	1	2	0	1
48h	0	0	0	0	0	0	0	0
	0	0	0	0	0	1	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	2	0	2
	0	0	0	0	1	1	0	0

Scoring Legend

1 = Mild focal or multifocal change

2 = Moderate multifocal change or mild locally-extensive change

3 = Moderate locally-extensive change

4 = Severe locally-extensive change

5.3.3 Effects on antibody production of antigen and adjuvant platform separately and in combination

Ideally, a vaccine will induce a localized immune response at the site of injection, so that if other vaccines are administered at the same time, for example in a different limb, they will not be affected by a heightened systemic response to the first vaccine. If other vaccine antigens are present, interference can occur due to competition for Mϕ's uptake of the antigen, resulting in the ineffective T and B cell priming, and thus lower immune responses. If vaccinating in two arms, there can be interference with circulating antibodies [147]. In order to ensure that the humoral responses being induced by our vaccine platform are the result of a localized immune response that is only induced by the presence of both components together (and thus would not have an effect on a separate vaccine administered at a different site), we split our vaccine antigen and the adjuvant platform and examined the responses to each component when injected together and at separate sites.

8-week old adult BALB/c mice were immunized intramuscularly with 20 μL of vaccine per injection site, boosted after three weeks, and the sera obtained at two week intervals in order to determine the PTd-specific IgG2a and IgG1 serum responses through ELISAs. Vaccine formulations included 0.1μg PTd, 10 μg CpG ODN, 20 μg IDR-HH1002, and 10 μg VIDO-EP#3, with the CpG and IDRP components pre-complexed.

The ELISA analysis showed that there was a strong induction of the IgG2a antibody response, with a median titre reaching 7.5×10^5, after immunization with PTd and the adjuvant platform together at one site (Fig 5.2A). This response was significantly higher than that seen after immunization with PTd alone, or with PTd at one site and the adjuvant platform in the opposing limb (median titres up to 1.7×10^3, and 1.0×10^3, respectively). The IgG2a response seen to PTd alone was nearly identical to that seen when the adjuvant and antigen components were injected in separate limbs.

The IgG1 results followed a similar trend as the IgG2a. The titres seen after immunization with PTd alone or PTd in one leg and adjuvants in the other, were very comparable, with median titres up to 3.2×10^5 and 2.0×10^5, respectively (Fig 5.2B). There was a significantly greater IgG1

response when mice are immunized with the combined antigen and adjuvant combination at the same site, reaching a median titre of $1.1x\ 10^6$.

These results indicate that the antibody response induced to the vaccine is the result of a localized immune response that requires the presence of both components at the injection site to be induced.

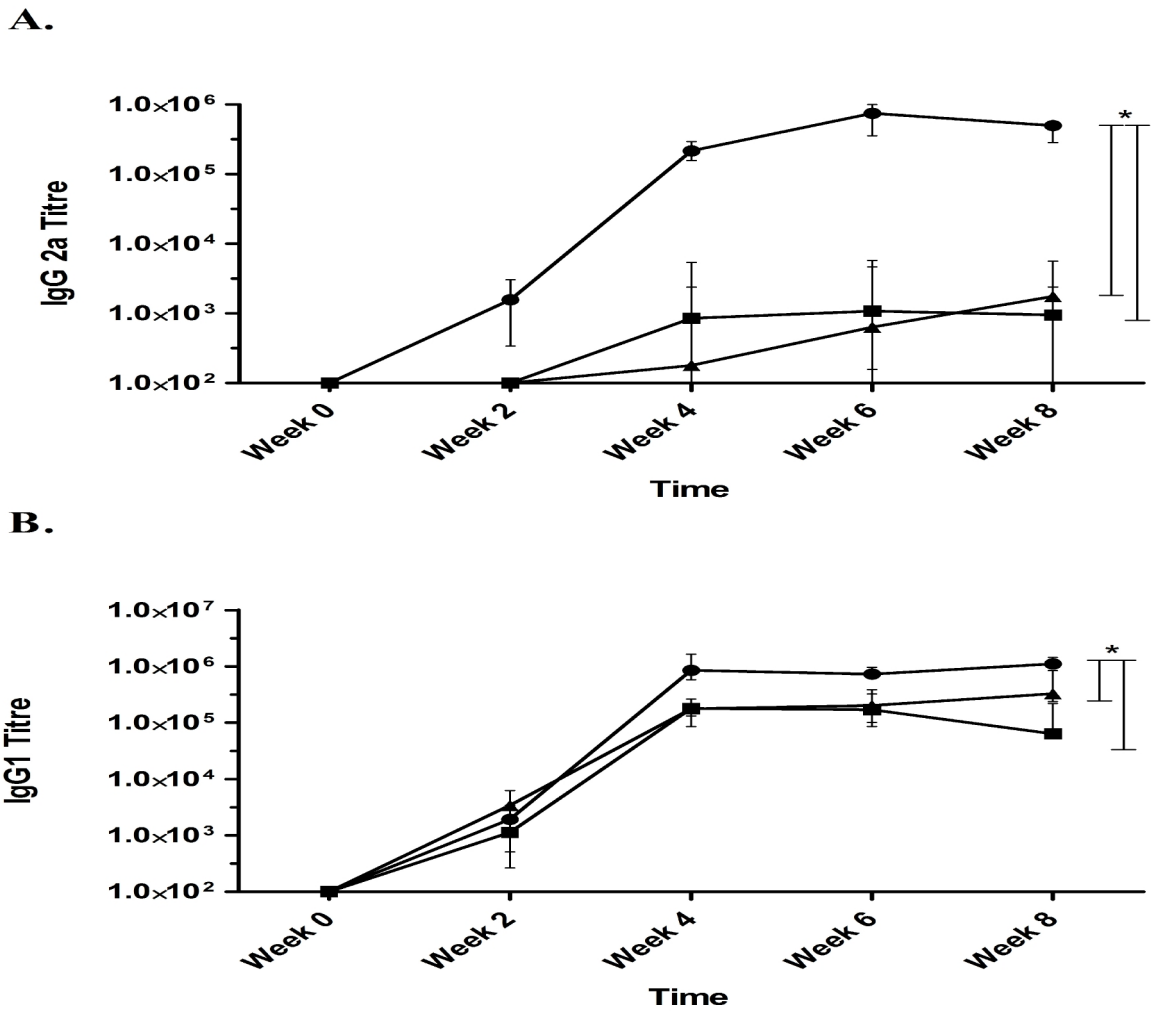


Figure 5.2 Adult IgG2a and IgG1 humoral immune responses to vaccination with antigen and adjuvant at separate and distinct sites.

Adult BALB/c mice (*n* = 10 mice per group) were immunized intramuscularly with PTd (0.1μg) + CpG C (10μg)+ IDR-HH1002 (20μg)+ VIDO-EP#3 (10μg), with the IDRP & CpG components complexed – in the Right Leg (●), PTd (0.1μg) alone in the Right Leg and CpG C (10μg) + IDR-HH1002 (20μg)+ VIDO-EP#3 (10μg), with the IDRP & CpG components complexed – in the Left Leg (■), or PTd (0.1μg) alone in the Left Leg (▲) at 8 weeks of age. An identical secondary immunization was given after 3 weeks. Sera were collected every 2 weeks after the initial immunization for 10 weeks. Muscle biopsies of the injection site were taken at 10 weeks and histologically examined. Median titres are shown. **A)** There was a statistically significant difference in IgG2a titres found between mice immunized with adjuvant platform and antigen together in the same leg, as compared to those given either PTd alone, or separated PTd and adjuvant components ($p<0.001$). **B)** There was a statistically significant difference in the IgG1 titres between mice immunized with the adjuvant platform and antigen together in the same leg, as compared to those given either PTd alone, or the separated antigen and adjuvant components in different legs ($p<0.001$).

5.3.4 Polyphosphazene safety profile in BALB/c mice

In order to examine the safety of the polyphosphazene component, high doses of polyphosphazene alone (50μg, 150μg, or 300μg) were given to adult BALB/c mice. After a dose of 300μg given subcutaneously, the bodies of the mice became visibly swollen and the mice died within 5 days. At a dose of 150μg, mice survived however they showed clinical signs such as a rough coat and lethargy. These mice were boosted after 7 days and punch biopsies taken after 14 days and sent for histological analysis to Dr.Brendan O'Conner at Prairie Diagnostic Services. The histological analysis indicated that, at the standard vaccine doses used for our neonatal or adult mouse experiments, there were no lesions present at the injection site, and no significant histological changes seen. For mice that received very high doses of PP, there were severe lesions (epidermal necrosis) at the injection site, with deep perivascular dermatitis and mild dermal fibrosis (fibroblast proliferation in the deep dermins and subcutis). There was a mixed inflammatory cell infiltration, including neutrophils (heterophils), macrophages, lymphocytes and plasma cells. Mast cells were also commonly present in the dermis. These lesions were also present in mice vaccinated with a higher adjuvant platform dose including 0.1μg PTd, 10 μg CpG ODN, 75 μg IDR-HH18, and 50 μg VIDO-EP#3 after 6 days (Fig 5.3).

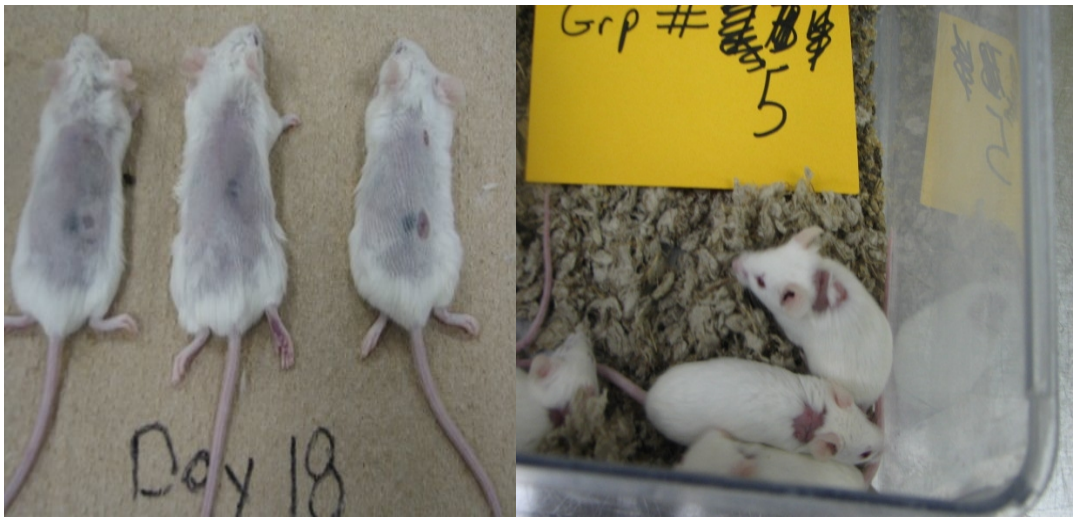


Figure 5.3 Visible lesions of mice post-vaccination with high doses of adjuvant platform.

Adult BALB/c mice (*n* = 20 mice per group) were immunized subcutaneously with PTd (0.1μg)+ CpG C (10μg) + IDR-HH18 (75μg) + VIDO-EP#3 (50μg), at 8 weeks of age. Samples (punch biopsies) were taken from the lesion at the injection site at 3, 6, 9, 12, 15, and 18 days post-immunization.

5.3.5 Replacing CpG ODN with Poly I:C in the adjuvant platform

The synthetic analog of double-stranded RNA, Poly I:C, is a TLR3 agonist, and may be a possible substitute for CpG ODN within our adjuvant platform. In order to examine the effects of replacing the CpG component with Poly I:C, 8-week old adult BALB/c mice were immunized subcutaneously with 50 μL of vaccine, boosted after four weeks, and the sera obtained at two week intervals in order to determine the PTd-specific IgG2a and IgG1 serum responses through ELISAs. Vaccine formulations included 0.1 μg PTd, 10 μg CpG ODN or Poly I:C, respectively, 20 μg IDR-HH1002, and 10 μg VIDO-EP#3, with the CpG (or Poly I:C) and IDRP components pre-complexed.

ELISA analysis showed that immunization with our adjuvant platform containing Poly I:C or CpG ODN induced significantly higher IgG2a titres, indicative of a Th1 immune response, as compared to the vaccine containing antigen alone (Fig 5.4A). The titre after immunization with the CpG ODN vaccine reached a median of $1.0 \times 10^{5,}$ while the comparable Poly I:C vaccine reached a highest median titre of 5.0×10^{3}. The IgG2a response after immunization with the CpG ODN platform was detectable after only two weeks, notably earlier than the antibody responses seen in mice given the adjuvant platform containing Poly I:C, which did not show a response until six weeks after the first immunization (and two weeks after the booster dose). The IgG1 response to vaccination with the adjuvant platforms containing Poly I:C or CpG ODN, indicative of a Th2 type response, was not significantly different, with both vaccine groups as well as the PTd alone group able to induce high IgG1 titres, reaching a median of 2.7×10^{5} to 1.9×10^{6} (Fig 5.4B).

Subsequently we wished to examine the possible effects of the route of immunization and the Poly I:C component dose on the humoral immune response. Thus another experiment was performed in which 7-day old neonatal BALB/c mice were immunized either intramuscularly (with 10μL) or intranasally (with 20μL) with our adjuvant platform, containing 1.0μg PTd, 2.37μg VIDO-EP#3, 4.74μg IDR-HH1002, and either a 2.37μg dose of CpG ODN, or various doses (ranging from 0.1μg to 10μg) of Poly I:C. Mice were boosted after four weeks, and sera obtained at 4, 6, and 10 weeks to determine the PTd-specific IgG2a and IgG1 serum responses through ELISAs.

After intramuscular immunization, all mice that received our adjuvant platform showed an IgG2a response, with no significant differences seen between the group containing CpG ODN and those groups containing Poly I:C (Fig 5.5A). There was no significant difference in response found between the various dosages of Poly I:C, and all of our adjuvant platform vaccine groups showed significantly higher titres than the PTd alone group. By week 10, a strong IgG2a response, with median titres ranging from 5.0×10^5 to 5.5×10^6, was seen in all of the adjuvant platform groups that were immunized intramuscularly. When immunized intranasally, there was a significantly higher IgG2a response in mice that received the adjuvant platform containing CpG ODN, as compared to either the same (2.37μg) or a higher (10μg) dose of Poly I:C or to antigen alone (Fig 5.5B). The titre in the mice that received the CpG ODN containing vaccine reached 3.1×10^5, as compared to 3.8×10^2 in the mice receiving vaccines with Poly I:C.

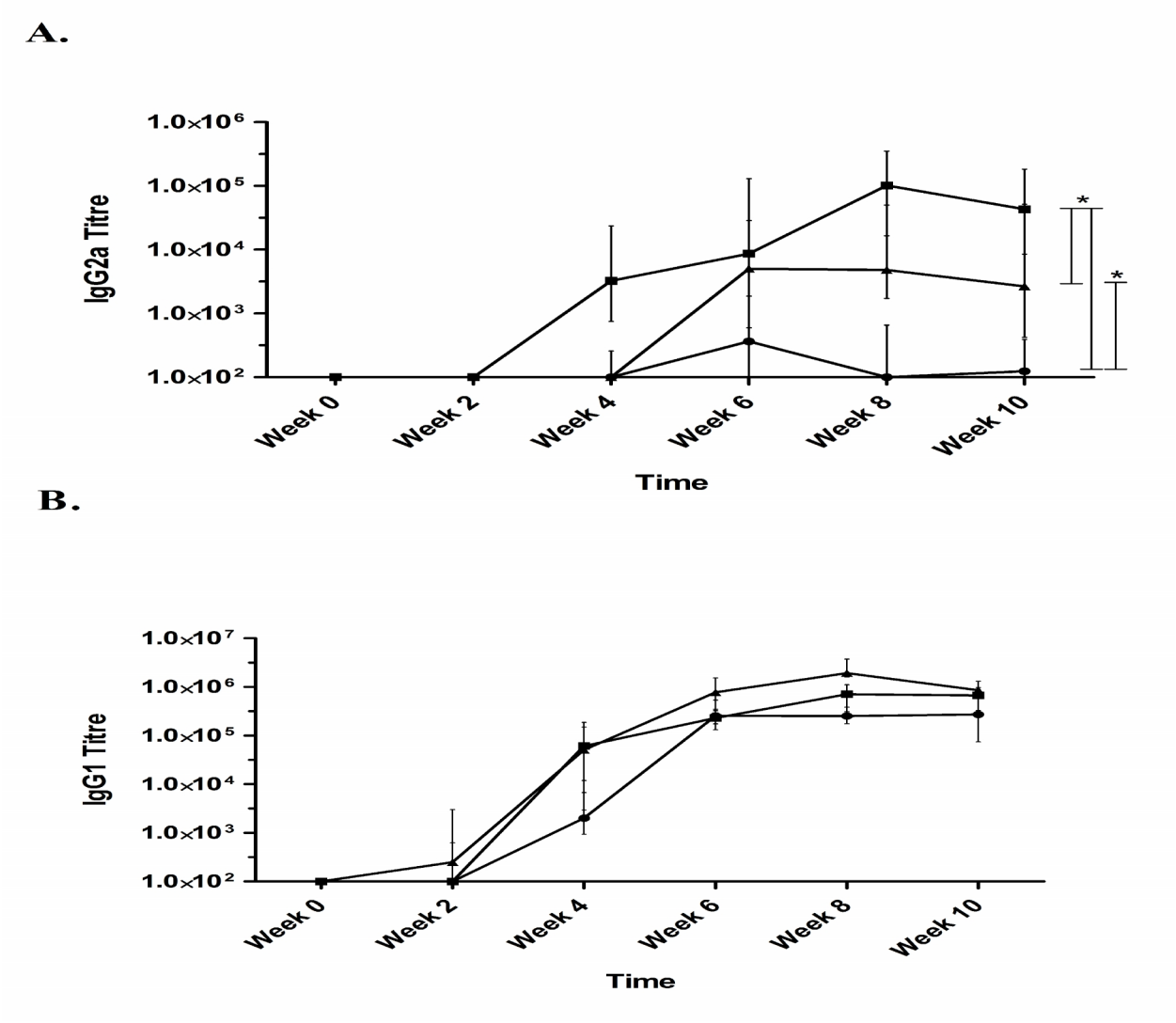


Figure 5.4 Adult IgG2a and IgG1 humoral responses to subcutaneous immunization with adjuvant platforms containing CpG ODN or Poly I:C.

Adult BALB/c mice (*n* = 10 mice per group) were immunized subcutaneously with 0.1μg PTd antigen alone (●), PTd(0.1μg)+CpG C(10μg)+IDR-1002(20μg)+VIDO-EP#3(10μg), with the IDRP & CpG components complexed (■), or PTd(0.1μg)+Poly I:C(10μg)+IDR-1002(20μg)+VIDO-EP#3(10μg), with the IDRP & Poly I:C components complexed (▲), at 8 weeks of age. An identical secondary immunization was given after 4 weeks. Sera was obtained before boost and at 2, 4, 6, 8, and 10 weeks post immunization, and used for ELISAs. The median titres are shown. **A)** There were statistically significant differences in the IgG2a titre between mice immunized with PTd + the adjuvant platform containing CpG ODN as compared to those that received PTd + adjuvant platform with Poly I:C ($p < 0.05$). Both adjuvant platform groups were statistically different from the mice that received PTd alone ($p < 0.001$ for the CpG ODN containing group and $p < 0.05$ for the Poly I:C containing group respectively). **B)** There were no statistically significant differences seen in the IgG1 titres between mice that were vaccinated with either adjuvant platform or PTd alone.

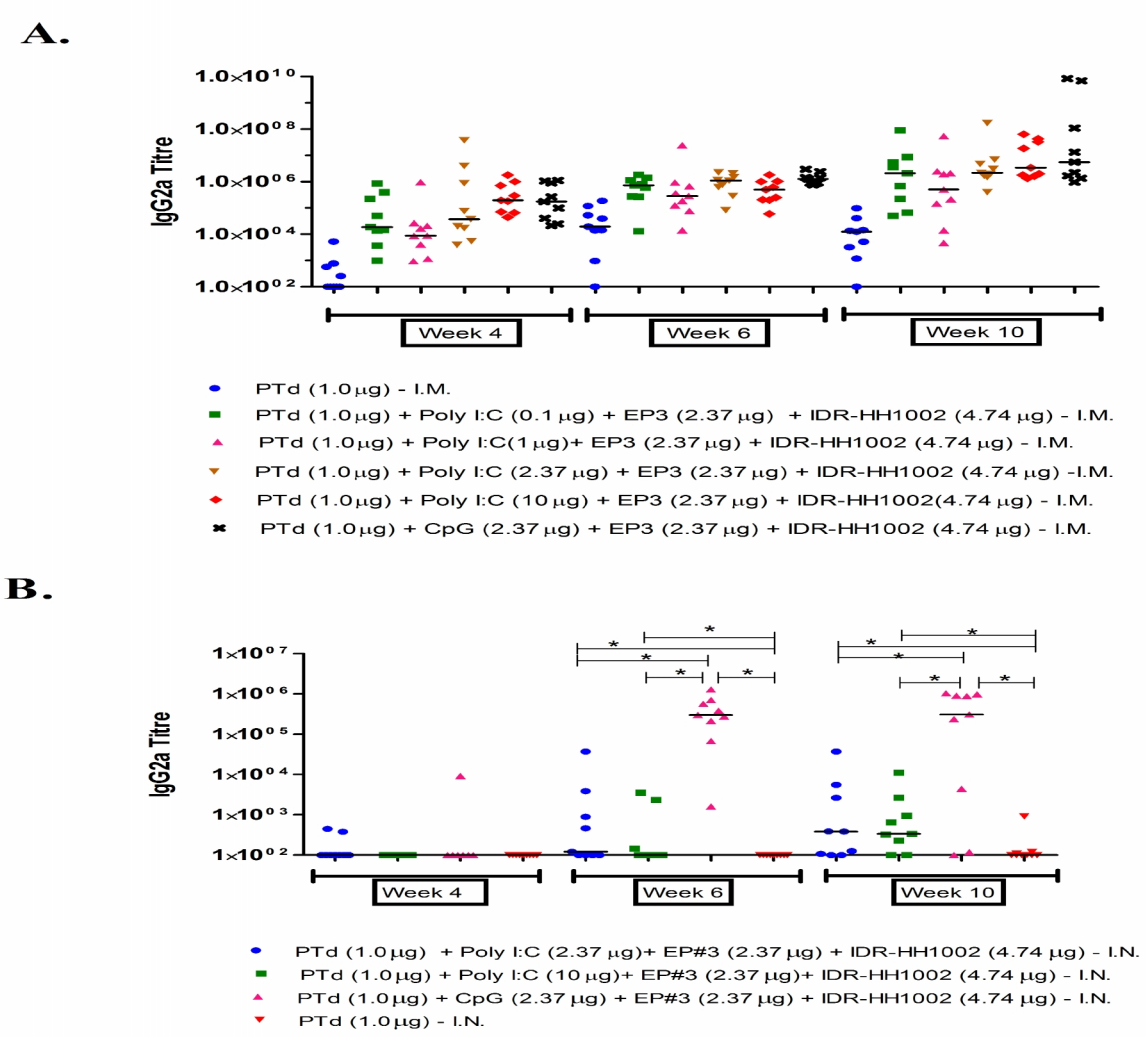


Figure 5.5 Neonatal IgG2a and IgG1 humoral responses to immunization through various routes with adjuvant platforms containing CpG ODN or Poly I:C.

Neonatal BALB/c mice (*n* = 9 mice per group) were immunized with intramuscularly or intranasally with 1.0µg PTd antigen alone, PTd (1.0µg)+ CpG C(2.37µg)+ IDR-HH1002(4.74µg)+ VIDO-EP#3(2.37µg), with the IDRP & CpG components complexed, or PTd (1.0µg)+ Poly I:C (either 0.1µg, 1µg, 2.37µg or 10µg)+ IDR-HH1002(4.74µg)+ VIDO-EP#3(2.37µg), with the IDRP & Poly I:C components complexed at 7 days of age. An identical secondary immunization was given after 4 weeks. Sera were collected at 4, 6, and 10 weeks after the initial immunization. The median titres are indicated. **5A**. No statistically significant differences in IgG2a titre were found between any adjuvant platform groups. All groups immunized with the adjuvant platform showed statistically significant differences from the mice treated with PTd alone ($p<0.05$). **5B.** A statistically significant difference was seen between mice that received the CpG ODN vaccine as compared to the Poly I:C version ($p<0.005$), or PTd alone ($p<0.001$).There were also statistically significant differences in the IgG2a titres between mice treated with the 2.37µg or 10µg dose of Poly I:C, as compared to PTd alone ($p<0.01$, and $p<0.05$, respectively).

5.4 Discussion

The re-emergence of vaccine-preventable diseases such as whooping cough in the developed world is worrisome. It emphasizes the need for the development of effectively adjuvanted vaccines that are able to induce appropriate immune responses in susceptible populations, such as neonates and the elderly. The adjuvants best suited for such a role will have to be both safe and effective. We recently developed a novel multi-component adjuvant platform and tested it with pertussis toxoid in adult and neonatal mice [139]. Here, we have shown here the versatility and safety of this adjuvant platform.

To examine the versatility of the adjuvant platform, HBsAg was combined with our platform and tested in adult mice. The adjuvant platform was able to induce significantly stronger IgG2a titres and equally strong IgG1 titres as when the commercial vaccine adjuvant Alum was given with the same antigen dose (Fig 5.1A/B). Even when the adjuvant dose was decreased by ten-fold, the adjuvant platform was able to induce titres equal to those seen with Alum. These results were supported by previous research, which indicated that the CpG ODN component was effective in allowing lower doses of HBsAg to be used in vaccination in mice [148]. Thus, the adjuvant platform was effective with other antigens as a humoral stimulator in mice.

Practical concerns must also be addressed when designing an adjuvant platform. During infancy, there are several vaccines given in developed countries, often simultaneously. This makes the type of immune response being induced by a vaccine even more important, since if the immune response is non-specific and systemic, it may interfere with, or increase the side effects of, a vaccine that is given at the same time at a different injection site. The histology of the injection site in adult mice was examined after intramuscular injection with the PTd vaccine formulation that had either been separated into its antigen and adjuvant platform components, or was combined together (Fig 5.2A/B). The combined vaccine platform showed mild locally induced inflammation at the injection site with an influx of heterophils by 7 hours post-injection, which remained through 48 hours, indicating a vaccine-induced inflammatory response had been created. However when the adjuvant platform and antigen components were administered separately, the injection site inflammation was greatly reduced, with only a very mild reaction seen at the site, indicating that when not given in combination with an antigen, the adjuvant platform does not act through inducing systemic inflammation. This indicates that there may be a

lack of interference when giving the platform at one site and a different vaccine at another. The humoral immune response of the separated vaccine antigen and adjuvant platform components was also examined (Fig 5.3A/B). Both the IgG2a and IgG1 responses were significantly higher in the combined adjuvant and antigen groups than when the components were separated. For IgG2a, only in combination was the antigen and adjuvant platform able to induce a strong antibody response, and while there was a strong IgG1 response seen even with the components separately, this can be attributed to the fact that the PTd antigen alone is capable of inducing strong IgG1 antibody responses. These results supported the histological data, and further confirm that the immune response being seen after immunization with our vaccine is a specific response, which requires the presence of both the antigen and adjuvant platform to be induced. The safety profile of some the PP component of the adjuvant platform was examined histologically post-vaccination, and it was found that at the recommended dose there was no ill-effect, although when administered in extremely high doses, there were toxic effects of the polyphosphazene observed.

Combination adjuvants have proven highly effective, and the current trend is towards the use of multiple TLR ligands as adjuvants in order to try and engage several inflammatory mechanisms simultaneously and create synergy between the adjuvants. In order to increase the versatility of the adjuvant platform, we attempted to replace some of the individual components. It has been found that the TLR 3 ligand, the synthetic dsRNA Poly I:C, when complexed with IDR-1002, was able to induce comparable MCP-1 production from human PBMCs *in vitro* (data not shown). Thus we attempted to replace the CpG ODN component of our adjuvant platform with Poly I:C for the subcutaneous vaccination of adult mice (Fig 5.4A/B). The humoral response indicated that the CpG ODN component was more effective in increasing the Th-1 type IgG2a titres as compared to Poly I:C. However, there was no difference seen in the IgG1 titres achieved.

It has been previously shown that optimal protection using TLR ligands such as CpG ODN is achieved when the adjuvant is delivered through the same route as the infectious agent [87]. Thus, we examined other routes of immunization in order to examine the effects this had on the immune responses induced. (Fig 5.5A/B). When given to neonatal mice intramuscularly, Poly I:C within our adjuvant platform was able to induce the same high titres as when the CpG ODN

component was used, indicating that this may be a route of immunization for which the replacement of the expensive CpG ODN component with Poly I:C would be feasible. However, when given intranasally, the IgG2a humoral results mirror those seen when the vaccines were given subcutaneously, and the adjuvant platform containing Poly I:C induces significantly lower titres than a platform containing CpG ODN. These results emphasize the importance of route of administration in the immune responses induced, and show that it may be possible in certain situations, to alter the components of our adjuvant platform to adjust to the route of infection and the particular pathogen being dealt with.

We have developed a multi-component adjuvant platform that is able to induce antigen-specific localized site responses with several antigens. This platform is safe, inducing only mild inflammation at therapeutic doses, and is able to be used through several routes of administration, including subcutaneously, intramuscularly, and intranasally. The platform is also versatile, with the ability to replace components and still achieve strong humoral responses through some routes of administration, making it a strong candidate for further research in the search for good neonatal adjuvants.

CHAPTER 6: GENERAL DISCUSSION

There are several issues that make it difficult to achieve successful specific immune responses to vaccines in neonates. There is an inherent Th2 bias in the infant immune system, making the induction of strong and lasting Th1 type humoral and cell-mediated immune responses difficult to achieve. Another important issue is the presence of maternal antibodies which can interfere with vaccination and the achievement of a sufficient primary immune response. Thus, the traditional adjuvants and routes of immunization used in adults, such as alum or intramuscular immunization, may not be appropriate for use in the human infant. Therefore, the development of an appropriate vaccine platform is of vital importance in order to be able to protect infants from debilitating infectious diseases.

A successful neonatal vaccine should not induce tolerance to the antigen or elicit allergic responses. In particular, a candidate neonatal vaccine must be extremely safe in order to avoid adding to current circulating theories in the general population, many who may regard vaccines as dangerous or as having severe side effects. These impressions can make a vaccine unattractive and less likely to be accepted for use by parents. Towards this end, dose-sparing should be used in order to minimize the amounts of antigen required, and thorough testing in neonatal animal models such as the mouse and pig should be performed before moving into any human trials. A gold standard vaccine for human neonates would be one that is able to create the pathogen-appropriate "balanced" or "mixed" Th1/Th2, or, alternatively, Th1/Th2 biased responses and that can generate long-lasting protective immunity. However, there is a functional immaturity of some neonatal cells, which results in a Th2 cytokine milieu being present after stimulation, making it difficult for antibody secreting cells to survive until the infant has reached a certain age.

A balanced immune response is one that includes elements of both humoral and cell mediated immunity in a manner that effectively protects by reducing infection and/or pathogenesis. In the case of some diseases, such as those caused by infection with *Leishmania*, there is a clear delineation of the required type of immune response, with a Th1 response resulting in successful protection against or clearance of disease, and a Th2 response creating

disease susceptibility and leading to non-healing lesions [149]. Other diseases, such as pertussis, appear to be best combated with an immune response incorporating a more even distribution of the Th1 and Th2 responses. As previously discussed, a cell-mediated immune response mediated by IFN-γ helps to most effectively clear *B. pertussis* infection, however passive immunization experiments have shown that a humoral response featuring Ab alone is also capable of conferring some level of protection [23]. It appears that an ideal immune response for pertussis would thus incorporate a mixture of features of both the Th1 and Th2 response, including strong humoral and cell-mediated immunity. Although Th1 immune responses are required to confer protection against numerous infectious diseases, a one-sided and prolonged Th1 type of response is not always ideal, and in many cases can be detrimental to the host. The cell-mediated immune response usually activates CTL in an antigen-specific fashion, with the CTL in turn destroying cells displaying the antigen (e.g. virus-infected cells). Cell-mediated immune responses stimulate cells to secrete a variety of cytokines that influence the function of other cells involved in the adaptive and innate immune responses. It can also results in the activation of macrophages and NK cells, enabling them to destroy intracellular pathogens. The cell-mediated immune response can cause significant pathologies when excessively activated, such as in the case of *Helicobacter pylori* infection, where a prolonged Th1 response is associated with peptic ulcer development in the antrum of the stomach [150]. A strong Th1 response has also been implicated in the pathogenesis of several other autoimmune diseases, including Crohn's disease and multiple sclerosis [151, 152]. Thus, when attempting to design a strategy for immune deviation, one must consider that merely shifting a predominantly Th2 response to a Th1 or vice-versa may result in the development of new pathologies instead of, or in addition to, the desired therapeutic effects.

Although the infant immune response is predisposed to the Th2 type, it is possible to achieve strong Th1 responses, sometimes even approaching the levels seen in adults, through the use of appropriate adjuvants with vaccine antigens. The most successful adjuvant additions to a neonatal vaccine will most likely involve a combination of adjuvants that work through several different immune pathways, ideally achieving a synergistic effect. This concept of using several different adjuvants to attempt to promote a balanced response in the neonate led to the development of the adjuvant platform examined for use with the antigen PTd. Through this study

and those of our collaborators, we demonstrated that each individual adjuvant component used was able to induce an immune response in combination with the PTd antigen that is greater than that seen with the antigen alone. The responses appear to work through several mechanisms, including increased antibody production and shift in the antibody subtype produced towards a Th1 type, upregulation of IL-8 and the MCP-1 chemokine, which result in the attraction to the site of infection of monocytes, memory T cells and DC, and the possibility of antigen encapsulation and a depot effect allowing sustained induction of the immune response [66, 153]. One hypothesis is that the adjuvant platform is enabling the B cells in neonatal mice to reach a "stimulation threshold" necessary for optimal antibody induction, and thus they successfully produce strong antibody titres earlier than when using an alum adjuvant. The secondary IgG2a response induced by our adjuvant platform in mice immunized as neonates was also extremely long lived. Since mice exposed as neonates to antigen often produce IgG1-dominated secondary antibody responses, this induction of Th1 memory by our vaccine is promising [154]. Vaccination with our adjuvant platform thus promoted a shift in the immune response in both adult and neonatal BALB/c mice in the Th1 direction [139]. The adjuvant platform is also able to successfully address the issue of MAb interference [141].

Our experiments also showed strong Th2 type Ab production following vaccination with the adjuvant platform. The resulting IgG1 titres were equivalent to, and sometimes greater than, the IgG2a. The strength of the IgG1 response may, at least in part, be explained by the mouse model and antigen used. The BALB/c mouse is a Th2-biased model, with the MHC haplotype $H2^d$ [155, 156]. This genetic background has been shown to effect the type of immune response induced by some pathogens or antigens, with these mice often exhibiting predominantly Th2 type responses to exogenous antigen stimulation [156, 157]. However, under certain experimental conditions, it has also been demonstrated that BALB/c mice do not always exhibit Th2 responses and, depending on factors such as the dose of antigen used for priming, the innate biases of a mouse strain can be overcome [157]. Another possible factor in the strength of the IgG1 antibody response seen is the PTd antigen. Our data indicates that PTd antigen itself induces a greater IgG1 response than IgG2a. We consistently saw little to no detectable IgG2a response to PTd alone in adult mice, and very low IgG2a titres in neonates, who received a 10-fold higher antigen dose. This hypothesis is further supported by our data examining the use of a different antigen, HBsAg, in combination with the adjuvant platform. After immunizing mice

with HBsAg plus alum as an adjuvant, IgG2a titres of up to approximately $1x10^4$ were successfully induced. By comparison, when mice were immunized with PTd plus alum in another experiment, the maximum IgG2a titre induced was approximately $5x10^2$. The adjuvant alum is a well-characterized promoter of Th2 and IgG1 antibody responses, thus it is unlikely to be responsible for the increased IgG2a titres seen [158]. It appears that the HBsAg itself is a better inducer of IgG2a antibody production than PTd. This mixture of a Th2 biased mouse strain, and an antigen that tends to produce an IgG1 as opposed to an IgG2a antibody response, may explain the results seen after vaccination with the adjuvant platform. The fact that the platform is still able to induce a very strong IgG2a response in a Th2 biased mouse model using an antigen that preferentially induces IgG1 responses in this model suggests that the adjuvant platform is indeed successfully shifting the immune response in these mice towards the Th1 direction.

Given that our adjuvant platform induced a sustained cell-mediated immune response and decreased Th2 cytokines, this indicates that it is able to promote a more balanced response in the neonate, possibly through the inhibition of, or decrease in, the Th2 type cytokine milieu present after immunization. Our results indicated low amounts of IL-5 produced in mice immunized with antigen alone or the Quadracel® vaccine, with ablation of this production in mice that received the adjuvant platform. This disappearance of detectable IL-5 and IL-5-producing cells may appear to be at odds with the significant increase in IgG1 titres seen using the adjuvant platform. A Th2 response is initially characterized by an increase in cytokines such as IL-4, IL-5, and IL-13. These cytokines create an appropriate milieu for the interaction between Th2 type helper T cells and cognate B naïve cells, which induces the B cells to differentiate, isotype switch, and produce Th2 type antibodies such as IgE and IgG1 in the ensuing humoral response. However, while IL-5 has been shown to be a correlate of the Th2 response, it is not the only factor that can drive the secretion of IgG1. It has been shown *in vitro* that murine $CD4^+$ Th1 clones are able to induce IgG1 production, and that this antibody production is IL-5 independent , indicating the possibility of more than one pathway for the induction of IgG1 synthesis [159]. It has also been noted there is a lack of direct evidence showing that IL-5 contributes to the generation of *in vivo* antibody responses [160]. Moreover, B cells have been shown to respond and secrete IgG1 to a mixture of IL-4 and IL-6 independent of IL-5. Both IL-5 and IL-6 were found *in vitro* to independently stimulate increases in steady-state levels of immunoglobulin and J-chain mRNA

and proteins, as well as to induce differentiation of a murine B-cell lymphoma cell-line into strong Ab-secreting cells [161]. These two cytokines appear to at least partially function through distinct molecular pathways. In our experiments, the production of IL-6 was detected at a consistent level (approximately 400pg/ml) from re-stimulated splenocytes of adult mice immunized with our adjuvant formulation, as well as in mice that received antigen alone or Quadracel® (data not shown). This IL-6 may play a role in the induction of the IgG1 responses seen after vaccination with antigen alone. Studies have also found that $CD4^+$ Th1 and Th2 cells can differ in their ability to induce isotype specific Ab synthesis, with some clones being superior to others [162]. This indicates that the ability of a T cell to secrete a characteristic cytokine such as IFN-γ (seen at high levels in all of our vaccinated groups), does not necessarily predict the functional capacity of that cell to induce antibody synthesis [162]. It has also been shown that the ability of Th2 cells to induce T-dependent B cell responses *in vivo* is not unique and that, following immunization, both Th1 and Th2 cells migrate into B cell follicles to support B cell clonal expansion and Ab production [163]. This leads to a hypothesis that the adjuvant platform is stimulating Th1 $CD4^+$ T cells, that are then inducing the production of both IgG1 and IgG2a responses. Another possibility is that the adjuvants are working synergistically to increase both the IgG1 and IgG2a responses, as has been reported previously for bovine-respiratory syncytial virus-specific IgG titres using vaccine combinations of PP and CpG ODN [116]. Finally, studies have shown that using different adjuvants and immunization regimes can drastically affect the humoral response without necessarily influencing cytokine production patterns by T-cells [164].

In both the humoral and cell-mediated responses in adult and neonatal mice, the ratio of the characteristic Th1:Th2 type response is increased after vaccination with our adjuvant platform. This ability to induce strong and balanced humoral as well as cell-mediated responses in both adult as well as neonatal mice, culminated in the protection of immunized mice against challenge with live *B. pertussis*. The adjuvant platform, when combined with only two antigens, was able to surpass the protective efficacy of Quadracel®, the currently used vaccine in Canada for infant immunization, which includes five pertussis antigens combined with an alum adjuvant. When the antigen included in the vaccine platform was changed, a dose sparing effect and a

strong Th1 immune response was still seen in adult mice, as compared to alum, indicating that this platform has versatility and possible applications beyond that of just pertussis. Variation in the routes of administration also indicated further ways in which the adjuvant platform may be tailored to deal with other pathogens, with the platform showing promise as both a traditional intra muscular and a mucosal adjuvant. This mucosal ability is particularly important, as it can be a difficult area in which to achieve strong immune responses, and there are many pathogens which enter through mucosal surfaces. The safety of the platform was demonstrated through its lack of histological effects when it is administered without antigen, indicating that it causes a site-specific localized immune response only when antigen is present, and not an increased systemic response that could interfere with subsequent or simultaneous other vaccinations in the neonate.

The issue of costs was examined through the replacement of the CpG ODN component, technology to which there is limited access, with another TLR ligand Poly I:C. This tweaking of the adjuvant platform showed promise as an intramuscular adjuvant, however there were limitations in its abilities to function when used subcutaneous or mucosally. However, it may still be possible to replace and exchange various components of the adjuvant platform and achieve successful immune responses depending on the pathogen being addressed, its route of infection, and the immune response required to successfully combat infection.

In summary, we have developed a novel vaccine formulation against *B. pertussis*, which is able to induce strong humoral and cell-mediated immune responses, especially Th1 responses, in both adult and neonatal mice, with the immune responses induced being long-lasting and protective against infection. We have also demonstrated that our novel adjuvant platform itself is adaptable for use with other vaccine antigens and through other routes of administration, and it may be possible to adjust the platform components while maintaining efficacy.

CHAPTER 7: CONCLUSION

7.1 Overall Conclusions

We have developed a novel vaccine against *B. pertussis* through the co-formulation of multiple adjuvants, including CpG ODN, PP, and IDRP, along with the *B. pertussis* antigen, PTd. This vaccine formulation is able to induce strong, long-lived Th1 and Th2 type humoral immune responses in BALB/c adult and neonatal mice, with an especially notable increase in the Th1 type IgG2a antibody response as compared to immunization with commercial pertussis vaccines or the adjuvant alum. The cell mediated immune response in neonates and adults after vaccination with the novel vaccine shows a decreased IL-5 response while a strong IFN-γ response is maintained, confirming the mixed Th1/Th2 response seen humorally. When an additional pertussis antigen, PRN, is included in the vaccine formulations, protection against *B. pertussis* bacterial challenge can be achieved, with complete clearance of bacteria in many animals, which is superior to the results seen with commercial vaccines in the same model. The adjuvant platform is demonstrably versatile, and can be used with the HBsAg to induce a strong Th1/Th2 response in adult mice, similar to that seen using PTd, with the IgG2a type antibodies being significantly increased as compared to immunization using the standard vaccine adjuvant alum. Finally, the novel vaccine platform has been demonstrated to recruit immune cells such as neutrophils in a localized manner, with an inflammatory response and increased vasodilation seen at the site of injection, and not in a systemic manner, with the presence of both the vaccine antigen and adjuvant together required for the influx of immune cells to occur.

7.2 Future Research

Further characterization of the cell-mediated immune response is necessary in order to determine the mechanism of protection involved using this adjuvant platform. Examination of the effects of the adjuvant platform on the DC subsets as well as the $CD4^+$ and $CD8^+$ T cells induced and their cytokine production may be useful in order to better understand the exact role these cells are playing in the protection of the mice against infection with *B. pertussis*. Further studies in other animal models, such as pigs, are necessary to ensure that the platform is effective within a better correlate of the human immune system, and a more extensive safety profile of the vaccine must be compiled. Finally, clinical trials must be performed first in animals, and eventually humans, for the overall efficacy and safety of the platform to be determined.

CHAPTER 8: REFERENCES

8.1 References:

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