Vaccine-induced protective immunity to *Bordetella pertussis*

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Declaration of Authorship

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Abstract

Whooping cough (pertussis) is an infectious disease caused by the respiratory pathogen *Bordetella pertussis* that is re-emerging in many developed countries, despite extensive vaccine coverage. One of the proposed reasons for this resurgence is that commercial acellular pertussis (aP) vaccines do not induce appropriate or long-lasting immunity to *B. pertussis* infection due to the choice of alum as the adjuvant. This project aimed to evaluate the mechanisms of vaccine-induced immunity to *B. pertussis* and to explore the use of novel adjuvants for aP vaccines that promote stronger cell-mediated and memory immune responses to *B. pertussis*, with the intention of informing the rational design of third-generation aP vaccines.

Toll-like receptor (TLR) agonists have potent immunomodulatory activity and can function as effective adjuvants, capable of promoting robust cellular immunity to co-administered antigens *in vivo*. The present study examined the hypothesis that TLR agonists alone or in combination with other adjuvants could enhance the immunogenicity and protective efficacy of aP vaccines. Indeed, it was found that replacing alum with the TLR9 agonist CpG or the novel TLR2 agonist LP1569 in an experimental aP vaccine induced more potent Th1 and Th17 cell responses, and was more effective at promoting rapid *B. pertussis* clearance from the lungs. Furthermore, addition of CpG or LP1569 to a commercial aP vaccine already formulated with alum enhanced antigen-specific IFN-γ and IgG2c responses. Finally, it was shown that LP1569 was a more potent inducer of antigen-specific Th1 and Th17 cell responses than CpG, suggesting it could be the more appropriate adjuvant for an aP vaccine in humans.

Cyclic diguanylate monophosphate (c-di-GMP) is a bacterial intracellular signalling molecule which signals through the adaptor molecule stimulator of interferon gene (STING) to induce type I interferon (IFN) production. C-di-GMP has also been shown to activate NFκB and can induce production of IL-12, IFN-γ and IL-8 by human dendritic cells (DCs). Furthermore, it was reported that nasal delivery of c-di-GMP reduced the severity of *B. pertussis* infection by enhancing Th1 polarising cytokine and nitric oxide production by alveolar macrophages. Recent evidence also suggests that TLR and STING agonists can function synergistically to induce potent Th1-polarised immune responses. The present study investigated if the combination of a TLR2 and a STING agonist might be a more effective adjuvant than either alone for an aP vaccine. The results showed that the combination of c-di-GMP and LP1569 induced more potent production of Th1 and Th17 polarising cytokines by bone marrow-

derived DCs and macrophages than either agonist alone. An evaluation of the TLR2 and STING agonists *in vivo* revealed that they were a highly effective adjuvant combination for promoting antigen-specific Th1 and Th17 responses in mice immunised with a model antigen. Furthermore, immunising mice with an experimental aP vaccine adjuvanted with the TLR2-STING agonist combination conferred greater protection against *B. pertussis* challenge, and recruited greater numbers of $CD4^+$ resident memory T (T_{RM}) cells to the lungs of mice, than an experimental alum-adjuvanted aP vaccine. This finding suggests that a TLR2-STING agonist combination is more effective than alum for promoting protective immune responses, including T_{RM} cells specific for *B. pertussis*.

This study also revealed that intranasally administering an aP vaccine formulated with c-di-GMP and LP1569 induced local IgA and very potent Th17 responses, and protected against respiratory challenge with *B. pertussis*. Furthermore, intranasal immunisation with this experimental aP vaccine induced significantly greater recruitment of $CD4^+$ T_{RM} cells to the lungs than intraperitoneal immunisation, indicating this route of administration may be the most effective for promoting long-term memory responses to *B. pertussis*.

Developing a booster aP vaccine containing a novel adjuvant to enhance Th1 and Th17 cell responses would be logistically simpler than redesigning the current combined paediatric vaccine for infant immunisation. However, it has not yet been established if the Th2 responses induced with alum-adjuvanted aP vaccines are set following primary immunisation or can be redirected to Th1 responses by boosting with a vaccine containing a novel adjuvant. In the present study, it was found that following priming with an experimental aP vaccine containing the Th2-promoting adjuvant alum, it is difficult to enhance Th1 and Th17 cell responses with an experimental aP vaccine containing a TLR2 or TLR2-STING agonist combination. Furthermore, priming with an experimental aP vaccine containing a TLR2-STING agonist combination and boosting with an experimental alum-adjuvanted aP vaccine did not induce robust Th1 and Th17 cell responses. The findings suggest that priming and boosting with an experimental aP vaccine containing a TLR2-STING agonist combination is required for potent cell-mediated immunity to *B. pertussis*.

Collectively, the findings from this study suggest that a TLR agonist, or a TLR2-STING agonist combination, are more appropriate adjuvants for aP vaccines than alum, capable of inducing potent Th1, Th17 and IgG2c responses *in vivo.* Furthermore, the data indicates that intranasal immunisation may be the optimal route of vaccine administration for the establishment of long-term immunity to *B. pertussis*.

Publications

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Abbreviations

ACT	Adenylate cyclase toxin
Alum	Aluminium hydroxide
aP	Acellular pertussis
APC	Antigen-presenting cell
BCG	Bacillus Calmette-Guérin
BCR	B cell receptor
β-GAL	β-galactosidase
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CAF01	Cationic adjuvant formulation no. 1
CDC	Centers for Disease Control
c-di-AMP	Cyclic-di-adenosine monophosphate
c-di-GMP	Cyclic diguanylate monophosphate
CDN	Cyclic dinucleotide
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GMP-AMP synthase
ClfA	Clumping factor A
DCs	Dendritic cells
DNT	Dermonecrotic toxin
dPT	Chemically detoxified pertussis toxin
ds	Double-stranded
EBAO	Ethidium Bromide-Acridine Orange
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FHA	Filamentous haemagglutinin
FIM	Fimbriae
FMO	Fluorescence minus one
GC	Germinal center
GPI	Global Pertussis Initiative
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase

HSV	Herpes simplex virus
IDR	Innate defence regulator peptide 1002
IFN	Interferon
IFNAR	Type I IFN receptor
Ig	Immunoglobulin
IL	Interleukin
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
IRF	IFN-regulatory factor
i.t.	Intratracheal
KLH	Keyhole limpet hemocyanin
LNs	Lymph nodes
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LT	Heat-labile toxin
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLRs	NOD-like receptors
NO	Nitric oxide
OMVs	Outer membrane vesicles
OPD	o-Phenylenediamine dihydrochloride
PAMP	Pathogen-associated molecular pattern
PCB	Phosphate citrate buffer
PCEP	Polyphosphazene
PLG	Poly-lactide-co-glycolide
Prn	Pertactin
PRR	Pattern recognition receptor
PT	Pertussis toxin
RLRs	Retinoic acid-inducible gene-I (RIG-I)-like receptors
RPMI	Roswell Park Memorial Institute

rPT	Genetically inactivated pertussis toxin
s.c.	Subcutaneous
SCID	Severe combined immunodeficient
SFM	Serum-free media
SLE	Systemic lupus erythematosus
ss	Single-stranded
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
Tcf	Tracheal colonisation factor
T_{CM}	Central memory T
TCR	T cell receptor
TCT	Tracheal cytotoxin
T_{EM}	Effector memory T
Tfh	Follicular helper T cells
Th	T helper
TLRs	Toll-like receptors
Treg	Regulatory T
TRIF	TIR-domain-containing-adaptor protein inducing IFN-β
T_{RM}	Resident memory T
WHO	World Health Organisation
wP	Whole cell pertussis
WT	Wild-type

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Introduction

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1.1 Background and history of *B. pertussis*

The Gram-negative bacterium *Bordetella pertussis* causes whooping cough (pertussis), a respiratory tract infection that remains to this day endemic, despite high vaccination coverage. The World Health Organisation (WHO) estimated that there were 195,000 deaths as a result of *B. pertussis* infection in 2008, and that in total there were around 16 million cases of pertussis worldwide that same year [1]. Humans are the only known host of *B. pertussis*, and this bacterium is spread from person to person by aerosol transmission and colonises the airways, causing damage to the epithelium and impairing normal respiratory function. Studies using immunocompromised mice have demonstrated disseminated disease [2, 3], however, systemic infection is extremely rare in humans, with infection remaining localised to the respiratory tract [4]. Despite this, infection is associated with high morbidity and mortality, primarily in unimmunised infants [5]. The first pertussis epidemic was described in 1578 by Guillaume de Baillou [6], however, the *B. pertussis* organism was only identified in the early 1900s by Bordet and Gengou [7]. Pertussis vaccines were not commonly used until the late 1940s.

1.2 Pertussis disease course and post-exposure prophylaxis

A typical *B. pertussis* infection lasts 4-8 weeks [8] and the disease course can be subdivided into three phases; the catarrhal phase, the paroxysmal phase and the convalescent phase [6]. Upon infection, there is an initial incubation period which usually lasts 6-20 days. During this period, *B. pertussis* invades and virulence factors such as tracheal cytotoxin (TCT), cause damage to the airway epithelium and the alveoli, which impedes normal ciliary movement [9]. The first stage of disease is the catarrhal phase which characteristically last 1-2 weeks. Symptoms such as coughing first appear during this phase, and increase in severity as the disease advances. The risk of transmission is also highest during this stage. The ensuing paroxysmal phase can last anywhere from 3-6 weeks and is characterised by spontaneous coughing fits followed by posttussive whooping. In children, posttussive vomiting is also common and asphyxia has been seen to occur in infants [10, 11]. The final convalescent phase of *B. pertussis* infection can persist for several months, and during this stage, symptoms gradually decrease in severity. Typical systemic symptoms observed over the course of *B. pertussis* infection include lymphocytosis (caused by pertussis toxin (PT), a virulence factor of *B. pertussis*), however, fever rarely occurs [8].

Macrolide antibiotics are typically used to treat *B. pertussis* infection, however, only early administration, during the catarrhal phase, will lessen the duration and gravity of disease [12, 13]. While the original treatment course involved 14 days of antibiotics, it has since been shown that 7 days is as effective [14]. Erythromycin is usually the primary antibiotic dispensed, however, it has a number of adverse side effects including diarrhoea and vomiting [12]. Furthermore, erythromycin-resistant *B. pertussis* strains have been detected in the US [15], France [16] and China [17]. Azithromycin and clarithromycin have been shown to be as effective as erythromycin, but are associated with fewer side effects so may be more suitable treatments [13, 18]. However, with the emergence of macrolide-resistant *B. pertussis* strains becoming more prominent, there is an urgent need for novel therapies to treat this highly infectious disease.

1.3 Vaccines against *B. pertussis*

The first vaccines to be introduced were the whole cell pertussis (wP) vaccines, which consisted of a heat or chemically detoxified preparation of the bacteria, administered with or without aluminium salts (alum) as an adjuvant [8]. These vaccines were very effective and a significant decrease in the incidence of pertussis was seen relatively soon after their introduction [19]. However, wP vaccines were very reactogenic, largely due to the high content of lipopolysaccharide (LPS), a highly immunostimulatory bacterial cell wall component that activates innate immune cells via Toll-like receptor 4 (TLR4) [20, 21]. Immunisation with wP vaccines was associated with a number of local and systemic side effects, including whole limb swelling, fevers and rare cases of seizures [22, 23]. These together with reputed neurological complications in a small number of vaccinated children and associated adverse publicity following litigation cases in the UK against the vaccine manufacturers, led to a loss of confidence in the vaccine. The ensuing decrease in vaccine uptake resulted in an increase in the number of cases of pertussis in the UK and other countries [19]. This motivated efforts to replace the wP vaccines with safer acellular pertussis (aP) vaccines, prepared with selected *B. pertussis* antigens and delivered with an adjuvant to enhance their immunogenicity [24]. After successful testing in clinical trials, the first aP vaccines were introduced in the 1980s, first in Japan [25], and nearly a decade later in Europe and the USA [26]. These subunit vaccines are now commonly used in most developed countries worldwide. Indeed, the current vaccination strategy in Ireland [27], the UK [28] and the US [29, 30] involves three immunisations with an aP vaccine between 2 and 6 months of age, with follow up booster vaccinations around 4 years

and 11 years of age. However, they have not yet been introduced into routine vaccination protocol in most developing countries, primarily for reasons of cost.

1.4 Vaccine antigens and their roles in protection

B. pertussis was initially believed to be exclusively an extracellular pathogen and this assumption has delayed progression of our understanding of the mechanism of protective immunity. As with a number of other extracellular pathogens, such as *Corynebacterium diphtheriae* and *Clostridium tetani* that cause diphtheria and tetanus respectively, humoral immunity, especially antibodies, were believed to be the key protective factors crucial for preventing infection [31, 32]. Indeed, passive transfer studies in mice have demonstrated that anti-*B. pertussis* antibodies in human colostrum or sera could protect naïve mice against intracerebral challenge with live *B. pertussis* [33]. Furthermore, it has been demonstrated that newborn piglets that passively receive antibodies to *B. pertussis* through colostrum from their vaccinated mothers were protected against respiratory infection [34]. During development of the aP vaccines, a focus was placed on selecting virulence factors that induced robust antibody responses, with little regard for the potential role of cell-mediated immunity in protection against *B. pertussis* infection.

A number of *B. pertussis* antigens were found to be immunogenic and induced potent antibody responses in mice when administered with adjuvants. PT is immunogenic in mice [35] and is the only antigen shown to definitely confer protection to *B. pertussis* in humans; it was tested as a single component vaccine in clinical trials [36, 37]. PT is too toxic for human use in its active form, and therefore chemical detoxification or inactivation by genetic recombination has been employed in vaccine formulations. Genetic engineering of PT has produced non-toxic versions of the protein that retain their immunogenicity, and they have been shown to be more immunogenic than chemically detoxified PT (dPT) [38]. PT has been shown to be an important virulence factor of *B. pertussis;* PT deficient strains are inefficient at colonising the respiratory tract [39]. Furthermore, it has been established that this virulence factor is responsible for the systemic symptoms associated with *B. pertussis* infection including lymphocytosis and histamine sensitivity [40, 41], and moreover it is the cause of mortality in neonatal mice [42, 43].

Filamentous haemagglutinin (FHA) is a *B. pertussis* surface protein important for mediating adhesion of the bacteria to the respiratory epithelial cells of humans [44-46] and mice [35, 47]. Kimura *et al.* demonstrated that immunisation of mice with FHA in the presence of alum

induced the production of high titres of FHA-specific immunoglobulin (Ig) G in the serum and the lungs [48]. The induction of antibody correlated with a reduction in bacterial colonisation of the lungs and trachea following intranasal (i.n.) challenge [48]. Pertactin (Prn) is a 69 kilodalton membrane protein of *B. pertussis* that has been shown to protect mice against respiratory challenge with *B. pertussis* through the production of agglutinating antibodies [49]. There is also evidence to suggest that it enhances the efficacy of aP vaccines that include FHA and PT in children [50]. Finally, a set of agglutinogens known as fimbriae (FIM), which also play a role in adhesion [51], have also been included in a five-component vaccine with PT, FHA and Prn. Antibodies specific for FIM2 and FIM3 are induced by immunisation with wP and five-component aP vaccines and there is circumstantial evidence that they contribute to protection against *B. pertussis* [52].

The current aP vaccines used in most developed countries contain either three or five purified antigens adsorbed to alum and co-formulated with diphtheria and tetanus toxoids (DTaP), usually as part of the paediatric vaccine combinations. The aP vaccines have proven to be safe, with a number of clinical trials demonstrating a significantly lower incidence of adverse side effects when compared with wP vaccines [53-56]. Results from phase III clinical trials in Sweden [54] and Italy [53] indicated that aP vaccines containing higher numbers of antigens were the most effective. In the NIH-sponsored phase III clinical trials carried out in Italy and Sweden in the mid-1990s, certain of the aP vaccines were found to be more effective than the wP vaccine [53, 54, 57]. However, many researchers believe that the Connaught Laboratories wP vaccine used in these trials was suboptimal and that wP vaccines manufactured in Europe were actually more effective than any aP vaccine [58]. Edwards and colleagues compared the efficacies of thirteen aP vaccines with a wP vaccine [59]. Each of the DTaP vaccines examined contained inactivated PT, most contained FHA and others included either FIM2/3 or Prn alone, or a combination of the two. All of the DTaP vaccines induced significantly higher antigen-specific antibody responses than the wP vaccine examined. Unfortunately, despite the fact that aP vaccines induce potent antibody responses, they do not seem to confer the same level of protective immunity to *B. pertussis* as the original wP vaccines and an increase in the number of cases has been seen in many areas with high vaccine coverage in recent years.

One explanation for the relative failure of these vaccines is that humoral immunity is not the essential arm of the adaptive immune response required for protection. There is increasing evidence to suggest that *B. pertussis* is capable of infecting and surviving intracellularly within mammalian cells such as human macrophages [60, 61], and murine macrophages recovered from bronchoalveolar lavage samples [62, 63]. This has provided circumstantial evidence that

cellular immune responses, in addition to humoral immunity, may play a crucial role in protection. Indeed, recent studies in the mouse respiratory challenge model have demonstrated that in addition to innate immune responses, both cellular and humoral arms of the adaptive immune responses are required for clearance of a primary infection with *B. pertussis* and for optimum protective immunity induced by vaccination [64-66]. Furthermore, it appears that humoral immunity alone is not capable of conferring long-term protection to this respiratory pathogen [66-68].

1.5 Other virulence factors of *B. pertussis* as vaccine antigens

In addition to the antigens discussed in section 1.4, *B. pertussis* has several other virulence factors that are currently not included in any commercial aP vaccines, including TCT, dermonecrotic toxin (DNT), LPS, and tracheal colonisation factor (Tcf) [69]. One other virulence factor which has been investigated as a potential vaccine antigen is adenylate cyclase toxin (ACT). ACT is a bifunctional protein that has cyclase and haemolytic activities, both of which have been shown to be crucial for the initiation of *B. pertussis* infection [42, 70]. Indeed, Guiso *et al.* proposed that ACT is predominantly responsible for the development of pulmonary lesions during *B. pertussis* infection [71]. It has been reported that individuals infected with *B. pertussis* produce antibodies to ACT [72], and furthermore, a number of studies in mice have shown that immunisation with purified ACT protects them from *B. pertussis* infection [71, 73]. Moreover, no ACT negative *B. pertussis* strains have been identified to date, possibly due to the crucial role this virulence factor plays in establishing infection in the host. This indicates that ACT could be an important antigen for inclusion in aP vaccines.

1.6 The innate immune response to *B. pertussis* infection

Phagocytes, including dendritic cells (DCs) and macrophages, are the major effector cells of the innate immune system. They engulf and destroy pathogens, as well as producing pro-inflammatory cytokines to recruit and activate cells of the adaptive immune response. Receptors on the surface of phagocytes (pattern recognition receptors; PRRs) recognise specific conserved patterns expressed on the surfaces of pathogens (pathogen-associated molecular patterns; PAMPs). These PAMPs are not present in mammalian cells and therefore, engagement with PRRs allows cells to broadly distinguish self from non-self, and identify the

organism as foreign. The rapid detection of microbial agents is essential for the effective initiation of host defence mechanisms against infection.

1.6.1 Neutrophils

Neutrophils are one of the first lines of defence against bacterial infections, and have been shown to be important in protecting against several bacterial pathogens of the respiratory tract including *Pseudomonas aeruginosa* [74] and *Klebsiella pneumoniae* [75]. One of the main functions of these granulocytes is to phagocytose and kill bacteria and *in vitro* studies have demonstrated that human neutrophils can engulf and kill *B. pertussis* organisms [76]. Surprisingly, depleting neutrophils in a murine model of primary *B. pertussis* infection did not enhance disease severity [77]. However, they are important in the control of infection in immune mice or in mice passively immunised with immune serum [77]. Evidence of their importance in host immunity is demonstrated in that PT and ACT, two virulence factors of *B. pertussis*, inhibit early neutrophil recruitment to the respiratory tract and phagocytosis by neutrophils [77-79]. Recruitment is prevented by inhibiting the production of certain neutrophil-attracting chemokines including KC, MIP-2 and LIX [77-79].

1.6.2 Macrophages

Macrophages are another phagocytic population involved in local innate immunity against *B. pertussis* in the respiratory tract. Carbonetti *et al.* demonstrated that depletion of airway macrophages resulted in enhanced bacterial infection [80]. Their importance can be attributed in part to their capacity to efficiently phagocytose and kill *B. pertussis,* however, there is increasing evidence that *B. pertussis* are capable of surviving within macrophages [60, 61]. Lamberti *et al.* showed that while a high percentage of *B. pertussis* engulfed by human macrophages were destroyed within acidic compartments, some were capable of evading killing by remaining in non-acidic vacuoles [61]. An increase in intracellular bacterial number was observed over the subsequent 48 hours as *B. pertussis* replicated safely within the macrophages [61]. As *B. pertussi*s is capable of surviving within macrophages, production of antimicrobial effector molecules such as nitric oxide (NO) by these phagocytic cells is crucial for killing the invading bacteria. NO production by activated murine macrophages is an effective antimicrobial effector mechanism utilised in the clearance of several pathogens [81]. Indeed, iNOs knockout mice have been shown to be more susceptible to *B. pertussis*

respiratory challenge than wild-type (WT) mice [82]. It has also been shown that macrophages from mice previously immunised with the wP vaccine produce NO when stimulated with killed *B. pertussis* [83, 84]. This NO production correlated with increased macrophage activation and suggested that wP vaccines may function in part by enhancing the ability of macrophages to kill intracellular bacteria. The ability of *B. pertussis* to survive intracellularly within macrophages has been credited, at least in part, to the virulence factor ACT which has been shown to interfere with oxidative responses and inhibit intracellular killing [85, 86].

1.6.3 Natural killer cells

Natural killer (NK) cells are innate lymphocytes which primarily function in the immune response to viral infections and tumours, as they have the ability to recognise and kill abnormal host cells, such as virally infected cells. NK cells can also function in the immune response to bacterial pathogens, as they are an early source of interferon-gamma (IFN-γ). The importance of IFN-γ has been attributed in part to its role in activating macrophages to kill intracellular bacteria by producing NO [87]. Depletion of NK cells during *B. pertussis* infection has been shown to result in a lethal infection in mice, involving dissemination of bacteria to the liver from the lungs [3]. Furthermore, IFN-γ depletion with monoclonal antibodies was shown to result in greater lung bacterial burden [88]. In an attempt to evade this early IFN-γ production, *B. pertussis* employs PT to interfere with NK cell chemotaxis to the lungs [89].

1.6.4 γδ T cells

γδ T cells are innate-like T cells that respond very early in infection. They have been shown to play protective roles at mucosal surfaces against numerous bacterial, viral and parasitic infections and this has been attributed to their ability to readily produce IFN-γ and interleukin-17 (IL-17), which can recruit and activate other innate immune cells including macrophages, NK cells and neutrophils [90-95]. Despite their pro-inflammatory role in multiple infections, Zachariadis *et al.* reported that γδ T cells play an immunomodulatory role in *B. pertussis* infection [96]. Early infiltration of neutrophils, macrophages and NK cells was augmented in γδ T cell receptor (TCR) knockout mice compared with WT mice [96]. While this correlated with a significant decrease in *B. pertussis* colonisation of the lungs early in infection, both WT and γδ $TCR^{-/-}$ mice cleared the infection at the same rate, however, the γδ $TCR^{-/-}$ mice suffered from severe pulmonary inflammation and extensive lung damage. Furthermore, while the Th1-type

immune response generated in the γδ TCR$^{-/-}$ mice was as robust as in the WT mice, the FHA-specific IL-4 production and IgG1 antibody titres were also significantly enhanced [96]. This indicates that γδ T cells play a role in determining the adaptive immune response induced, as well as regulating the initial innate inflammatory response.

1.7 The adaptive immune response to *B. pertussis* infection

The T and B cells of the adaptive immune system are reliant on the innate immune system for activation. Detection of a pathogen by an antigen presenting cell (APC) such as a macrophage or DC results in subsequent uptake and degradation of the pathogen to small components. These small peptide antigens are presented by APCs to the TCR on T lymphocytes. The key molecules involved in presentation on APCs are the major histocompatibility complex class I and class II (MHC class I or II) molecules, with the surface molecules CD80, CD86 and CD40 playing a role in co-stimulation.

T and B cells mediate cellular and humoral immunity respectively. They possess unique TCR and B cell receptors (BCR) capable of recognising specific antigens which allows them to tailor pathogen-specific responses. This leads to more efficient elimination of invading organisms from the body and prevents reinfection.

1.7.1 T cells

T cells derive from lymphoid progenitors in the thymus. Each T cell possesses a unique TCR on its surface allowing it to recognise and respond to a specific antigen. Proliferation and maturation of naïve T cells into effector T cells requires three signals and occurs in secondary lymphoid organs such as the spleen and lymph nodes (LNs). Signal one involves engagement of the TCR with MHC class I or MHC class II on an APC presenting the T cells' cognate antigen. Signal two involves co-stimulation of T cells by the interaction of costimulatory molecules; CD28 on T cells with CD80 and CD86 on APCs. The cytokine environment comprises signal three and ultimately determines which subtype of effector T cell differentiates. The effector T cell family are broadly divided into CD4^{+} and CD8^{+} T cells. CD4^{+} T cells are involved in various immune cell processes including providing signals to B cells to drive activation, differentiation and antibody class switching [97], whereas CD8^{+} (cytotoxic) T cells can induce programmed cell death in target cells [98].

1.7.2 $CD4^+$ T cell subtypes

$CD4^+$ T cells can be divided into different subtypes and the best characterised are the T helper 1 (Th1), Th2 and Th17 cells (Figure 1.1). The presence of IL-12 in the microenvironment during T cell maturation induces expression of the transcription factor T-bet in naïve T cells, resulting in differentiation into Th1 cells [99]. Th1 cells are important in the immune response to intracellular pathogens. Viruses and certain bacteria such as Mycobacteria, are capable of evading degradation and surviving intracellularly within phagocytes [100]. IFN-γ is the main cytokine produced by Th1 cells and it is capable of activating macrophages and stimulating antibacterial mechanisms to kill intracellular pathogens e.g. NO production [101, 102]. IFN-γ has also been implicated in directing antibody class switching towards the IgG2a isotype *in vitro* and *in vivo* [103, 104]. The chemokine receptors CXCR3 and CCR5 are preferentially expressed on human Th1 cells [105, 106].

Infection with parasites, such as helminths, induces potent IL-4 production which drives Th2 cell differentiation by upregulating the transcription factor GATA3 [107-109]. Th2 cells predominantly produce IL-4, IL-5 and IL-13, express the chemokine receptor CCR4, and are important for humoral responses, especially in promoting the production of IgE and IgG1 antibodies by B cells [103, 105]. However, Th2 cells and their cytokines have also been implicated in a number of allergic conditions including asthma [110]. Indeed, treating asthmatic patients with a soluble IL-4 receptor inactivated IL-4 in the circulation and resulted in anti-inflammatory effects [111].

Activation of the transcription factor RORγT is driven by IL-6 and TGF-β in the microenvironment, and results in Th17 cell differentiation [112]. Furthermore, the cytokines IL-1β and IL-23 can also help promote their differentiation, and are involved in Th17 cell expansion and maintenance [113, 114]. Th17 cells primarily function to defend against bacterial and fungal infections through the production of inflammatory mediators such as IL-17, IL-21, IL-22, GM-CSF and TNF [115]. IL-17 is involved in recruiting neutrophils which are very efficient at phagocytosing and degrading extracellular pathogens [116, 117]. However, Th17 cells have also been implicated in a growing number of autoimmune diseases including multiple sclerosis [118], psoriasis [119] and rheumatoid arthritis [120]. Th17 cells predominantly express the chemokine receptor CCR6 which facilitates their migration to different sites around the body [121].

Regulatory T (Treg) cells are a distinct subtype of $CD4^+$ T cell that are involved in maintaining homeostasis and resolution of the immune response following pathogen clearance (Figure 1.1). There are two types of Treg cells that express the transcription factor Foxp3, and both typically express CD25. Natural (thymic) Treg cells become committed during their development in the thymus, and inducible (peripheral) Treg cells, differentiate from naïve T cells in the periphery when TGF-β or retinoic acid is present in the microenvironment. $Foxp3^+$ Treg cells characteristically produce the anti-inflammatory cytokines IL-10 or TGF-β, and function to limit pro-inflammatory T cell function. Similar to Th17 cells, Treg cells usually express the chemokine receptor CCR6 [121].

Follicular helper T (Tfh) cells are a newly discovered subset of $CD4^+$ T helper cells whose primary function is in germinal centre (GC) formation [122, 123] and activation of B cells to produce antibodies (Figure 1.1) [124, 125]. They have been shown to survive as long-term memory cells [126, 127] and assist with the induction of memory B cell responses [128]. Tfh cells are distinguished from other T cells by the expression of various cell surface receptors including CXCR5, PD-1, ICOS and CD40L [129-131]. Surface expression of CXCR5 allows these cells to respond to CXCL13 and migrate to the B cell follicles of secondary lymphoid tissues to interact with B cells [124, 125, 132] In contrast to Th1, Th2 and Th17 cells, the differentiation of Tfh cells is controlled by the master transcription factor Bcl6 [129, 130, 133-136] and the cytokines IL-6, IL-21 and IL-27 have been implicated in driving their differentiation [137, 138]. In the absence of IL-6 and IL-21, there is a massive reduction in Tfh differentiation which results in impaired humoral immunity [139, 140]. Tfh cells produce a number of B cell helper cytokines including IL-4, IL-10 and IL-21 [141]. IL-21 is highly expressed by Tfh cells [141, 142], but is also produced by other $CD4^+$ T helper cell subsets [139, 143-145]. It is important in facilitating B cell proliferation and differentiation into long-lived antibody-secreting plasma cells, as well as GC formation and maintenance [139, 146-148].

Tfh cells play an important role in the immune response to a number of pathogens. They have been implicated in the generation of a strong primary antibody response and effective memory responses to influenza virus in mice [149, 150], and infection with *Toxoplasma gondii,* where IL-21 has been shown to be necessary for optimal cellular and humoral immunity to the parasite [151]. However, Tfh cells have also been implicated in the development of various autoimmune diseases including bullous pemphigoid [152], systemic lupus erythematosus [153, 154] and rheumatoid arthritis [155].

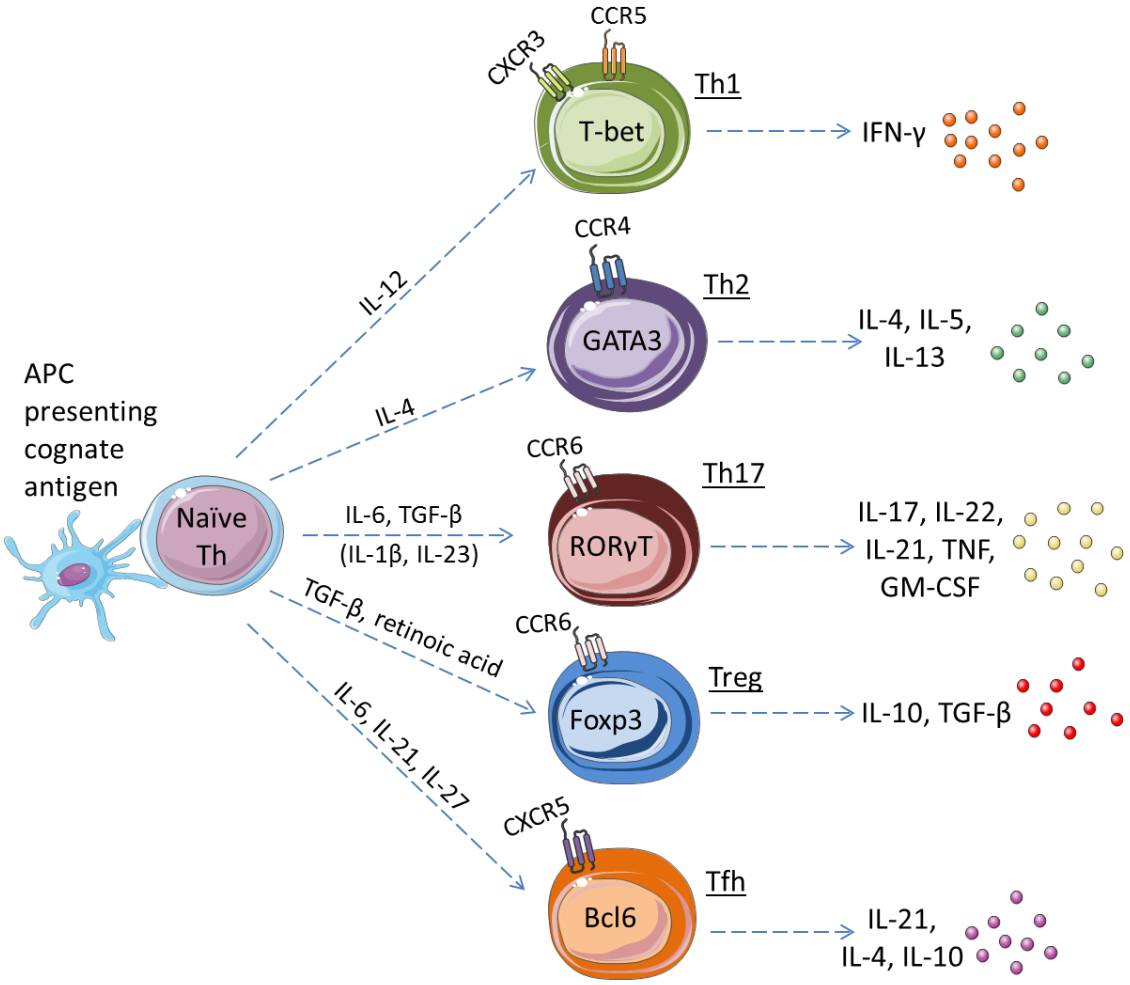

Figure 1.1 CD4$^+$ T cell differentiation and cytokine production

There are several different subtypes of CD4$^+$ T cells, and cytokines in the microenvironment determine the differentiation patterns. Briefly, Th1 cells differentiate from naïve T cells in the presence of IL-12. They predominantly produce IFN-γ and function in the immune response to intracellular pathogens. Th17 cells differentiate in response to IL-6 and TGF-β, and their presence is maintained by IL-1β and IL-23. They can produce IL-17, IL-21, IL-22, TNF and GM-CSF, and protect against bacterial and fungal infections. The presence of IL-4 in the microenvironment drives Th2 cell differentiation and this CD4$^+$ T cell subtype plays a role in parasitic infection, but has also been implicated in allergic responses. Treg cells are induced following TGF-β or retinoic acid stimulation and function to inhibit pro-inflammatory T cell responses by producing IL-10 and TGF-β. The newly discovered Tfh cells differentiate from naïve T cells in the presence of IL-6, IL-21 and IL-27, and they facilitate B cell proliferation and antibody production by producing IL-21, IL-4 and IL-10. (Figure adapted from [156] and [157]. Images of cells modified from Servier Medical Art)

1.7.3 Cytotoxic T cells

$CD8^+$ T cells primarily function in the immune response to intracellular pathogens, such as viruses, but are also capable of killing 'altered self' e.g. tumour cells [98]. When a host cell becomes infected or begins behaving abnormally, such as unrestrained proliferation observed in tumour cells, MHC class I molecules presenting aberrant antigens are expressed on its surface [98]. $CD8^+$ T cells are capable of recognising this peptide-MHC complex and once activated can induce programmed cell death within the target cell by two mechanisms. The first method involves the release of cytotoxic effector proteins including perforin and granzymes. These proteins enter the cytosol of the target cell and initiate the caspase cascade, which ultimately results in fragmentation of nuclear DNA and cell death. Cytotoxic T cells can also induce apoptosis in damaged or infected cells by engaging specific death-associated receptors on the target cell surface e.g. FAS ligand on the $CD8^+$ T cell engages FAS on the surface of the target cell, and activates the caspase cascade resulting in target cell death [98]. $CD8^+$ T cells have been shown to be important in the immune response to numerous viral pathogens including influenza virus [158] and herpes simplex virus 2 (HSV-2) infection [159].

1.7.4 Memory T cell subsets

Typically naïve T cells encounter antigens in secondary lymphoid organs such as the LNs. Upon activation, they rapidly proliferate and differentiate into either effector or memory T cells. Effector T cells promptly migrate to inflamed tissues to assist with pathogen elimination. In contrast, memory T cells do not have immediate effector function, but persist after infection has been cleared and recirculate in the bloodstream, acting as a surveillance system in case of secondary challenge with the same pathogen, when they can provide immediate protection [98]. Initially, the memory T cell population was divided into two subtypes of cells, central memory T (T_{CM}) and effector memory T (T_{EM}) cells, both of which recirculate between the blood, LNs and tissue after pathogen clearance. They are distinguished by the expression of CCR7, a chemokine receptor which directs cell homing to secondary lymphoid organs [160]. Resting T_{CM} cells express high levels of CCR7 and CD62L, and are more sensitive to antigenic stimulation than naïve T cells. Upon encountering cognate antigen, they rapidly proliferate to repopulate the memory T cell population, and can then differentiate into T_{EM} cells. In contrast, resting T_{EM} cells lack CCR7 and express CD44, in addition to an array of surface markers and chemokine receptors which allow them to migrate to inflamed tissues during infection [160, 161]. The T_{EM} cell population can be subdivided into Th1-type and Th2-type T_{EM} cells. Th1-type

T_{EM} cells are characterised by expression of CXCR3 and CCR5, and production of IFN-γ upon encountering cognate antigen [162]. In contrast, Th2-type T_{EM} cells typically express high levels of CCR4, and produce IL-4 when activated. T_{CM} cells can also be classified based on expression of chemokine receptors. Similarly, $CXCR3^+$ T_{CM} represent pre-Th1 type T_{EM}, and $CCR4^+$ T_{CM} cells, pre-Th2 type T_{EM} cells [162].

In recent years, a third subtype of memory T cell was identified. In contrast to T_{EM} and T_{CM} cells, these resident memory T (T_{RM}) cells remain localised in the tissues after infection has been cleared and do not recirculate to the bloodstream and secondary lymphoid organs. Indeed, T_{RM} cells have been shown to be present in tissues at many barrier sites of the body, including the skin [163, 164], gut [165, 166] and lung [167, 168]. Furthermore, these cells have also been found at non-barrier sites within the body, including the brain [169], kidneys [170] and heart [171]. Although T_{RM} cells are distinct from T_{EM} cells, they do share a number of features including high expression of CD44, low expression of CD62L and CCR7, and the ability to produce cytokines [172]. CD69 is the primary marker used to identify T_{RM} cells as all that have been discovered so far express this C-type lectin. CD69 enables cell retention in tissues by downregulating surface expression of the sphingosine-1-phosphate receptor 1, which directs migration to the blood [173, 174]. CD103 expression on T_{RM} cells has also been described during infection of the intestines and lungs [175, 176]. However, expression of this integrin appears to be flexible, with reports of T_{RM} cells lacking CD103 in the liver [168] and secondary lymphoid organs [177]. Both $CD4^+$ and $CD8^+$ T_{RM} cells have been identified, however, $CD8^+$ T_{RM} cells and their functions have been described in greater detail. The reason for this is the majority of studies on T_{RM} cells to date have utilised viral models of infection, where they were originally identified [165, 178], and as viral infection typically generates substantially more $CD8^+$ than $CD4^+$ T cells, this focus has led to a large knowledge gap between the two subtypes.

The role T_{RM} cells play in protecting against infectious diseases is still being elucidated, but there are several reports that $CD8^+$ and $CD4^+$ T_{RM} cells are protective against secondary viral infection in various tissues, including the lungs and skin of mice [178, 179]. Indeed, i.n. challenge with a sublethal dose of influenza virus resulted in the robust recruitment of $CD8^+CD103^+$ T_{RM} cells, in addition to $CD8^+$ T_{EM} and T_{CM} cells, in the lungs of mice [179]. Furthermore, these mice were protected from subsequent lethal i.n. challenge with influenza virus. In contrast, while intraperitoneal (i.p.) immunisation with a sublethal dose of influenza virus similarly generated robust levels of $CD8^+$ T_{EM} and T_{CM} in the lungs, no $CD103^+$ T_{RM} cells were induced and these mice were not protected from lethal infection [179]. Furthermore, there is evidence that $CD4^+$ T_{RM} are recruited to the lungs and female reproductive tracts of

mice during viral infection, and that these cells confer protection against infection [167, 180]. In addition to their induction following infection, there are reports that T_{RM} cells are expanded following vaccination. Indeed, Connor and colleagues demonstrated that Bacillus Calmette-Guérin (BCG) vaccination recruits T_{RM} cells to the lungs, and showed that these $CD4^+$ T_{RM} cells were sufficient to confer protection against BCG infection [181].

Tailoring vaccines to promote the development and maintenance of these protective resident cells could be an ideal strategy to promote long-term immunity, however, T_{RM} cells have been implicated in the development of autoimmune conditions including psoriasis [182, 183], contact dermatitis [184] and inflammatory bowel disease [185]. More studies need to be performed to fully elucidate the mechanisms involved in rendering these cells pathogenic.

1.7.5 The role of T cells in protection against *B. pertussis* infection

Studies in animal models and in convalescent and vaccinated children have provided evidence of a role for T cells in immunity to *B. pertussis* infection. Respiratory infection of mice and children with *B. pertussis* induces strong Th1 responses [64, 88, 186] and long-term, but not lifelong, immunity [187]. IFN-γ is the key pro-inflammatory cytokine produced by Th1 cells and this cytokine plays a crucial role in preventing bacterial spread from the respiratory tract [2]. Mice lacking IFN-γ receptors (IFN-$\gamma R^{-/-}$ mice) develop a lethal infection associated with systemic dissemination of the bacteria from the lungs to the liver and other organs [2]. A newly developed baboon infection model has also recently highlighted the importance of Th17 cells in *B. pertussis* infection [188]. Inoculation of baboons with *B. pertussis* resulted in the induction of a robust IL-17 response, as well as induction of the cytokines IL-6, IL-23 and IL-1β, which are associated with differentiation of Th17 cells. Similarly, peripheral blood mononuclear cells collected from convalescent baboons produced IL-17 following *ex vivo* stimulation with heat-killed bacteria. These data suggest that a *B. pertussis*-specific memory Th17 response is important for adaptive immunity and long-lived protection against pertussis.

The wP vaccines were shown to primarily induce potent Th1 responses, and accordingly, their ability to confer protection against *B. pertussis* was significantly reduced in IFN-$\gamma^{-/-}$ mice [189]. Immunisation with wP vaccines also induces IL-17-secreting $CD4^+$ T cells, however, the contribution of Th17 cells to wP-mediated protection against *B. pertussis* infection was significantly lower than that of Th1 cells [189]. In contrast, aP vaccines promote robust induction of Th2 cells in mice and in children [65, 190, 191]. Although Th2 cells are considered

to be important for helping B cell production of antibody, studies carried out by the Mills laboratory have demonstrated that Th2 cells are not responsible for the protection conferred with aP vaccines in mice [189]. They reported that IL-4$^{-/-}$ mice immunised with the aP vaccine clear *B. pertussis* infection as quickly as immunised WT mice, despite having defective Th2 responses [189]. Furthermore, they found that protection induced by aP vaccines in mice was significantly reduced in IL-17$^{-/-}$ but not IFN-γ$^{-/-}$ mice, suggesting that the aP vaccines mediate protection by inducing Th17, but not Th1 or Th2 cells [189]. As IFN-γ has been shown to be crucial for controlling *B. pertussis* infection [2], this evidence indicates that the significant feature that explains the superior efficacy of wP over aP vaccines, is their ability to induce Th1 cells [189].

Treg cells have a negative impact in *B. pertussis* infection and it was shown that this infectious pathogen could exploit their anti-inflammatory properties to subvert the host immune response. Indeed, ACT has been shown to augment LPS-induced IL-10 production by bone marrow-derived DCs (BMDCs) and macrophages (BMDMs) [192]. Furthermore, immunisation of mice with the model antigen keyhole limpet hemocyanin (KLH) adjuvanted with ACT induced antigen-specific IL-10 and IL-5 production *in vivo,* which is indicative of Treg and Th2 cells. CD8^{+} T cells also have a negative influence on clearance of *B. pertussis* infection. Adoptive transfer of purified CD8^{+} T cells to irradiated mice resulted in enhanced bacterial load in the lungs when compared with mice that received non-immune T cells [64]. Furthermore, a number of the mice died from infection.

1.7.6 The potential role of T_{RM} and Tfh cells in *B. pertussis* infection

It has not yet been investigated if Tfh cells are important in combatting *B. pertussis* infection, however, they have been shown to play a role in protection against tuberculosis. Tuberculosis is a persistent lung infection caused by the bacterium *Mycobacterium tuberculosis,* which is characterised by the formation of granulomas containing *M. tuberculosis*-infected macrophages, in both humans and animals [193, 194]. Slight *et al.* demonstrated that in a murine model of *M. tuberculosis* infection, organised lymphoid structures containing CXCR5^{+} T cells formed in the lungs, and in CXCR5$^{-/-}$ animals, lung bacterial burden was enhanced when compared to WT mice [195].

Similarly, the role of T_{RM} cells in long-term protection against *B. pertussis* infection has not been examined to date. However, they have been shown to function in the immune response

against other bacteria. Indeed, Sakai *et al.* observed that an IFN-γ-producing $CD4^+$ T cell population that localises to the lung parenchyma is generated during *M. tuberculosis* infection [196]. T cell deficient mice that received these $CD4^+CD69^{hi}CXCR3^{hi}$ cells by adoptive transfer were significantly more protected from subsequent infection than control mice that received no T cells.

1.7.7 B cells

B cells develop from haematopoietic precursors in the bone marrow, and like T cells, each B cell possesses a BCR on its surface which is specific for a unique antigen. In contrast to T cells, the BCR can recognise peptide in its native conformation and upon encountering its cognate antigen, a B cell can differentiate into an antibody-producing plasma cell or a memory cell. Antibodies can initiate an immune response by neutralisation or opsonisation of a pathogen, or by activating the complement system.

5 different antibody classes exist in both humans and mice; IgM, IgA, IgD, IgG and IgE. Human IgG is the most abundant class in the circulation, and contains a number of subclasses; IgG1, IgG2, IgG3 and IgG4 [98]. In contrast, mice express IgG1, IgG2a (or IgG2c depending on mouse strain [197]), IgG2b and IgG3, and these are not direct homologues of the human isotypes [198]. Additionally, while mice express one IgA isotype, humans can express two, IgA1 and IgA2. IgA is the most abundant antibody subclass at mucosal surfaces in both humans and mice, and plays an important role in neutralising pathogens and secreted toxins at these barrier sites. The functions of antibody isotypes vary greatly depending on subclass, however, the overall primary functions of antibodies include neutralisation, opsonisation, complement activation, mast cell and NK cell activation (Table 1.1).

Human antibody classes	**Primary function**
IgM	Complement system activation
IgA: IgA1 and IgA2	Virus and toxin neutralisation at mucosal surfaces
IgD	Not well characterised, potential role in activating basophils and mast cells [199]
IgG: IgG1, IgG2, IgG3, IgG4	Neutralisation (IgG1, IgG2, IgG3, IgG4), opsonisation (IgG1, IgG3), complement system activation (IgG3, IgG1) and NK cell activation (IgG1, IgG3)
IgE	Mast cell activation

Table 1.1 Human antibody subclasses and primary functions
(Adapted from Figure 9.19 in [98], and [198, 199]).

1.7.8 The role of B cells in protection against *B. pertussis* infection

The importance of B cells and antibodies in clearing *B. pertussis* infection has been demonstrated in a number of studies using knockout mice [2, 65]. Mahon *et al.* showed that $Ig^{-/-}$ mice suffered from a persistent chronic lung infection post aerosol challenge and failed to clear the bacteria from the lungs [2]. Antibodies against *B. pertussis* virulence factors are capable of preventing bacterial colonisation by blocking adherence of the bacteria to human epithelial cells [200]. However, it is becoming increasingly evident that distinct IgG subclasses have diverse roles in anti-bacterial immunity. T cell derived cytokines have distinct roles in Ig class switching in mice, with IFN-γ (derived from Th1 cells) associated with murine IgG2a/IgG2c [197, 201, 202], and Th2 cytokines associated with IgG1 production [203, 204]. The wP vaccine predominantly induced antigen-specific IgG2a, whereas the aP vaccine induced antibody responses dominated by IgG1 [205]. The failure of aP vaccines to induce these antibody subclasses may contribute to their more limited efficacy compared with wP vaccines. While the role of IgA in clearance of *B. pertussis* infection has not been well characterised, this isotype has been shown to enhance binding, phagocytosis and killing of *B. pertussis* organisms by human polymorphonuclear leukocytes, indicating it could be important in pathogen elimination [206, 207].

1.8 Resurgence of pertussis

Outbreaks of pertussis are on the rise, with a significant number of cases reported, especially during 2012, in many countries that have high vaccination coverage, including the USA [208, 209], Australia [210], Ireland [211] and England and Wales [212] (Figure 1.2). Pertussis incidences in these countries have decreased since the 2012 epidemics, however, the numbers of reported cases each year still remain worryingly high (Table 1.2).

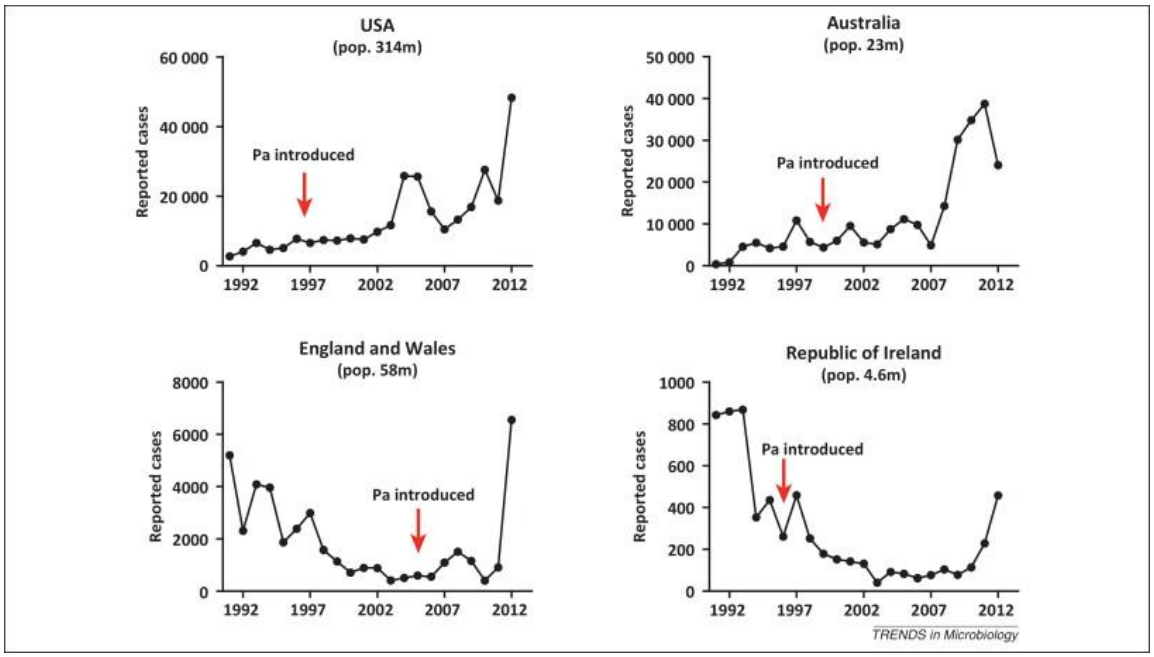

Figure 1.2 Re-emergence of pertussis

The reported number of cases of pertussis in the USA, Australia, England and Wales, and Ireland, from 1991-2012. The population of each country in 2012 is included below the title. The arrows indicate when the aP vaccination programme was introduced in each country. (Taken from [213]).

	2012	**2013**	**2014**	**2015**
Ireland	458	173	73	118
UK and N. Ireland	11,980	6,090	4,046	5,207
USA	48,277	28,639	32,971	18,166*
Australia	23,855	12,319	11,842	22,508

Table 1.2 Reported number of pertussis cases per year from 2012-2015

*Provisional number of cases reported. (Data obtained from the Centers for Disease Control (CDC) [214-216] and WHO [217]).

A number of explanations have been proposed for this re-emergence, including antigenic variation in one or more of the antigens in current aP vaccines. The Netherlands has seen a dramatic increase in the incidence of pertussis since 1996, despite extensive use of aP vaccines since their introduction [218]. A study by Mooi and colleagues, designed to investigate whether the strains of *B. pertussis* in circulation in the mid-1990s express the same antigenic variants of surface proteins as those in circulation in the early 1950s before introduction of the wP vaccine, demonstrated polymorphisms in the genes encoding PT and Prn [219]. The strains of *B. pertussis* now in circulation express distinct variants of PT and Prn than those from the

early 1950s (which were the strains included in the wP vaccine). This provided evidence that vaccination has put selective pressure on *B. pertussis* resulting in the appearance of strains antigenically distinct from those in the vaccine. PT and Prn are key antigens in aP vaccines and have been shown to be important for conferring protective immunity to *B. pertussis* [220-222]. Variation in these proteins could explain low efficacy of the aP vaccines in humans. The emergence of antigenically distinct variants of Prn and PT has also been seen in other countries, including Finland [223], Canada [224], Australia [225], the UK [226] and the USA [227], and corresponds with an increase in pertussis activity in these areas. Indeed, in Australia, the percentage of *B. pertussis* isolates that are Prn negative has increased from 5% in 2008, to 76% in 2012 (Figure 1.3) [225].

An alternative explanation for the resurgence of whooping cough is that the aP vaccines do not induce the appropriate arm of the immune response crucial for preventing colonisation with *B. pertussis*, because inappropriate antigens or adjuvants are included in current vaccines. It was previously believed that potent antibody responses were the key factor in protective immunity against *B. pertussis* and the virulence factors selected for use in aP vaccines were chosen based on this assumption. However, studies in mice, recently complemented by those in baboons, have shown that T cells do play a fundamental role in protection induced by natural infection or immunisation with wP vaccines [189, 228]. The wP vaccine contained significantly more antigens than the aP vaccine and perhaps the antigen combination selected for the aP vaccine is not effective at inducing T cell responses. A more likely explanation is the choice of alum as the adjuvant, which is known to boost antibody responses, but is less effective at promoting cell-mediated immunity, especially Th1 responses. Furthermore, the current aP vaccine formulation may be ineffective at inducing immunological memory. Several outbreaks have been reported which support waning immunity as the primary cause of pertussis resurgence, with evidence that children who were initially immunised with wP vaccines had longer lasting protection against *B. pertussis* than those primed with aP vaccines [229, 230]. Furthermore, a recent study that examined waning immunity in children in California demonstrated that after receiving a fifth dose of the DTaP vaccine, the odds of contracting pertussis increased by an average of 42% each year [231].

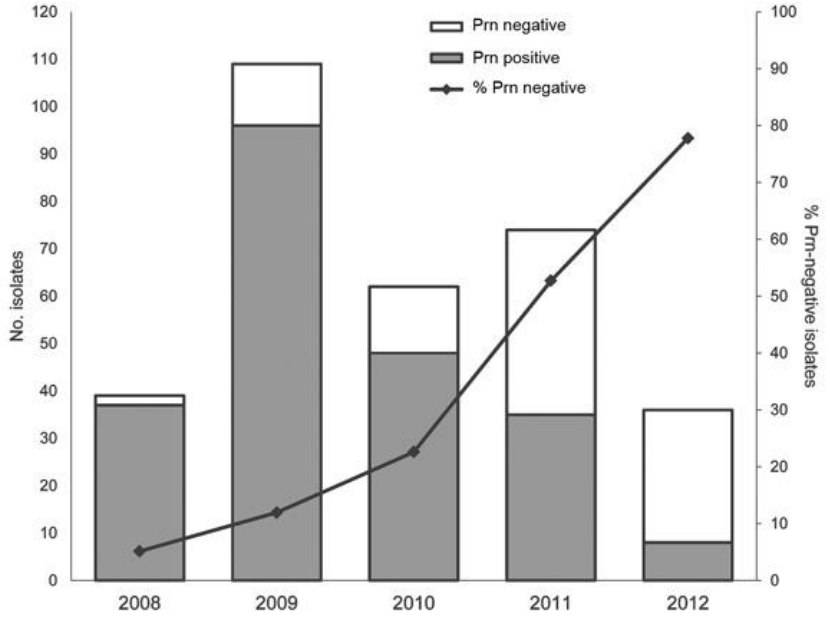

Figure 1.3: The emergence of Prn-negative *B. pertussis* strains in Australia from 2008-2012.

The graph displays the numbers of *B. pertussis* isolates obtained (left Y axis) and percentages of Prn-negative isolates (right Y axis) identified in Australia during the period of 2008-2012. The number of isolates expressing Prn is represented by the grey bars, and the white bars represent the number of isolates not expressing Prn. 320 *B. pertussis* isolates were obtained from 5 different regions in Australia over the course of the study, and Western blotting was used to identify the presence or absence of Prn. The percentage of *B. pertussis* isolates that were Prn-negative increased each year: 5% in 2008, 12% in 2009, 23% in 2010, 53% in 2011 and 78% in 2012. (Figure taken from [225]).

1.9 Adjuvants

Adjuvants are compounds which enhance the immunogenicity of purified or recombinant antigens, which alone would be incapable of activating innate immune responses required for directing adaptive immunity [232]. One of the reasons the wP vaccines were so successful is that they possess intrinsic adjuvanticity due to inherent moieties that are capable of stimulating TLRs and other innate immune pathways e.g. LPS signals through TLR4 and is a very potent adjuvant [233]. The aP vaccines on the other hand, are composed of purified or recombinant bacterial antigens, which lack immunostimulatory activity and are poorly immunogenic and therefore require formulation with an adjuvant. A limited number of adjuvants have been licensed for human use to date (Table 1.3).

Adjuvant	Component	Year licensed	Vaccines included in
Alum	Aluminium mineral salts	1926	Diphtheria, tetanus, pertussis, IPV, Hep. A and B, HPV, meningococcal, pneumococcal
MF59	Oil-in-water emulsion	1997	Influenza (seasonal and pandemic)
Virosome	Liposomes	2000	Hep. A and influenza
AS04 (MPL and alum)	Alum-adsorbed TLR4 agonist	2005	Hep. B, HPV
AS03	Oil-in-water emulsion	2009	Influenza (pandemic)
Thermo-reversible	Oil-in-water emulsion	2010	Influenza (pandemic)
AS01 (MPL and QS21)	Liposomes + immunostimulants	2015	Malaria

Table 1.3 Vaccine adjuvants licensed for human use in Europe and North America
Abbreviations: IPV, inactivated poliomyelitis vaccine; Hep. A, hepatitis A; HPV, human papilloma virus; MPL, monophosphoryl lipid A; QS21, Quillaja saponaria 21. (Adapted from [234-236] and [237]).

1.9.1 Alum

Alum was the first adjuvant licensed for use in humans. As it had a history of safety with other vaccines, it was chosen for the aP vaccines developed in the 1980s and 1990s. The adjuvant activity of alum was first demonstrated in the 1920s and due to their relatively low toxicity and good safety, these aluminium-based compounds are still the most common adjuvants in human vaccines today [232]. Aluminium phosphate and aluminium hydroxide have both been used for aP vaccines, but it has been shown that the choice of salt can have major consequences on the efficacy. Denoel *et al.* demonstrated that antigen desorption from aluminium phosphate is much more rapid than from aluminium hydroxide, particularly in the case of Prn, and this correlated with decreased efficacy of an aluminium phosphate vaccine in mice [238].

The mechanism of action of alum as an adjuvant is not yet fully understood and indeed is widely disputed. Alum was thought to act mainly by depot formation at the site of injection, thus causing slow release of antigen and allowing for longer interaction time between antigen and APC [239]. It has also been shown to increase antigen uptake and presentation by DCs [240], the key APC for activating naïve T cells. In addition, there is evidence that alum may function as an adjuvant by activating the NLRP3 inflammasome to induce production of IL-1β [241, 242]. This has been disputed by others and part of the reason for the anomalies may lie in the choice of assays to measure adjuvant activity. Many studies employed antibody as a

readout and some of these failed to find a role for NLRP3. Ross *et al.* demonstrated that alum promotes inflammasome activation and consequent IL-1β production and thereby promotes Th17 responses, which mediates protection induced with aP vaccines [189]. Although alum is capable of promoting potent antibody as well as Th2 and Th17 responses, it fails to induce effective Th1 responses which are crucial for clearance of *B. pertussis* from the respiratory tract [189]. Indeed, alum has been shown to impair the induction of Th1 responses by directly inhibiting IL-12p35 expression and therefore, IL-12 production by DCs [243]. The inability of current aP vaccines to induce appropriate cell-mediated immunity appears to reflect the choice of alum as the adjuvant and this may explain their failure to prevent colonisation of the respiratory tract. A number of current studies in animal models are focused on improving the quality of the immune responses induced with aP vaccines by switching from alum to Th1-inducing adjuvants, such as TLR agonists [189, 244].

1.9.2 TLR agonists

TLRs are a subtype of PRR, mammalian homologues of the Toll receptors initially identified in *Drosophila* [245]. 13 murine TLRs have been identified to date, 10 of which are expressed in humans. TLRs 1, 2, 4, 5, 6 and 10 are found on the cell surface, whereas TLR3, 7/8 and 9 are located intracellularly, within the cytoplasm or endosomes (Figure 1.4) [246]. Each TLR recognises specific PAMPs. TLR2 dimerises with either TLR1 or TLR6 to recognise triacylated or diacylated lipoproteins respectively [247-249]. The ligand for TLR10 is currently unknown, however, this orphan receptor appears to be capable of heterodimerising with TLR1 or TLR2 and eliciting anti-inflammatory responses [250, 251]. TLR4 recognises LPS, a component of Gram-negative bacterial cell walls [252, 253]. Flagellin, the primary constituent of bacterial flagellum, signals through TLR5 [254]. Viral double-stranded (ds) RNA and single-stranded (ss) RNA signal through TLR3 and TLR7/8 respectively [255-257], and unmethylated CpG motifs in bacterial DNA signal through TLR9 [258]. The specific ligands for murine TLR11, TLR12 and TLR13 have not yet been confirmed. However, TLR11 and TLR12 are capable of binding the protein profilin expressed by *T. gondii* [259, 260], and furthermore, TLR11 can also recognise flagellin, expressed by *Salmonella typhimurium.* Finally, TLR13 has been shown to engage a specific bacterial ribosomal RNA sequence [261].

With the exception of TLR10, signalling has been well described for all human TLRs. Signalling through TLR1-9, apart from TLR3, results in recruitment of the adaptor molecule myeloid differentiation primary response gene 88 (MyD88) to the cytoplasmic portion of the TLR.

MyD88 activation initiates a signalling cascade involving a number of kinases, ultimately resulting in activation of the transcription factor Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and pro-inflammatory cytokine production [262-265]. The MyD88-independent or TIR-domain-containing-adaptor protein inducing IFN-β (TRIF) pathway is utilised by TLR3 and TLR4 only [266, 267] and results in type I IFN (IFN-α, IFN-β) secretion through activation of the IFN-regulatory factor (IRF) family of transcription factors (Figure 1.4).

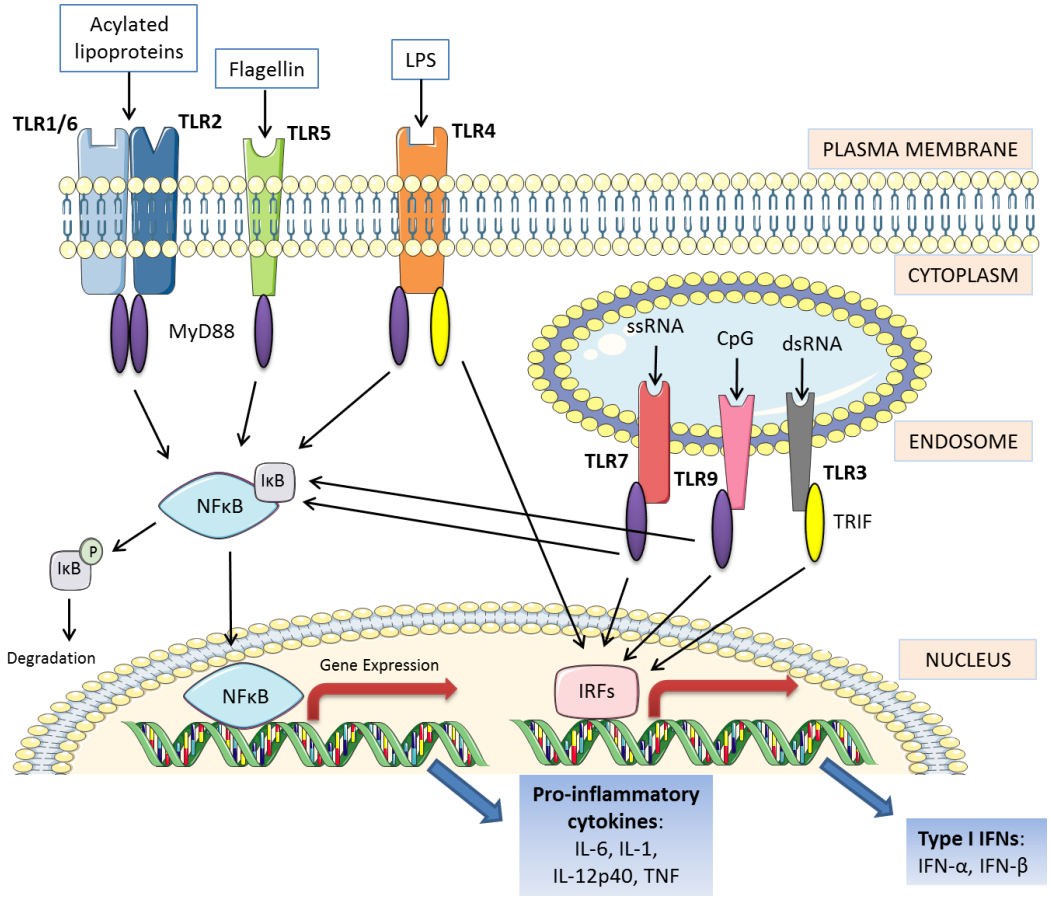

Figure 1.4 TLR ligands and signalling

The ligand for each of the 10 human TLRs has been identified, with the exception of the orphan receptor TLR10. Briefly, TLR2 is located on the cell surface, can heterodimerise with TLR1 or TLR6 and recognises acylated lipopeptides. TLR4 binds LPS, and TLR5 can engage flagellin, both of which are bacterial components. The endosomally located TLR7 and TLR3 recognise ssRNA and dsRNA respectively, while TLR9 can engage CpG motifs. Downstream signalling of all the TLRs, except TLR4, involves recruitment of the adaptor molecule MyD88 to the intracellular domain of the TLR signalling complex, and results in activation of the transcription factor NFκB and production of pro-inflammatory cytokines including IL-12 and IL-6. TLR4 signalling involves the adaptor molecule TRIF and activation of the IRF family of transcription factors, resulting in type I IFN (IFN-α and IFN-β) production. The TLR10 signalling pathway has not been well characterised to date so was not included in this diagram. (Figure adapted from [268]. Images of cells modified from Servier Medical Art).

1.9.3 TLR agonists as adjuvants for a pertussis vaccine

The toxicity of wP vaccines, including the induction of fever and rarer cases of seizures, has been linked with LPS in the vaccine [21], and efforts are being made to develop less toxic wP vaccines by reducing the LPS content [269]. However, one of the reasons the wP vaccines are so efficacious is due to the presence of LPS, which activates TLR4 signalling pathways, and has been shown to be important for protection against *B. pertussis* infection [20]. Indeed, immunisation of TLR4-defective mice with a wP vaccine failed to confer protective immunity against *B. pertussis* [20]. LPS is too toxic for human use, but a derivative, monophosphoryl lipid A (MPL), which binds to TLR4 and retains certain of the immunostimulatory properties of LPS without the toxicity issues, can promote similar immune responses [270]. AS04, a combination of MPL and alum, has been approved for use in human vaccines since 2005 and is currently used in some viral vaccines, such as hepatitis B (Table 1.3) [271]. Geurtsen *et al.* demonstrated that mice immunised with an aP vaccine adjuvanted with MPL had reduced lung colonisation after challenge when compared with mice immunised with the aP vaccine containing alum [272]. Furthermore, spleen cells from mice immunised with the MPL-containing aP vaccine had decreased production of *B. pertussis*-specific IL-5 *ex vivo* when compared with the mice given the alum-adjuvanted vaccine, indicating that MPL can skew the immune response away from Th2 (Figure 1.5). Moreover, Brummelman and colleagues demonstrated that addition of a meningococcal LPS derivative, LpxL1, to a commercial alum-adjuvanted aP vaccine enhanced the antigen-specific IFN-γ response and IgG2a to IgG1 ratio in mice, compared with mice immunised with the commercial alum-adjuvanted aP vaccine alone, indicating an overall enhancement of Th1 responses (Figure 1.5) [273].

Evidence of the benefits of using TLR agonists as adjuvants for pertussis vaccines is not confined to TLR4, CpG oligonucleotides from bacterial DNA, that signal through TLR9, are potent inducers of Th1 responses [274], and also promote induction of Th17 cells (Figure 1.5). Asokanathan *et al.* demonstrated that immunisation of mice with dPT, FHA and Prn in combination with both CpG and alum enhanced IFN-γ production by spleen cells and NO production by peritoneal macrophages when compared with mice immunised with the antigens in combination with alum alone [244]. Furthermore, mice that had been immunised with the aP vaccine that included CpG and alum had no detectable bacteria in the lungs 7 days after aerosol challenge, whereas bacteria were still present in mice immunised with the antigens in combination with alum [244]. Similarly, Kindrachuk *et al.* showed that mice immunised i.n. with dPT formulated with a complex of CpG and the synthetic innate defence regulator peptide HH2 (CpG-HH2) produced high levels of PT-specific IgG1 and IgG2a

antibodies [275]. In contrast, mice that received dPT with CpG alone (dPT-CpG) produced modest IgG1 and IgG2a production, and mice immunised with dPT-HH2 alone produced high levels of IgG1 antibody, but no IgG2a antibodies [275]. These results indicate that the CpG-HH2 combination induced a balanced Th1/Th2 response.

A novel TLR1/2 binding lipoprotein from *B. pertussis,* BP1569, was recently identified by the Mills lab. BP1569 was shown to have immunostimulatory properties *in vivo* and *in vitro* in TLR4 defective mice and cells, and in addition, was shown to be an antigenic component of *B. pertussis* [276]. Due to an inability to completely remove LPS from the lipoprotein, an LPS-free lipopeptide version of BP1569, LP1569, was synthesised. If LP1569 retains the immunostimulatory capacity of BP1569, it could have the potential to act as an adjuvant for a pertussis vaccine.

The ability of TLR agonists to redirect the immune response induced by alum-adjuvanted aP vaccines in mice is promising for the future development of pertussis vaccines, as addition of a TLR agonist to an existing vaccine would be logistically simpler than starting with a completely new formulation. A number of TLR agonists have already been tested in phase II and phase III clinical trials [277]. These molecules are chemically stable and have low production costs, and therefore are ideal as adjuvants for future generation vaccines against *B. pertussis*.

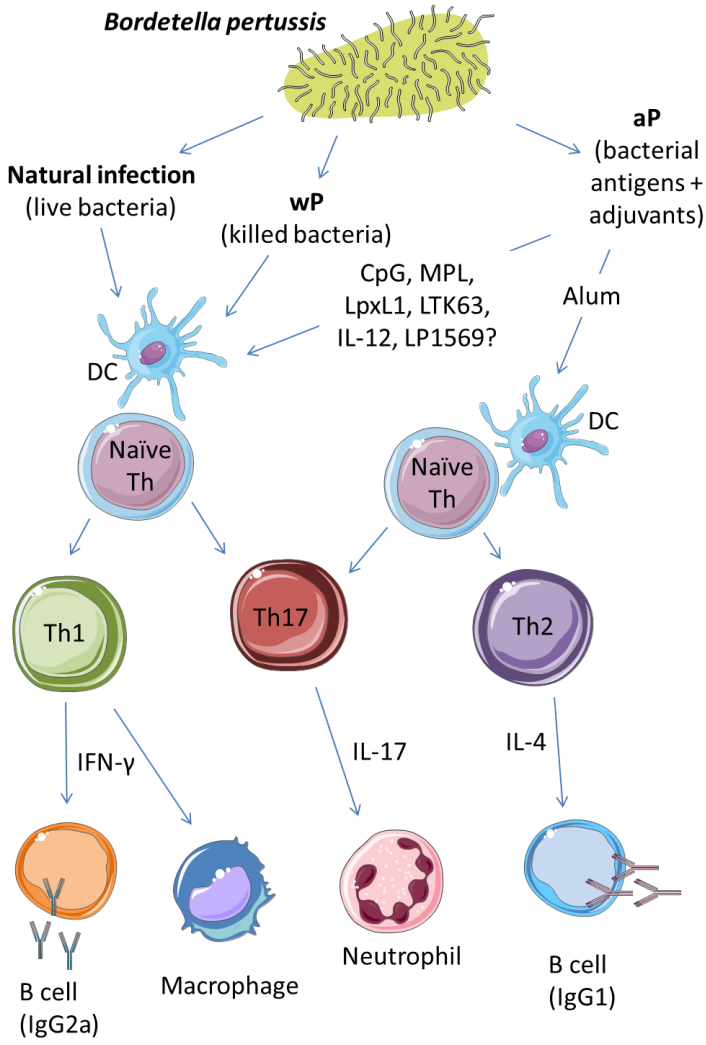

Figure 1.5 Mechanisms of natural and vaccine-induced immunity to *B. pertussis*

Upon *B. pertussis* infection, APCs such as DCs phagocytose *B. pertussis* and break down antigen into antigenic peptides. These activated DCs present *B. pertussis* antigens to naïve T cells, and produce the Th polarising cytokines IL-1β, IL-23, and IL-12, to induce Th17 and Th1 cell differentiation respectively. The resultant Th1 cells produce IFN-γ which activates macrophages to produce NO and effectively kill intracellular pathogens, and induces production of the IgG2a antibody isotype by B cells to efficiently opsonise *B. pertussis.* Th17 cells produce IL-17 which facilitates neutrophil recruitment and activation, and these granulocytes are proficient at phagocytosing and killing bacteria. Similar to natural infection, the wP vaccine induces a mixed Th1 and Th17 cell response which explains its high efficacy. In contrast, the aP vaccine predominantly induces a Th2 cell response due to the presence of the adjuvant alum in the vaccine, although it also promotes Th17 cell differentiation. Th2 cells produce IL-4 which induces antibody class switching to the IgG1 isotype, but no macrophage activation which is crucial for *B. pertussis* clearance. As described in sections 1.9.3, 1.9.5 and 1.12, substituting alum in the aP vaccine for certain TLR agonists including CpG, MPL or LpxL1, the cytokine IL-12, or LTK63 from *E. coli,* enhances protection against *B. pertussis* by directing the immune response towards Th1, and inducing potent cell-mediated immunity. (Figure adapted from [278]).

1.9.4 STING agonists and their potential as vaccine adjuvants

While the TLR family is the best characterised family of PRRs, several alternative receptor families exist for intracellular detection of foreign nucleic acids including the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and the NOD-like receptors (NLRs) [279]. Stimulator of interferon genes (STING) is an endoplasmic reticulum (ER) associated protein which is capable of directly binding to cytosolic cyclic dinucleotides (CDNs) [280]. Upon CDN binding, STING translocates from the ER to the perinuclear Golgi apparatus where it activates the mitogen-activated protein kinase (MAPK), TANK-binding kinase 1 (TBK1) [281]. The STING-TBK1 complex then migrates to endolysosomal compartments where TBK1 phosphorylates and activates IRF3, for upregulation of type I IFN [282], and NFκB, for upregulation of pro-inflammatory cytokines (Figure 1.6) [283].

The CDNs were first described as small molecule second messengers secreted by Gram-negative bacteria, which regulate diverse bacterial cell processes including cellulose synthesis, motility and metabolism [284-286]. However, some CDNs have recently been identified as having immunostimulatory properties. Indeed, cyclic diguanylate monophosphate (c-di-GMP), a bacterial intracellular signalling molecule, has been shown to induce immune responses in human and mouse cells *in vitro* [287]. Furthermore, there is evidence that c-di-GMP has the capacity to act as an adjuvant *in vivo;* mice subcutaneously (s.c.) immunised with a *Staphylococcus aureus* antigen formulated with c-di-GMP, had significantly lower bacterial load in the livers and spleens following a lethal dose of *S. aureus,* than mice immunised with c-di-GMP alone, or the PBS-immunised control mice [288]. While the mechanism by which c-di-GMP acts as an adjuvant has not been fully elucidated, it has been shown to enhance antigen uptake by pinocytosis and receptor-mediated endocytosis when administered i.n. [289]. Furthermore, TNF signalling has been shown to be essential for the adjuvant activity of c-di-GMP [290].

Another bacterially derived CDN, cyclic-di-adenosine monophosphate (c-di-AMP), has also been shown to be a potent mucosal adjuvant [291]. CDNs have also been shown to be prophylactically protective against infectious pathogens. I.n. administration of c-di-GMP to mice before i.n. challenge with *B. pertussis,* induced a potent innate immune response and protected mice from infection [292]. Furthermore, c-di-AMP mediates innate resistance to *M. tuberculosis* infection [293]. Moreover, both c-di-GMP and c-di-AMP have been shown to activate the NLRP3 inflammasome, however, the exact mechanisms for this have yet to be

elucidated, although this activation has been shown to be independent of STING signalling [294].

In addition to the bacterially-derived CDNs, CDNs can also be synthesised within the host cell upon detection of foreign nucleic acids. Cyclic GMP-AMP synthase (cGAS), a cytosolic protein, catalyses the production of another CDN called cyclic GMP-AMP (cGAMP), from ATP and GTP, upon binding dsDNA sequences greater than 30 base pairs in length [295]. Like c-di-GMP and c-di-AMP, cGAMP is capable of activating STING to induce a type I IFN response [296-298], and has also been shown to have adjuvant properties in mice [299].

The evidence so far indicates that STING agonists have good potential as adjuvants for infectious disease vaccines. Indeed, the adjuvant activity of the cationic polymer chitosan, has been shown to be dependent on the cGAS-STING pathway. Immunisation of WT mice with the H1 fusion antigen from *M. tuberculosis* and chitosan*,* induced antigen-specific IFN-γ production and IgG2c antibodies *in vivo*, however, these Th1-type immune responses were significantly impaired in $STING^{-/-}$ mice [300]. Furthermore, these responses were also inhibited in mice lacking the type I IFN receptor ($IFNAR^{-/-}$), indicating that STING-dependent type I IFN production is essential for the adjuvant activity of chitosan [300]. Chitosan has been investigated in a number of clinical trials and has been shown to be safe, indicating it could be used as an adjuvant for human vaccines in the future [301].

1.9.5 Cytokines as adjuvants for aP vaccines

Cytokines have the ability to skew the immune response, and studies in infection and cancer models have shown that IL-1, IL-12 and GM-CSF can enhance the immunogenicity of soluble antigens. Mahon *et al.* showed that IL-12, the cytokine responsible for driving Th1 differentiation, is capable of acting as an effective adjuvant for a laboratory-prepared aP vaccine containing dPT, FHA and Prn (Figure 1.5) [302]. Mice immunised with this experimental aP vaccine cleared bacteria as rapidly as mice immunised with a wP vaccine, on day 5 post challenge, whereas mice immunised with the aP vaccine without IL-12 did not clear the infection until day 9 post challenge. They also showed that stimulation of murine macrophages *in vitro* with killed whole bacteria (equivalent to the wP vaccine) induced IL-12 production, which promotes induction of Th1 cells, whereas stimulation of macrophages with the antigens FHA, PT or Prn (contents of the aP vaccine) did not induce IL-12 production by macrophages.

This, together with the fact that LPS is a potent inducer of IL-12 production, may in part explain why wP but not aP vaccines induce Th1 responses.

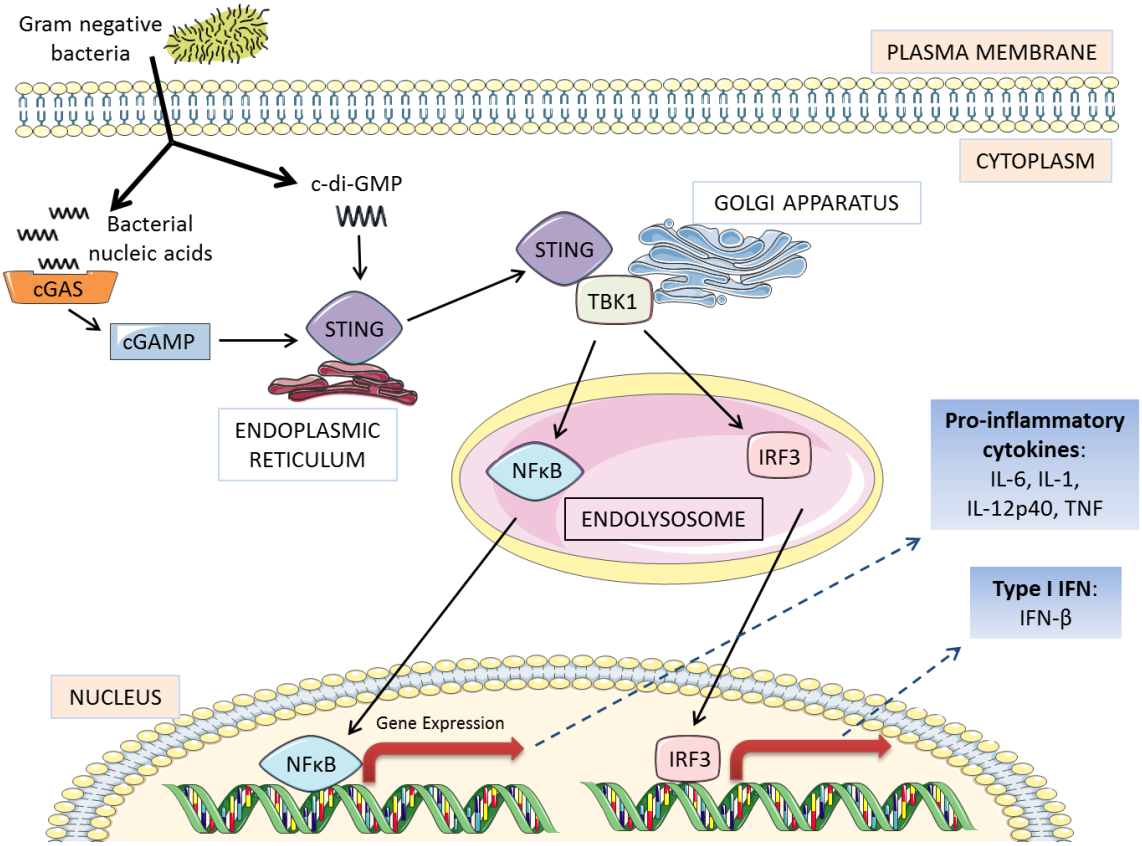

Figure 1.6 Simplified schematic of the STING signalling pathway

CDNs can be produced by Gram-negative bacteria, or by the host cell upon detection of cytoplasmic bacterial nucleic acids. The host cell-derived CDN cGAMP is synthesised by the cytosolic protein cGAS upon binding of bacterial DNA. An ER-associated adaptor protein called STING, is capable of directly binding cGAMP, and bacterially-derived CDNs such as c-di-GMP, and upon activation, STING translocates to the Golgi apparatus where it activates TBK1. The STING-TBK1 complex can then migrate to perinuclear endosomes where TBK1 phosphorylates NFκB and IRF3, inducing pro-inflammatory cytokine, and type I IFN production, respectively.

1.10 Live attenuated *B. pertussis* vaccines

Genetically attenuated bacteria are still capable of colonising the host and inducing an immune response without causing severe disease, and this could potentially be a very effective method of inducing protective immunity to *B. pertussis*. Indeed a live attenuated strain of *B. pertussis,* BPZE1, has been developed, that presents no threat to the host due to the introduction of several mutations into 3 key virulence factors [303]. A single i.n. immunisation with BPZE1 was shown to induce a potent Th1 response and to protect infant (3 week old) mice significantly more than two doses of an aP vaccine from *B. pertussis* and *B. parapertussis* infection [303]. Furthermore, immunisation with BPZE1 has been shown to promote robust Th17 cell responses, and $CD4^+$ but not $CD8^+$ T cells have been shown to be crucial for BPZE1-mediated protection, presumably through the production of IFN-γ and IL-17 [304]. BPZE1 has also been shown to induce systemic *B. pertussis*-specific IgG [303] and local IgA responses [305] in immunised mice. Long-term protection induced with BPZE1 has also been shown to be substantially better than that conferred with an aP vaccine; 12 months after immunisation, infant and adult mice immunised i.n. once with BPZE1 were significantly more protected from i.n. challenge with *B. pertussis,* than mice immunised twice i.p. with an aP vaccine [306].

BPZE1 was engineered with the intention of being administered to infants as a single-dose, i.n. vaccine, prior to the scheduled course of DTaP vaccinations, and indeed, studies investigating the efficacy of this option have been promising. Neonatal mice i.n. primed with BPZE1 maintain their Th1 and Th17 cell profile, and a greater IgG2a/IgG1 antibody ratio, despite two subsequent i.p. booster immunisations with aP vaccines [307]. Furthermore, mice i.n. immunised with BPZE1 and boosted once with an aP vaccine were significantly more protected from i.n. challenge with *B. pertussis,* than mice that were i.n. administered BPZE1 once, or i.p. immunised with an aP vaccine once [307].

The safety of BPZE1 was evaluated using immunodeficient mouse strains in advance of clinical studies in humans. IFN-$\gamma R^{-/-}$ mice die from infection with live *B. pertussis,* and this is associated with dissemination of the bacteria from the lungs to the liver [2], however, no dissemination was observed in IFN-$\gamma R^{-/-}$ mice infected with the BPZE1 strain [308]. Furthermore, infection with live attenuated BPZE1 was well tolerated in neonatal mice, whereas infection with virulent *B. pertussis* resulted in significant mortality [308]. Finally, this vaccine has been evaluated in several phase I clinical trials in humans and has been shown to be immunogenic and well tolerated, indicating it has substantial potential as a vaccine candidate for pertussis [309, 310].

1.11 The use of outer membrane vesicles in pertussis vaccination

Outer membrane vesicles (OMVs) containing antigens in their native conformation, are naturally secreted from the surface of most Gram-negative bacteria during normal cell processes [311]. *B. pertussis* cells growing in liquid culture have been shown to release OMVs containing multiple *B. pertussis* antigens including an analog of LPS, lipooligosaccharide (LOS), Prn and PT [312]. As these OMVs have inherent adjuvanticity and immunogenic properties (they contain some of the main immunogens included in aP vaccines), they could be a valuable asset in the development of third-generation aP vaccines. Indeed, administering OMVs by the i.n. route has been shown to confer protection against *B. pertussis* i.n. challenge, as has administration by the i.p. route when the OMVs are adjuvanted with alum [313]. Furthermore, immunising mice with *B. pertussis* OMVs formulated with diphtheria and tetanus toxoids have been shown to induce as potent Th1 and Th17 responses as a wP vaccine, and a greater IgG2a to IgG1 ratio than seen with an aP vaccine [314]. OMV vaccines have been licensed against serogroup B *Neisseria meningitidis* [315] and two were shown to have good efficacy during epidemics in Norway [316] and Cuba [317]. However, the current vaccines are strain-specific and a focus has been placed on developing an OMV vaccine that is broadly protective against across all Neisseria serotypes [318].

1.12 Intranasal administration of aP vaccines

Since *B. pertussis* infects the respiratory tract, nasally delivered vaccines may be a more effective approach than using conventional parenterally delivered vaccines for inducing local mucosal immunity at the site of infection and preventing bacterial colonisation. I.n. immunisation with FHA and recombinant PT (rPT) formulated with chitosan induced significantly greater local IgA responses in mice than i.p. immunisation with FHA and rPT adjuvanted with alum, whereas the systemic IgG responses were as potent [319]. Furthermore, LTK63, a non-toxic mutant of the *Eschericia coli* heat-labile toxin (LT) which lacks the ADP-ribosylating activity of the native protein, has been shown to be an effective mucosal adjuvant for nasal delivery of an aP vaccine in mice (Figure 1.5) [320]. I.n. immunisation of mice with a two-component aP vaccine supplemented with LTK63 enhanced antigen-specific IFN-γ, IL-4 and IL-5, serum IgG and lung secretory IgA. Immunised mice had significantly lower bacterial load in the lungs following aerosol challenge with *B. pertussis* when compared to mice immunised with the soluble antigens alone. Although, LT-based adjuvants are unlikely to be used for nasal vaccines in humans due to the possibility of inducing Bell's palsy [321], these

studies do provide proof-of-principle that nasal vaccination is a viable approach for inducing mucosal immunity with pertussis vaccines. Finally, the live attenuated pertussis vaccine candidate BPZE1, which was already discussed in section 1.10, is administered by the i.n. route and has been shown to be safe in humans and highly effective at promoting local and systemic responses [303, 305, 309]. Indeed, a live attenuated influenza vaccine that is administered i.n. has been licensed for human use, so there is a strong possibility that an i.n. *B. pertussis* vaccine could also be introduced [322].

1.13 Particulate antigen delivery systems

An alternative approach for improving the immunogenicity of soluble antigens is to incorporate them into particulate antigen delivery systems; this enhances uptake by APCs and reduces systemic dissemination of the antigens. Incorporation of dPT with CpG and the innate defence regulator peptide 1002 (IDR) into polyphosphazene (PCEP) microparticles enhanced the immunogenicity of the antigen and enhanced protection against i.n. challenge with *B. pertussis* [323]. Additionally, on day 7 post-infection, mice immunised with the particulate formulation had similar bacterial counts to mice immunised with a licensed alum-adjuvanted aP vaccine (Quadracel), which contained additional antigens to dPT. High levels of IFN-γ, IL-12, and TNF were observed in the lung tissue of the PCEP-vaccinated mice, whereas IL-10 concentrations were significantly higher in mice immunised with the alum-adjuvanted aP vaccine, suggesting that the microparticle-encapsulated antigens induced a strong Th1 response, while the aP vaccine with alum predominantly promoted a Th2 response.

Conway *et al.* have also demonstrated that immunising mice twice by the i.p. or intramuscular (i.m.) route with dPT and FHA formulated in poly-lactide-co-glycolide (PLG) microparticles conferred a high level of protection against *B. pertussis* infection, that was as robust as that induced when FHA and dPT were administered i.p. with alum [324]. Furthermore, immunising with this PLG microparticle formulation by the i.p. and i.m. route induced a robust Th1-type immune response, whereas immunisation with dPT and FHA in nanoparticles induced primarily a Th2 immune response, indicating microparticles were more favourable for pertussis vaccination [324]. Furthermore, a single i.p. administration of *B. pertussis* FIM entrapped within PLG microparticles induced potent antigen-specific antibody production in mice and protected them from infection as efficiently as a single i.p. immunisation of alum-adjuvanted FIM [325]. This evidence suggests that microparticles may be effective delivery systems for

enhancing immune responses to *B. pertussis* antigens and beneficially, the PLG polymer has already been approved for human use by the FDA [326].

1.14 Current strategies employed to protect vulnerable neonates

It has been recognised since the early 1900s that infants too young to be vaccinated are protected to some extent from diseases such as diphtheria, measles and poliomyelitis by IgG antibodies passively transferred from the mother across the placenta, however, this does not appear to be the case with pertussis [327-329]. Studies have been underway since then to investigate if maternal immunisation could induce early protection against pertussis while the infants' immune system is still developing. Indeed, studies in mice showed that maternal immunity did have a role in protecting infants from *B. pertussis* challenge [330]. Furthermore, newborn piglets whose mothers were immunised with an inactivated *B. pertussis* strain and who were allowed to suckle colostrum for 4-5 days after birth, had significantly greater *B. pertussis*-specific IgG and secretory IgA in serum and bronchoalveolar lavage fluid than piglets born to unvaccinated mothers, and were more protected from intrapulmonary *B. pertussis* challenge [34]. These studies confirm that passive transfer of antibodies from mother to infant can protect them from *B. pertussis* infection, however, in contrast to humans, transfer only occurs in pigs through colostrum ingestion and not transplacentally, limiting the ability to analyse antibody transfer in this model [34].

Several studies in humans have now reported that Tdap immunisation during pregnancy is safe, with little to no adverse outcomes observed in infants or mothers [331-333]. Indeed, maternal immunisation has been shown to correlate with high antigen-specific IgG antibody titres in neonates at birth which can last into the first 2 months of life, and could potentially provide infants with greater protection against pertussis until they are vaccinated [332, 334, 335]. However, it has been reported that immunisation in the third trimester of pregnancy is the most effective time for inducing high PT-specific IgG antibody titres at birth [336]. A number of countries worldwide including the UK [337], US [30], Ireland [27], New Zealand [338] and Australia [339] are now recommending for women to be vaccinated with TdaP during pregnancy. Indeed, maternal immunisation against pertussis has been in practise in the USA since 2008 [30], and in 2012 the UK introduced a maternal pertussis vaccination programme following an outbreak where 14 infants died from pertussis [337, 340].

Cocooning is another strategy that has been implemented in many countries in an attempt to prevent vulnerable infants from getting infected with *B. pertussis*. This approach involves

indirectly protecting neonates from pertussis by vaccinating their family and caretakers, thus limiting the potential for transmission. Indeed, the WHO, the CDC and the Global Pertussis Initiative (GPI) have recommended this scheme [341-343], however, strict adherence to the schedule and vaccination of all potential contacts does not always occur, and this is necessary for this strategy to be successful [344, 345]. There is also evidence that the cocoon programme is not very efficient or cost-effective, and furthermore, it is resource and labour intensive, indicating it may not be the most effective method of protecting neonates [346, 347].

1.15 Aims of the project

The currently licensed, alum-adjuvanted aP vaccine confers ineffective and short-lasting immunity against the respiratory pathogen *B. pertussis* and as a result, the incidence of whooping cough has been increasing in many countries with high vaccine coverage in recent years. The goal of this study was to evaluate the efficacy of aP vaccines formulated with TLR agonists and a novel TLR-STING agonist combination, with the aim of informing the design of future aP vaccines. In detail, the project examined:

- If the TLR agonists CpG or LP1569 were more effective adjuvants than alum for an aP vaccine, capable of inducing potent cellular and humoral immunity and memory responses to *B. pertussis*.
- If addition of CpG or LP1569 to a commercially available alum-adjuvanted aP vaccine could skew the induced immune response away from a Th2 and towards a Th1 phenotype.
- If a TLR-STING agonist combination was a more effective adjuvant than a TLR agonist alone.
- If substitution of alum for a novel TLR-STING agonist combination enhanced the efficacy of an experimental aP vaccine.
- If i.n. administration of an experimental aP vaccine formulated with a TLR-STING agonist combination conferred greater protection than i.p. immunisation by inducing more appropriate local immunity to *B. pertussis* and enhancing memory responses.
- If administration of a booster aP vaccine formulated with a Th1-promoting adjuvant could redirect the immune response induced after initial immunisation with an alum-adjuvanted aP vaccine and enhance cellular immunity.

The hypothesis of this study was that a TLR agonist or TLR-STING agonist combination would be a more efficient adjuvant for an aP vaccine, capable of inducing more appropriate cellular and humoral immunity, and memory responses, than an alum-adjuvanted aP vaccine. Consequently, these studies will contribute to the field by providing novel information on potential adjuvants for third-generation aP vaccines.

Chapter 2:

Materials and Methods

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2.1 Materials

2.1.1 Complete Roswell Park Memorial Institute (cRPMI) medium

RPMI-1640 medium (RPMI; Sigma-Aldrich)

10% heat inactivated fetal calf serum (FCS; Sigma-Aldrich)

100 mM L-glutamine (Sigma-Aldrich)

100 U/ml penicillin / 100 µg/ml streptomycin (Sigma-Aldrich)

50 µM β-mercaptoethanol (Sigma-Aldrich) (*ex vivo* T cell culture only)

2.1.2 Red blood cell lysis buffer

4.35 g Ammonium chloride (NH_4Cl; Sigma-Aldrich)

Dissolved in 500 ml ddH_2O

2.1.3 Stainer-Scholte (S&S) liquid medium

L-glutamic acid (monosodium salt) ($C_5H_8NNaO_4.xH_20$)	10.72 g
L-proline ($C_5H_9NO_2$)	0.24 g
Sodium chloride (NaCl)	2.5 g
Potassium phosphate monobasic (KH_2PO_4)	0.5 g
Potassium chloride (KCl)	0.2 g
Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	0.1 g
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.02 g
Tris (Trizma base; $C_{14}H_{11}NO_3$)	1.525 g

Made up to 1 litre with ddH_2O (pH 7.3-7.4) and sterilised by autoclaving.

(All of the above reagents were obtained from Sigma-Aldrich.)

2.1.4 Supplement (200X)

0.4 g L-cystine ($C_6H_{12}N_2O_4S_2$) - Dissolved in 1 mL 37% Hydrogen chloride (HCl; Sigma-Aldrich)

0.1 g Ferrous sulfate heptahydrate ($FeSO_4.7H_20$; Sigma-Aldrich)

0.2 g L-ascorbic acid ($C_6H_8O_6$; Sigma-Aldrich)

0.04 g Nicotinic acid ($C_6H_5NO_2$; Sigma-Aldrich)

1.0 g L-glutathione (reduced) ($C_{10}H_{17}N_3O_6S$; Sigma-Aldrich)

Made up to 50 mL with ddH_2O and filter sterilised.

2.1.5 1% Casein salt solution

6.0 g NaCl

10.0 g BactoCasamino acids (Becton Dickinson)

Made up to 1 L with ddH_20 (pH 7.0-7.2) and autoclaved at 115°C for 20 minutes.

2.1.6 Bordet-Gengou blood agar plates

5 ml Glycerol ($C_3H_8O_3$; Fisher Chemical)

15 g Bordet-Gengou agar (Becton Dickinson)

Made up to 500 mL with ddH_2O and autoclaved at 121^oC for 20 minutes.

2 ml Cephalexin (10 mg/mL; Sigma-Aldrich)

100 ml horse blood (pre-warmed to 37°C; Cruinn)

2.1.7 ELISA buffers and other reagents

ELISA wash buffer

500 ml 20X PBS

9.5 L dH_2O

5 ml Tween 20 (Sigma-Aldrich)

Phosphate buffered saline (PBS) 20X

320 g NaCl (1.4M)

46 g Sodium hydrogen phosphate (Na_2HPO_4; 0.08M)

8 g KH_2PO_4 (0.01M)

8 g KCl (0.03M)

2 L dH_2O

pH 7.0

ELISA blocking solutions

Cytokine ELISA:	BD kits: 10% (w/v) milk (Marvel)
	R&D kits: 1% (w/v) Bovine serum albumin (BSA; Sigma-Aldrich)
IFN-β ELISA:	PBS/10% FCS (Sigma-Aldrich)
Antibody ELISA:	0.1% BSA

Phosphate citrate buffer (PCB)

20.38 g anhydrous citric acid ($C_6H_8O_7$; Sigma-Aldrich)

31.54 g Na_2HPO_4 (Sigma-Aldrich)

Made up to 2 L with dH_2O

pH 5.0

ELISA substrate solution

1 o-Phenylenediamine dihydrochloride (OPD) tablet (10mg; Sigma-Aldrich)

25 ml PCB

7 µl H_2O_2

ELISA stop solution

26.74 ml 18M Sulfuric acid (H_2SO_4; Sigma-Aldrich)

473.26 l dH_2O

Carbonate buffer (IFN-β ELISA)

0.84 g Sodium bicarbonate ($NaHCO_3$; Sigma-Aldrich)

0.356g Sodium carbonate (Na_2CO_3; Sigma-Aldrich)

100 ml dH_20

pH 9.4-9.6

2.1.8 ELISA antibodies

(a) Cytokine ELISA antibodies	Capture dilution	Blocking	Detection dilution	Top working standard	Supplier
IL-12p70	1:120	1% BSA	1:60	1500 pg/ml	R&D Systems
IL-1β	1:120	1% BSA	1:60	1000 pg/ml	R&D Systems
IL-23	1:120	1% BSA	1:180	2500 pg/ml	R&D Systems
IL-17	1:120	1% BSA	1:60	1000 pg/ml	R&D Systems
IL-10	1:120	1% BSA	1:60	2000 pg/ml	R&D Systems
IL-6	1:500	10% milk	1:500	5000 pg/ml	BD Pharmingen
IL-5	1:500	10% milk	1:500	2500 pg/ml	BD Pharmingen
IL-12p40	1:1000	10% milk	1:500	5000 pg/ml	BD Pharmingen
IFN-γ	1:1000	10% milk	1:500	10 ng/ml	BD Pharmingen
Strep-HRP	-	-	1:1000	-	BD Pharmingen
IFN-β	1:1000	PBS/10% FCS	1:2000	20 U/ml	Refer to 2.2.16

(b) Antibody ELISA antibodies	Blocking	Detection dilution	Supplier
Total IgG	0.1% BSA	1:5000	Sigma-Aldrich
IgG1	0.1% BSA	1:4000	BD Pharmingen
IgG2c (HRP-conjugated)	0.1% BSA	1:1000	Bio-Rad Laboratories
IgA (HRP-conjugated)	0.1% BSA	1:1500	Southern Biotech

2.1.9 FACS antibodies

Antibody	Fluorochrome	Clone	Isotype	Supplier
CD80	BV650	16-10A1	Arm. Hamster, IgG	BioLegend
CD80	PE	16-10A1	Arm. Hamster, IgG	BioLegend
CD86	FITC	GL-1	Rat IgG2a, κ	BD Pharmingen
CD86	APC/Cy7	GL-1	Rat IgG2a, κ	BioLegend
CD40	PE	1C10	Rat IgG2a, κ	eBioscience
CD40	FITC	3/23	Rat IgG2a, κ	BD Pharmingen
MHC II	APC	M5/114.15.2	Rat IgG2b, κ	eBioscience
MHC II	eFluor450	M5/114.15.2	Rat IgG2b, κ	eBioscience
CD11c	BV785	N418	Arm. Hamster, IgG	BioLegend
CD4	APC-eFluor780	RM4-5	Rat IgG2a, κ	eBioscience
CD3	BV650	17A2	Rat IgG2b, κ	BioLegend
CD8	PeCy7	53-6.7	Rat IgG2a, κ	eBioscience
CD69	FITC	H1.2F3	Arm. Hamster, IgG	eBioscience
CD44	BV605	IM7	Rat IgG2b, κ	BioLegend
CD62L	PECF594	MEL-14	Rat IgG2a, κ	BD Bioscences
CD103	BV785	M290	Rat IgG2a, κ	BD Bioscences
B220/CD45R	AF700	RA3-6B2	Rat IgG2a, κ	eBioscience
CD11b	AF780	M1/70	Rat IgG2b, κ	eBioscience
IFN-γ	Pe-Cy7	XMG1.2	Rat IgG1, κ	eBioscience
IL-17	eFluor450	eBio17B7	Rat IgG2a, κ	eBioscience
Ly6C	FITC	AL-21	Rat IgM, κ	BD Pharmingen
GR1 (Ly6G)	PE	RB6-8C5	Rat IgG2b, κ	eBioscience
F4/80	Pe-Cy5	BM8	Rat IgG2a, κ	eBioscience
CD16/CD32 (Fcγ block)	Purified	93	Rat IgG2a, λ	eBioscience

2.1.10 Other FACS reagents

Reagents	Supplier	Concentration
Live/Dead Aqua	Life Technologies	1:600 (PBS)
PMA	Sigma-Aldrich	5 ng/ml
Anti-CD3	BD Pharmingen	1 µg/ml
Ionomycin	Sigma-Aldrich	500 ng/ml
Brefeldin A	Sigma-Aldrich	5 µg/ml
Paraformaldehyde (PFA)	Thermo Scientific	2%
Collagenase D	Sigma-Aldrich	1 mg/ml
Deoxyribonuclease I (DNAse I)	Sigma-Aldrich	20 U/ml
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich	-
Saponin	Sigma-Aldrich	0.05%
Foxp3 staining kit	eBioscience	-
FACS CompBeads	BD Biosciences	1:10 (PBS)

2.1.11 Adjuvants

Adjuvant	Solubility	Supplier	Concentration *in vitro*	Concentration *in vivo*
CpG 1668	ddH_2O	Sigma-Aldrich Genosys	1, 5 or 25 µg/ml	50 µg/mouse
LP1569	DMSO	EMC Microcollections	0.04, 0.2, 1, 5 or 25 µg/ml	50 µg/mouse
Aluminium hydroxide (Alhydrogel)	-	Brenntag Biosector, Denmark	-	100 µg/mouse
c-di-GMP	Endotoxin-free water	Invivogen	1 or 10 µg/ml	10 µg/mouse

2.1.12 Antigens

Antigen	Supplier	Concentration *in vitro*	Concentration *in vivo*
FHA	Kaketsuken, Kumamoto, Japan	0.5 or 2 µg/ml	Refer to figure legend
PT	LIST laboratories (#181)	-	Refer to figure legend
PT-9K/129G	Novartis Vaccines, Siena	-	Refer to figure legend
rPT	LIST Laboratories (#184)	-	Refer to figure legend
Prn	LIST Laboratories (#187)	1 or 2 µg/ml	Refer to figure legend
Sonicated *B. pertussis*	-	1 or 10 µg/ml	-
KLH	Calbiochem	2, 50 or 100 µg/ml	1 µg/mouse

2.1.13 Other reagents

Reagent	Supplier
Lipofectamine 2000 (lipofectamine)	Invitrogen
Ethidium Bromide-Acridine Orange (EBAO)	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
PBS	Sigma-Aldrich

2.2 Methods

2.2.1 Cell counting

Cells were diluted 1:10 in 50% (v/v) trypan blue/PBS and a disposable haemocytometer was filled with 10 µl of cell suspension. Viable cells exclude the stain and appear white under a light microscope. These were counted and the cell number was determined using the formula: #cells / #squares * dilution factor *10^4 / ml.

For counting samples where the red blood cells were not previously lysed, cells were diluted 1:10 in EBAO and 10 µl was pipetted into a disposable haemocytometer (Hycor Biomedical, UK). The numbers of viable (green) and dead cells (red) were counted using a fluorescence microscope. Live cells were counted and cell number determined using the same formula as before.

2.2.2 BMDC culture

Femurs and tibiae were isolated from naïve C57BL/6 or IFNAR$^{-/-}$ mice. The surrounding tissues were removed and the ends of the bones were cut open. A 27 G needle was used to flush out the bone marrow with cRPMI. Cells were centrifuged at 1,300 rpm for 5 minutes and resuspended in 2 ml of pre-warmed, filter-sterilised red blood cell lysis buffer. Cell aggregates were broken up by gently pipetting the lysis buffer up and down. After 2 minutes, 23 ml of cRPMI was added and cells were centrifuged as before. Supernatants were decanted and the pellet was resuspended in 10 ml cRPMI and counted using Trypan blue as described in section 2.2.1. Cells were cultured at a concentration of 0.5-0.7 x 10^6 cells/ml in T175 tissue culture flasks (25 ml per flask) in cRPMI containing 20 ng/ml GM-CSF. The GM-CSF was obtained from culture supernatants of the GM-CSF transfected J558 plasmacytoma cell line. On day 3, 25 ml cRPMI + 20 ng/ml GM-CSF were added to each flask. On day 6, supernatants were gently removed and 25 ml pre-warmed, sterile PBS was added to each flask. The flasks were agitated gently and the PBS then transferred to a 50 ml centrifuge tube containing 10 ml cRPMI. 25 ml of pre-warmed EDTA (Sigma-Aldrich) was then added to each flask and the flask was incubated at 37°C for 5 minutes. Afterwards, the EDTA solution was gently pipetted up and down to detach loosely adherent cells and transferred to a 50 ml tube containing 10 ml of cRPMI. Cells were centrifuged and all pellets pooled in a final volume of 10 ml cRPMI. Cells were counted and cultured at a concentration of 0.5-0.7 x 10^6 cells/ml in T175 tissue culture flasks (25 ml per flask) in cRPMI containing 20 ng/ml GM-CSF. On day 8, 25 ml cRPMI + 20 ng/ml GM-CSF was

added to each flask. On day 10, loosely adherent cells were removed by gentle pipetting and then centrifuged down. Supernatants were decanted and cell pellets from all flasks were pooled in 10 ml medium. Cells were counted and plated out at a concentration of 1 x 10^6 cells/ml in cRPMI in 96 well plates. These BMDCs were left to adhere for at least 30 minutes and then treated as required on the same day.

2.2.3 BMDM culture

For the generation of BMDMs from C57BL/6 mice, the bone marrow was isolated as described in section 2.2.2. Bone marrow cells were cultured in petri dishes at 1 x 10^6 cells/ml in cRPMI supplemented with 20% v/v of M-CSF in the form of supernatant obtained from culture of the M-CSF transfected L929 cell line. On day 3, an additional 2 ml of M-CSF was added to the culture. On day 6, loosely adherent cells were removed by washing with PBS and adherent macrophages were gently scraped in cRPMI, counted and seeded at the required concentration in tissue culture plates. BMDMs were allowed to adhere and rest for at least 3 hours before stimulation.

2.2.4 Lipofectamine transfection of cells

Cells were plated out at a concentration of 1 x 10^6 cells/ml in cRPMI in 96 well plates and left to settle for 30 minutes before treatment. Lipofectamine was used at a final concentration of 1 µg/ml (stock concentration of 1 mg/ml). The c-di-GMP-lipofectamine complex was prepared as follows and describes the volume required to treat one well of cells on a 96 well plate:

(a) Lipofectamine was diluted 1:200 in serum-free media (SFM) to a total volume of 25 µl. This was mixed gently and incubated for 5 minutes at room temperature.
(b) C-di-GMP was separately diluted to the desired concentration in SFM to a total volume of 25 µl.
(c) After 5 minutes, the lipofectamine and c-di-GMP dilutions were combined (total volume of 50 µl), mixed gently, and incubated for 20 minutes at room temperature to allow the complex to form.
(d) After 20 minutes, the transfection mix was added to 200 µl cells in a 96 well plate to give a total volume 250 µl (total dilution of 1:1000 of stock lipofectamine of 1 mg/ml).
(e) Cells were incubated at 37°C in a CO_2 incubator for 24 hours and then cytokine concentrations in supernatants were quantified by ELISA.

2.2.5 Mice

All mice were maintained according to European Union and Irish Department of Health and Children regulations. Experiments were performed under licence from the Health Products Regulatory Authority and with approval from the Trinity College Dublin Comparative Medicine Unit. C57BL/6 mice were purchased from Harlan, or were bred in house in the Comparative Medicine Unit in Trinity College Dublin. IFN-$\gamma^{-/-}$, IL-17A$^{-/-}$ and IFNAR$^{-/-}$ mice were bred in house. All animals used were age-matched females, were 6-12 weeks old at the initiation of experiments and were housed under specific pathogen-free conditions. Mice were sacrificed by asphyxiation with CO_2.

2.2.6 Group numbers in animal studies

In each immunisation experiment, there were 20 mice per experimental group (e.g. for experiments containing 4 treatment groups overall, 80 mice were used in total). 4 mice in each experimental group were sacrificed before aerosol challenge with *B. pertussis* to examine the antigen-specific T cell and antibody responses induced with the various experimental vaccines. The remaining 16 mice in each group were challenged with *B. pertussis,* and 4 mice per group were sacrificed at each timepoint post infection to examine *B. pertussis* burden in the lungs.

2.2.7 Immunisation schedule

The standard immunisation schedule used in these studies was to immunise mice twice at 0 and 4 weeks, and challenge them with *B. pertussis* 2 weeks after the last immunisation. An accelerated schedule (immunise at 0 and 3 weeks, challenge at 4 weeks) was used in two studies only.

2.2.8 Vaccines used in animal studies

Typically, mice were immunised twice at 0 and 4 weeks (unless otherwise stated) with commercially available aP or wP vaccines or experimental aP vaccines. The commercial aP vaccine used was either Infanrix (GSK; diphtheria and tetanus toxoids and aP adsorbed to alum; the pertussis component comprising detoxified PT, FHA and Prn) or Adacel (Sanofi Pasteur; diphtheria and tetanus toxoids and aP adsorbed to alum; the pertussis component comprising detoxified PT, FHA, Prn, FIM2 and FIM3). The wP vaccine used in these studies was

either obtained from the Serum Institute of India, or was the WHO International Standard pertussis whole cell vaccine, 94/532 from NIBSC, Herts., UK.

Experimental aP vaccines consisted of the *B. pertussis* antigens FHA, Prn or PT which was either genetically or chemically inactivated. The genetically detoxified PT used was either GMP-grade genetically detoxified PT (PT-9K/129G), supplied by GSK Vaccines, Siena, Italy or a PT mutant (rPT) obtained from LIST laboratories (#184). The chemically detoxified PT used was purchased from LIST as active PT (#181) and then detoxified by the protocol described in section 2.2.9.

In the majority of these studies, rPT was included in the experimental aP vaccines as pertussis vaccines containing this form of PT have previously been shown to be more immunogenic than those containing dPT [348], however, dPT was used in one study when rPT was unavailable. Varying the types of PT used was not ideal as chemically treating PT results in loss of immunogenicity due to conformational changes in the toxin, while PT remains in its native conformation following genetic engineering. However, T cell responses induced with the different forms of PT should be similar as the TCR recognises processed antigen, but it is possible that B cell responses and antibody production could be affected as the BCR recognises peptide in its native conformation. For this reason, PT-specific antibody responses were not prioritised in these studies.

2.2.9 Detoxification of PT

For experiments involving dPT, active PT (salt-free) was purchased from LIST laboratories (#181) and detoxified as follows. Lyophilised, salt-free PT was dissolved in PBS with 0.5% (w/v) formaldehyde and 0.05 M lysine for a final PT concentration of 0.5 µM. After 48 hours rotation at 37°C, the toxin was dialysed into 4 L of PBS at 4°C with constant stirring for 31 hours with 3 changes of PBS (protocol obtained from Sutherland et al [349]).

2.2.10 Immunisation by the i.p. route

For i.p. immunisations, mice were scruffed and experimental vaccines were administered into the peritoneum in a total volume of 200 µl. Mice were returned to their cage of origin and monitored for 10 minutes to ensure no adverse effects due to immunisation.

2.2.11 Immunisation by the i.n. route

For i.n. immunisations, mice were lightly anaesthetised with isoflurane to minimise their stress prior to immunisation. Each mouse was placed in a transparent isoflurane chamber and the isoflurane gas was turned on and set to a concentration of 5%. The mouse was left in the chamber for about 90 seconds and monitored until its breathing slowed to around 45 breaths per minute. The mouse was then removed from the isoflurane chamber and placed on its back on a heating pad. The experimental vaccine (which was prepared in a total volume of 20 µl) was administered by placing two 10 µl droplets on the mouse nares which were subsequently inhaled during the natural breathing process. After immunisation, the mouse was kept on the heat pad until it started to move. It was then transferred back into its cage of origin and monitored until it had completely recovered from the anaesthetic. The entire procedure was carried out under a laminar flow hood to maintain a sterile environment.

2.2.12 Immunisation s.c. into the footpad

Mice were injected s.c. into each footpad with reagents dissolved in sterile PBS in a total volume of 25 µl (50 µl/mouse). The lymphatics of the foot drain preferentially to the popliteal LNs located behind the knee. Popliteal LNs were removed 7 days after immunisation. Cells were restimulated (as described in the figure legends) and cytokine concentrations in supernatants were quantified by ELISA.

2.2.13 Culturing of *B. pertussis*

Frozen stocks of *B. pertussis* (BP338) were thawed, 100 µl was lawned onto Bordet-Gengou agar plates and the bacteria were grown on these blood agar plates for 3-4 days. After that, liquid cultures were established by inoculating conical flasks containing 100 mL S&S liquid medium and supplement (1X) with *B. pertussis* (half a Bordet-Gengou plate of bacteria per conical flask). The liquid cultures were incubated at 37°C in a shaking incubator for 24 hours. Following liquid culture, the bacteria were centrifuged at 10,500 rpm for 20 minutes with the brake off (4-20°C). The bacterial pellets were resuspended in 10 ml 1% casein salts per flask (pre-heated to 37°C).

2.2.14 *B. pertussis* aerosol challenge

Respiratory infection of mice was performed two weeks after the second immunisation unless otherwise stated and was performed by aerosol challenge. 5 x 10^8 colony forming units (CFU) /ml or 1 x 10^9 CFU/ml of *B. pertussis* were aerosolised using a Pari TurboBoy SX nebuliser. Mice were randomised and exposed to the aerosolised bacteria for 10 minutes in a perspex chamber, followed by 10 minutes rest in the chamber. The challenge dose was confirmed by isolating the lungs from 4 mice 2 hours post challenge and performing CFU counts. The course of *B. pertussis* infection was followed by performing CFU counts on lungs from groups of 4 mice at intervals after challenge.

2.2.15 CFU counts of lungs and trachea

The lungs or trachea were aseptically removed and homogenised in 1 ml (full lungs) or 500 µl (half lung or full trachea) of 1% casein solution. Undiluted and serially diluted homogenates (100 µl) from individual lungs or tracheas were spotted in duplicate onto Bordet-Gengou agar plates and the number of CFU was calculated after 5-6 days incubation at 37°C. The counts were converted into Log_{10} CFU for graphical representation of lung bacterial load.

2.2.16 Preparation of sonicated *B. pertussis* for *ex vivo* restimulation

The *B. pertussis* liquid culture was centrifuged at 4,000 rpm for 30 minutes and washed with sterile PBS (4,000 rpm for 30 minutes). The bacterial suspension was then diluted to 1 x 10^{10} CFU/ml in sterile PBS and was sonicated in 3 ml quantities in 15 ml conical tubes using 10 x 15 second pulses on ice. The sonicated *B. pertussis* suspension was then centrifuged at 10,000 rpm for 15 minutes and the supernatant was collected. Protein concentration was measured using the Pierce BCA protein kit and stored at -80°C. The sonicated *B. pertussis* protein was used at 1 or 10 µg/ml for restimulation of cells.

2.2.17 Detection of antigen-specific T cell responses to *B. pertussis*

Spleen or LN cells from four immunised mice per group were isolated from their respective organs 6 weeks post primary immunisation. They were processed by forcing them through a 40 µM filter using the barrel of a 5 ml syringe. Cells were pelleted and resuspended in an appropriate volume and counted using EBAO. Once counted, spleen or LN cells were cultured

at a concentration of 2 x 10^6/ml or 1 x 10^6/ml respectively, at 37°C and 5% CO_2 with the antigens FHA (0.5 or 2 µg/ml), Prn (1 or 2 µg/ml) or sonicated *B. pertussis* (1 or 10 µg/ml). Stimulation with PMA (25 ng/ml) and anti-mouse CD3 (1 µg/ml) or medium only were used as positive and negative controls. Supernatants were removed after 72 hours and IFN-γ, IL-17 and IL-5 concentrations were determined by two-site ELISA.

2.2.18 Cytokine ELISA

Cytokine concentrations were determined using ELISA kits from BD Pharmingen or R&D systems. High binding certified 96-well plates were coated with 50 µl of diluted capture antibodies in PBS (1 µg/ml; dilutions as per table 2.1.8a) and incubated overnight at 4°C. Plates were then washed in wash buffer 4 times and non-specific binding sites blocked by adding 180 µl of blocking solution for 2 hours at room temperature. After 2 hours, plates were washed again in wash buffer 4 times and 50 µl of test supernatants were added to wells along with serially diluted standard recombinant protein for each cytokine. Supernatants were diluted 1:10 for IL-6 and 1:50 for IL-12p40 or left undiluted for all other cytokines. Plates were then incubated overnight at 4°C. Next, plates were washed in wash buffer 4 times followed by addition of 50 µl/well of biotinylated detection antibody (1 µg/ml; dilutions as per table 2.1.8a). Plates were then incubated for 1 hour at 37°C in the dark and then washed in wash buffer 4 times. Plates were incubated with 50 µl/well horseradish peroxidase (HRP)-conjugated streptavidin at room temperature for 20 minutes. After washing in wash buffer, 50 µl of substrate solution was added to each well. The colour was allowed to develop and the reaction was stopped by adding 25 µl stop solution once the standard curves for each cytokine had developed.

Plates were then read at 492 nm using a microtitre plate reader and the cytokine concentrations determined with reference to the standard curve prepared using recombinant cytokines of known concentrations.

2.2.19 IFN-β ELISA

High-binding 96-well plates were coated with a 1:1000 dilution of rat anti-mouse IFN-β monoclonal antibody (Santa Cruz, Heidelberg, Germany) in carbonate buffer and incubated at 4°C overnight. On day 2, plates were washed 3 times in wash buffer and then blocked with PBS/10% FCS for 2 hours at 37°C. The blocking solution was removed and 50 µl undiluted

supernatants were added to the plate, along with a recombinant IFN-β standard (PBL Biomedical Laboratories, Piscataway, NJ) which was serially diluted in PBS/10% FCS and the plate was incubated at 4°C overnight. On day 3, the plates were washed 3 times in wash buffer and incubated at 4°C overnight with 50 µl/well of rabbit anti-mouse IFN-β polyclonal antibody (PBL Biomedical Laboratories, Piscataway, NJ) diluted 1:2000 in PBS/10% FCS. On day 4, the plates were washed 3 times and then incubated for 2 hours with 50 µl/well of anti-rabbit HRP (Sigma-Aldrich) diluted 1:2000 in PBS/10% FCS. The plates were washed again, developed and read as described in section 2.2.15.

2.2.20 Antibody ELISA

Sera were obtained from immunised mice by cardiac puncture and antigen-specific serum IgG1, IgG2c and total IgG antibodies were quantified by ELISA. IgA antibody titres in lung homogenates were also quantified by the same ELISA protocol.

Medium binding certified 96-well plates were coated with 50 µl/well of FHA (1 µg/ml), Prn (1 µg/ml), PT (1 µg/ml) or *B. pertussis* sonicate (5 µg/ml) and incubated overnight at 4^{o}C. Plates were then washed in wash buffer 4 times and non-specific binding sites blocked by adding 180 µl of blocking solution (0.1% BSA in PBS) for 2 hours at room temperature. After 2 hours, plates were washed again in the wash buffer and 50 µl of serum (diluted 1:50 for IgG1 and IgG2c, or 1:200 for IgGtot) or lung homogenate (diluted 1:5) was added to the first column of wells on the 96 well plate. These samples were then serially diluted 1:2 in 0.1% BSA across the plate to generate an 11-point titration curve. Plates were incubated for 2 hours at 37°C. Next, plates were washed in wash buffer 4 times, followed by addition of 50 µl/well of biotinylated IgGtot or IgG1 detection antibody, or HRP-conjugated anti-mouse IgG2c or IgA detection antibody (dilutions as per table 2.1.8b) diluted in 0.1% BSA. Plates were then incubated for 1.5 hours at room temperature in the dark. After incubation, plates were washed in wash buffer 4 times and incubated with 50 µl/well HRP-conjugated streptavidin (BD Pharmingen; 1:1000 in 0.1% BSA) at room temperature for 20 minutes, with the exception of IgG2c and IgA which can be developed immediately as the antibodies are HRP-conjugated. After washing in wash buffer, plates were developed and read using the same method as for cytokine ELISA in section 2.2.15. Antibody levels are expressed as the mean endpoint titre (± SE), determined by extrapolation of the linear part of the titration curve to 2 SE above the background value obtained with non-immune mouse serum.

2.2.21 Preparation of organs for flow cytometry

Isolated lungs were placed on the lid of a petri dish and chopped up finely using a clean sterile scalpel. Lungs were then transferred to a 1.5 ml sterile Eppendorf tube containing 1 ml HBSS and the enzymes Collagenase D and DNAse I. Lungs were incubated for 1 hour at 37°C to facilitate tissue breakdown and release of cells. After incubation, the digested lungs were forced through 70 µM cell strainers using the barrel of a 5 ml syringe. Cells were centrifuged at 1,300 rpm for 5 minutes and the supernatant was removed. The pellet was resuspended in 1 ml of red blood cell lysis buffer to lyse the red blood cells. After 1 minute, 9 ml of cRPMI was added to neutralise the reaction. Cells were centrifuged at 1,300 rpm for 5 minutes and resuspended in an appropriate volume of cRPMI for counting with trypan blue.

Spleens and LNs were processed by forcing them through a 40 µM cell strainer using the barrel of a 5 ml of 1 ml syringe respectively. Cells were centrifuged at 1,300 rpm for 5 minutes and the supernatant was removed. The pellet was resuspended in 1 ml of red blood cell lysis buffer to lyse the red blood cells. After 1 minute, 9 ml of cRPMI was added to neutralise the reaction. Cells were centrifuged at 1,300 rpm for 5 minutes and resuspended in an appropriate volume of cRPMI for counting with trypan blue.

Once counted, if intracellular cytokines were to be analysed, cells were either stimulated for 1 hour with Brefeldin A or 4-6 hours with PMA, Ionomycin and Brefeldin A.

2.2.22 Surface staining for flow cytometry

Single cell suspensions isolated from *in vivo* studies or obtained from *in vitro* cultures, were transferred to FACS tubes at 1 x 10^6 cells/tube. The cells were washed in PBS and centrifuged at 1,300 rpm for 5 minutes. They were incubated with 50 µl of Live/Dead aqua stain for 20 minutes on ice. After incubation, cells were washed in PBS and centrifuged at 1,300 rpm for 5 minutes. They were incubated with surface antibodies (table 2.1.9) at the appropriate dilutions and anti-CD16/CD32 (Fcγ block; 1:200 in PBS) for 20 minutes on ice in the dark. Cells were washed and fixed with 50 µl 2% PFA for 15 minutes at room temperature. After fixing, cells were washed with PBS and resuspended in 150 µl PBS for acquisition. Refer to section 2.2.22 for flow cytometry gating strategies.

2.2.23 Intracellular staining for flow cytometry

Intracellular staining was employed in order to detect soluble antigens such as cytokines. Cells were first stained with surface antibodies, as described in section 2.2.19. After fixing, cells were washed once with PBS and then with 1 ml permeabilisation buffer (0.05% saponin in PBS). They were then stained with 50 µl of intracellular cytokine antibodies appropriately diluted in permeabilisation buffer. After 15 minutes, cells were washed twice with PBS and re-suspended in 150 µl PBS for acquisition. Fluorescence minus one (FMO) and isotype controls were prepared as appropriate.

Stained cell suspensions were acquired using the LSRFortessa (BD) flow cytometer according to the manufactures instructions. The flow cytometer was calibrated using single-stained compensation beads (BD Biosciences, 552843). FACS data analysis was performed using FlowJo (Treestar) software.

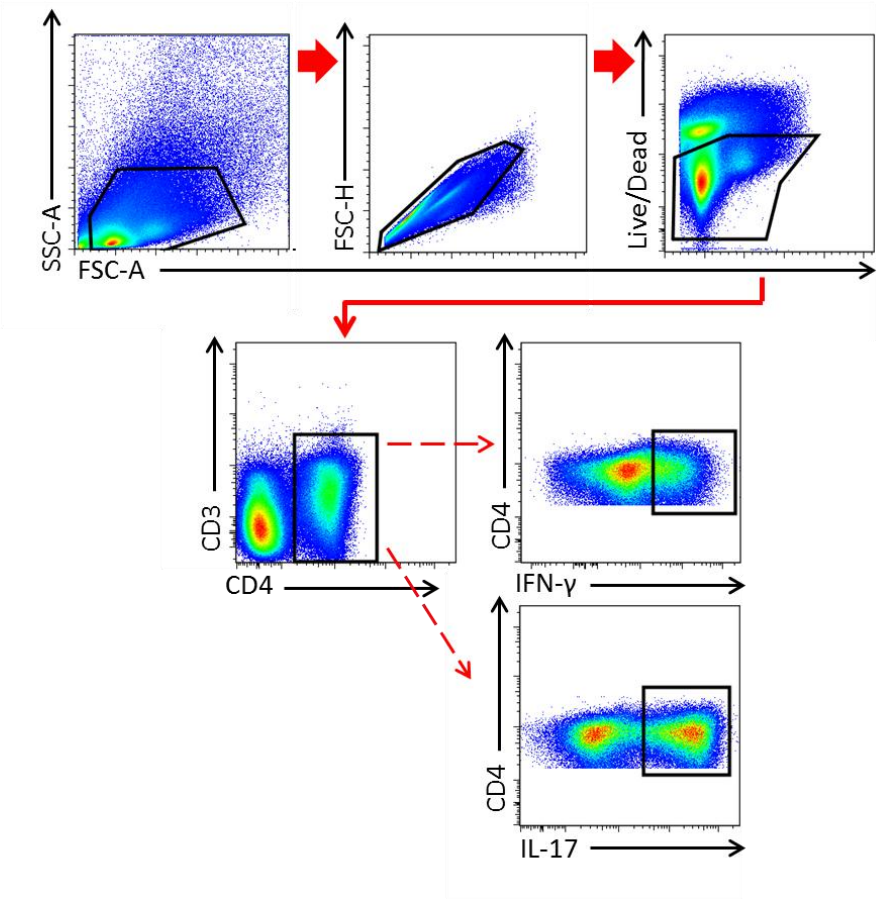
2.2.24 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5. One or two-way analysis of variance (ANOVA) followed by the Bonferroni post test was used to analyse the statistical significance between three or more groups. *P* values less than 0.05 were considered to be statistically significant.

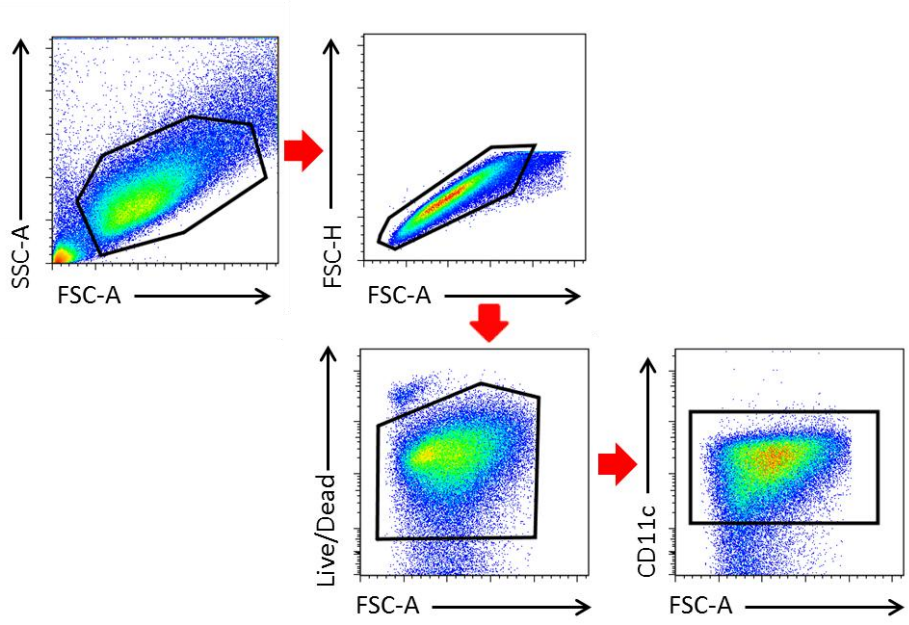
Normality was examined using the Shapiro-Wilk test, however, it was not possible to accurately assess whether the data followed a normal distribution due to small sample sizes in the studies (n=4 for all studies).

2.3 Flow cytometry gating strategies

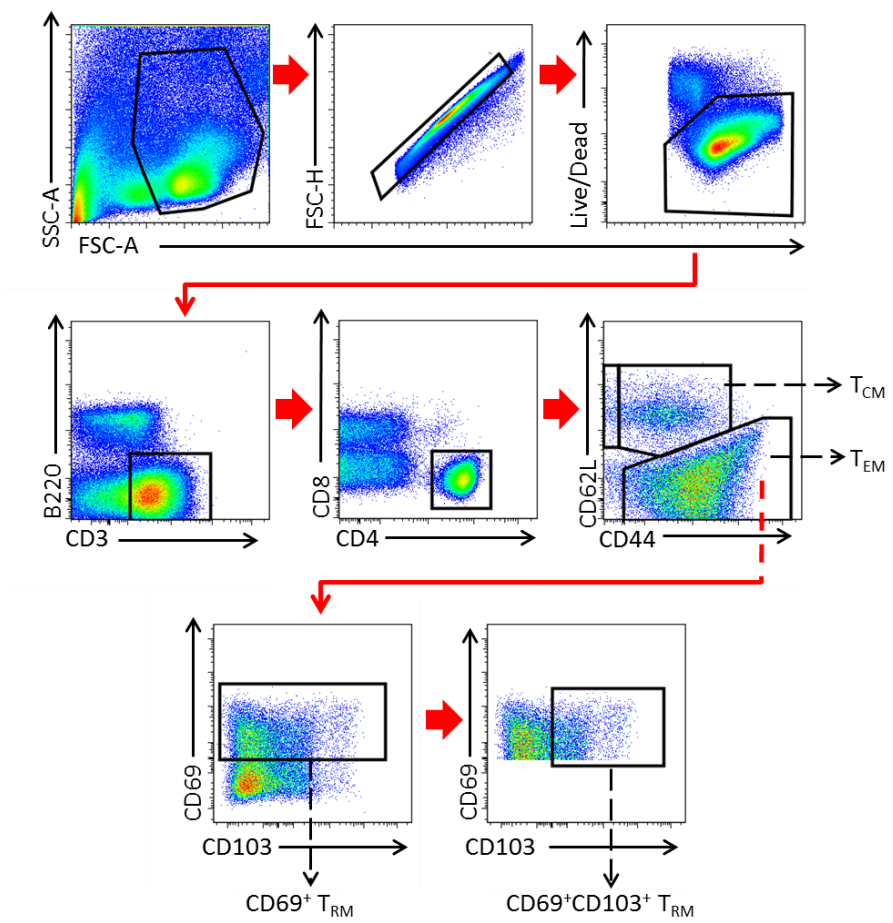
2.3.1 IFN-γ^{+} and IL-17^{+} CD4^{+} T cells



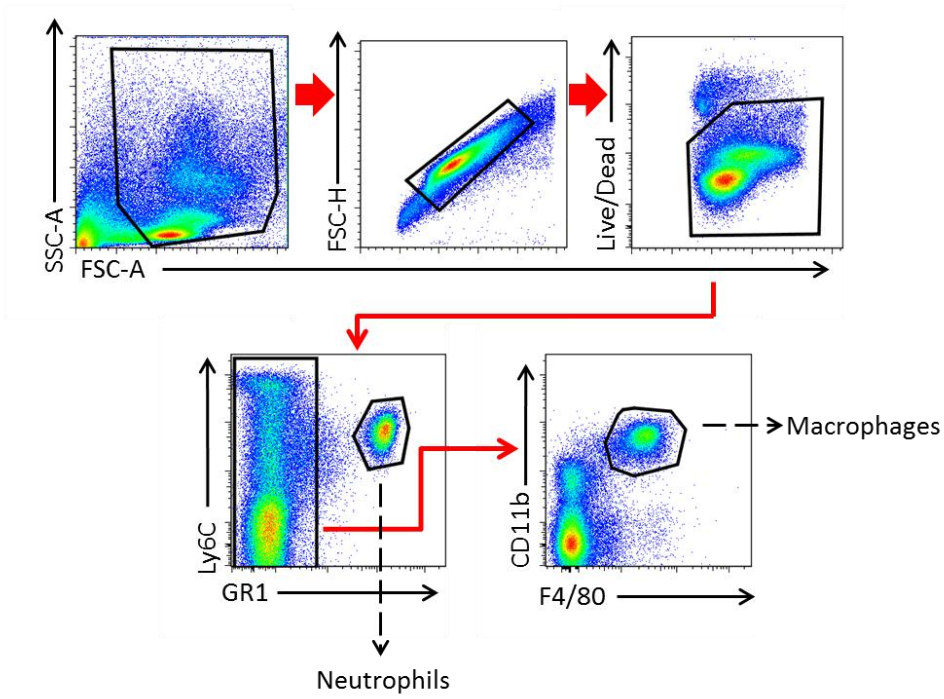
2.3.2 DC maturation



2.3.3 CD4^{+} T_{RM} cells



2.3.4 Macrophage and neutrophil gating



Chapter 3:

The use of TLR agonists as adjuvants for aP vaccines

Chapter 3: The use of TLR agonists as adjuvants for aP vaccines

3.1 Introduction

Whooping cough is a respiratory infection caused by the Gram-negative bacterium *B. pertussis,* which can be lethal in unimmunised infants. Studies have shown that IFN-γ, the key pro-inflammatory cytokine produced by Th1 cells, plays a key role in protective immunity to *B. pertussis* infection [88, 350]. Indeed, mice defective in the IFN-γ receptor suffer from disseminated infection and often die from the disease [2]. Additionally, natural infection with *B. pertussis* induces IL-17 production [351], and there is compelling evidence that Th17 responses are important in addition to Th1 responses in a baboon model of infection [188].

The first pertussis vaccines to be introduced were the wP vaccines, which consisted of a heat or chemically detoxified preparation of the bacteria, administered with or without alum as an adjuvant [8]. These vaccines were very effective as they induced potent Th1 and Th17 responses [189], and a significant decrease in the incidence of pertussis was seen relatively soon after their introduction [19]. However, adverse side effects associated with immunisation with wP vaccines resulted in their replacement with the safer aP vaccines [53-55]. Since the introduction of aP vaccines in the 1990s, the incidence of whooping cough has been increasing in a number of countries with high vaccination coverage, including the Netherlands, England, Australia, and Ireland [211, 212, 218, 352]. One suggested reason for this re-emergence is that the aP vaccine confers ineffective immunity to *B. pertussis,* due to the use of alum as the adjuvant. The alum-adjuvanted aP vaccine predominantly induces a potent Th2 response [189]. While it also has the capacity to induce protective Th17 and antibody responses, it is inefficient at promoting Th1 responses which, in combination with Th17, are required for optimal protection against *B. pertussis* [189]. A number of studies in mouse models have shown that replacing alum with Th1-promoting adjuvants in experimental aP vaccines can enhance protective immunity to *B. pertussis.* Indeed, the Mills laboratory have previously shown that IL-12, the cytokine responsible for driving Th1 differentiation, is an effective adjuvant in a laboratory-prepared aP vaccine, conferring protection against *B. pertussis* infection in a mouse model [302].

The ability to drive potent Th1 and Th17-biased responses are essential characteristics of potential adjuvants for aP vaccines. Several TLR agonists, including CpG oligodeoxynucleotides (TLR9), R848 (TLR7/8) and MPL (TLR4) are capable of acting as adjuvants, driving robust cell-

mediated immunity *in vivo* [353-356]. This property of TLR agonists indicates that they could act as effective adjuvants for an aP vaccine. Indeed, Geurtsen *et al.* previously demonstrated that mice immunised s.c. with an aP vaccine adjuvanted with MPL, were more protected from *B. pertussis* infection when compared with mice immunised with an aP vaccine containing alum [272].

CpG oligodeoxynucleotides from bacterial DNA signal through TLR9 to activate DCs, which induces potent Th1 responses [274], and also promote induction of Th17 cells [357]. Indeed, CpG has already been examined as a vaccine adjuvant in a number of murine models of infectious diseases and cancer and was shown to be effective [358-360]. Furthermore, the safety of CpG has been evaluated in clinical trials, and has been shown to be a suitable adjuvant for human use [361-363].

BP1569 is a novel TLR2 binding lipoprotein isolated from *B. pertussis* that has been shown to have potent immunostimulatory activity *in vitro*; it can induce the production of a number of Th1 and Th17 polarising cytokines by BMDCs [276]. If BP1569 is shown to have adjuvant properties, it could be a very useful addition to an aP vaccine as it is also an antigenic component of *B. pertussis* [276]. Due to difficulty in removing residual LPS, an LPS-free lipopeptide version of BP1569, LP1569, was synthesised to further investigate the properties of this lipoprotein *in vivo*. If LP1569 retains the same properties as BP1569, it could be a very effective adjuvant for an aP vaccine, having dual properties as both a *B. pertussis* antigen and adjuvant.

The primary goal of vaccination is to induce effective and long-lasting immunity to a specific pathogen, however, well-defined biomarkers of long-term protection have yet to be established. The generation of potent humoral immune responses and antibodies were initially thought to mediate protective immunity, however, there is evidence that humoral immunity alone cannot generate long-term protection against *B. pertussis* infection [66-68], and indeed, circulating antibodies titres have been shown to decline to undetectable levels rapidly after immunisation [364, 365]. Th1 and Th17 cells have been shown to play an important role in vaccine-induced immunity to *B. pertussis* in mice [189], and furthermore, a study in a baboon model of infection demonstrated that Th1 and Th17 cells persisted in the circulation for up to 2 years after infection, indicating that the establishment of persistent T cells may be a more appropriate indicator of memory [188].

$CD4^+$ and $CD8^+$ T_{CM} ($CD44^-CD62L^+$), T_{EM} ($CD44^+CD62L^-$) and T_{RM} ($CD44^+CD62L^-CD69^+$) cells are subsets of memory T cells generated during infection, that remain poised to prevent

reinfection, and the role these cells play in protection against numerous pathogens is becoming clearer. Indeed, Sakai *et al.* reported that during *M. tuberculosis* infection, an IFN-γ-producing $CD4^+$ T cell population is generated that becomes localised in the lung parenchyma [196]. Furthermore, adoptive transfer of these T_{RM} cells into T cell deficient ($TCR\alpha^{-/-}$) mice infected with *M. tuberculosis* significantly reduced lung bacterial burden, compared with $TCR\alpha^{-/-}$ mice that received no T cells. Interestingly, there is evidence that vaccination can induce recruitment and proliferation of these $CD4^+$ lung-resident memory T cells. Connor and colleagues observed a significant increase in this population in the lung tissue of mice s.c. immunised with the BCG vaccine [181]. Furthermore, by using fingolimod to inhibit migration of antigen-activated $CD4^+$ T cells out of the LNs after immunisation, they showed that these $CD4^+$ T_{RM} cells were sufficient to protect against BCG i.n. challenge. However, mice treated with fingolimod prior to BCG immunisation were not protected from subsequent challenge, indicating that these memory T cells migrate from the LNs to the lung after immunisation. To date, the memory phenotype of $CD4^+$ T cell subtypes induced with *B. pertussis* infection and vaccination has not been investigated.

The aim of this study was to assess the immunogenicity and protective efficacy of an aP vaccine adjuvanted with either the TLR9 agonist CpG, or the novel TLR2 agonist LP1569, in a mouse model of *B. pertussis* infection. Furthermore, the ability of these experimental aP vaccines formulated with TLR agonists to induce $CD4^+$ T_{RM} cell recruitment to the lungs was examined. The hypothesis being tested was that CpG and LP1569 would elicit more appropriate cellular and humoral immune responses to *B. pertussis* infection than an alum-adjuvanted aP vaccine, and therefore, would generate better protection and confer longer lasting immunity to this pathogen. The primary goal of this study was to identify a more effective adjuvant than alum for promoting appropriate cellular immunity to *B. pertussis.*

3.2 Results

3.2.1 Aerosol challenge with live *B. pertussis* induces Th1 and Th17 cell expansion.

There is considerable evidence that natural infection with *B. pertussis* induces expansion of Th1 and Th17 cells *in vivo* [88, 350, 351]. In order to confirm this, mice were challenged with an aerosol of live *B. pertussis* and the lungs were isolated on day 0, 7, 15 and 21 post infection. Lung cells were stained with antibodies specific for CD4 and CD3 markers and intracellular cytokines, to determine the subtypes of T cells induced by infection, by FACS analysis. The percentages and corresponding numbers of $CD4^+$ T cells producing IFN-γ (Th1 cells) increased by day 21 post infection (Figure 3.1A and 3.1B). A large increase in the percentage and absolute numbers of $CD4^+$ T cells producing IL-17 (Th17) cells was observed by day 15 post infection. These results indicate that both Th1 and Th17 cells expand in the lungs of mice after natural infection with *B. pertussis*.

3.2.2 Immunising mice with a wP vaccine induces a systemic Th1 and Th17 cell response, recruits more $CD4^+$ T_{RM} cells to the lungs, and confers greater protection against *B. pertussis* infection, than an aP vaccine.

Having shown that Th1 and Th17 cells expand in the lungs of mice after aerosol challenge (Figure 3.1A and 3.1B), the ability of an aP and a wP vaccine to induce T cell responses and protect against *B. pertussis* infection was assessed. Mice were immunised i.p. twice at 0 and 4 weeks with a wP or an aP vaccine, or with PBS, and were challenged with live *B. pertussis* 2 weeks after the second immunisation. Mice immunised with a wP vaccine had lower CFU counts in the lungs post challenge than mice immunised with an aP vaccine, indicating they were more protected from *B. pertussis* challenge (Figure 3.2).

In order to investigate the immune responses induced with the two vaccines, the spleens and sera from four mice in each experimental group were taken 1 day before aerosol challenge and their antigen-specific T cell and antibody responses were examined. Spleen cells from mice immunised with a wP vaccine produced significantly more antigen-specific IFN-γ and IL-17 than spleen cells from mice immunised with the aP vaccine (Figure 3.3A). Significantly more antigen-specific IL-5 was produced by spleen cells from mice immunised with an aP vaccine, than spleen cells from mice immunised with a wP vaccine. Mice immunised with a wP vaccine had significantly more *B. pertussis*-specific IgG2c and total IgG titres in sera, than mice

immunised with an aP vaccine (Figure 3.3B). There were no significant differences in the IgG1 antibody titres in sera between mice immunised with an aP vaccine or a wP vaccine.

The lungs were isolated in addition to the spleens and sera 1 day before aerosol challenge to investigate the recruitment of memory T cells after immunisation. Lung cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for analysis by flow cytometry. Mice immunised with a wP vaccine had a marked increase in the percentage of $CD4^+$ T_{EM} cells infiltrating into the lungs when compared with mice immunised with an aP vaccine or control mice (Figure 3.4A). While the percentages of $CD4^+$ T_{RM} and $CD4^+CD103^+$ T_{RM} were similar between the three groups of mice, it was clear there were more cells in the lungs of wP-immunised mice (Figure 3.4B and 3.4C). Consistently, the absolute numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells recruited to the lungs of mice immunised with a wP vaccine were significantly elevated compared with the PBS-immunised control mice, or mice immunised with an aP vaccine (Figure 3.4D). Indeed, mice immunised with an aP vaccine had similar numbers of these $CD4^+$ memory T cell subtypes in the lungs to control mice. These findings indicate that immunisation with a wP vaccine recruits significantly more $CD4^+$ T_{EM} and T_{RM} cells to the lungs, than immunisation with an aP vaccine. Furthermore, immunisation with an aP vaccine does not enhance infiltration of $CD4^+$ T_{RM} cells to the lungs over the numbers seen in naïve mice.

To examine if this dramatic increase in lung memory T cells recruited by wP immunisation is maintained after infection, mice were aerosol challenged with *B. pertussis* 2 weeks after the second immunisation and 7 days after challenge, lung cells were stained with markers for memory T cells. After aerosol challenge, the percentages of $CD4^+$ $T_{EM,}$ T_{RM} and $CD103^+$ T_{RM} cells in the lungs of mice immunised with a wP vaccine were considerably greater than those seen in aP-immunised mice or the PBS-immunised control mice (Figure 3.5A, 3.5B and 3.5C). The corresponding numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs after infection was consistent with the percentages, with numbers of these $CD4^+$ memory T cell subtypes in the lungs being greatest in mice immunised with a wP vaccine (Figure 3.5D). Interestingly, the numbers of $CD4^+$ T_{EM} and T_{RM} cells in the lungs of the PBS-immunised control mice 7 days after aerosol challenge appeared to be slightly elevated when compared with those in the lungs of aP-immunised mice. This may suggest that immunisation with aP vaccines inhibits recruitment of T_{RM} cells to the lungs after natural infection.

Overall, the results of this study indicate that immunising mice with a wP vaccine induces greater Th1, Th17 and IgG2c responses, and confers greater protection against *B. pertussis* infection, than immunising mice with an aP vaccine. Furthermore, immunisation with a wP

vaccine recruits significantly more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells to the lungs of mice, than immunisation with an aP vaccine, and this enhancement is maintained after *B. pertussis* challenge.

3.2.3 The TLR9 agonist CpG activates DCs and induces production of Th1 and Th17 polarising cytokines.

The results from the previous two studies showed that Th1 and Th17 cells were expanded in the lungs of mice after aerosol challenge (Figure 3.1A and 3.1B), and that mice immunised with an efficacious wP vaccine induced more systemic Th1 and Th17 cell responses than a less protective aP vaccine (Figure 3.2A and 3.3A). This suggested that an effective adjuvant for an aP vaccine should induce potent antigen-specific IFN-γ and IL-17 responses. As it has been reported that the TLR9 agonist CpG can induce potent Th1 and Th17 responses to other antigens [274, 357], it was hypothesised that it could potentially act as an effective adjuvant for an aP vaccine.

To first investigate if the TLR9 agonist CpG can activate murine DCs and induce production of Th1 and Th17 polarising cytokines, BMDCs were stimulated with CpG for 24 hours and the expression of T cell co-stimulatory markers, and the production of T cell polarising cytokines, was examined. CpG-stimulated BMDCs had enhanced expression of CD80, CD86, CD40 and MHC class II when compared with DCs stimulated with medium only (Figure 3.6A). CpG also induced the production of IL-12p40, IL-12p70, IL-1β, IL-23 and IL-6 production by murine DCs (Figure 3.6B). These results indicate that CpG can activate BMDCs and induce the production of Th1 and Th17 polarising cytokines.

3.2.4 CpG is a more effective adjuvant than alum for an aP vaccine, inducing antigen-specific IFN-γ, IL-17 and IgG2c responses, and conferring as good protection against B. pertussis infection as a wP vaccine.

Having shown that CpG induced Th1 and Th17 polarising cytokine production *in vitro* (Figure 3.6B), the ability of CpG to act as an adjuvant for an aP vaccine *in vivo* was examined. Mice were immunised i.p. twice at 0 and 3 weeks with an experimental CpG-adjuvanted aP vaccine (Ag + CpG), an experimental alum-adjuvanted aP vaccine (Ag + alum) or PBS as a control and were challenged with live *B. pertussis* 1 week after the last immunisation. Mice that were immunised with Ag + CpG before challenge had significantly lower CFU counts in the lungs post

challenge than mice that were immunised with Ag + alum, indicating they were more protected from *B. pertussis* infection (Figure 3.7A). Analysis of the areas under the curves (Figure 3.7B) confirmed that immunisation with Ag + CpG (28.63) was more effective at conferring protection against *B. pertussis* infection than immunisation with Ag + alum (64.20). All the immunised mice were more protected from aerosol challenge than the control mice.

Having shown that an experimental CpG-adjuvanted aP vaccine was more effective than an experimental alum-adjuvanted aP vaccine at conferring protection against *B. pertussis* infection (Figure 3.7A), the efficacy of an experimental CpG-adjuvanted aP vaccine was compared with that of a wP vaccine. Mice were immunised i.p. twice at 0 and 3 weeks with an experimental CpG-adjuvanted aP vaccine (Ag + CpG), an experimental alum-adjuvanted aP vaccine (Ag + alum), a commercial wP vaccine or PBS as a control and were challenged with live *B. pertussis* 1 week after the last immunisation. Mice immunised with Ag + CpG before aerosol challenge had similar bacterial burden in the lungs post challenge to mice immunised with a wP vaccine, and had significantly lower CFU counts in the lungs on days 3 and 7 post infection than mice that were immunised with Ag + alum (Figure 3.8A). Analysis of the areas under the curves (Figure 3.8B) confirmed that mice immunised with Ag + CpG (13.10) were as protected from *B. pertussis* challenge as mice immunised with a wP vaccine (15.04), and were more protected than mice immunised with Ag + alum (37.66) before challenge. All immunised mice were significantly more protected from *B. pertussis* infection than the control mice.

To examine the immune responses induced with the vaccines, the spleens and sera from four mice in each experimental group were isolated 1 day prior to aerosol challenge and their antigen-specific T cell and antibody responses were examined. Spleen cells from mice immunised with Ag + CpG produced significantly more antigen-specific IFN-γ and IL-17 than spleen cells from mice immunised with Ag + alum (Figure 3.9A). Significantly more *B. pertussis*-specific IFN-γ and IL-17 was produced by spleen cells from mice immunised with a wP vaccine, than mice immunised with Ag + CpG or Ag + alum. Spleen cells from mice immunised with Ag + alum produced considerably more antigen-specific IL-5 than mice immunised with Ag + CpG or a wP vaccine. Mice immunised with Ag + CpG had significantly more IgG2c antibodies in sera compared with mice immunised with Ag + alum, but these titres were not as high as those observed in the sera of mice immunised with a wP vaccine (Figure 3.9B). Robust antigen-specific IgG1 titres were seen in the sera of mice immunised with Ag + alum and a wP vaccine. Mice immunised with a wP vaccine had the highest total IgG titres in sera, and mice immunised with Ag + CpG and Ag + alum had similar levels of serum total IgG antibodies.

Having shown that a Th1/Th17-promoting wP vaccine induces greater memory T cell recruitment to the lungs than an aP vaccine (Figure 3.4 and 3.5), it was hypothesised that substitution of alum for the Th1-promoting adjuvant CpG in an aP vaccine would enhance infiltration of memory T cells into the lungs. The lungs were isolated in addition to spleens and sera 1 day before aerosol challenge and lung cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for analysis by flow cytometry. Mice immunised with Ag + CpG had similar percentages of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} recruited to the lungs after immunisation as mice immunised with Ag + alum (Figure 3.10A, 3.10B and 3.10C). In contrast, wP-immunised mice had substantially higher percentages of memory T cells recruited to the lungs, than mice immunised with Ag + alum or Ag + CpG. The absolute numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} infiltrating into the lungs was comparable between mice immunised with Ag + alum and Ag + CpG, however, the numbers of these memory T cell subtypes recruited to the lungs of mice immunised with a wP vaccine was significantly greater (Figure 3.10D). Moreover, the numbers of memory T cells recruited to the lungs of mice after immunisation with Ag + CpG and Ag + alum, was the same as numbers in the lungs of the PBS-immunised control mice. These results indicate that immunising mice with an experimental aP vaccine adjuvanted with CpG is no more effective at recruiting $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} to the lung tissues, than immunising with an experimental alum-adjuvanted aP vaccine.

The results of this study indicate that an experimental CpG-adjuvanted aP vaccine is more effective than an experimental alum-adjuvanted aP vaccine, and is as effective as a wP vaccine, at conferring protection against *B. pertussis* infection in mice. Furthermore, it was found that an experimental aP vaccine formulated with CpG induces robust Th1, Th17 and IgG2c responses *in vivo*. However, immunisation with an experimental CpG-adjuvanted aP vaccine does not recruit more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} to the lungs than immunisation with an experimental alum-adjuvanted aP vaccine.

3.2.5 An aP vaccine formulated with CpG protects mice from *B. pertussis* infection through IFN-γ-dependent mechanisms.

Having shown that an experimental CpG-adjuvanted aP vaccine induced Th1 and Th17 cell responses (Figure 3.9A) and enhanced antigen-specific IgG2c antibody titres in sera *in vivo* (Figure 3.9B), experiments were designed to examine if protection conferred with an experimental CpG-adjuvanted aP vaccine was mediated through IFN-γ or IL-17-dependent mechanisms. WT, IFN-$\gamma^{-/-}$ and IL-$17^{-/-}$ mice were immunised i.p. twice with a laboratory-

prepared aP vaccine formulated with CpG (Ag + CpG), and were aerosol challenged 2 weeks after with live *B. pertussis*. WT and IL-17$^{-/-}$ mice immunised with Ag + CpG had similar bacterial burden in the lungs post challenge on all days examined (Figure 3.11A). In contrast, IFN-γ$^{-/-}$ mice immunised with Ag + CpG had significantly higher CFU counts in the lungs on days 3 and 14 post challenge than WT mice. Analysing the areas under the curves (Figure 3.11B) revealed that WT mice immunised with Ag + CpG were the most protected from *B. pertussis* infection (22.84) and then the IL-17$^{-/-}$ mice (25.17), whereas the IFN-γ$^{-/-}$ mice were the least protected from infection (58.16). This result indicates that IFN-γ is a crucial component of the immune response to *B. pertussis* infection in mice.

The pro-inflammatory cytokines IFN-γ and IL-17 are well known to function in the recruitment and activation of various innate immune cells, predominantly macrophages and neutrophils respectively [101, 116]. Therefore, the numbers of neutrophils and macrophages in the lungs of the immunised WT and knockout mice were examined by flow cytometry post aerosol challenge with *B. pertussis*. Immunisation of WT and IL-17$^{-/-}$ mice with Ag + CpG enhanced macrophage numbers in the lungs on day 3 post challenge, when compared with the PBS-immunised control mice (Figure 3.11C). In contrast, the IFN-γ$^{-/-}$ mice immunised with Ag + CpG had similar numbers of macrophages in the lungs to the PBS-immunised control mice. Neutrophil numbers were similar between the control mice, and the WT and IFN-γ$^{-/-}$ mice immunised with Ag + CpG, whereas the IL-17$^{-/-}$ mice immunised with Ag + CpG had two-fold lower numbers of neutrophils in the lungs post challenge, although this did not reach statistical significance.

The findings here suggest than an experimental CpG-adjuvanted aP vaccine confers protection by promoting macrophage recruitment to the lungs, and that IFN-γ is important in facilitating macrophage recruitment or expansion. In contrast, neutrophils do not appear to play as crucial a role in *B. pertussis* clearance. Overall the results of this study indicate that an experimental aP vaccine formulated with CpG protects mice from *B. pertussis* infection through IFN-γ-dependent mechanisms.

3.2.6 The TLR2 agonist LP1569 activates murine DCs and induces pro-inflammatory cytokine production.

BP1569, a novel TLR2 binding lipoprotein from *B. pertussis* identified by the Mills laboratory, was shown to have immunostimulatory properties *in vitro* and to be an antigenic component

of *B. pertussis* [276]. However, completely removing LPS from BP1569 proved difficult and in order to carry out more extensive studies, LP1569, an LPS-free lipopeptide version of BP1569 was synthesised.

To examine if LP1569 could activate innate immune cells and induce production of Th1 and Th17 polarising cytokines similar to BP1569, BMDCs were stimulated with increasing concentrations of LP1569 for 24 hours. LP1569 enhanced expression of CD80, CD86, CD40 and MHC class II on DCs (Figure 3.12A). LP1569 also induced production of IL-12p40, IL-1β and IL-6 in a dose dependent manner (Figure 3.12B). These findings indicate that LP1569 is capable of inducing the production of innate Th1 and Th17 polarising cytokines, and expression of CD80, CD86, CD40 and MHC class II on BMDCs.

3.2.7 LP1569 acts as an effective adjuvant for an experimental aP vaccine, inducing potent antigen-specific IFN-γ, IL-17 and IgG2c production, and protecting mice from *B. pertussis* infection.

Having shown that the TLR2 agonist LP1569 drives production of T cell polarising cytokines by DCs (Figure 3.12B), the capacity of LP1569 to act as an adjuvant for an experimental aP vaccine *in vivo* and confer protection against *B. pertussis* infection was examined. Mice were immunised i.p. twice at 0 and 5 weeks with PBS as a control, or an experimental aP vaccine consisting of FHA, Prn and dPT alone with no adjuvant (Ag) or formulated with LP1569 as the adjuvant (Ag + LP1569), before aerosol challenge with live *B. pertussis*.

Mice immunised with Ag + LP1569 had no detectable bacteria in the lungs on days 3, 7 and 10 post infection, indicating they were significantly protected from *B. pertussis* infection (Figure 3.13A). In contrast, mice immunised with Ag had significantly more *B. pertussis* colonisation in the lungs post challenge compared with mice immunised with Ag + LP1569, and indeed had similar lung CFU counts to the PBS-immunised control mice on day 14 post challenge. Analysing the areas under the curves (Figure 3.13B) confirmed that mice immunised with Ag + LP1569 (5.507) were the most protected from *B. pertussis* infection, but that mice immunised with Ag without adjuvant (28.56) were more protected from infection than the PBS-immunised control mice (44.08).

In order to examine the immune responses induced with Ag + LP1569, the spleens and sera from four immunised mice in each experimental group were isolated 1 day prior to aerosol challenge and antigen-specific T cell and antibody responses were examined. Spleen cells from

mice immunised with Ag + LP1569 had significantly enhanced FHA and Prn-specific IFN-γ and IL-17 production, and significantly reduced IL-5 production, when compared with mice immunised with Ag alone or PBS (Figure 3.14A). FHA-specific IgG2c, IgG1 and total IgG antibody titres in sera were significantly higher in mice immunised with Ag + LP1569, than mice immunised with Ag alone (Figure 3.14B). Taken together, the findings of this study indicate that the TLR2 agonist LP1569 is an effective adjuvant for an experimental aP vaccine, inducing potent Th1, Th17 and IgG2c responses in mice.

3.2.8 Immunising mice with an aP vaccine formulated with LP1569 induces robust antigen-specific IFN-γ, IL-17 and IgG2c responses, and confers greater protection against *B. pertussis* infection than immunising mice with an alum-adjuvanted aP vaccine.

Having shown that LP1569 has adjuvant activity *in vivo* (Figure 3.13A), the efficacy of an aP vaccine adjuvanted with LP1569 was compared with that of an alum-adjuvanted aP vaccine. Mice were immunised i.p. twice at 0 and 4 weeks with PBS as a control, or an experimental aP vaccine formulated with either LP1569 (Ag + LP1569) or alum (Ag + alum) before aerosol challenge with live *B. pertussis.* Mice immunised with Ag + LP1569 appeared to be more protected from *B. pertussis* early in infection than mice immunised with Ag + alum, however by day 14 post infection, both groups of immunised mice had similar CFU counts in the lungs (Figure 3.15A). Analysis of the areas under the curves (Figure 3.15B) confirmed that mice immunised with Ag + LP1569 (39.36) were more protected from *B. pertussis* infection than mice immunised with Ag + alum (47.58). All immunised mice were significantly more protected from *B. pertussis* challenge than the control mice.

In order to examine the immune responses induced with the vaccines, four mice in each experimental group were sacrificed 1 day prior to aerosol challenge and spleens and sera were collected. Spleen cells from mice immunised with Ag + LP1569 produced significantly more FHA-specific IFN-γ and IL-17, and less antigen-specific IL-5, than spleen cells from mice immunised with Ag + alum (Figure 3.16A). Mice immunised with Ag + LP1569 also had significantly higher IgG2c antibody titres in sera than mice immunised with Ag + alum, however, serum IgG1 and total IgG levels were similar between the two immunised groups of mice (Figure 3.16B). The results of this study indicate that an experimental aP vaccine formulated with LP1569 induces robust Th1, Th17 and IgG2c responses, and confers greater protection against *B. pertussis* infection than an experimental alum-adjuvanted aP vaccine.

3.2.9 Addition of CpG or LP1569 to a commercial aP vaccine enhances antigen-specific IFN-γ and IgG2c production.

Having identified that both CpG and LP1569 are effective adjuvants for experimental aP vaccines (Figure 3.7A, 3.8A, 3.13A and 3.15A), it was investigated if addition of these adjuvants to the commercial aP vaccine which is already formulated with alum (Infanrix), could improve its efficacy and enhance *B. pertussis* clearance in immunised mice. The premise here was that childhood immunisation against pertussis involves administration of a combination vaccine against a number of diseases. Unbundling this vaccine to replace alum with a more suitable Th1-promoting adjuvant for the pertussis component would be a difficult undertaking, whereas addition of a Th1/Th17-promoting adjuvant to the commercial aP vaccine already containing alum might be a more suitable option if it could enhance Th1 responses.

Mice were immunised twice at 0 and 4 weeks with the commercial aP vaccine formulated with alum (Infanrix; aP + alum), the commercial aP vaccine supplemented with either CpG (aP + alum + CpG) or LP1569 (aP + alum + LP1569), or with PBS as a control, and were aerosol challenged with live *B. pertussis* 2 weeks after the last immunisation. All immunised mice were significantly more protected from *B. pertussis* challenge than the PBS-immunised control mice (Figure 3.17A). There were no significant differences in the CFU counts of mice immunised with aP + alum or aP + alum + LP1569. On day 3 post infection, the bacterial burden in the lungs was significantly lower in mice immunised with aP + alum + CpG compared with mice immunised with aP + alum. All immunised mice cleared *B. pertussis* infection by day 7, with the exception of one mouse immunised with aP + alum that had detectable bacteria in the lungs on day 10 post challenge. Analysis of the areas under the curves (Figure 3.17B) indicated that mice immunised with aP + alum + CpG (8.063) were more protected from *B. pertussis* infection, than mice immunised with aP + alum + LP1569 (10.34), or mice immunised with aP + alum (16.00).

To examine the T cell and antibody responses induced with the aP vaccines supplemented with different adjuvants, inguinal LNs and sera were isolated from four mice in each experimental group 1 day before aerosol challenge. LN cells from mice immunised with aP + alum + CpG or aP + alum + LP1569 had significantly enhanced FHA-specific IFN-γ, and reduced antigen-specific IL-5 production when compared with LN cells from mice immunised with aP + alum (Figure 3.18A). No antigen-specific IL-17 was detected in any group. Mice immunised with aP + alum + CpG and aP + alum + LP1569 had more antigen-specific IgG2c antibodies in sera compared with mice immunised with aP + alum, however, these did not reach statistical significance (Figure 3.18B). Antigen-specific IgG1 titres in sera were as robust in mice

immunised with aP + alum + LP1569 as mice immunised with aP + alum. These findings suggest that addition of CpG or LP1569 to the commercial alum-adjuvanted aP vaccine Infanrix enhances Th1 and IgG2c responses, and simultaneously reduces Th2 responses.

3.2.10 An experimental aP vaccine adjuvanted with LP1569 induces more potent cellular and humoral immune responses in mice than an aP vaccine containing CpG.

The results from the studies so far indicate that both CpG and LP1569 are potent Th1-promoting adjuvants (Figure 3.9A, 3.14A and 3.16A), capable of conferring protection against *B. pertussis* infection (Figure 3.7A, 3.8A, 3.13A and 3.15A). To determine if CpG or LP1569 is a more effective adjuvant, mice were immunised i.p. twice at 0 and 4 weeks with an experimental aP vaccine adjuvanted with CpG (Ag + CpG), or LP1569 (Ag + LP1569), or with PBS as a control and were aerosol challenged with live *B. pertussis* 2 weeks after the last immunisation. Mice immunised with Ag + CpG and Ag + LP1569 had comparable levels of *B. pertussis* colonisation in the lungs post infection (Figure 3.19A), and analysis of the areas under the curves (Figure 3.19B) supported the finding that mice immunised with Ag + CpG (21.02) and mice immunised with Ag + LP1569 (18.30) were similarly protected from infection.

To examine which adjuvant induced more potent T cell and antibody responses, spleens and sera were isolated from four mice in each experimental group 1 day before aerosol challenge. Spleen cells from mice immunised with Ag + LP1569 produced more antigen-specific IFN-γ and IL-17 than spleen cells from mice immunised with Ag + CpG (Figure 3.20A). Spleen cells from mice immunised with Ag + CpG produced significantly more antigen-specific IL-5 than cells from mice immunised with Ag + LP1569, however these levels were still very low. FHA-specific IgG2c titres in sera were similar between mice immunised with Ag + CpG and Ag + LP1569, however, IgG1 antibody levels in the sera of mice immunised with Ag + LP1569 were significantly higher than in the sera of mice immunised with Ag + CpG (Figure 3.20B). The findings of this study indicate that LP1569 is a more potent adjuvant that CpG, inducing more robust Th1 and Th17 cell responses.

3.2.11 A single immunisation with an experimental LP1569-formulated aP vaccine recruits more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells to the lungs than an experimental alum-adjuvanted aP vaccine.

Having shown that LP1569 is a potent adjuvant for an aP vaccine (Figure 3.13, 3.15 and 3.19), capable of promoting potent Th1 and Th17 cell responses *in vivo* (Figure 3.14A, 3.16A and 3.20A), it was investigated if a single immunisation with an experimental aP vaccine formulated with LP1569 was sufficient to more efficiently protect mice from *B. pertussis* infection than an experimental alum-adjuvanted aP vaccine. Mice were immunised i.p. once with PBS as a control, or an experimental aP vaccine composed of two *B. pertussis* antigens (FHA and rPT) formulated with alum (Ag + alum) or LP1569 (Ag + LP1569). 3 weeks after immunisation, mice were aerosol challenged with live *B. pertussis.* Mice immunised with Ag + LP1569 and Ag + alum had similar CFU counts in the lungs after challenge, and there were no significant differences between them on any days examined (Figure 3.21A). Analysis of the areas under the curve (Figure 3.21B) indicated that mice immunised with Ag + alum (76.04) were more protected from aerosol challenge than mice immunised with Ag + LP1569 (86.47). The spleens and sera were isolated 1 day before aerosol challenge to examine the antigen-specific T cell and antibody responses, however, no cytokine production by spleen cells, or serum IgG antibodies were detected in any of the mice.

It was previously shown that an experimental CpG-adjuvanted aP vaccine could not induce greater infiltration of memory T cells into the lungs than an experimental alum-adjuvanted aP vaccine (Figure 3.10). Having shown that LP1569 is a more potent inducer of both Th1 and Th17 cell responses than CpG (Figure 3.20A), it was investigated if an experimental LP1569-formulated aP vaccine was more capable of recruiting $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} to the lung tissues than an experimental alum-adjuvanted aP vaccine. 10 days after aerosol challenge, the lungs were isolated from four mice in each experimental group and lung cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for flow cytometry analysis. Mice immunised with Ag + LP1569 had considerably more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs after *B. pertussis* challenge than mice immunised with Ag + alum (Figure 3.22A, 3.22B and 3.22C). Interestingly, the infected PBS-immunised control mice also had higher percentages of these memory T cells in the lungs than mice immunised with Ag + alum. This trend was reflected in the absolute numbers, supporting the data that unimmunised, infected mice have greater numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs than mice immunised with Ag + alum (Figure 3.22D). The results of this study suggest that immunising mice with an experimental aP vaccine formulated with LP1569 recruits more $CD4^+$ T_{EM}, T_{RM} and

$CD103^+$ T_{RM} cells to the lungs than immunising mice with an experimental alum-adjuvanted aP vaccine, and following aerosol challenge with *B. pertussis,* these populations expand to a greater extent. Furthermore, infected control mice have more memory T cells in the lungs than infected mice that were immunised with an experimental alum-adjuvanted aP vaccine, indicating that the Th2 response induced with this experimental vaccine may inhibit recruitment of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells to the lungs.

Overall, the findings from this study indicate that while immunising mice twice with an experimental LP1569-adjuvanted aP vaccine confers greater protection against *B. pertussis* infection than immunising twice with an experimental alum-adjuvanted aP vaccine, a single immunisation is not sufficient to confer enhanced protective immunity. Interestingly, immunisation with an experimental aP vaccine formulated with LP1569 induces the infiltration of substantially more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells into the lungs after aerosol challenge with *B. pertussis,* than does immunisation with an experimental alum-adjuvanted aP vaccine.

3.3 Discussion

As Th1 and Th17 cells have been shown to be involved in mediating clearance of *B. pertussis* from the respiratory tract [64, 189], an effective adjuvant for an aP vaccine should be capable of inducing these T cell subtypes *in vivo*. In this study, two TLR agonists, CpG and LP1569, were shown to potently induce cellular immune responses and to be effective adjuvants for aP vaccines.

Consistent with previous studies [274, 357], the TLR9 agonist CpG was shown to activate murine DCs and induce production of IL-12p40, IL-12p70, IL-1β, IL-23 and IL-6 *in vitro*. Additionally, the results from the current study demonstrated that CpG was an effective adjuvant for an aP vaccine, capable of inducing potent Th1, Th17 and IgG2c responses *in vivo*. Furthermore, not only were mice immunised with an experimental CpG-adjuvanted aP vaccine significantly more protected from *B. pertussis* infection than mice immunised with an experimental alum-adjuvanted aP vaccine, but they were as protected as mice immunised with a wP vaccine. This study also showed that IFN-$\gamma^{-/-}$ mice immunised with an experimental CpG-adjuvanted aP vaccine were more susceptible to aerosol challenge with *B. pertussis* than IL-$17^{-/-}$ mice, whereas IL-$17^{-/-}$ mice that were immunised with an experimental CpG-adjuvanted aP vaccine had similar bacterial burden in the lungs post challenge to immunised WT mice. These findings indicate that CpG confers protection against *B. pertussis* infection by inducing IFN-γ *in vivo* and in the absence of IFN-γ, the protective effect of CpG is lost.

It has been reported that IFN-γ is crucial for activating macrophages to kill intracellular bacteria in *B. pertussis* infection [2], and consistent with this, immunised IFN-$\gamma^{-/-}$ mice had decreased numbers of macrophages in the lungs when compared with the immunised WT and IL-$17^{-/-}$ mice. Together, these results support a previous study that demonstrated that a CpG-adjuvanted aP vaccine facilitates macrophage recruitment and activation. Indeed, Asokanathan *et al.* showed that mice immunised with an experimental aP vaccine consisting of FHA, Prn and dPT formulated with CpG, had enhanced NO production by macrophages compared with mice immunised with these antigens alone, or formulated with alum [244]. Furthermore, this enhanced macrophage activation correlated with increased protection against *B. pertussis* challenge.

The present study here also showed that IL-$17^{-/-}$ mice immunised with an experimental CpG-adjuvanted aP vaccine had decreased neutrophil recruitment to the lungs, but were still as protected as the WT mice. This result suggested that neutrophils are not as important as macrophages in aP vaccine-mediated immunity. This is consistent with the observations of

Andreasen *et al.* who showed neutrophils do not play a huge role in primary infection of mice, but do play a role in previously infected mice [77]. Together these results demonstrate that an experimental CpG-adjuvanted aP vaccine is more effective than an experimental alum-adjuvanted aP vaccine at conferring protection against *B. pertussis* infection by inducing IFN-γ production and enhancing macrophage responses.

LP1569, the novel TLR2 agonist lipopeptide from *B. pertussis,* was shown to induce production of Th1 and Th17 polarising cytokines by DCs *in vitro* and was an effective adjuvant for an aP vaccine *in vivo.* Mice immunised with an experimental aP vaccine supplemented with LP1569 were significantly more protected from aerosol challenge with *B. pertussis* and had enhanced cell-mediated and humoral immune responses than mice immunised with an aP vaccine lacking the adjuvant. Furthermore, an experimental LP1569-adjuvanted aP vaccine was found to significantly enhance antigen-specific IFN-γ and IL-17 production in the spleen when compared with an experimental alum-adjuvanted aP vaccine.

Current aP vaccines are administered as part of a combination vaccine for childhood immunisation and unbundling this vaccine to replace alum as the adjuvant for the pertussis component would be a difficult task. It would be preferable to add a Th1-promoting adjuvant, such as CpG or LP1569, to the commercial aP vaccine already containing alum as this may skew the immune response induced away from Th2 and towards Th1, thus enhancing the efficacy of the vaccine. A previous study by Asokanathan and colleagues demonstrated that immunisation of mice with several *B. pertussis* antigens formulated with a combination of CpG and alum as adjuvants induced strong peritoneal macrophage activation and IFN-γ production by spleen cells [244]. Furthermore, mice immunised with this experimental aP vaccine adjuvanted with CpG and alum had no detectable bacteria in the lungs 7 days after aerosol challenge, whereas mice immunised with the alum-adjuvanted aP vaccine were still colonised with *B. pertussis*. Moreover, Sugai *et al.* showed that mice immunised three times with DPT formulated with both alum and CpG had significantly enhanced IgG2a in sera compared with mice immunised with a DPT-alum combination [366]. Consistent with these reports, the present study demonstrated that mice immunised with a commercial alum-adjuvanted aP vaccine combined with CpG or LP1569, had enhanced antigen-specific IFN-γ, reduced IL-5 production and higher serum IgG2c antibody titres than mice immunised with the commercial alum-adjuvanted aP vaccine alone, indicating that immunisation with CpG and LP1569 could promote Th1 responses, even in the presence of Th2-driving adjuvants. However, an increase in protective efficacy was difficult to observe due to the high protection already conferred with the commercial aP vaccine alone in this experiment.

These experiments complemented previous literature that CpG is an effective adjuvant for aP vaccines, and provided new data on the potential of LP1569 as an alternative adjuvant for alum in an aP vaccine. Both CpG and LP1569 are capable of inducing potent Th1 and Th17 responses *in vivo* resulting in efficient clearance of *B. pertussis* infection. CpG and LP1569 are also capable of skewing the immune response away from Th2 when added to the commercial aP vaccine Infanrix which is already formulated with alum. Additionally, CpG has already been evaluated for use in humans in several clinical trials including phase III for cancer [367] and phase I/II for Hepatitis B [361, 368] where it proved safe and effective at increasing the immunogenicity of co-administered antigens. Finally, a direct comparison of the two adjuvants showed that LP1569 elicited more robust Th1 and Th17 cell responses against *B. pertussis* antigens than CpG. This may be due to the potential property of LP1569 as a *B. pertussis* antigen, in addition to its adjuvant activity. Despite the potent *in vivo* activity of LP1569, a single immunisation with an experimental aP vaccine formulated with the TLR2 agonist, was not more effective at protecting mice from *B. pertussis* infection than an experimental alum-adjuvanted aP vaccine.

There is evidence that wP vaccines induce more durable protection against *B. pertussis* infection than aP vaccines [187], however, the mechanism of protection has not been well characterised. The present study found that a wP vaccine recruited significantly more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells to the lungs than a commercial aP vaccine, when mice were examined before and after *B. pertussis* challenge. As the T cell responses induced with these two vaccines were very distinct, it was postulated that the induction of these memory T cells was dependent on potent Th1 and Th17 cell responses, which did not occur following immunisation with an aP vaccine. Therefore, it was hypothesised that an aP vaccine containing a Th1-promoting adjuvant would facilitate greater memory T cell recruitment to the lungs than an alum-adjuvanted aP vaccine. However, an experimental CpG-adjuvanted aP vaccine, which induced potent Th1 responses, was shown to be no more capable of enhancing infiltration of $CD4^+$ memory T cells into the lungs than that seen with an experimental alum-adjuvanted aP vaccine. In contrast, mice immunised with an experimental aP vaccine formulated with LP1569 had significantly more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs after *B. pertussis* challenge, than mice immunised with an experimental alum-adjuvanted aP vaccine. As LP1569 was shown to be a more potent inducer of Th17 responses than CpG, it is possible that IL-17, and not IFN-γ, is the key cytokine involved in establishing $CD4^+$ memory T cell populations. Indeed, Christensen *et al.* observed that depleting IL-17 reduced the infiltration of $CD4^+$ T_{EM} cells to the lungs of mice immunised with a *Streptococcus pyogenes* virulence factor

formulated with a Th1/Th17-promoting adjuvant [369]. Interestingly, it was also observed that after *B. pertussis* challenge, mice immunised with a commercial aP vaccine appeared to have slightly lower numbers of memory T cells in the lungs than the PBS-immunised control mice. Moreover, after *B. pertussis* infection, mice immunised with an experimental alum-adjuvanted aP vaccine had substantially less $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} in the lungs than unimmunised mice. These results suggested that while Th17 cell responses may be important for the recruitment of $CD4^+$ T_{EM} and T_{RM} cells to the lungs, Th2 cells may impair this recruitment. This could explain the poor memory responses induced with aP vaccines despite multiple booster vaccinations [231]. If this is the case then immunisation with an experimental LP1569-adjuvanted aP vaccine, or natural infection with *B. pertussis,* which induce mixed Th1/Th17 cell responses *in vivo*, could be more effective at generating long-term immunity to *B. pertussis* than immunisation with an alum-adjuvanted aP vaccine.

Taken together, these studies demonstrated that the TLR agonists CpG and LP1569 are more suitable adjuvants for aP vaccines than alum, capable of inducing protective cellular and humoral immune responses against *B. pertussis.* Furthermore, Th17-promoting adjuvants appear to be more efficient at inducing migration of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} to the lungs than Th1 or Th2-promoting adjuvants; the Th1/Th17-promoting adjuvant LP1569 has a greater capacity to recruit memory $CD4^+$ T cells to the lungs than the Th1-promoting adjuvant CpG or the Th2-promoting adjuvant alum. This indicates that adjuvants like LP1569 may be more effective for use in pertussis vaccines as they appear to induce long-lived memory responses, in addition to potent cellular immunity.

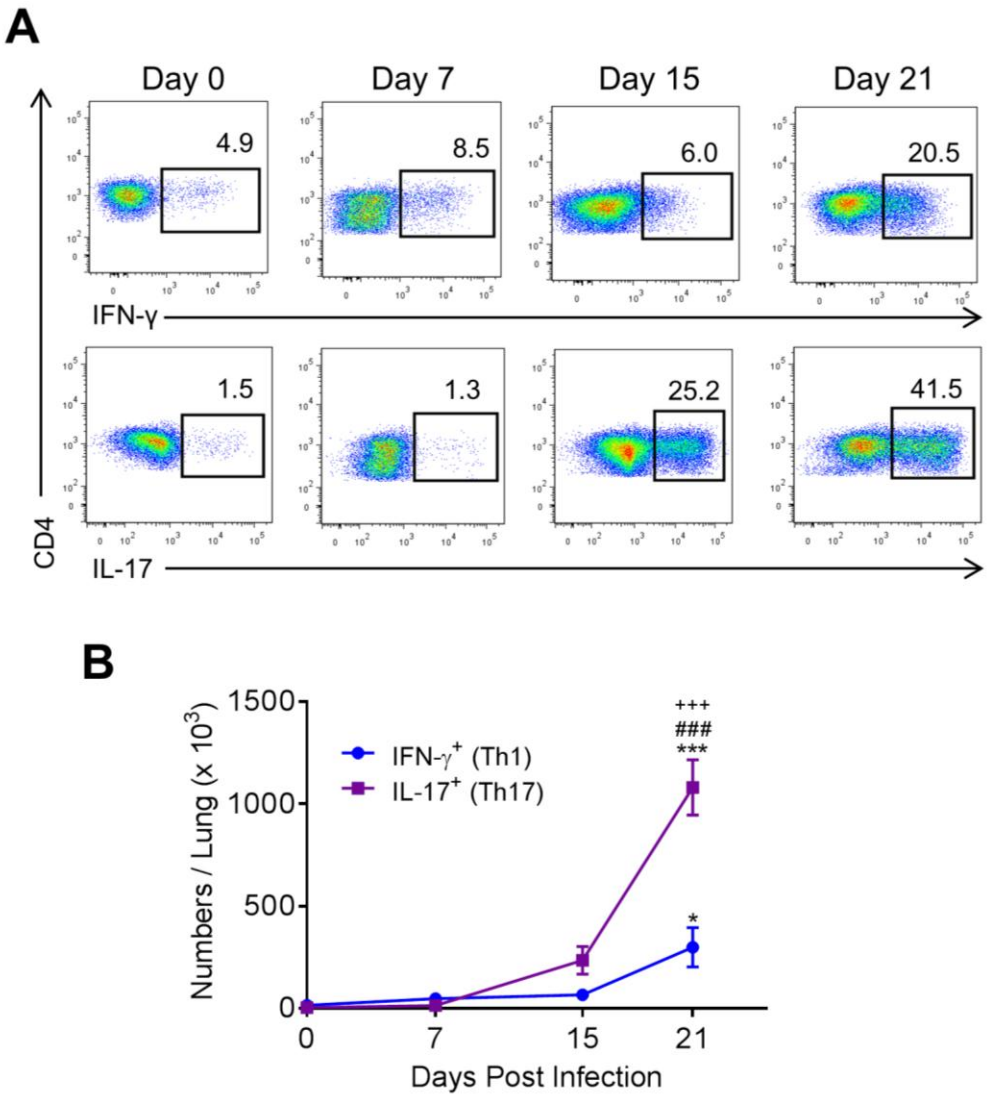


Figure 3.1 Aerosol challenge with live *B. pertussis* induces Th1 and Th17 cell expansion.
C57BL/6 mice were aerosol challenged with live *B. pertussis* and lungs were isolated over the course of infection. Lung cells were stimulated with PMA (5 ng/ml), Ionomycin (500 ng/ml), and Brefeldin A (5 µg/ml) for 4 hours before being stained for flow cytometry. (A) Representative FACS plots (n=4) of the percentages of $CD4^+IFN\text{-}\gamma^+$ Th1 cells and $CD4^+IL\text{-}17^+$ Th17 cells present in the lungs post infection. (B) Absolute numbers of Th1 and Th17 cells over the course of infection. Results shown are mean ± SEM values (n=4 mice). *$p < 0.05$, ***$p < 0.001$ versus day 0; ###$p < 0.001$ versus day 7; +++$p < 0.001$ versus day 15 by two-way ANOVA with the Bonferroni post test.

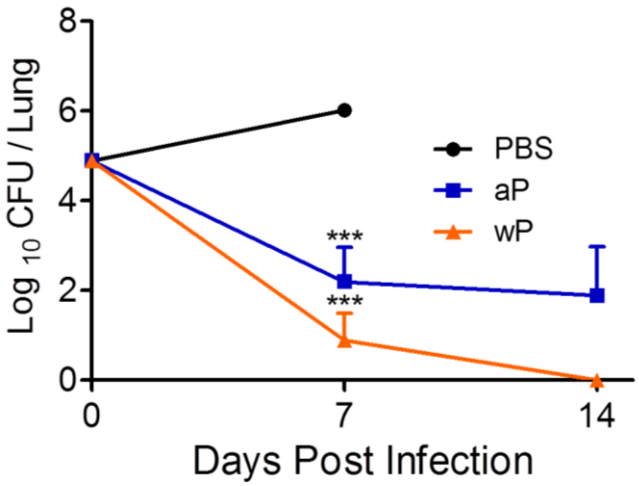


Figure 3.2 A wP vaccine confers greater protection against *B. pertussis* challenge than an aP vaccine.

C57BL/6 mice were immunised i.p. twice (0 and 4 weeks) with an aP vaccine (Adacel; 1/10th human dose), a wP vaccine (Serum Institute of India; 1/10th human dose) or with PBS as a control. Mice were aerosol challenged with live *B. pertussis* 2 weeks after the last immunisation. Four mice from each experimental group were sacrificed on day 7, and four mice from the aP and wP groups on day 14 post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. Results shown are mean ± SEM CFU counts (n=4 mice). ***$p < 0.001$ versus PBS by two-way ANOVA with the Bonferroni post test.

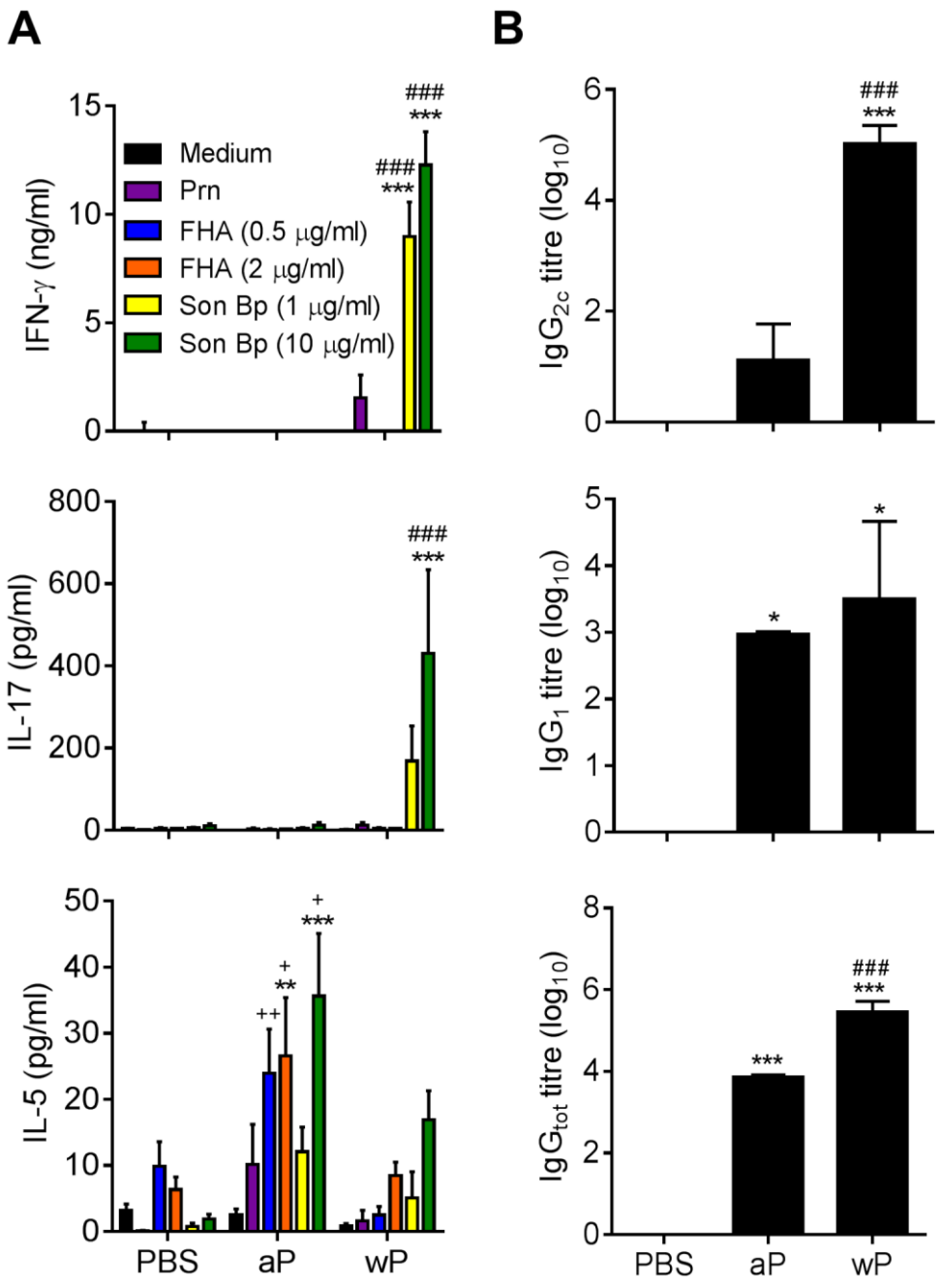


Figure 3.3 The wP vaccine induces more robust Th1 and Th17 cell responses and *B.* pertussis-specific IgG2c antibodies than the aP vaccine.

Mice were immunised i.p. twice as described in Figure 3.2. Spleens and sera from four mice in each experimental group were isolated 1 day prior to aerosol challenge. (A) Spleen cells (2 x 10^6 cells/ml) were cultured with medium or the antigens Prn (2 μg/ml), FHA (0.5 or 2 μg/ml), or sonicated *B. pertussis* (Son Bp; 1 or 10 μg/ml) and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results are shown as mean ± SEM values (n=4 mice). ***p* < 0.01, ****p* < 0.001 versus PBS; ###*p* < 0.001 versus aP; +*p* < 0.05, ++*p* < 0.01 versus wP by two-way ANOVA with the Bonferroni post test. (B) *B. pertussis*-specific antibody titres in sera were quantified by ELISA. Results are shown as mean ± SEM values (n=4 mice). **p* < 0.05, ****p* < 0.001 versus PBS; ###*p* < 0.001 versus aP by one-way ANOVA with the Bonferroni post test.

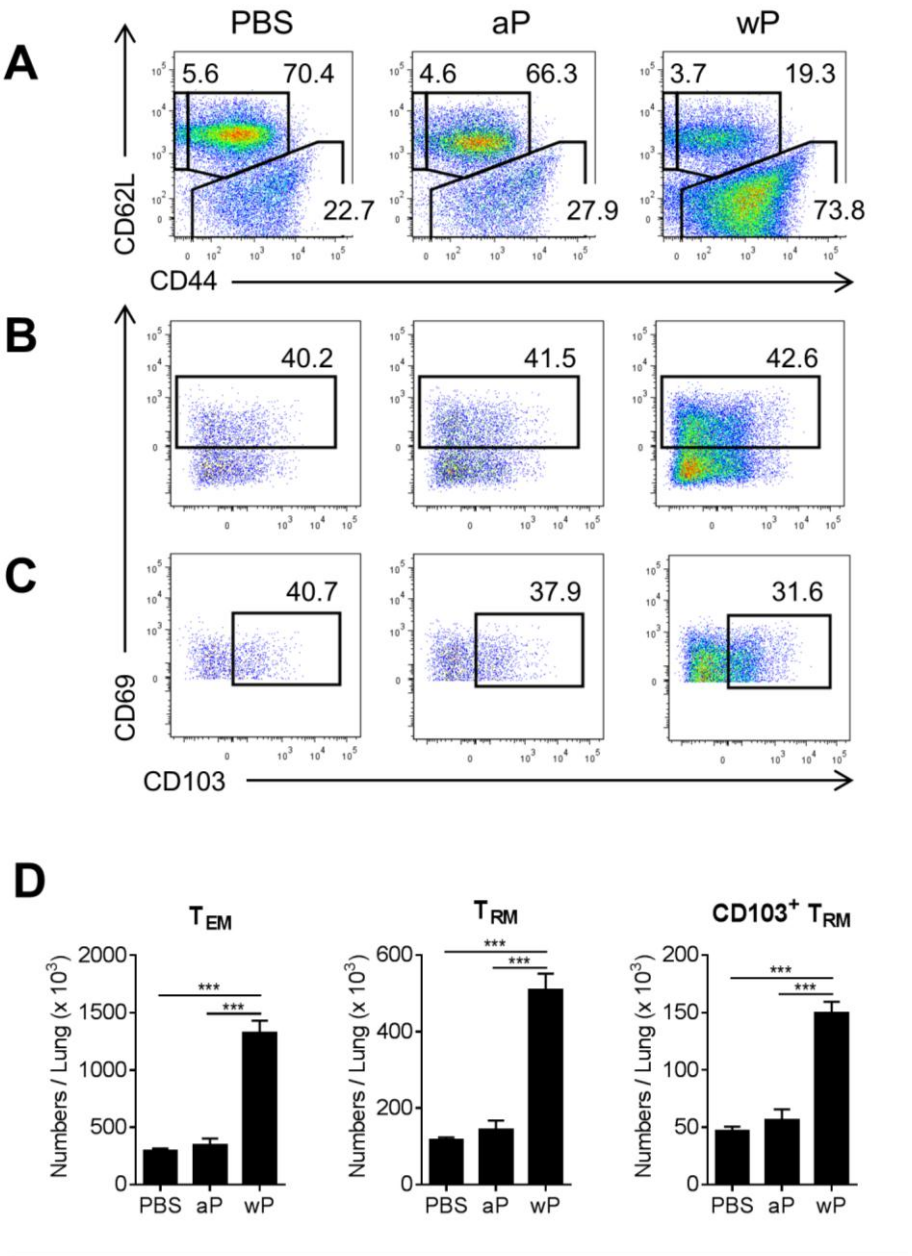


Figure 3.4 T_{RM} cells are recruited to the lungs of mice following peritoneal immunisation with a wP vaccine, but not with an aP vaccine.

Mice were immunised i.p. twice as described in Figure 3.2. The lungs were isolated 2 weeks after the last immunisation and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing the (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+CD103^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). ***$p < 0.001$ by one-way ANOVA with the Bonferroni post test.

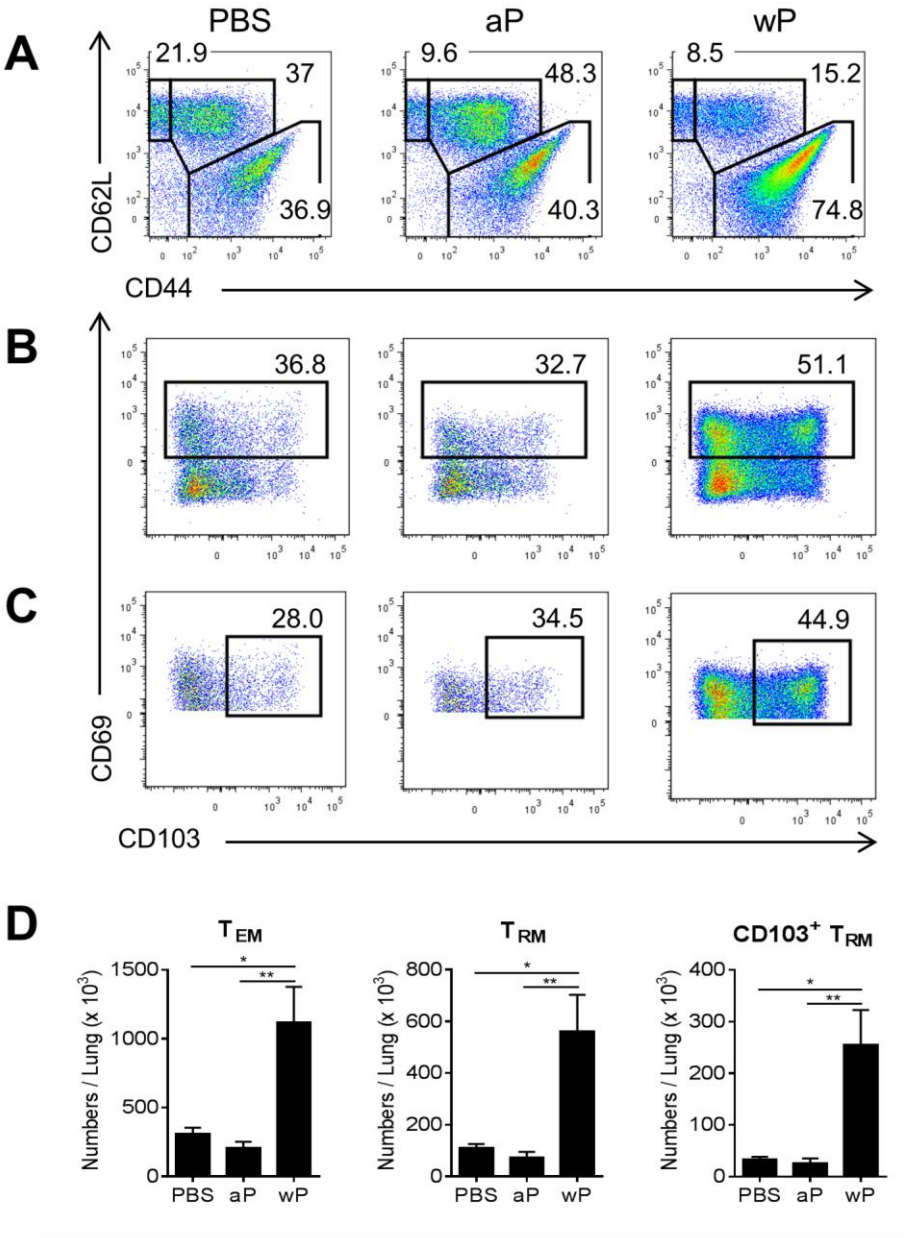


Figure 3.5 Mice immunised with the wP vaccine maintain higher levels of lung T_{RM} cells than mice immunised with the aP vaccine after aerosol challenge with *B. pertussis.*

Mice were immunised i.p. twice as described in Figure 3.2. 2 weeks after the last immunisation, mice were aerosol challenged with live *B. pertussis.* The lungs were isolated 7 days post infection and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing the (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+CD103^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). *$p < 0.05$, **$p < 0.01$ by one-way ANOVA with the Bonferroni post test.

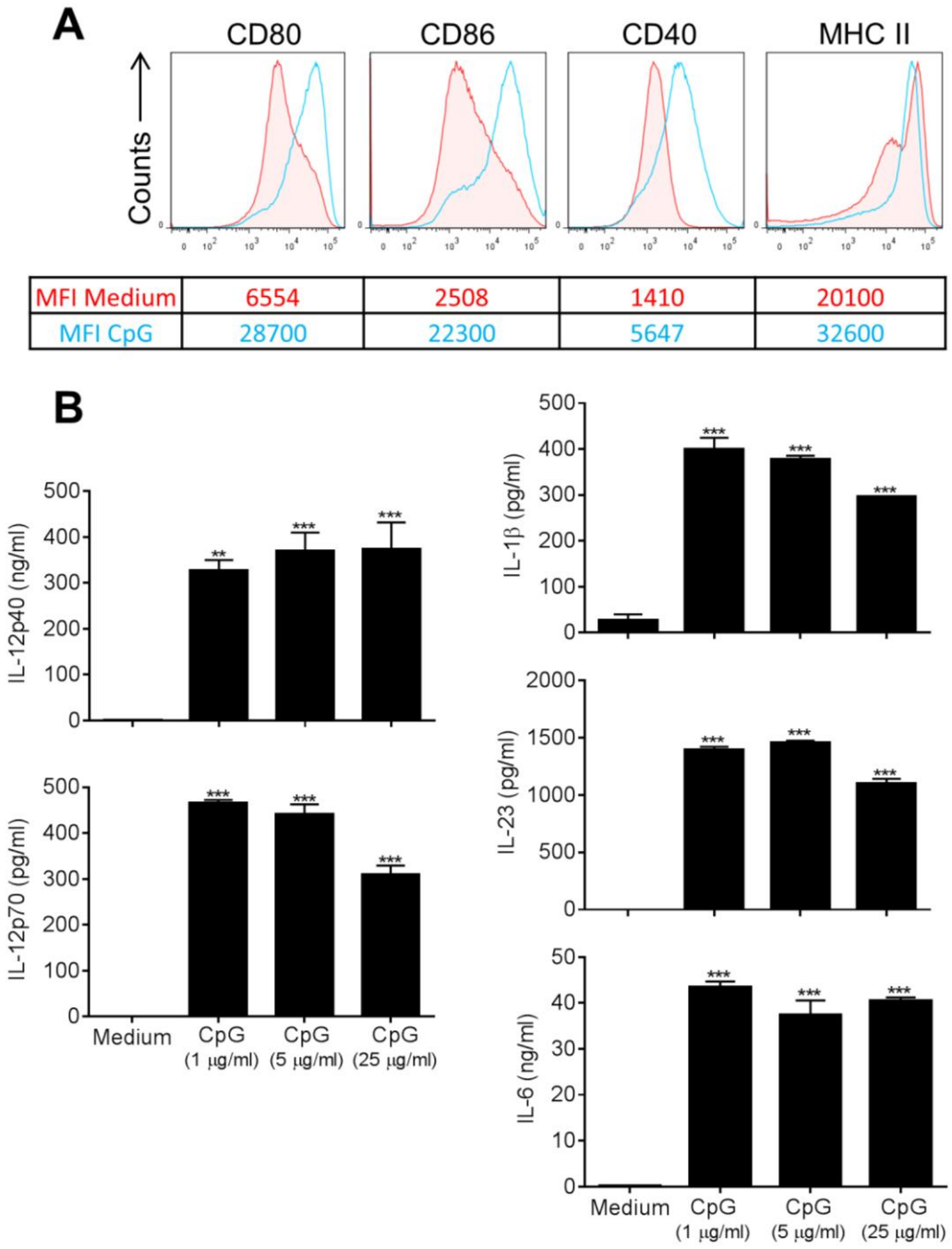
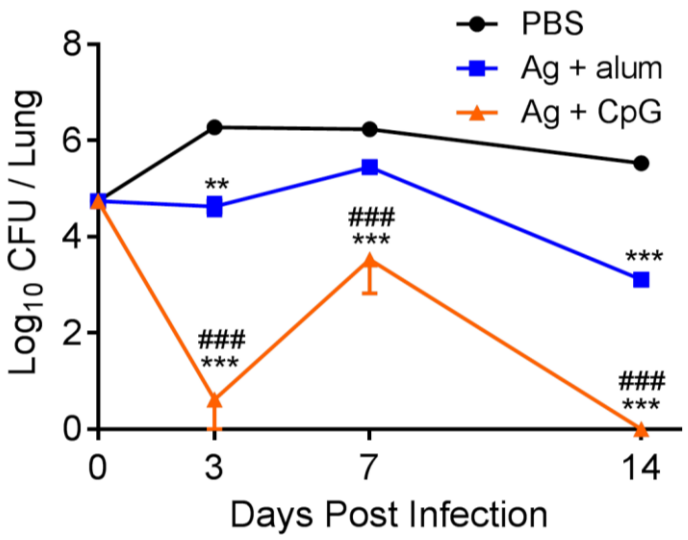


Figure 3.6 The TLR9 agonist CpG activates BMDCs and induces production of Th1 and Th17 polarising cytokines.

BMDCs (1 x 10^6 cells/ml) from C57BL/6 mice were stimulated with increasing concentrations of CpG (1, 5 or 25 μg/ml) or medium as a control for 24 hours. After 24 hours, cells were stained for FACS analysis and cytokine concentrations in the supernatants were quantified by ELISA. (A) FACS plots and mean fluorescence intensity (MFI) showing CD80, CD86, CD40 and MHC class II expression on BMDCs stimulated with CpG (5 μg/ml) versus medium only. (B) Production of IL-12p40, IL-12p70, IL-1β, IL-23 and IL-6 by BMDCs stimulated with increasing concentrations of CpG. ***p < 0.001 versus medium only by one-way ANOVA with the Bonferroni post test. Results shown are mean ± SEM values and are representative of three independent experiments (n=3).

A

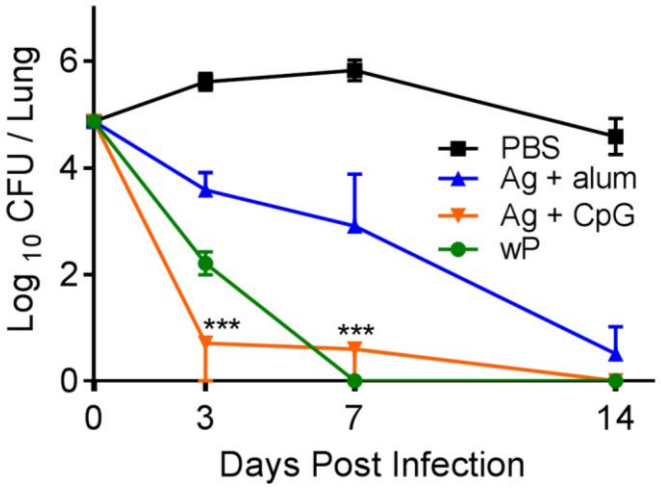


B

AUC	PBS	Ag + alum	Ag + CpG
	82.72	64.20	28.63

Figure 3.7 CpG is a more effective adjuvant than alum for an experimental aP vaccine.
C57BL/6 mice were immunised i.p. twice (0 and 3 weeks) with PBS or the antigens FHA (2.5 µg/mouse) and PT-9K/129G (1 µg/mouse) formulated with alum (100 µg/mouse) or CpG (50 µg/mouse). Mice were aerosol challenged with live *B. pertussis* 2 weeks after the last immunisation. Four mice from each group were sacrificed on days 3, 7 and 14 post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. (A) Results shown are mean ± SEM CFU counts (n=4 mice). $^{**}p < 0.01$, $^{***}p < 0.001$ versus PBS; $\#\#\#p < 0.001$ versus Ag + alum by two-way ANOVA with the Bonferroni post test. (B) Total area under the curve (AUC) was calculated using GraphPad Prism.

A



B

AUC	PBS	Ag + alum	Ag + CpG	wP
	75.09	37.66	13.10	15.04

Figure 3.8 An experimental aP vaccine formulated with CpG is as effective as a wP vaccine at conferring protection against *B. pertussis* infection.

C57BL/6 mice were immunised i.p. twice (0 and 3 weeks) with PBS, wP (WHO International Standard pertussis whole cell vaccine, 94/532 from NIBSC, Herts., UK; 1/5th human dose) or an experimental aP vaccine composed of FHA (2.5 µg/mouse) and PT-9K/129G (1 µg/mouse) formulated with alum (100 µg/mouse) or CpG (50 µg/mouse). Mice were aerosol challenged with live *B. pertussis* 1 week after the last immunisation. Four mice from each group were sacrificed on days 3, 7 and 14 post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. (A) Results shown are mean ± SEM CFU counts (n=4 mice). ***$p < 0.001$ versus Ag + alum by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

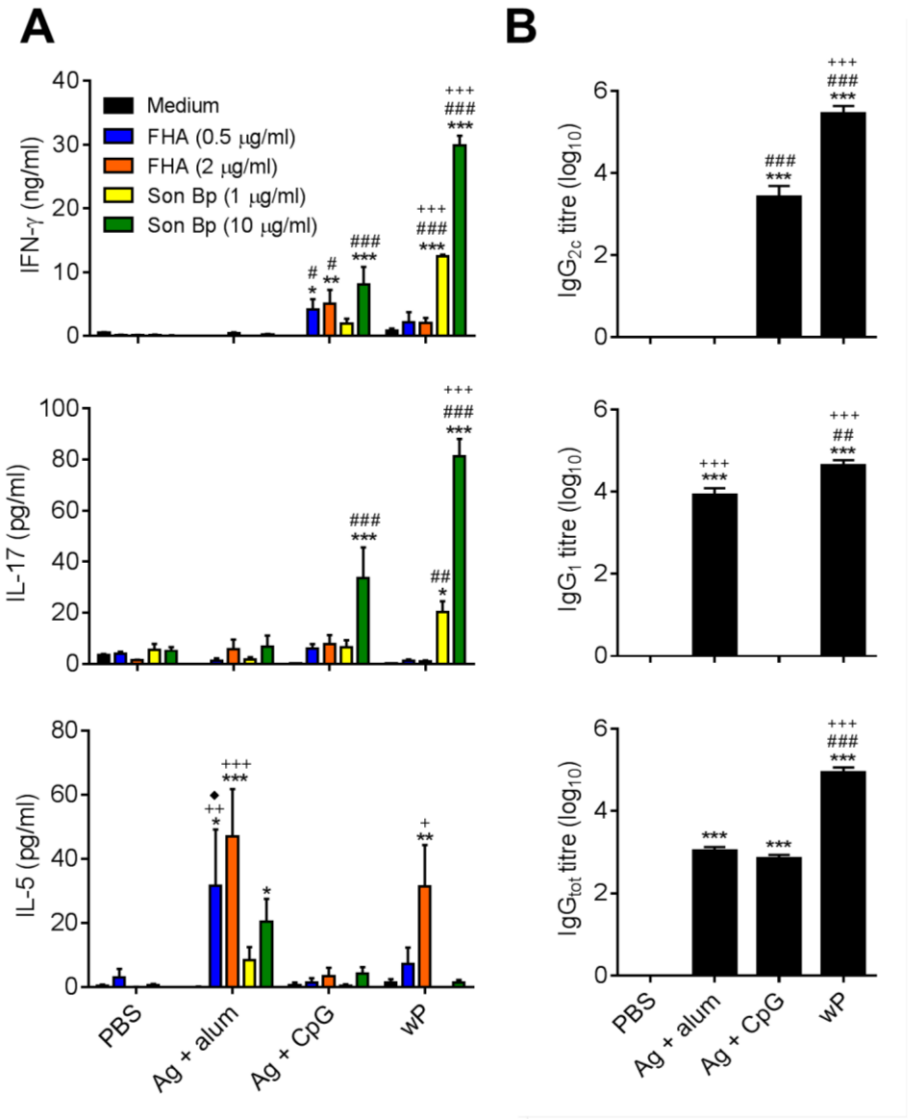


Figure 3.9 A CpG-adjuvanted aP vaccine induces greater antigen-specific IFN-γ, IL-17 and IgG2c production than an aP vaccine formulated with alum.

Mice were immunised i.p. twice as described in Figure 3.8. Four mice in each experimental group were sacrificed 1 day prior to aerosol challenge and spleens and sera were isolated. (A) Spleen cells (2 x 10^6 cells/ml) were cultured with medium or the antigens FHA (0.5 or 2 μg/ml) or Son Bp (1 or 10 μg/ml) for 72 hours and then cytokine concentrations in supernatants were quantified by ELISA. Results in each panel are mean ± SEM values (n=4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus PBS; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus Ag + alum; +$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$ versus Ag + CpG; ◆$p < 0.05$ versus wP by two-way ANOVA with the Bonferroni post test. (B) *B. pertussis*-specific antibody titres were quantified by ELISA. Results shown are mean ± SEM values (n=4 mice). ***$p < 0.001$ versus PBS; ###$p < 0.001$ versus Ag + alum; +++$p < 0.001$ versus Ag + CpG by one-way ANOVA with the Bonferroni post test.

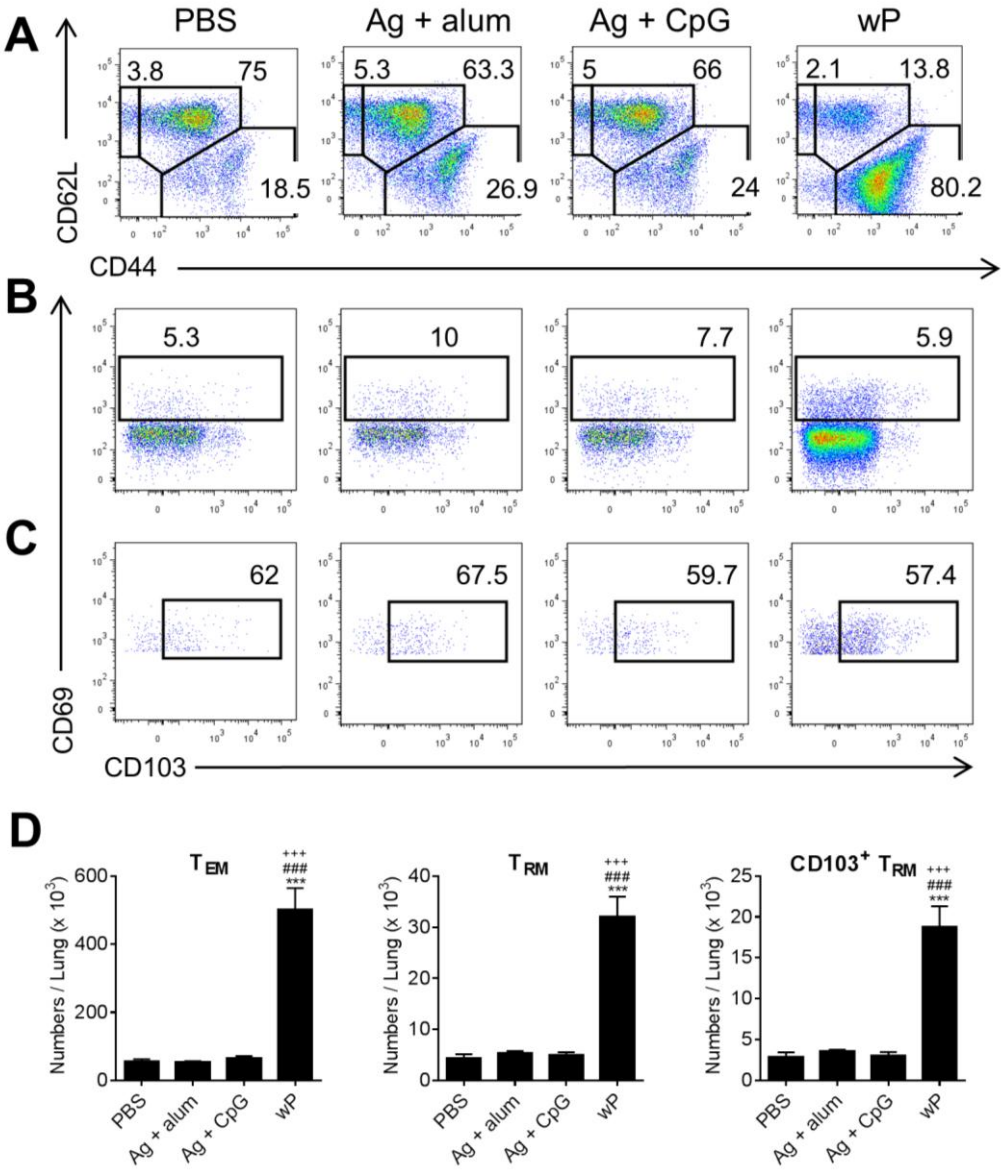
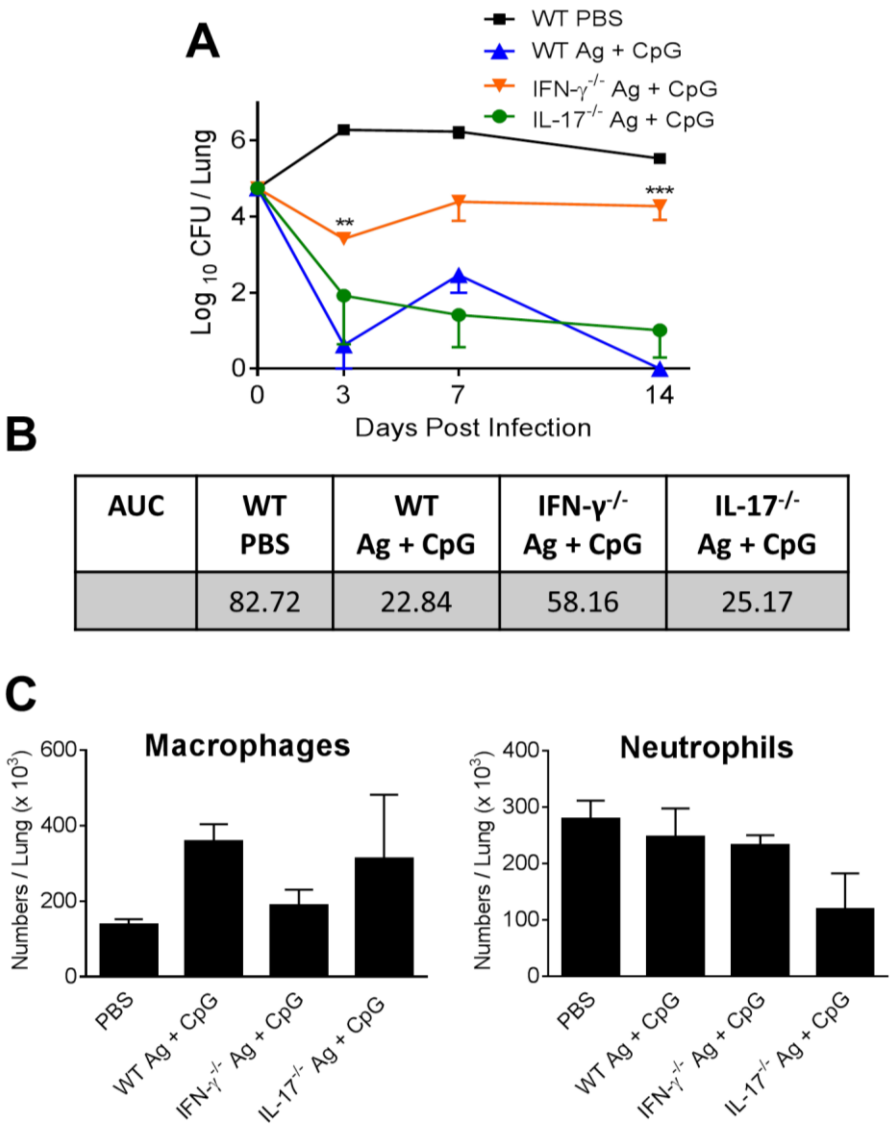
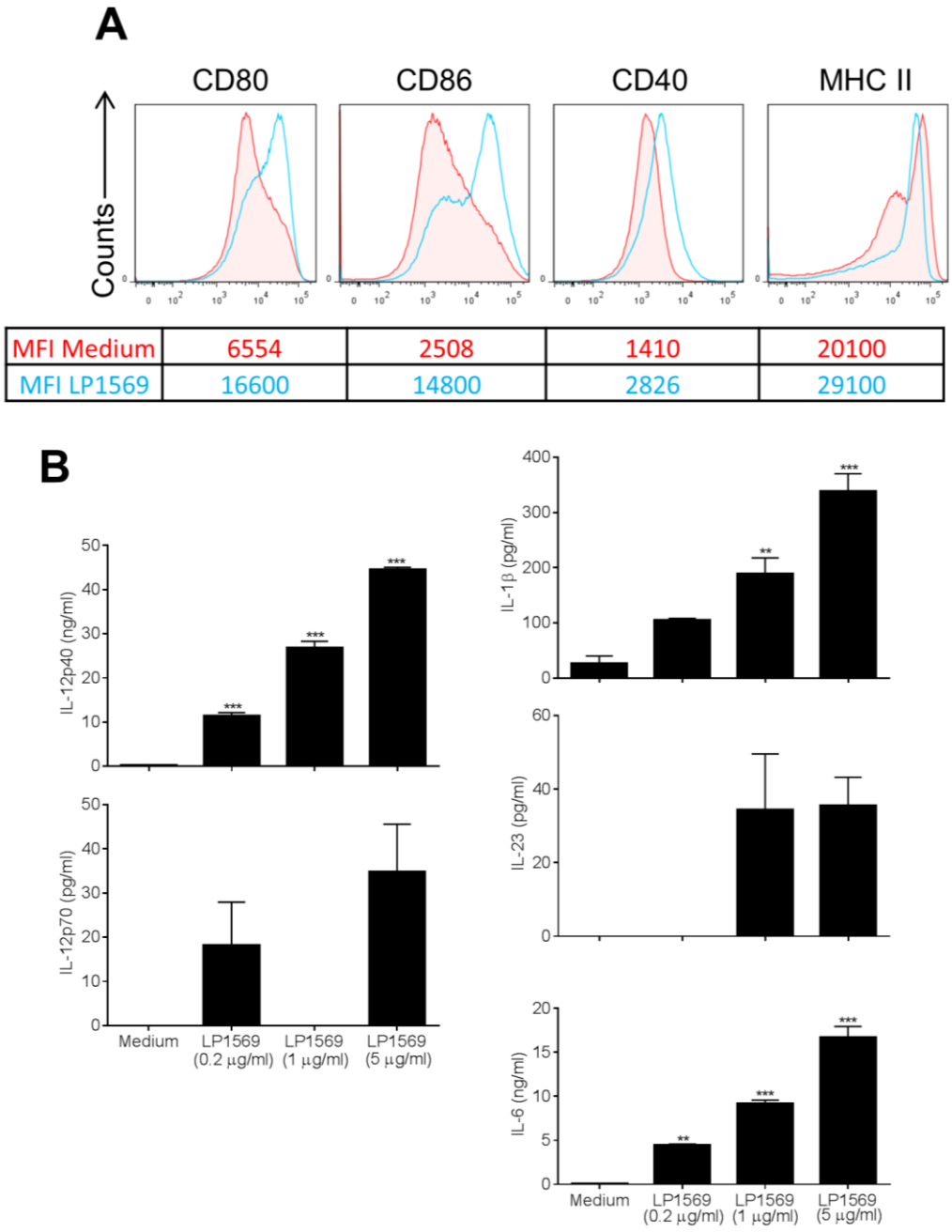


Figure 3.10 An experimental aP vaccine adjuvanted with CpG does not induce better infiltration of CD4$^+$ T_{EM} and T_{RM} cells to the lungs than an aP vaccine formulated with alum.
Mice were immunised i.p. twice as described in Figure 3.8. The lungs were isolated 2 weeks after the last immunisation and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing the (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+CD103^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). ***$p < 0.001$ versus PBS; ###$p < 0.001$ versus Ag + alum; +++$p < 0.001$ versus Ag + CpG by one-way ANOVA with the Bonferroni post test.



AUC	WT PBS	WT Ag + CpG	IFN-γ$^{-/-}$ Ag + CpG	IL-17$^{-/-}$ Ag + CpG
	82.72	22.84	58.16	25.17

Figure 3.11 An experimental aP vaccine formulated with CpG protects mice from *B. pertussis* infection through IFN-γ-dependent mechanisms.
C57BL/6 (WT), IFN-γ$^{-/-}$ and IL-17$^{-/-}$ mice were immunised twice (0 and 3 weeks) with the laboratory-prepared Ag + CpG vaccine described in Figure 3.8, or with PBS as a control, and were challenged with live *B. pertussis* 1 week after the last immunisation. Four mice from each group were sacrificed on days 3, 7 and 14 post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. (A) Results shown are mean ± SEM CFU counts (n=4 mice). $^{**}p < 0.01$, $^{***}p < 0.001$ versus WT Ag + CpG by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism. (C) 3 days post aerosol challenge, the lungs from four infected mice per experimental group were isolated and half of the lungs were stained for FACS analysis to determine the absolute numbers of infiltrating neutrophils (Ly6C^{+}, GR1^{+}) and macrophages (GR1^{-}, CD11b^{+}, F4/80^{+}). Results shown are mean ± SEM (n=4 mice). No significant differences were determined between any groups by one-way ANOVA with the Bonferroni post test.

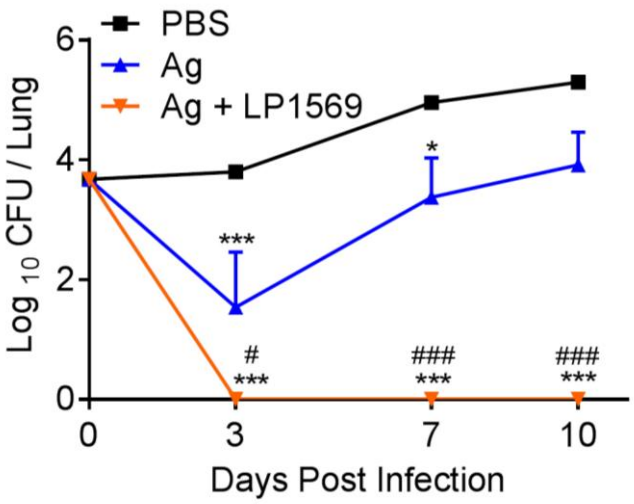


MFI Medium	6554	2508	1410	20100
MFI LP1569	16600	14800	2826	29100

Figure 3.12 The TLR2 agonist LP1569 activates BMDCs and induces production of Th1 and Th17 polarising cytokines.

BMDCs from C57BL/6 mice were stimulated with increasing concentrations of LP1569 (0.2, 1 or 5 µg/ml) or medium as a control for 24 hours. After stimulation, cells were stained for analysis by FACS and cytokine production was quantified by ELISA on cell supernatants. (A) FACS plots and MFI showing CD80, CD86, CD40 and MHC class II expression on BMDCs stimulated with LP1569 (5 µg/ml) versus medium only. (B) Production of IL-12p40, IL-12p70, IL-1β, IL-23 and IL-6 by BMDCs stimulated with increasing concentrations of LP1569. $**p < 0.01$, $***p < 0.001$ versus medium by one-way ANOVA with the Bonferroni post test. Results shown are mean ± SEM values and are representative of three independent experiments (n=3).

A



B

AUC	PBS	Ag	Ag + LP1569
	44.08	28.56	5.507

Figure 3.13 LP1569 acts as an adjuvant for an experimental aP vaccine and protects mice from *B. pertussis* infection.
C57BL/6 mice were immunised i.p. twice (0 and 5 weeks) with an experimental aP vaccine composed of FHA, Prn and dPT alone (Ag; 0.2 µg/mouse) or Ag formulated with LP1569 (50 µg/mouse) or PBS as a control. They were challenged by exposure to live *B. pertussis* 2 weeks after the second immunisation. Four mice from each group were sacrificed on days 3, 7 and 10 post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. (A) Results shown are mean ± SEM CFU counts (n=4 mice). *$p < 0.05$, ***$p < 0.001$ versus PBS; #$p < 0.05$, ###$p < 0.001$ versus Ag by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

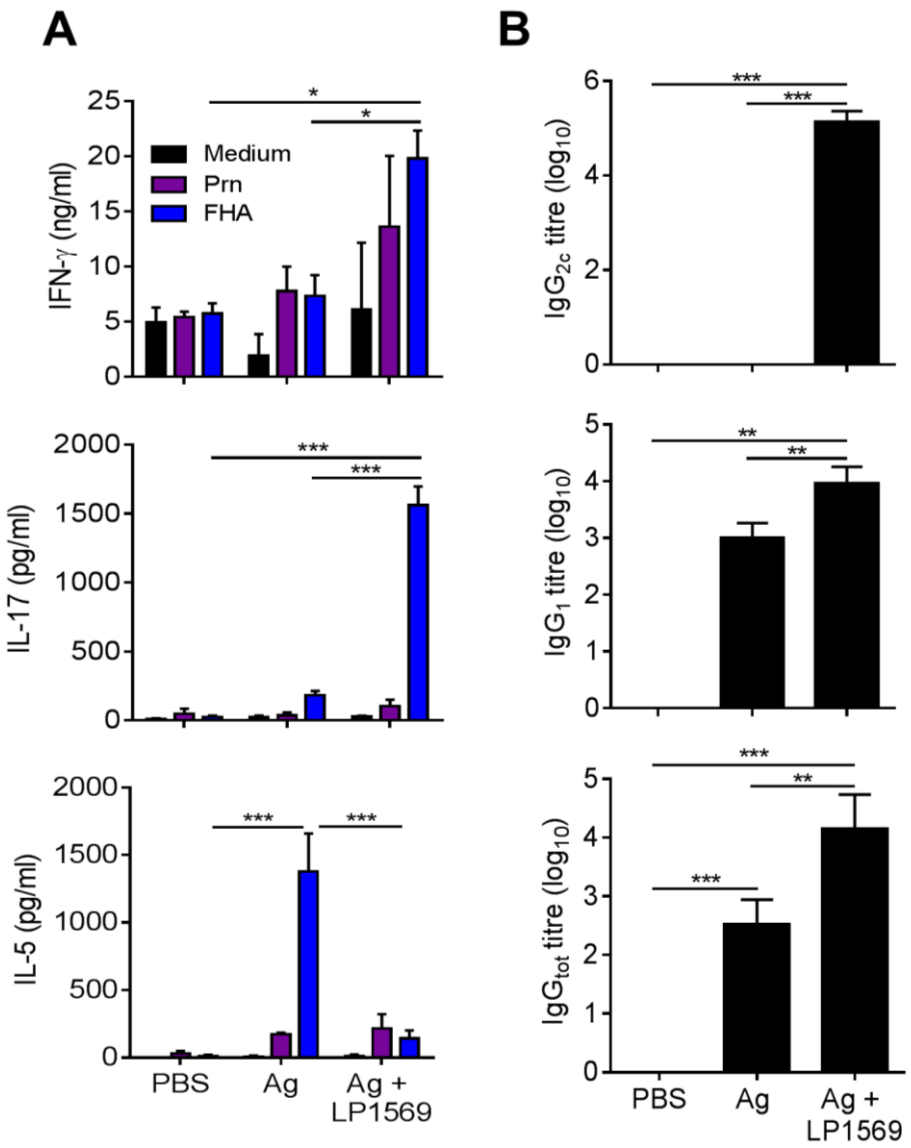
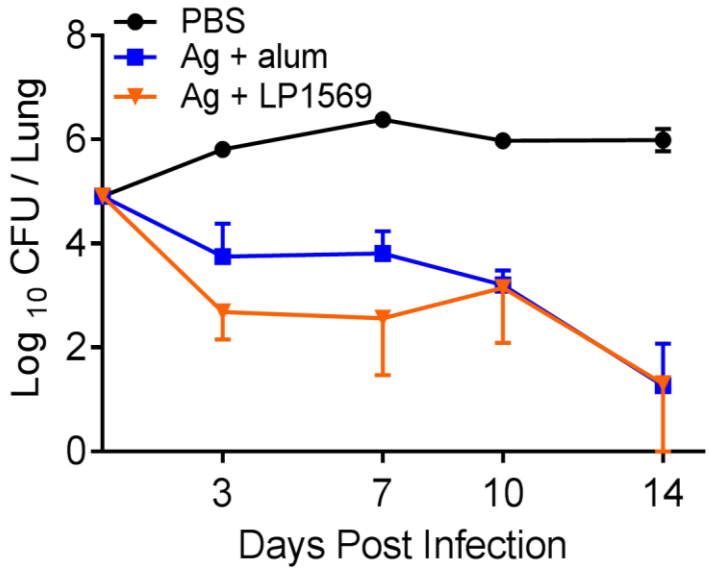


Figure 3.14 Adjuvantation of an experimental aP vaccine with LP1569 enhances antigen-specific IFN-γ, IL-17 and IgG2c production.

Mice were immunised as described in Figure 3.13. Spleens and sera were isolated from four mice in each experimental group 1 day prior to aerosol challenge. (A) Spleen cells (2 x 10^6 cells/ml) were cultured with medium or the antigens FHA (2 µg/ml) or Prn (1 µg/ml) for 72 hours. Cytokine concentrations were quantified by ELISA on cell supernatants. Results shown in each panel are mean ± SEM values (n=4 mice). *$p < 0.05$, ***$p < 0.001$ by two-way ANOVA with the Bonferroni post test. (B) FHA-specific antibody production in sera was quantified by ELISA. Results shown are mean ± SEM values (n=4 mice). **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	Ag + alum	Ag + LP1569
	82.95	47.58	39.36

Figure 3.15 Mice immunised with an aP vaccine containing LP1569 are more protected early in *B. pertussis* infection than mice administered an alum-adjuvanted aP vaccine.
C57BL/6 mice were immunised i.p. twice (0 and 4 weeks) with an experimental aP vaccine composed of rPT and FHA (Ag; 0.4 µg/mouse) formulated with alum (100 µg/mouse) or LP1569 (50 µg/mouse) or with PBS as a control. Mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation. Four mice from each group were sacrificed on days 3, 7, 10 and 14 post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. (A) Results shown are mean ± SEM CFU counts (n=4 mice). No significant differences between the vaccinated groups by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

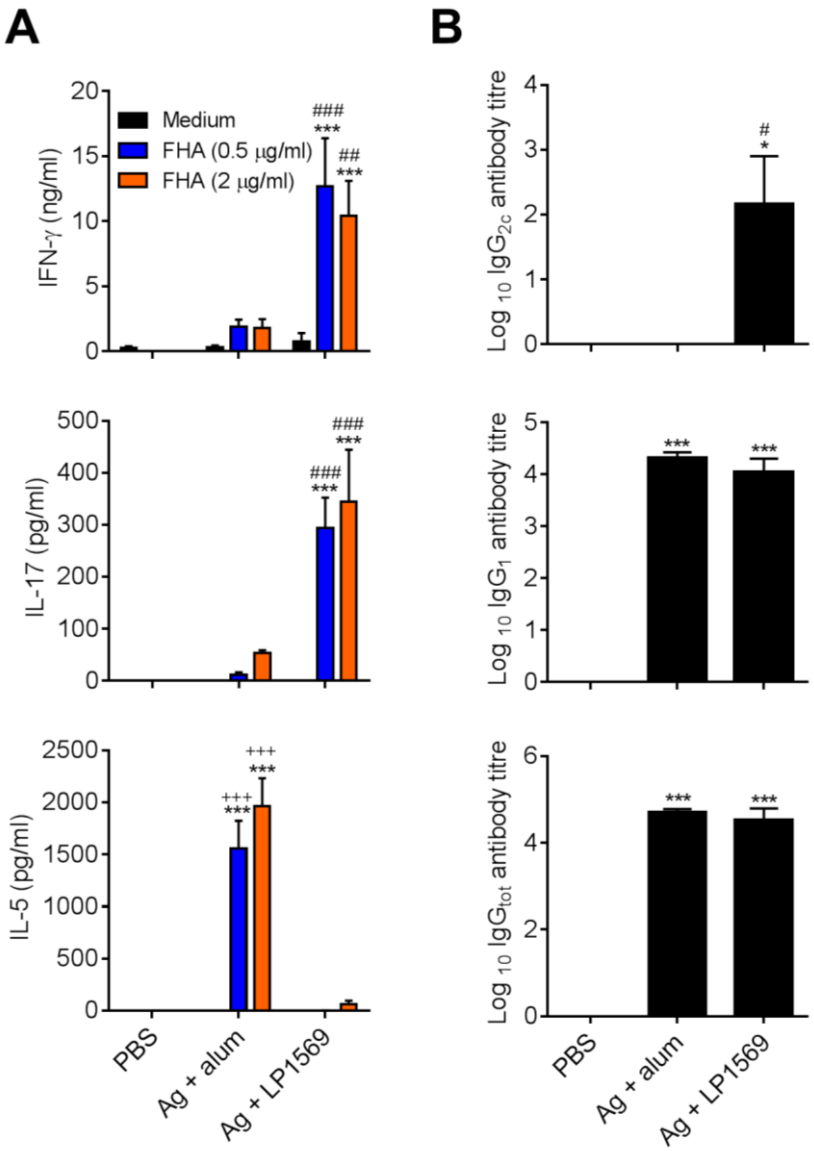
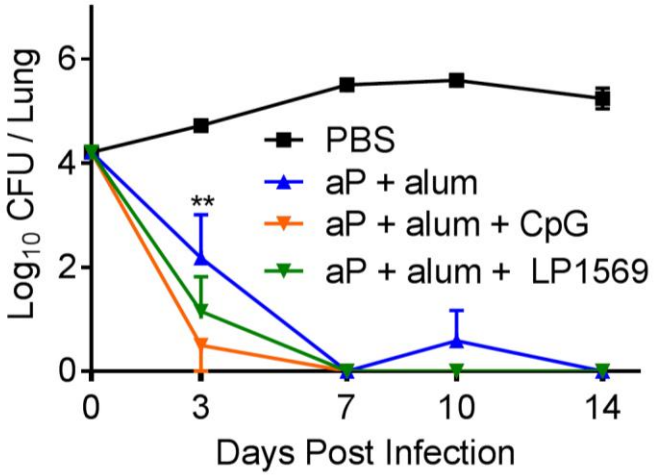


Figure 3.16 An LP1569-adjuvanted aP vaccine is more effective than an alum-adjuvanted aP vaccine at inducing FHA-specific IFN-γ, IL-17 and IgG2c production.
Mice were immunised i.p. twice as described in Figure 3.15. Spleens and sera from four mice in each group were isolated 1 day prior to aerosol challenge. (A) Spleen cells (2 x 10^6 cells/ml) were cultured with FHA (0.5 or 2 μg/ml) or medium as a negative control and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). ***p < 0.001 versus PBS; ##p < 0.01, ###p < 0.001 versus Ag + alum; +++p < 0.001 versus Ag + LP1569 by two-way ANOVA with the Bonferroni post test. (B) FHA-specific antibody production in sera was quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). *p < 0.05, ***p < 0.001 versus PBS; #p < 0.05 versus Ag + alum by one-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	aP + alum	aP + alum + CpG	aP + alum + LP1569
	72.17	16.00	8.063	10.34

Figure 3.17 Addition of CpG or LP1569 to the commercial aP vaccine enhances protection against *B. pertussis* infection.

C57BL/6 mice were immunised i.p. twice (0 and 4 weeks) with the commercial aP vaccine Infanrix (aP + alum; 1/25th human dose), aP + alum with CpG (50 µg/mouse), aP + alum with LP1569 (50 µg/mouse) or PBS as a control. Mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation. Four mice from each experimental group were sacrificed on days 3, 7, 10 and 14 post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. (A) Results shown are mean ± CFU counts (n=4 mice). $^{**}p < 0.01$ versus aP + alum + CpG by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

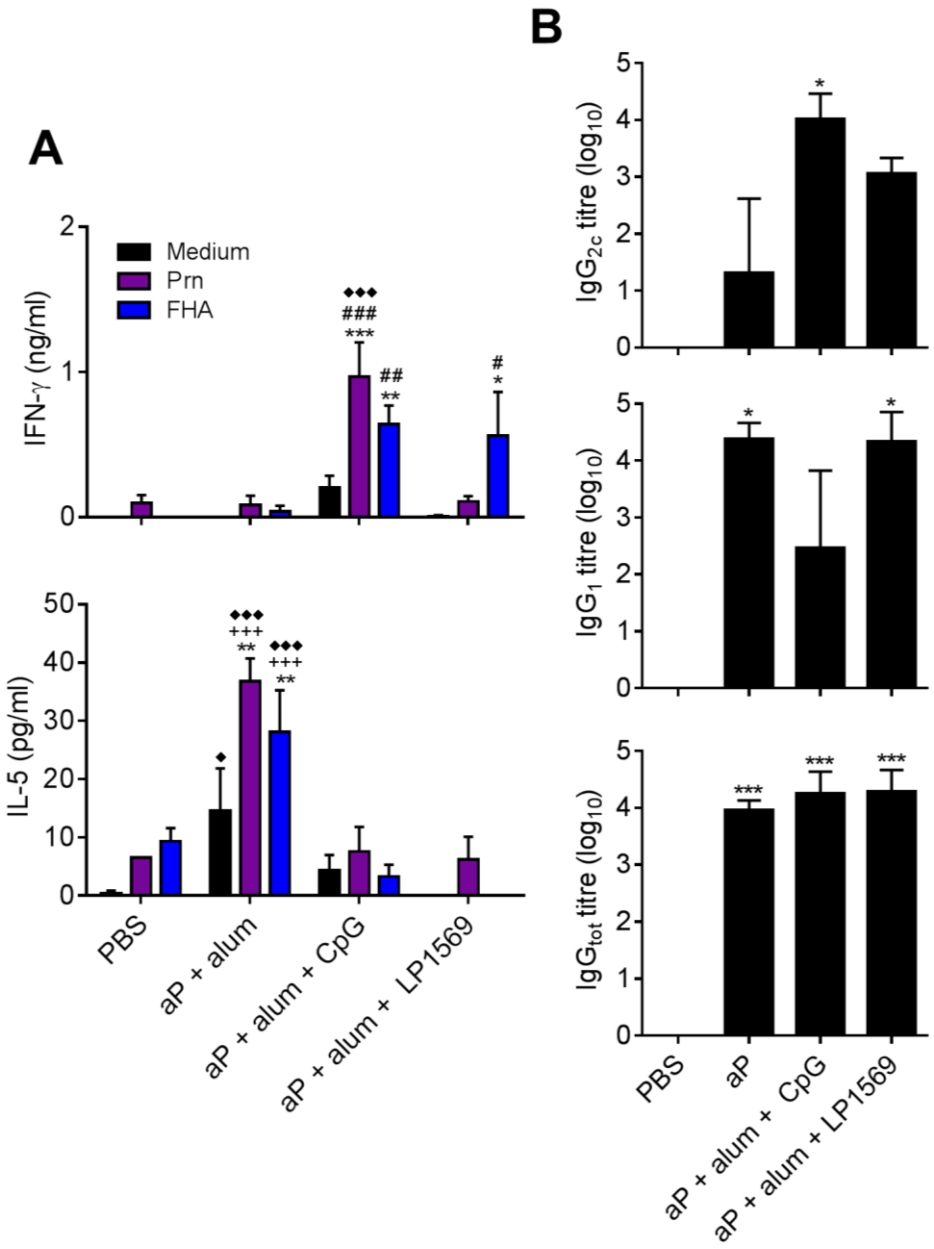
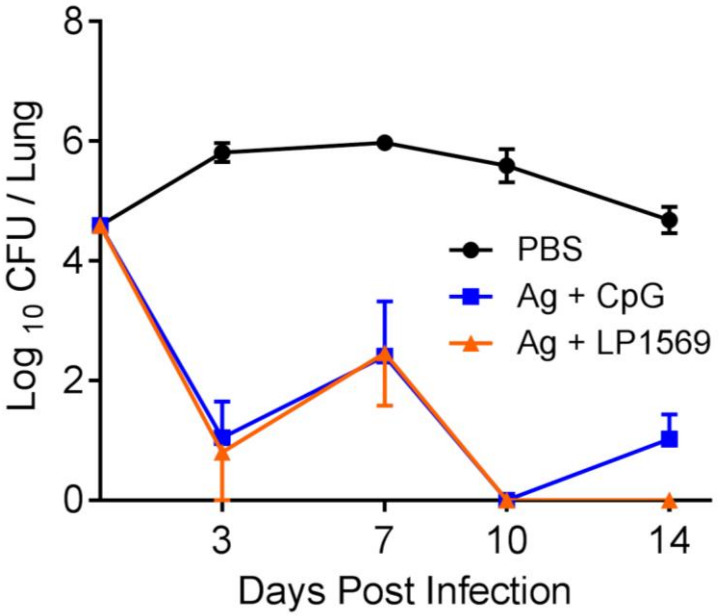


Figure 3.18 Adjuvantation of the commercial aP vaccine with CpG or LP1569 enhances antigen-specific IFN-γ and IgG2c production.

Mice were immunised i.p. twice as described in Figure 3.17. (A) Inguinal LNs were isolated from four immunised mice per group 1 day prior to aerosol challenge. LN cells (1 x 10^6 cells/ml) were cultured with the antigens FHA (2 µg/ml), Prn (1 µg/ml) or medium as a negative control and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results are shown as mean ± SEM (n=4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus PBS; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus aP + alum; +++$p < 0.001$ versus aP + alum + CpG; ◆$p < 0.05$, ◆◆◆$p < 0.001$ versus aP + alum + LP1569 by two-way ANOVA with the Bonferroni post test. (B) FHA-specific antibody production in sera was quantified by ELISA. Results are shown as mean ± SEM (n=4 mice). *$p < 0.05$, ***$p < 0.001$ versus PBS by one-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	Ag + CpG	Ag + LP1569
	77.04	21.02	18.30

Figure 3.19 CpG and LP1569 as adjuvants for an aP vaccine confer similar levels of protection against *B. pertussis* infection.
C57BL/6 mice were immunised i.p. twice (0 and 4 weeks) with an experimental aP vaccine composed of rPT, Prn and FHA (Ag; 0.2 µg/mouse) formulated with CpG (50 µg/mouse) or LP1569 (50 µg/mouse) or were immunised with PBS as a control. Mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation. Four mice from each group were sacrificed on days 3, 7, 10 and 14 post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. (A) Results shown are mean ± CFU counts (n=4 mice). No significant differences were determined between the immunised groups by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

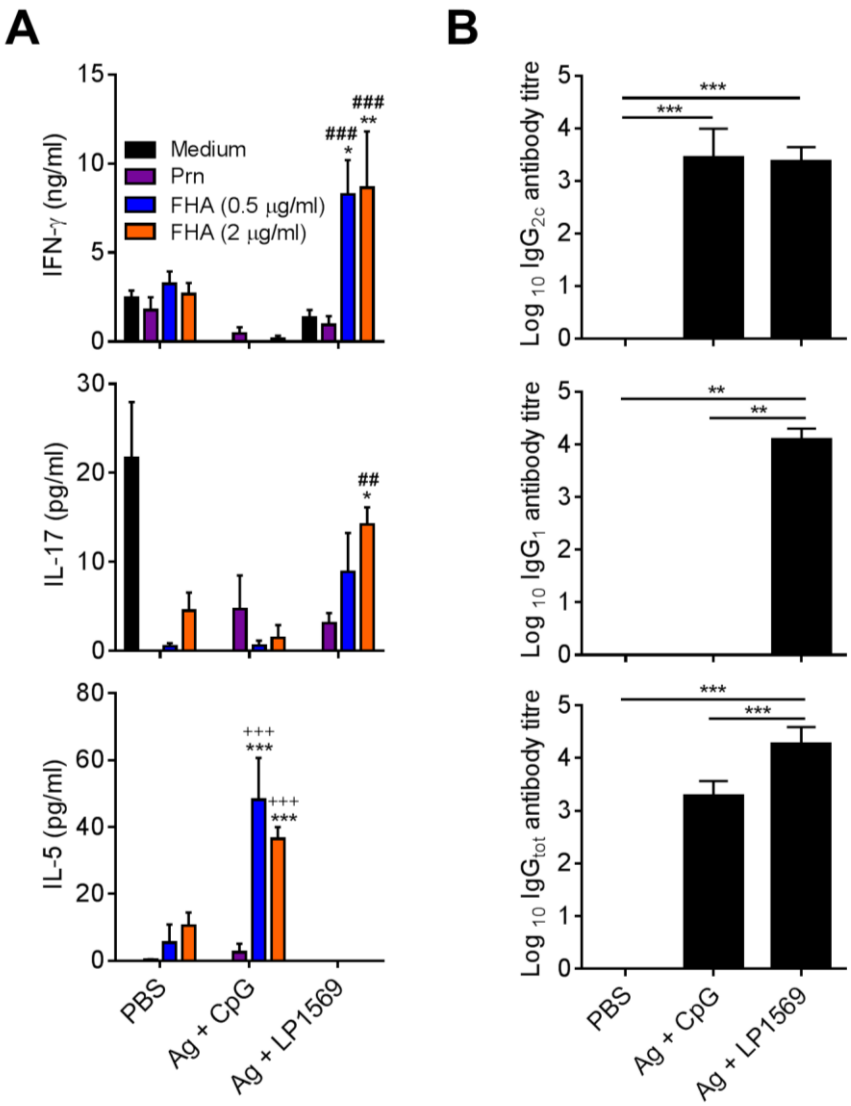
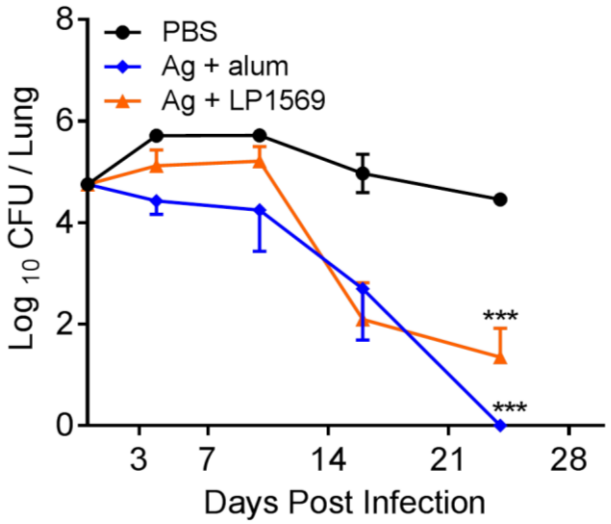


Figure 3.20 An aP vaccine adjuvanted with LP1569 induces more potent cellular and humoral immune responses to *B. pertussis* than an aP vaccine containing CpG.

Mice were immunised twice as described in Figure 3.19. Spleens and sera were isolated from four immunised mice per experimental group 1 day prior to aerosol challenge. (A) Spleen cells (2 x 10^6 cells/ml) were cultured with the antigens FHA (0.5 or 2 μg/ml), Prn (1 μg/ml) or medium as a negative control and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results are shown as mean ± SEM (n=4 mice). *p < 0.05, **p < 0.01, ***p < 0.001 versus PBS; ##p < 0.01, ###p < 0.001 versus Ag + CpG; +++p < 0.001 versus Ag + LP1569 by two-way ANOVA with the Bonferroni post test. (B) FHA-specific antibody production in sera was quantified by ELISA. Results are shown as mean ± SEM (n=4 mice). **p < 0.01, ***p < 0.001 by one-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	Ag + alum	Ag + LP1569
	125	76.04	86.47

Figure 3.21 A single immunisation with an experimental LP1569-adjuvanted aP vaccine does not confer greater protection against *B. pertussis* infection than immunising once with an experimental alum-adjuvanted aP vaccine.

Mice were immunised i.p. once with PBS as a negative control or FHA and rPT (Ag; 1 µg/mouse) formulated with LP1569 (50 µg/mouse) or alum (100 µg/mouse). 3 weeks after immunisation, mice were aerosol challenged with live *B. pertussis.* Four mice from each group were sacrificed at intervals post infection. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. (A) Results shown are mean ± CFU counts (n=4 mice). ***$p < 0.001$ versus PBS by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

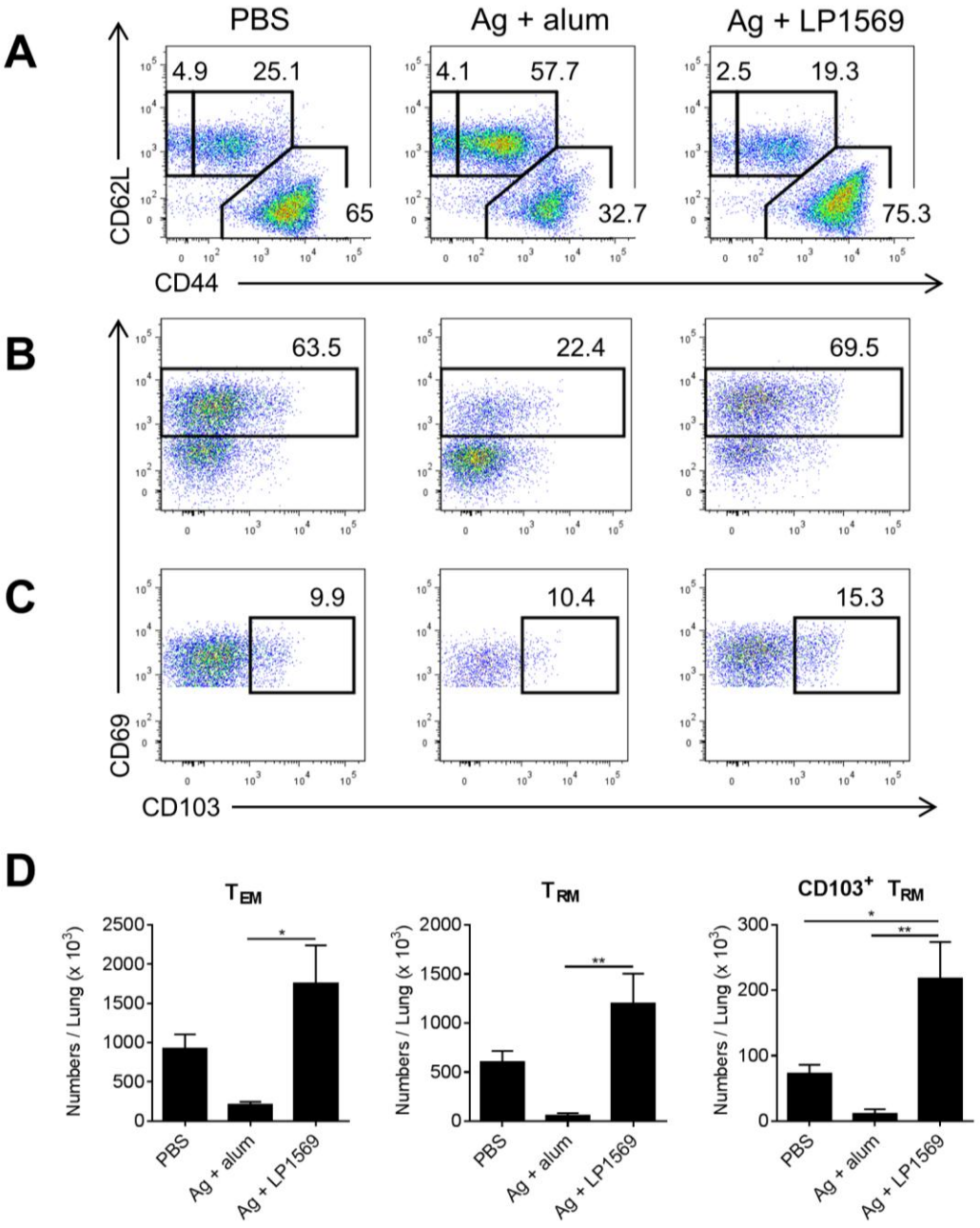


Figure 3.22 After *B. pertussis* challenge, mice immunised with an experimental LP1569-adjuvanted aP vaccine have greater numbers of $CD4^+$ T_{EM} and T_{RM} cell in the lungs than mice immunised with an experimental alum-adjuvanted aP vaccine.

Mice were immunised i.p. once as described in Figure 3.21. 3 weeks after immunisation, mice were aerosol challenged with live *B. pertussis* and 10 days later the lungs were isolated. Lung cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+CD103^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA with the Bonferroni post test.

Chapter 4:

Exploiting synergy between STING and TLR agonists as adjuvants for a pertussis vaccine

Chapter 4: Exploiting synergy between STING and TLR agonists as adjuvants for a pertussis vaccine

4.1 Introduction

C-di-GMP is a signalling molecule that was first discovered in *Acetobacter xylinum* and shown to regulate cellulose synthesis [284]. However, there is growing recognition of a role for this second messenger in a number of diverse bacterial processes including cell survival, motility, host colonisation and biofilm formation [370-374]. More recently, it has been observed that c-di-GMP has immunostimulatory properties and is capable of inducing the expression of type I IFN and NFκB-dependent inflammatory cytokines by signalling through the adaptor molecule, STING [280]. Indeed, Karaolis *et al.* demonstrated that c-di-GMP could induce the production of a range of pro-inflammatory cytokines and chemokines, including IFN-γ, IL-12p40, IL-1β, TNF, IL-8 and IP10 by murine and human DCs *in vitro* [287]. Furthermore, expression of cell surface molecules including CD80, CD86 and MHC class II on DCs was enhanced by c-di-GMP stimulation and these c-di-GMP-stimulated DCs had a greater ability to activate T cells compared with untreated DCs [287].

It has recently been shown that c-di-GMP has the ability to stimulate and modulate the host immune response *in vivo*. Indeed, i.p. injection of mice with c-di-GMP resulted in an influx of granulocytes and monocytes to the peritoneal cavity [287] and i.n. administration of this STING agonist induced a transient inflammatory response in the lungs, involving activation of lung-resident DCs to produce pro-inflammatory cytokines [375]. Furthermore, c-di-GMP has been shown to protect against a number of respiratory bacterial infections by vigorously enhancing innate immune responses *in vivo*. I.n. administration of c-di-GMP 24 hours prior to i.n. challenge with *B. pertussis* enhanced recruitment of innate immune cells, including neutrophils, macrophages, NK cells and DCs, to the lungs [292]. The production of type I cytokines by these cells (including IFN-γ, TNF, IL-12p70 and IL-6), coupled with enhanced NO production by macrophages, had a protective effect and led to a significant reduction in *B. pertussis* colonisation in the lungs of treated compared with untreated mice, 24 hours post challenge [292]. Similarly, i.n. administration of c-di-GMP 24 or 48 hours prior to intratracheal (i.t.) challenge with a lethal dose of *K. pneumoniae,* stimulated protective immunity against infection and extended survival [376]. The protective effect of c-di-GMP is not solely limited to its delivery at mucosal sites. C-di-GMP was shown to act as a prophylactic agent in the well-

characterised mouse model of *S. aureus*-induced mastitis; two intramammary treatments with c-di-GMP before *S. aureus* challenge protected mice against infection and reduced CFU counts in the tissues [287].

In addition to its immunomodulatory capabilities, c-di-GMP has been shown to act as a potent adjuvant, capable of eliciting robust cellular and humoral responses to co-administered antigens. I.n. immunisation of PsaA with c-di-GMP enhanced serum IgG2a, IgG1 and IgA levels and protected against mucosal infection in the well-established mouse *Streptococcus pneumoniae* colonisation model [375]. Similarly, i.n. administration of the model antigen β-galactosidase (β-Gal) co-administered with c-di-GMP resulted in significantly higher anti-β-Gal IgG titres in sera, particularly of the IgG2a isotype, and anti-β-Gal-specific IFN-γ in spleens, when compared with spleen cells from mice that received antigen alone [377]. These studies suggest that c-di-GMP induces a Th1-biased response to a co-administered antigen.

There is strong evidence that stimulating multiple TLRs simultaneously can amplify innate immune responses and enhance vaccine efficacy. Zhu *et al.* demonstrated that when three TLR agonists (MALP2, poly (I:C) and CpG) were used in combination as adjuvants for an HIV peptide vaccine, the protective efficacy conferred with the vaccine was significantly enhanced compared with a single TLR or double-TLR combinations [378]. Likewise, the combination of a TLR9 and TLR4 agonist (CpG and GLA) as an adjuvant for a subunit tuberculosis vaccine augmented the antigen-specific T cell responses and conferred greater protection against *M. tuberculosis* infection than a vaccine adjuvanted with either TLR agonist alone [379]. Recent data suggest that TLR and STING agonists can similarly function synergistically to induce potent Th1-polarised immune responses; Temizoz and colleagues demonstrated that treatment of murine DCs with a combination of CpG and cGAMP significantly enhanced IL-12p40 production [380]. Furthermore, immunisation of mice with CpG and cGAMP enhanced OVA-specific IFN-γ and IgG2c *in vivo* compared with mice immunised with either of the adjuvants alone [380]. Similarly, Yildiz *et al.* found that culturing mouse spleen cells with a combination of CpG and c-di-GMP synergistically enhanced the production of pro-inflammatory cytokines, type I IFN and augmented co-stimulatory molecule expression [381].

LP1569, the novel TLR2 agonist from *B. pertussis,* has been shown to act as a potent adjuvant for an experimental aP vaccine, inducing robust Th1 and Th17 cell responses *in vivo* [276]. This study proposed that the STING agonist c-di-GMP is capable of acting synergistically with LP1569 to enhance cell-mediated immunity *in vivo.* The aim of this study was to assess the immunogenicity and protective efficacy of an experimental aP vaccine adjuvanted with a novel

TLR2-STING agonist combination. The hypothesis being tested was that this TLR2-STING agonist combination would elicit more potent cellular, humoral and memory immune responses than a vaccine containing a TLR agonist on its own, and generate greater protective and longer-lasting immunity against *B. pertussis* infection.

4.2 Results

4.2.1 The STING agonist c-di-GMP induces the production of T cell polarising cytokines, and enhances expression of MHC class II and co-stimulatory molecules, on murine BMDCs.

There is substantial evidence that Th1 and Th17 cells have an important role in the clearance of *B. pertussis* infection in mice and baboons [188, 189]. In order to induce optimal protection against this pathogen, a vaccine that promotes a potent cellular immune response is necessary. Adjuvants play a key role in determining the type of adaptive immune response elicited by acting on innate immune cells, such as DCs, to induce the production of T cell polarising cytokines. To investigate if the bacterial signalling molecule c-di-GMP can induce the production of Th1 and Th17 polarising cytokines, murine BMDCs were cultured with increasing concentrations of c-di-GMP for 24 hours. As STING is an intracellular receptor [382], the transfection reagent lipofectamine was used in an attempt to enhance intracellular uptake of c-di-GMP. C-di-GMP is known to be a potent inducer of type I IFN [383], so IFN-β was measured as a positive control.

A low concentration of c-di-GMP induced modest production of IL-12p40 but no IL-23 by BMDCs, whereas significantly more IL-12p40 and IL-23 was produced with a high concentration of c-di-GMP (Figure 4.1). The addition of lipofectamine did not significantly enhance production of either IL-12p40 or IL-23 by BMDCs at either concentration of c-di-GMP examined. C-di-GMP induced production of IFN-β in a dose-dependent manner, and this production was further enhanced by the addition of lipofectamine. The concentrations of IL-1β and IL-12p70 were below the detection limit. These findings indicate that c-di-GMP is capable of inducing the production of Th1 and Th17 polarising cytokines by BMDCs.

To investigate if this STING agonist could also enhance expression of MHC class II and co-stimulatory molecules on the surface of BMDCs, murine BMDCs were cultured with two concentrations of c-di-GMP and after 24 hours, the expression of CD80, CD86, CD40 and MHC class II was examined by flow cytometry. Stimulation of BMDCs with a low concentration of c-di-GMP slightly enhanced expression of CD80, CD86 and CD40, whereas a high concentration of c-di-GMP induced robust expression of these molecules (Figure 4.2A). Both concentrations of c-di-GMP enhanced expression of MHC class II to a similar level. To examine if transfecting c-di-GMP into the cells further enhanced expression of these molecules, BMDCs were cultured with the lower concentration of c-di-GMP in the presence and absence of lipofectamine. There was a slight increase in expression of CD86 upon addition of lipofectamine, but CD80, CD40 and MHC class II expression remained the same (Figure 4.2B).

These data suggest that c-di-GMP can enhance expression of CD80, CD86, CD40 and MHC class II on the surface of BMDCs and that lipofectamine is not required for this enhancement.

There is evidence that type I IFNs induce co-stimulatory molecule expression on murine DCs and from human peripheral blood mononuclear cells [384-388]. To investigate if c-di-GMP-driven type I IFN is responsible for the increase in cell surface marker expression observed (Figure 4.2), BMDCs from IFNAR$^{-/-}$ and WT mice were cultured with two concentrations of c-di-GMP or medium as a negative control for 24 hours. Basal expression of CD80, CD86 and CD40 was higher in IFNAR$^{-/-}$ compared with WT BMDCs, whereas MHC class II was higher in WT BMDCs (Figure 4.3). The expression of CD86, CD40 and MHC class II was modestly lower in IFNAR$^{-/-}$ compared with WT BMDCs stimulated with a low concentration of c-di-GMP, however, CD80 expression was similar on WT and IFNAR$^{-/-}$ DCs. In contrast, at a high concentration of c-di-GMP, there was a slight decrease in expression of CD40 on IFNAR$^{-/-}$ compared with WT BMDCs, but CD80, CD86 and MHC class II expression was the same in the WT and IFNAR$^{-/-}$ BMDCs.

These results indicate that at high concentrations of c-di-GMP, type I IFN signalling is not crucial to induce expression of MHC class II and co-stimulatory molecules on murine BMDCs. Overall this study showed that c-di-GMP was capable of inducing the production of Th1 and Th17 polarising cytokines by murine BMDCs, and could also enhance expression of CD80, CD86, CD40 and MHC class II on the surface of these innate cells in a type I IFN-independent manner.

4.2.2 C-di-GMP and LP1569 synergistically induce production of Th1 and Th17 cell polarising cytokines, and enhance expression of MHC class II and co-stimulatory molecules, on BMDCs and BMDMs.

The results in Chapter 3 showed that the TLR2 agonist LP1569 was capable of inducing the production of Th1 and Th17 polarising cytokines, and enhancing expression of cell surface markers on BMDCs *in vitro* (Figure 3.12). Having demonstrated that c-di-GMP was similarly capable of activating BMDCs (Figure 4.1 and 4.2), the ability of a combination of c-di-GMP and LP1569 to synergistically enhance the production of Th1 and Th17 polarising cytokines, and the expression of MHC and co-stimulatory molecules, on BMDCs was assessed.

BMDCs were cultured with two concentrations of c-di-GMP combined with a fixed concentration of LP1569 and after 24 hours, cytokine production was quantified. The addition

of LP1569 with a high concentration of c-di-GMP significantly increased IL-12p40, IL-12p70, IL-1β, IL-23 and IFN-β production over that observed with LP1569 alone (Figure 4.4A). However, combining LP1569 with a low concentration of c-di-GMP did not enhance production of these cytokines above that induced with LP1569 alone. Flow cytometry analysis was also performed on the stimulated cells to examine expression of CD80, CD86, CD40 and MHC class II in response to the combination of agonists. In all cases, the combination of c-di-GMP and LP1569 markedly increased expression of BMDC surface markers when compared to either agonist alone (Figure 4.4B). These findings suggest that c-di-GMP and LP1569 synergistically enhance the production of IL-12p40, IL-12p70, IL-1β, IL-23 and IFN-β, and the expression of CD80, CD86, CD40 and MHC class II, on the surface of BMDCs.

In order to examine if transfecting BMDCs stimulated with c-di-GMP and LP1569 would further enhance production of Th1 and Th17 polarising cytokines, BMDCs were cultured with combinations of the two agonists in the presence and absence of lipofectamine. The addition of lipofectamine enhanced production of IL-12p40 and IL-12p70 when a low concentration of c-di-GMP was combined with LP1569, however, when a high concentration of c-di-GMP was used with LP1569 in the presence of lipofectamine, production of IL-12p40 and IL-12p70 was significantly impaired (Figure 4.5). Addition of lipofectamine significantly enhanced IL-1β production at all concentrations of c-di-GMP combined with LP1569, whereas IL-23 production was decreased at all concentrations of c-di-GMP upon addition of lipofectamine. These data indicate that at high concentrations of c-di-GMP combined with LP1569, lipofectamine does not enhance production of IL-12p40, IL-12p70, IL-1β and IL-23 by murine BMDCs.

Having shown that the synergistic induction of T cell polarising cytokines by c-di-GMP and LP1569 is dependent on the concentration of c-di-GMP (Figure 4.4A), the effect of LP1569 concentration on the production of T cell polarising cytokines by BMDCs was assessed. Murine BMDCs were stimulated with increasing concentrations of LP1569 and a fixed concentration of c-di-GMP for 24 hours and then cytokine production was quantified by ELISA. C-di-GMP and LP1569 synergistically induced production of IL-12p40 by BMDCs and this production was dependent on the concentration of LP1569 (Figure 4.6). C-di-GMP-induced production of IL-12p70, IL-1β and IL-23 was enhanced by increasing the concentration of LP1569 initially, but production of IL-12p70 began to plateau and production of IL-1β and IL-23 decreased at the highest concentration of LP1569 examined. These results suggest that the synergistic induction of T cell polarising cytokines by BMDCs stimulated with c-di-GMP and LP1569 is dependent on the concentration of LP1569. From these data, the optimal concentrations determined for

induction of Th1 and Th17 polarising cytokines by BMDCs with this TLR2 and STING agonist combination were 10 μg/ml of c-di-GMP and 1 μg/ml of LP1569.

The data so far demonstrated that c-di-GMP and LP1569 synergistically induce production of Th1 and Th17 polarising cytokines by murine BMDCs (Figure 4.4, 4.5 and 4.6). Macrophages are another type of innate immune cell that can produce cytokines which influence neighbouring T cell differentiation upon encountering an invading pathogen, and can also present antigen to memory T cells.

To investigate if c-di-GMP and LP1569 were also capable of synergistically inducing production of T cell polarising cytokines by macrophages, murine BMDMs were cultured with two concentrations of c-di-GMP and a fixed concentration of LP1569 for 24 hours and then the quantity of cytokines in supernatants was examined. C-di-GMP and LP1569 synergistically induced production of IL-12p40 and IL-6 by BMDMs and this production was dependent on the concentration of c-di-GMP (Figure 4.7). Addition of lipofectamine significantly inhibited production of these two cytokines by BMDMs. In contrast, the production of IL-10, an anti-inflammatory cytokine, increased in a c-di-GMP and lipofectamine-dependent manner. No IL-12p70, IL-1β or IL-23 was detected. These findings suggest that the combination of c-di-GMP and LP1569 induces production of IL-12p40, IL-6 and IL-10 production by murine BMDCs. Addition of lipofectamine inhibits production of IL-12p40 and IL-6, but enhances the production of IL-10 by BMDCs.

Overall, this study showed that c-di-GMP and LP1569 synergistically induced production of Th1 and Th17 polarising cytokines by murine BMDCs and BMDMs, and enhanced expression of co-stimulatory molecules and MHC class II on BMDCs.

4.2.3 A TLR2-STING agonist combination promotes antigen-specific Th1 and Th17 cell responses to a model antigen *in vivo*.

Having shown that the combination of c-di-GMP and LP1569 induces production of T cell polarising cytokines by BMDCs (Figure 4.4, 4.5 and 4.6) and BMDMs (Figure 4.7), the ability of this TLR2-STING agonist combination to induce antigen-specific Th1 and Th17 cells *in vivo* was assessed.

Mice were injected in the footpad with the model antigen KLH in PBS (KLH), or formulated with c-di-GMP (KLH + c-di-GMP), LP1569 (KLH + LP1569), or a combination of the two adjuvants

(KLH + c-di-GMP + LP1569). 7 days later, the popliteal LNs were isolated and the KLH-specific T cell responses were examined. LNs from mice immunised with KLH + c-di-GMP + LP1569 displayed robust KLH-specific IFN-γ and IL-17 production, and also had a marked reduction in IL-5 production, compared with LNs from mice immunised with PBS alone, KLH alone, KLH + c-di-GMP or KLH + LP1569 (Figure 4.8). LNs from mice immunised with KLH + c-di-GMP also produced significantly more KLH-specific IFN-γ than LNs from mice immunised with KLH or KLH + LP1569. LNs from mice that were immunised with KLH + LP1569 produced slightly more KLH-specific IFN-γ and IL-17 than the PBS or KLH-immunised control mice.

These findings indicate that the adjuvant combination of c-di-GMP and LP1569 induces robust Th1 and Th17 cell responses, but no Th2 responses, against a model antigen *in vivo.*

4.2.4 An experimental aP vaccine adjuvanted with a TLR2-STING agonist combination induces robust antigen-specific IFN-γ and IgG2c production, and confers protection against *B. pertussis* infection in mice.

Having shown that the combination of c-di-GMP and LP1569 induced antigen-specific Th1 and Th17 cells *in vivo* (Figure 4.8), the ability of this combination to act as an adjuvant for an experimental aP vaccine and confer protection against *B. pertussis* infection in mice was examined.

Mice were immunised with an experimental aP vaccine containing two *B. pertussis* antigens (Ag; FHA, rPT) formulated with c-di-GMP (Ag + c-di-GMP), LP1569 (Ag + LP1569) or a combination of the two adjuvants (Ag + c-di-GMP + LP1569) at 0 and 4 weeks, before aerosol challenge with live *B. pertussis*. Mice immunised with Ag + c-di-GMP + LP1569 had significantly lower CFU counts in the lungs on days 3, 7 and 10 post challenge than mice immunised with Ag + c-di-GMP, and on day 10 than mice immunised with Ag + LP1569 (Figure 4.9A). Analysis of the areas under the curves (Figure 4.9B) confirmed the higher efficacy of Ag + c-di-GMP + LP1569 (26.71) compared with Ag + LP1569 (43.43) and Ag + c-di-GMP (62.77).

In order to assess the immune responses induced with the various experimental vaccines, the spleens and sera from four immunised mice in each group were isolated 1 day prior to aerosol challenge and their antigen-specific T cell and antibody responses were examined. Mice immunised with Ag + c-di-GMP + LP1569 had significantly enhanced FHA-specific IFN-γ production when compared with mice immunised with Ag + c-di-GMP or Ag + LP1569 (Figure 4.10A). Similarly, FHA-specific IL-17 production by spleen cells appeared to be greatest in mice

immunised with Ag + c-di-GMP + LP1569, although this was non-significant by two-way ANOVA. Spleen cells from mice immunised with Ag + LP1569 produced slightly more antigen-specific IFN-γ than spleen cells from mice immunised with Ag + c-di-GMP or with PBS. Production of FHA-specific IL-5 by spleen cells was similar between the three immunised groups of mice. FHA-specific antibody titres were enhanced in the sera of mice immunised with Ag + c-di-GMP + LP1569, with significantly higher levels of the IgG2c isotype detected when compared with the sera of mice immunised with Ag + LP1569 or Ag + c-di-GMP (Figure 4.10B). Mice immunised with Ag + c-di-GMP + LP1569 or Ag + LP1569 had significantly more serum FHA-specific IgG1 than mice immunised with Ag + c-di-GMP, and antigen-specific total IgG titres were similar between the three groups of immunised mice.

The results of this study indicate that an experimental aP vaccine formulated with c-di-GMP and LP1569 induces a robust Th1 and Th17 cell response and confers greater protection against *B. pertussis* infection than an experimental aP vaccine formulated with either adjuvant alone.

4.2.5 An aP vaccine adjuvanted with a TLR2-STING agonist combination induces greater Th1, Th17 and T_{RM} cell responses, and confers greater protection against *B. pertussis* infection, than an experimental alum-adjuvanted aP vaccine.

Having shown that c-di-GMP and LP1569 were capable of acting as an effective adjuvant combination for an experimental aP vaccine *in vivo* (Figure 4.9), it was investigated if this combination was more effective than an experimental alum-adjuvanted aP vaccine at conferring protection against *B. pertussis* infection in mice.

Mice were immunised twice at 0 and 4 weeks with an experimental aP vaccine composed of two *B. pertussis* antigens (Ag; FHA, rPT) formulated with alum (Ag + alum) or the TLR2-STING agonist combination (Ag + c-di-GMP + LP1569), and were then challenged by aerosol with live *B. pertussis* 2 weeks after the second immunisation. Mice immunised with Ag + c-di-GMP + LP1569 had lower CFU counts in the lungs post challenge compared with mice immunised with Ag + alum indicating they were more protected from *B. pertussis* infection although this did not reach statistical significance (Figure 4.11A). Analysis of the areas under the curves (Figure 4.11B) confirmed that immunising with Ag + c-di-GMP + LP1569 (45.18) was more effective at conferring protection than immunising with Ag + alum (58.95).

To compare the immune responses induced with Ag + alum and Ag + c-di-GMP + LP1569, the spleens and sera from four mice in each experimental group were isolated 1 day prior to aerosol challenge, and the antigen-specific T cell and antibody responses were examined. Spleen cells from mice immunised with Ag + c-di-GMP + LP1569 produced significantly more antigen-specific IFN-γ and IL-17 than spleen cells from mice immunised with Ag + alum (Figure 4.12A). Mice immunised with Ag + c-di-GMP + LP1569 had significantly higher serum IgG2c titres compared with mice immunised with Ag + alum (Figure 4.12B). Robust antigen-specific IgG1 titres were detected in the sera of mice immunised with Ag + c-di-GMP + LP1569 and Ag + alum. Mice immunised with Ag + alum had significantly more antigen-specific IL-5 produced by spleen cells, and had significantly higher serum IgG1 titres than the PBS-immunised control mice.

A study in Chapter 3 reported that an experimental LP1569-adjuvanted vaccine induced greater recruitment of $CD4^+$ memory T cells to the lungs than an experimental alum-adjuvanted aP vaccine after *B. pertussis* challenge (Figure 3.22). To assess the infiltration of memory $CD4^+$ T cells into the lungs following immunisation with an experimental aP vaccine formulated with both c-di-GMP and LP1569, the lungs were isolated 1 day before aerosol challenge and lung cells were stained with markers indicative of T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+CD103^+$) cells. Mice immunised with Ag + c-di-GMP + LP1569 had a marked increase in the percentages of $CD4^+$ T_{EM} and $CD4^+CD103^+$ T_{RM} cells infiltrating into the lungs when compared with mice immunised with Ag + alum or the PBS-immunised control mice, however, the percentages of $CD4^+$ T_{RM} cells infiltrating were similar between the three groups of mice (Figure 4.13A, 4.13B and 4.13C). The absolute numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells recruited to the lungs of mice immunised with Ag + c-di-GMP + LP1569 were considerably enhanced compared with the PBS-immunised control mice, or mice immunised with Ag + alum, though only the $CD4^+$ T_{EM} population reached statistical significance (Figure 4.13D). Interestingly, mice immunised with Ag + alum appeared to have lower numbers of T_{RM} and $CD103^+$ T_{RM} cells in the lungs than the PBS-immunised control mice.

To investigate if mice immunised with Ag + c-di-GMP + LP1569 maintained higher memory T cell numbers in the lungs after infection, the lungs were isolated 7 days after aerosol challenge and stained with the same markers as described for the prechallenge day. After *B. pertussis* challenge, the percentages of $CD4^+$ T_{EM} and $CD103^+$ T_{RM} cells in the lungs was similar between mice immunised with Ag + c-di-GMP + LP1569, Ag + alum and the PBS-immunised control mice, however, mice immunised with Ag + alum appeared to have the lowest percentages of $CD4^+$

T_{RM} cells compared with the other two groups of mice (Figure 4.14A, 4.14B and 4.14C). The absolute numbers of lung $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells was similar between mice immunised with Ag + c-di-GMP + LP1569 and the PBS-immunised control mice, however, mice immunised with Ag + alum appeared to have lower numbers of these memory T cell subtypes (Figure 4.14D). These data suggest that immunising mice with an experimental aP vaccine containing a TLR2-STING agonist combination as the adjuvant induces greater memory T cell infiltration into the lungs, than does immunising with an experimental alum-adjuvanted aP vaccine. Furthermore, after *B. pertussis* challenge the memory T cells in the lungs of mice immunised with an experimental aP vaccine formulated with c-di-GMP and LP1569 expand to a greater extent.

Overall this study indicates that an experimental aP vaccine formulated with c-di-GMP and LP1569 protects mice against *B. pertussis* infection more than an experimental alum-adjuvanted aP vaccine. Furthermore, this TLR2-STING agonist-adjuvanted aP vaccine induces greater systemic Th1 and Th17 cell responses, and memory T cell recruitment to the lungs, than those seen with an aP vaccine containing a Th2-promoting adjuvant.

4.2.6 Intranasal immunisation with an experimental aP vaccine containing a TLR2-STING agonist combination induces potent Th17 and IgA responses, robust T_{RM} cell recruitment, and confers protection against *B. pertussis* infection in mice.

Immunisation by the i.n. route induces potent mucosal immunity against respiratory pathogens [389-391]. As *B. pertussis* is spread by aerosol transmission, it was hypothesised that i.n. administering an aP vaccine that induced robust mucosal immune responses could enhance protection against respiratory infection. Mice were immunised i.p. twice at 0 and 4 weeks with an experimental aP vaccine formulated with c-di-GMP and LP1569 (Ag + c-di-GMP + LP1569), or were i.n. immunised twice with Ag + c-di-GMP + LP1569 before aerosol challenge with live *B. pertussis.*

Both groups of immunised mice were significantly more protected from *B. pertussis* infection than the PBS-immunised control mice and rapidly cleared the infection (Figure 4.15A). Analysis of the areas under the curves (Figure 4.15B) showed that mice immunised i.n. with Ag + c-di-GMP + LP1569 were more protected from *B. pertussis* infection (15.55) than the mice immunised i.p. with Ag + c-di-GMP + LP1569 (21.07). There were no significant differences between the immunised groups of mice by two-way ANOVA.

Having shown that immunising i.n. with an experimental aP vaccine formulated with a STING-TLR2 agonist confers as good protection as i.p. delivery, the immune responses induced by this mucosal route of administration were investigated. The spleens from four mice in each experimental group were isolated 1 day prior to aerosol challenge and the antigen-specific T cell responses were examined. Spleen cells from mice immunised i.p. with Ag + c-di-GMP + LP1569 produced robust FHA and *B. pertussis*-specific IFN-γ, but low amounts of antigen-specific IL-17 (Figure 4.16). In contrast, immunising with Ag + c-di-GMP + LP1569 by the i.n. route induced robust Prn, FHA and *B. pertussis*-specific IL-17 production by spleen cells, which was significantly higher than the production seen in the mice immunised i.p. with Ag + c-di-GMP + LP1569. Only the *B. pertussis*-specific IFN-γ production by spleen cells in mice immunised i.n. with Ag + c-di-GMP + LP1569 was higher than the production seen in the PBS-immunised control mice.

The sera were also taken from four mice in each experimental group 1 day before aerosol challenge to examine the antigen-specific antibody responses induced by the different routes of administration. Mice immunised i.p. with Ag + c-di-GMP + LP1569 had substantially more FHA and Prn-specific IgG2c, IgG1 and total IgG titres in sera than the PBS-immunised control mice (Figure 4.17A and 4.17B). Low FHA-specific total IgG and no FHA-specific IgG2c or IgG1 titres were detected in the sera of mice i.n. immunised with Ag + c-di-GMP + LP1569. These i.n. immunised mice had significantly higher titres of Prn-specific IgG2c and IgG1 in sera compared with the PBS-immunised control mice, although these were not as robust as their i.p. immunised counterparts.

Mucosal immunisation has been reported to induce robust antigen-specific IgA production [369]. Similarly, IL-17 has been shown to be involved in secretory IgA induction in the airways [392]. Given the potent antigen-specific IL-17 response in mice i.n. immunised with Ag + c-di-GMP + LP1569, the induction of IgA responses in the lungs was examined. 3 days after aerosol challenge with live *B. pertussis*, the lungs were isolated and homogenised and the antigen-specific IgA production was quantified. Mice that received Ag + c-di-GMP + LP1569 i.n. had modest amounts of Prn, PT and *B. pertussis*-specific IgA in the lungs, although this was determined to be non-significant by one-way ANOVA (Figure 4.18). In contrast, no IgA was detected in the lungs of mice that were immunised i.p. with Ag + c-di-GMP + LP1569 or the PBS-immunised control mice.

In addition to spleens and sera, the lungs were isolated 1 day prior to *B. pertussis* challenge to examine recruitment of memory T cell subtypes following immunisation. Lung cells were

stained with markers for $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} and analysed by flow cytometry. Immunisation with Ag + c-di-GMP + LP1569 i.p. recruited greater $CD4^+$ T_{EM} cells to the lungs than the PBS-immunised control mice, however, the percentages of T_{RM} and $CD103^+$ T_{RM} cells were similar between the two groups of mice (Figure 4.19A, 4.19B and 4.19C). In contrast, there was substantially greater $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} infiltration into the lungs following i.n. immunisation with Ag + c-di-GMP + LP1569 compared with the i.p. immunised mice. The absolute numbers reflected this interesting result; mice i.n. immunised with Ag + c-di-GMP + LP1569 had significantly more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells infiltrating into the lungs, than mice immunised with Ag + c-di-GMP + LP1569 i.p. (Figure 4.19D).

The lungs were also isolated 7 days after aerosol challenge to investigate the memory $CD4^+$ T cell expansion in the lungs after infection. While the percentages of $CD4^+$ T_{EM} cells in the lungs of mice immunised with Ag + c-di-GMP + LP1569 i.p. were higher than the PBS-immunised control mice, the percentages of this memory T cell subtype in the lungs of mice i.n. immunised with Ag + c-di-GMP + LP1569 were the greatest (Figure 4.20A). Furthermore, mice i.n. immunised with Ag + c-di-GMP + LP1569 had a substantial increase in T_{RM} and $CD103^+$ T_{RM} cells in the lungs compared with mice immunised with Ag + c-di-GMP + LP1569 i.p. or the PBS control (Figure 4.20B and 4.20C). The absolute numbers were consistent with this, with mice i.n. immunised with Ag + c-di-GMP + LP1569 having significantly elevated numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs compared with the other two groups of mice (Figure 4.20D).

The results from this study indicate that i.n. immunisation with an experimental aP vaccine formulated with a TLR2-STING agonist combination switches the immune profile from Th1 to Th17, without impairing the protection conferred against *B. pertussis* infection. While it appears that i.n. is not as effective as i.p. immunisation for the induction of potent IgG responses, it seems to induce greater mucosal IgA responses than those seen when an experimental aP vaccine is administered by the parenteral route. Furthermore, i.n. immunisation with an experimental aP vaccine formulated with c-di-GMP and LP1569 appears to be the most effective method for recruiting and expanding large numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs of mice.

4.3 Discussion

In this study, the bacterial intracellular signalling molecule c-di-GMP, and the TLR2 agonist LP1569 were assessed for their ability to synergistically induce Th1 and Th17 responses *in vitro* and *in vivo*, with the aim of testing their efficacy as an adjuvant combination for an experimental aP vaccine. Consistent with previous studies, c-di-GMP was shown to induce production of IL-12p40 and type I IFN by murine BMDCs [287]. The production of the Th17 polarising cytokine IL-23 was also observed, though there are no previous reports of c-di-GMP inducing production of this cytokine *in vitro*. Furthermore, c-di-GMP was also found to be capable of enhancing expression of CD80, CD86, CD40 and MHC class II expression on the surface of BMDCs, as has been previously reported [287]. Type I IFNs have been shown to play a role in co-stimulatory molecule expression on murine DCs and from human peripheral blood mononuclear cells [384-388], so it was hypothesised that c-di-GMP enhances co-stimulatory molecule expression in a type I IFN-dependent manner. It was found that expression of CD80, CD86, CD40 and MHC class II in response to c-di-GMP on BMDCs, was only dependent on type I IFNs at low concentrations of c-di-GMP, and that high concentrations of c-di-GMP did not require type I IFN to induce expression.

It has been reported that TLR and STING agonists can act synergistically to induce potent Th1-polarised cytokines. A combination of CpG and cGAMP significantly enhanced IL-12p40 production by BMDCs [380]. Furthermore, mouse spleen cells treated with c-di-GMP complexed with CpG induced greater IL-12, TNF and IL-6 production than cells treated with either agonist alone [381]. The results of the present study showed that c-di-GMP and LP1569 can synergistically induce the production of not only Th1 polarising cytokines, but also the Th17 polarising cytokines IL-1β, IL-23 and IL-6 *in vitro*.

Both c-di-GMP and LP1569 have previously been shown to act as adjuvants, capable of enhancing the immune responses to co-administered antigens, in particular promoting Th1 responses [276, 287, 288, 377, 380, 393]. The results of the current study showed that immunising mice with a model antigen adjuvanted with this TLR2-STING agonist combination induced potent Th1 cell responses, but interestingly, this combination also induced robust Th17 cell responses, not seen with either adjuvant alone. These data suggested that c-di-GMP and LP1569 would be an effective adjuvant combination for an aP vaccine and indeed, studies using a murine model of *B. pertussis* infection were very promising; an experimental aP vaccine formulated with c-di-GMP and LP1569 conferred greater protection against *B. pertussis* infection than experimental aP vaccines adjuvanted with c-di-GMP or LP1569 alone. Finally, an experimental aP vaccine adjuvanted with the TLR2-STING agonist combination was shown to

be more effective at protecting mice against *B. pertussis* infection than an experimental alum-adjuvanted aP vaccine, inducing potent antigen-specific Th1 and Th17 cells, and IgG2c production.

A previous report in Chapter 3 showed that an experimental LP1569-adjuvanted aP vaccine induced greater numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs of infected mice than an experimental alum-adjuvanted aP vaccine, while an experimental CpG-adjuvanted aP vaccine did not. This was hypothesised to be due to the ability of LP1569 to promote more potent Th17 responses than CpG. In this study, it was shown that the adjuvant combination of c-di-GMP and LP1569 induced stronger Th1 and Th17 cell responses than an experimental LP1569-adjuvanted aP vaccine, indicating this combination vaccine may be more effective at inducing infiltration of memory $CD4^+$ T cell subtypes to the lungs. Indeed, mice immunised with an experimental aP vaccine formulated with a TLR2-STING agonist combination had greater trafficking of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells to the lungs before *B. pertussis* challenge, than an experimental alum-adjuvanted aP vaccine, and after infection, these cells expanded to a greater extent. These data suggest that an experimental aP vaccine containing a TLR2-STING agonist combination may confer longer lasting protection against *B. pertussis* infection than an experimental alum-adjuvanted aP vaccine by recruiting memory T cells to the lung.

Immunisation by the i.n. route generates potent mucosal immune responses and protects against respiratory infections [389-391]. Given that *B. pertussis* infects the airways, it was hypothesised that immunising i.n. with an aP vaccine containing a potent Th1/Th17-inducing adjuvant would be an effective method of eliciting a more appropriate and protective immune response against this pathogen. Indeed, nasal delivery of a single dose of the live attenuated *B. pertussis* vaccine BPZE1, was shown to induce a systemic Th1 response and confer long-lasting protection against *B. pertussis* infection in mice [306].

The results of the present study showed that mice immunised i.n. with an experimental aP vaccine formulated with a TLR2-STING agonist combination were as protected from *B. pertussis* challenge as mice immunised i.p. with the same experimental vaccine. Furthermore, the T cell response data obtained were very compelling; while immunising i.p. with an experimental aP vaccine formulated with c-di-GMP and LP1569 induced a robust Th1 and a lesser Th17 response, i.n. administering this experimental aP vaccine completely shifted the systemic $CD4^+$ T cell response to Th17. This is consistent with a recent report by Orr *et al.,* who demonstrated that while administering a tuberculosis subunit vaccine i.m. protects against aerosolised *M. tuberculosis* infection by inducing robust Th1 immunity in mice, i.n. delivery of

this vaccine switches the immune profile to Th17 in both the lungs and spleen, without affecting the protective efficacy of the vaccine [394]. Furthermore, it was also found in the present study that mice immunised i.n. with an experimental aP vaccine formulated with c-di-GMP and LP1569 did not have as robust levels of antigen-specific IgG2c, IgG1 or total IgG antibodies in sera, as mice immunised by the i.p. route. Similarly, Christensen and colleagues showed that i.n. immunisation of mice with a vaccine containing a Th1/Th17-promoting adjuvant induced IgG1 and IgG2a responses that were 10-100 fold below the responses observed in s.c. immunised mice [369].

There is evidence that while i.n. immunisation is not very effective at inducing systemic IgG responses, it elicits potent mucosal IgA immunity. Indeed, Christensen *et al.* observed that i.n. immunisation of mice with a vaccine containing a Th1/Th17-inducing adjuvant increased IgA secretion in the airways compared with s.c. immunisation [369]. The results of the present study showed that while IgA was completely absent in mice that were i.p. immunised with an experimental aP vaccine, mice immunised i.n. produced modest amounts of antigen-specific IgA in the lungs post challenge. Indeed, Th17 cells have been shown to indirectly enhance levels of secretory IgA in the airways by promoting expression of the receptor which facilitates transport of IgA to the lung epithelium [392]. Furthermore, it has been shown that depletion of IL-17 ablates lung IgA responses [369]. Given this, it was proposed that the robust systemic Th17 response observed in mice after i.n. immunisation facilitates IgA induction in the airways. The role for IgA in *B. pertussis* infection has not been well described, but this antibody isotype has been detected in the lungs of mice after primary infection [64] and moreover, IgA has been shown to facilitate the binding, phagocytosis and killing of *B. pertussis* organisms by human polymorphonuclear leukocytes indicating it could play an important role in clearance [206, 207].

In addition to the aforementioned data, there have been reports that i.n. immunisation may be a more effective method of inducing recruitment of memory T cells to the lungs. Indeed, Christensen *et al.* observed that significantly more IL-17$^+$ CD4$^+$ T_{EM} cells were recruited to the lungs of mice primed s.c. and boosted i.n. with a vaccine containing the Th1/Th17-promoting adjuvant cationic adjuvant formulation no. 1 (CAF01), than mice immunised s.c. twice with the same vaccine, which remained at naïve background level [369]. Furthermore, the IL-17$^+$ T_{EM} cells persisted in the lungs for at least 6 weeks after immunisation and adopted a T_{RM} (CD4$^+$CD44$^+$CD62L$^-$CD69$^+$) phenotype. In the present study, it was found that mice i.n. immunised with an experimental aP vaccine formulated with a TLR2-STING agonist combination recruited significantly more CD4$^+$ T_{EM}, T_{RM} and CD103$^+$ T_{RM} cells to the lungs than

mice immunised i.p. with the same experimental aP vaccine before *B. pertussis* challenge. Furthermore, these memory T cell populations expanded to a greater degree in the i.n. immunised mice after challenge. Considering that i.n. immunisation with an experimental aP vaccine containing a TLR2-STING agonist combination induces robust Th17 cell responses, and that there is evidence that IL-17 is important in the trafficking of $CD4^+$ T_{EM} cells to the lungs [369], these data support the previous hypothesis discussed in Chapter 3 that IL-17 production is essential for memory $CD4^+$ T cells recruitment. The results from this study indicated that i.n. immunisation was a significantly more effective route of administration for the induction of local memory $CD4^+$ T cell responses.

Overall, the experiments conducted in this study showed that immunisation of mice with an experimental aP vaccine formulated with a TLR2-STING agonist combination elicited a more potent Th1/Th17-biased response, and greater $CD4^+$ T_{RM} cell infiltration into the lungs, than mice that were immunised with an experimental alum-adjuvanted aP vaccine. It was also found that i.n. administration of an experimental aP vaccine containing a Th1-promoting adjuvant completely shifted the immune profile from Th1 to Th17 without affecting the protective efficacy of the vaccine. Furthermore, i.n. was shown to be significantly more effective than i.p. immunisation at inducing $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cell infiltration into the lungs. I.n. immunisation could potentially be a more favourable route of administration as *B. pertussis* is a respiratory pathogen, and this method of delivery can induce potent mucosal immunity [389-391]. However, the safety of administering an aP vaccine i.n. would need further investigation as there have been reports linking the i.n. immunisation of a number of vaccines with Bell's Palsy [320, 321, 395]. Nevertheless, a number of live attenuated influenza vaccines that are administered i.n. have been proven to be safe and are commercially available for human use, so the possibility of introducing a nasally administered *B. pertussis* vaccine could yet be achieved.

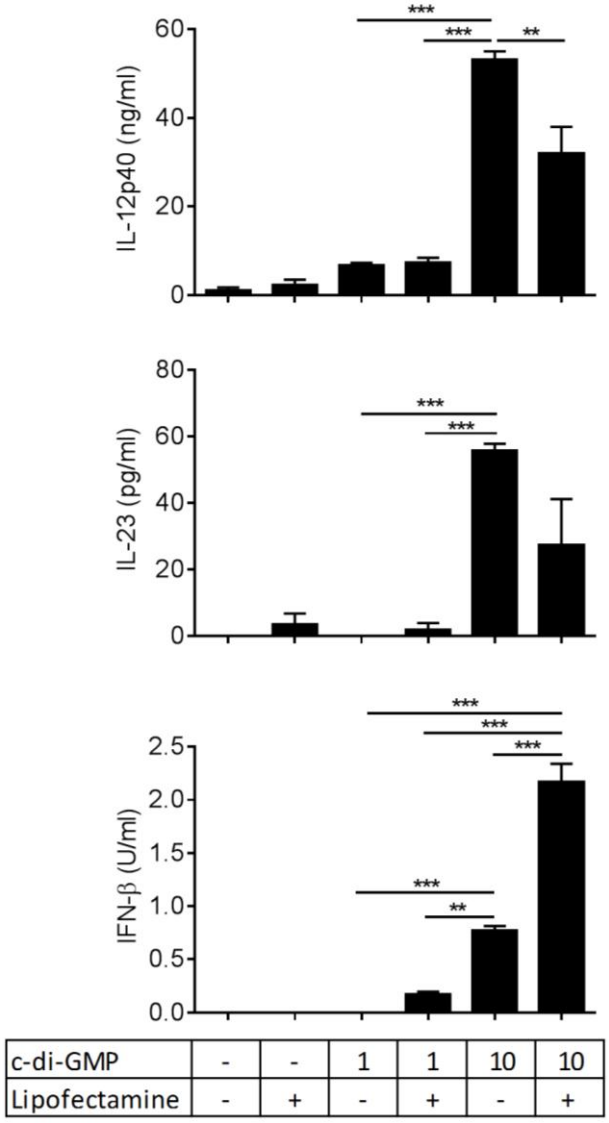


Figure 4.1 c-di-GMP induces production of Th1 and Th17 polarising cytokines, as well as type I IFN, by BMDCs.

BMDCs were cultured with c-di-GMP (1 or 10 μg/ml) in the presence or absence of the transfection reagent lipofectamine (1 μg/ml) or with medium as a control. After 24 hours, the production of IL-12p40, IL-23 and IFN-β was quantified in cell supernatants by ELISA. Data are the mean ± SEM of triplicate stimulations and are representative of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ by one-way ANOVA with the Bonferroni post test.

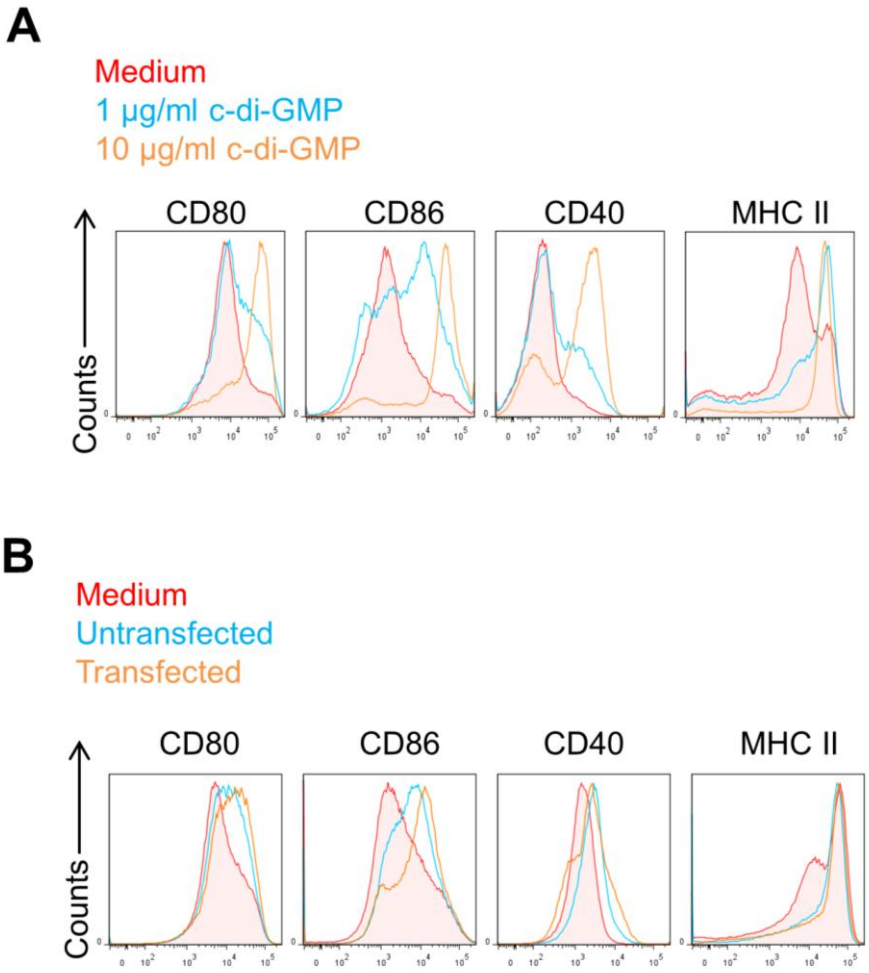


Figure 4.2 c-di-GMP enhances expression of MHC class II and co-stimulatory molecules on BMDCs in a dose-dependent manner.
BMDCs were cultured with c-di-GMP (1 or 10 µg/ml), in the presence or absence of lipofectamine (1 µg/ml) or with medium as a control. After 24 hours, expression of CD80, CD86, CD40 and MHC class II was assessed by flow cytometry. (A) Representative FACS plots showing the effects of different concentrations of c-di-GMP on BMDC surface marker expression. (B) FACS plots demonstrating the effect lipofectamine has on the ability of BMDCs stimulated with c-di-GMP (1 µg/ml) to express maturation markers and antigen-presenting molecules. Data are representative FACS plots from three independent experiments.

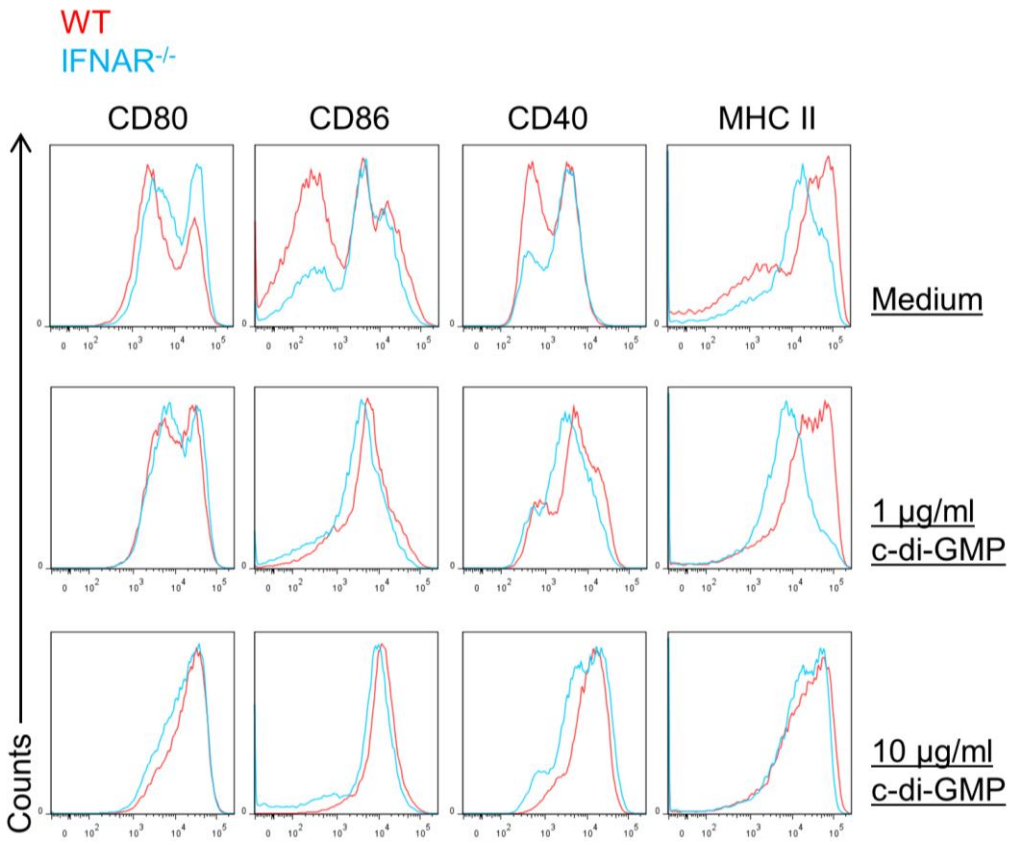


Figure 4.3 Type I IFN signalling plays a minor role in the ability of c-di-GMP to enhance expression of co-stimulatory molecules on the surface of BMDCs.
WT and IFNAR$^{-/-}$ BMDCs were cultured with c-di-GMP (1 or 10 ug/ml) or medium as a negative control for 24 hours. Expression of CD80, CD86, CD40 and MHC class II was assessed by flow cytometry. Data are representative FACS plots from three independent experiments.

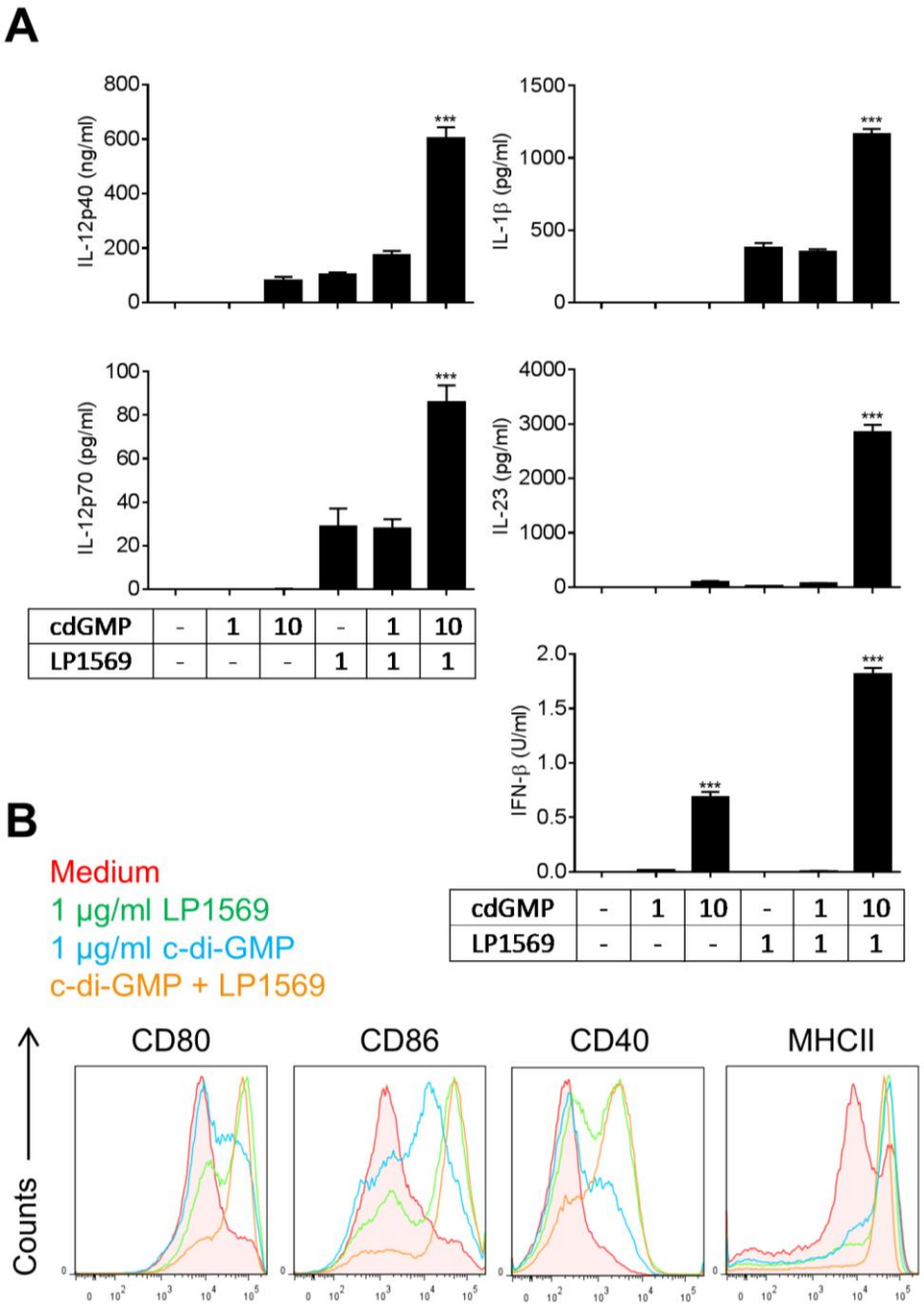


Figure 4.4 C-di-GMP and LP1569 enhance expression of MHC class II and co-stimulatory molecules and synergistically induce production of T cell polarising cytokines by BMDCs.
BMDCs were stimulated with c-di-GMP (cdGMP; 1 or 10 µg/ml), LP1569 (1 µg/ml) or a combination of the two agonists. (A) Production of Th1 and Th17 polarising cytokines and type I IFN was quantified by ELISA after 24 hours. Results are the mean ± SEM of triplicate stimulations and are representative of three independent experiments. ***$p < 0.001$ versus all other groups by one-way ANOVA with the Bonferroni post test. (B) Expression of the maturation markers CD80, CD86, CD40 and MHC class II was assessed by flow cytometry after 24 hours. Data are representative FACS plots from three independent experiments.

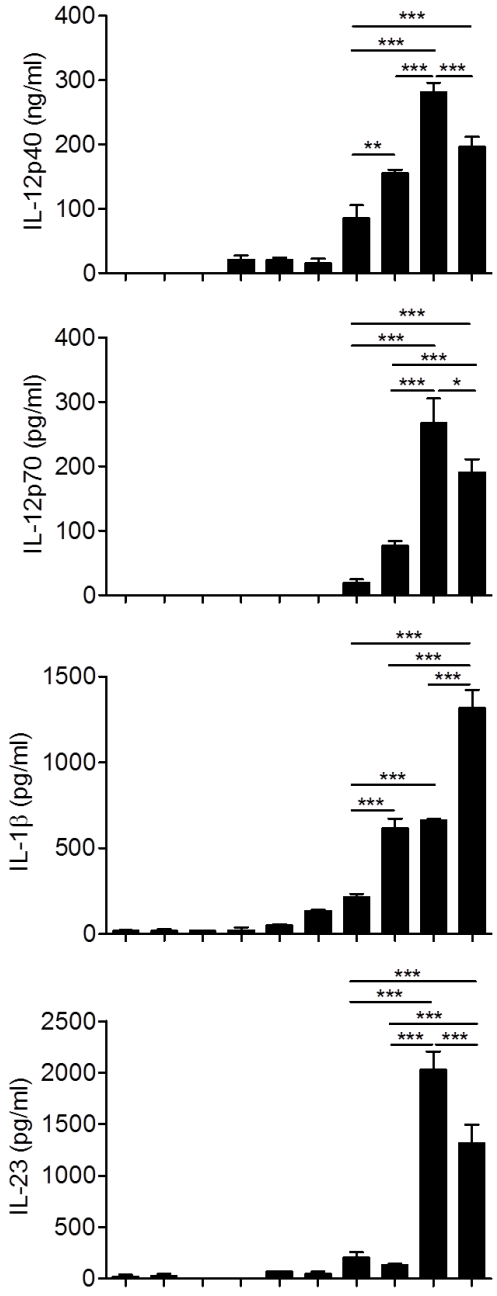


Figure 4.5 The transfection reagent lipofectamine does not enhance production of IL-12p40, IL-12p70 or IL-23 by BMDCs in response to stimulation with c-di-GMP and LP1569.
BMDCs were stimulated with c-di-GMP (1 or 10 µg/ml), LP1569 (1 µg/ml) or a combination of the two agonists in the presence or absence of lipofectamine (1 µg/ml). After 24 hours, the production of IL-12p40, IL-12p70, IL-1β and IL-23 was quantified by ELISA on cell supernatants. Data are the mean ± SEM of triplicate stimulations and are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA with the Bonferroni post test.

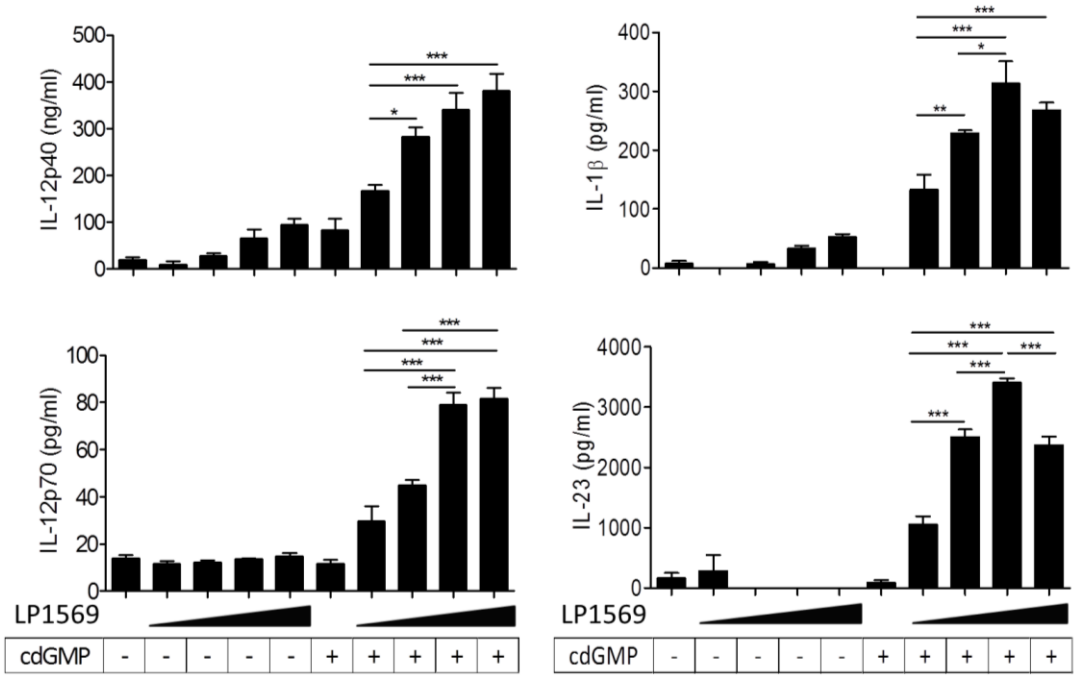


Figure 4.6 Synergistic induction of T cell polarising cytokines by c-di-GMP and LP1569 in BMDCs is dependent on the concentration of LP1569.

BMDCs were stimulated with increasing concentrations of LP1569 (0.04, 0.2, 1 or 5 µg/ml), c-di-GMP (cdGMP; 10 µg/ml) or a combination of the two agonists. After 24 hours, Th1 and Th17 cell polarising cytokine production was quantified by ELISA on cell supernatants. Data are the mean ± SEM of triplicate stimulations and are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA with the Bonferroni post test.

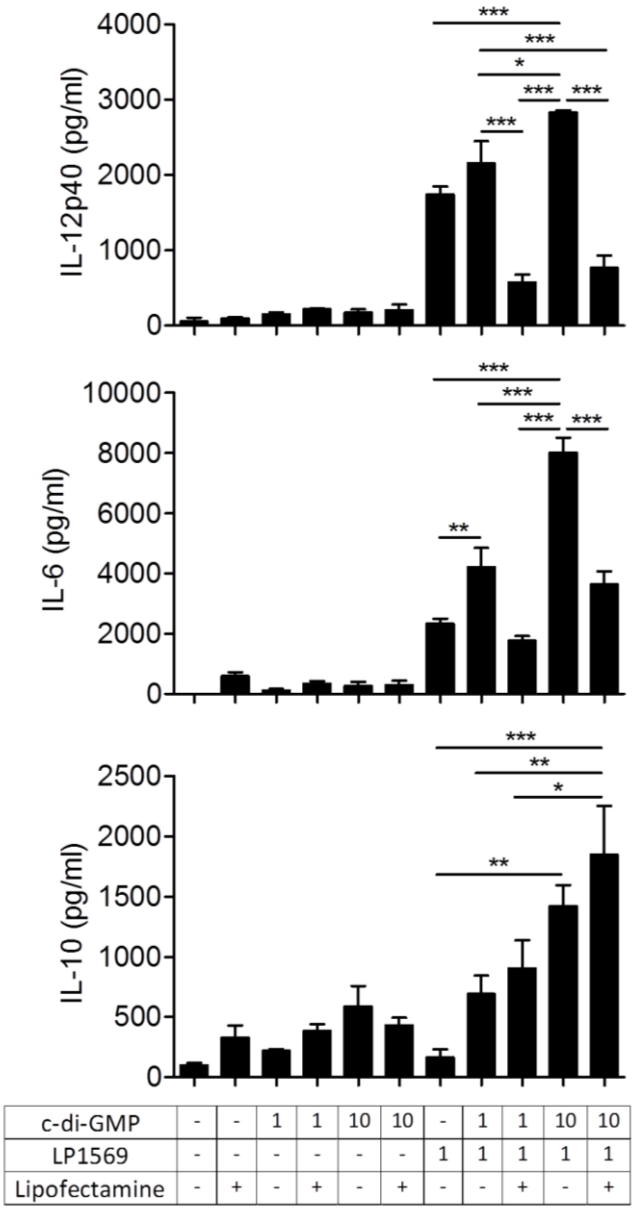


Figure 4.7 C-di-GMP and LP1569 synergistically induce production of T cell polarising cytokines by BMDMs, and this is not enhanced by addition of lipofectamine.
BMDMs were stimulated with c-di-GMP (cdGMP; 1 or 10 µg/ml), LP1569 (1 µg/ml) or a combination of the two agonists in the presence or absence of lipofectamine (1 µg/ml). After 24 hours, IL-12p40, IL-6 and IL-10 production by BMDMs was quantified by ELISA on cell supernatants. Data are the mean ± SEM of triplicate stimulations and are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA with the Bonferroni post test.

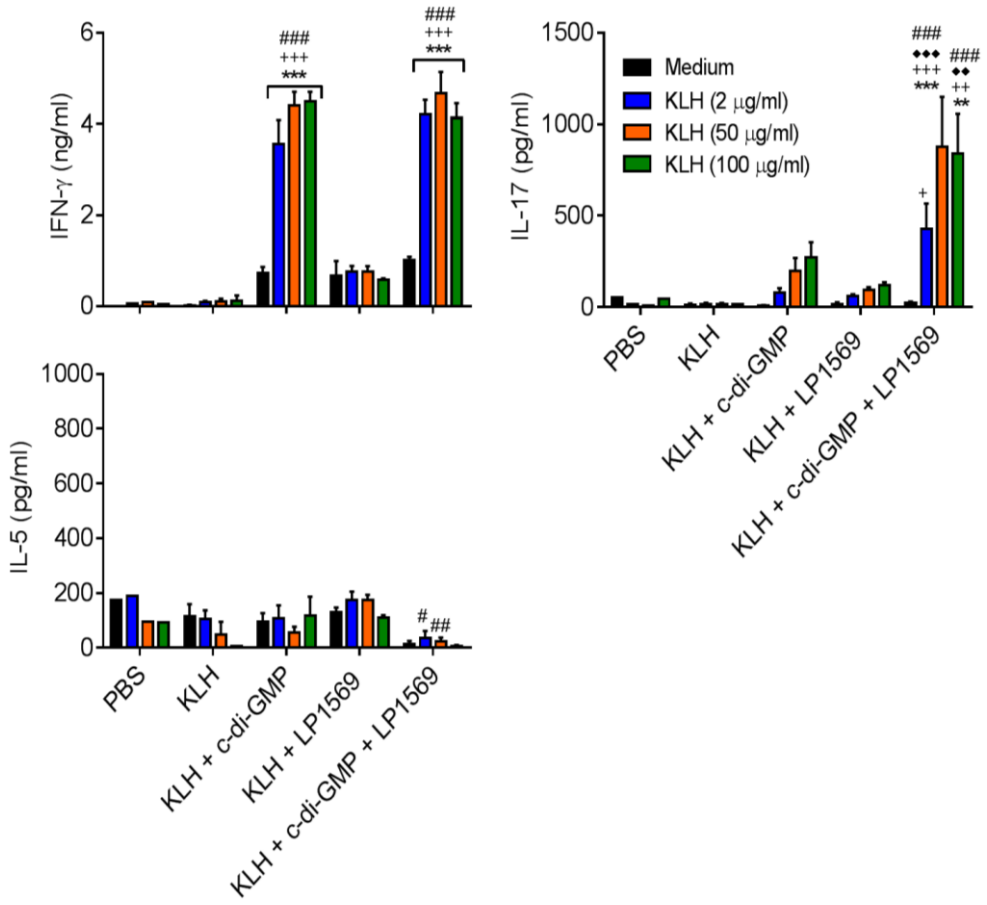
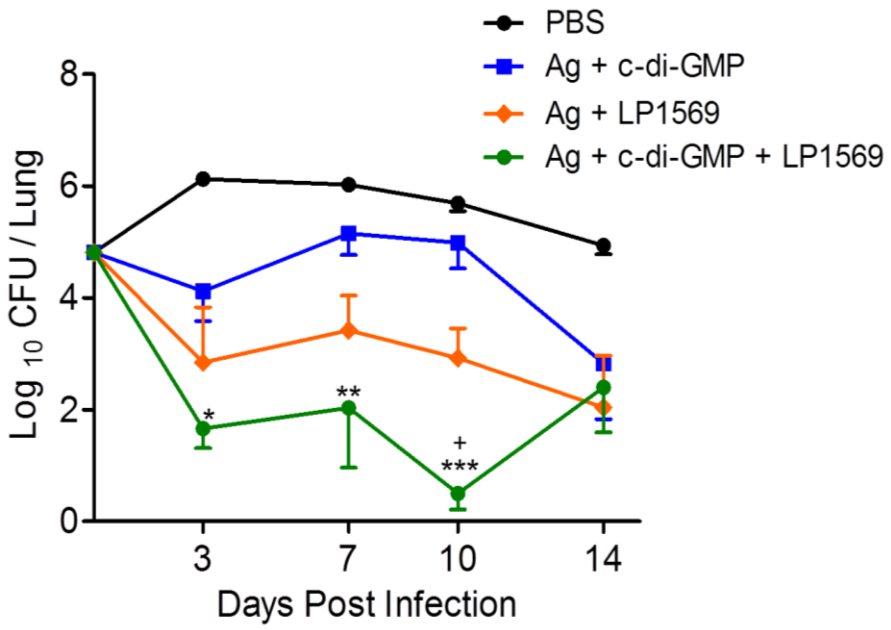


Figure 4.8 A TLR2-STING agonist combination promotes antigen-specific Th1 and Th17 responses to a model antigen *in vivo.*

Mice were injected in the footpad with PBS, KLH (1 µg/mouse), KLH + c-di-GMP (10 µg/mouse), KLH + LP1569 (50 µg/mouse) or KLH + c-di-GMP + LP1569. 7 days later, the popliteal LNs were isolated and cells were restimulated with increasing concentrations of KLH (2, 50, 100 µg/ml). After 72 hours, KLH-specific cytokine production in the supernatants was assessed by ELISA. Results are representative of the mean ± SEM of three mice per group. $**p < 0.01$, $***p < 0.001$ versus PBS; $++p < 0.01$, $+++p < 0.001$ versus KLH; ◆◆$p < 0.01$, ◆◆◆$p < 0.001$ versus KLH + c-di-GMP; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus KLH + LP1569 by two-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	Ag + c-di-GMP	Ag + LP1569	Ag + c-di-GMP + LP1569
	79.53	62.77	43.43	26.71

Figure 4.9 An experimental aP vaccine adjuvanted with a TLR2-STING agonist combination confers protection against *B. pertussis* infection.
Mice were immunised i.p. twice (0 and 4 weeks) with PBS as a control, or the antigens FHA and rPT (Ag; 0.2 µg/mouse) formulated with either c-di-GMP (10 µg/mouse), LP1569 (50 µg/mouse) or the two agonists combined. Immunised mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation and lungs were taken from four mice per group at intervals post infection to determine bacterial colonisation. (A) Results shown are mean ± SEM CFU counts (n=4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus Ag + c-di-GMP; +$p < 0.05$ versus Ag + LP1569 by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

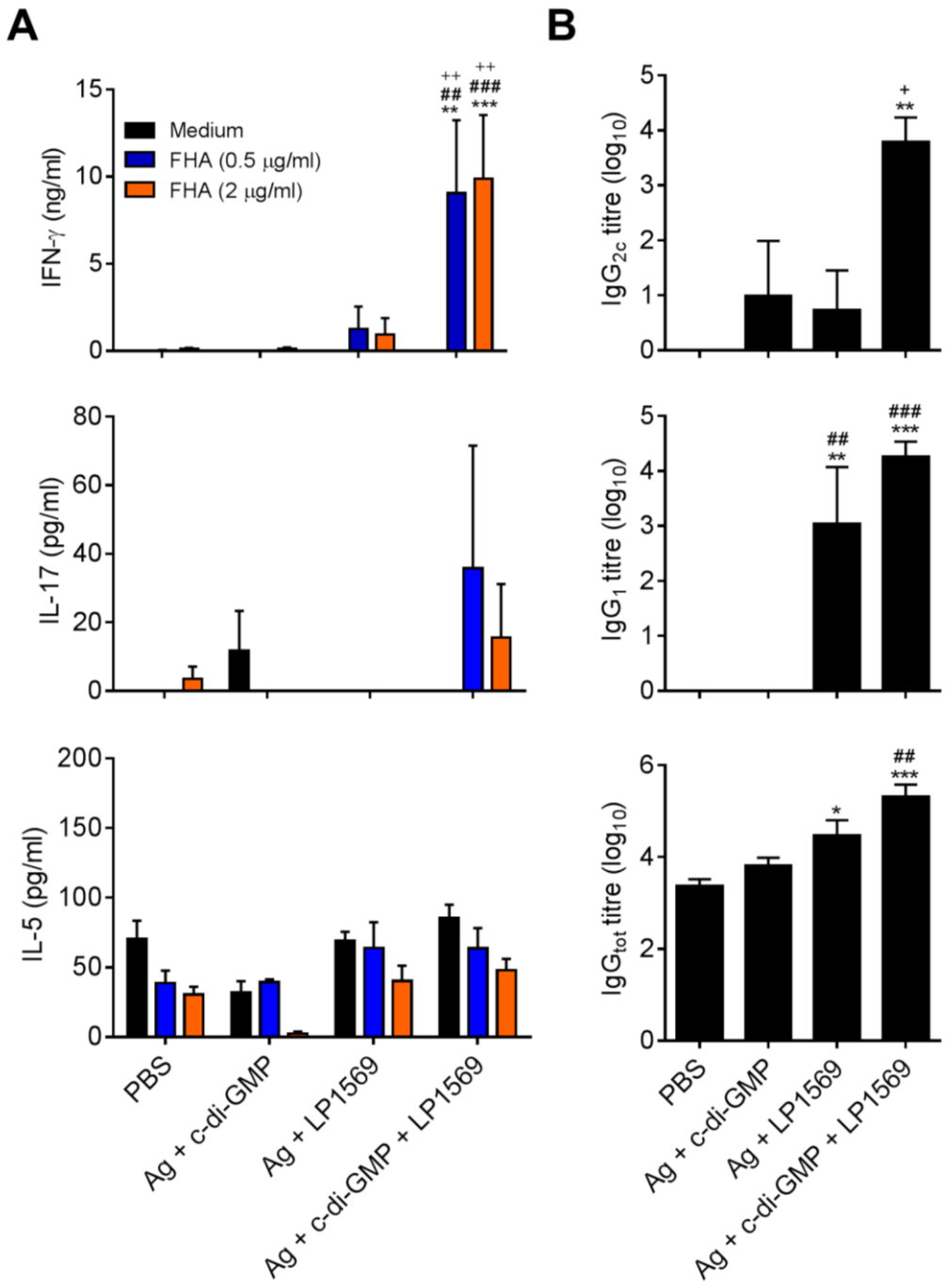
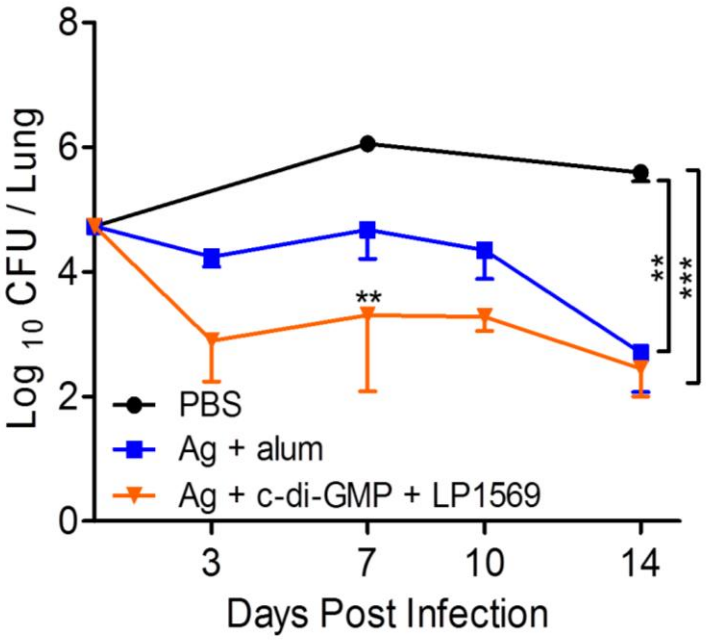


Figure 4.10 An experimental aP vaccine adjuvanted with a TLR2-STING agonist combination induces robust antigen-specific IFN-γ and IgG2c production.

Mice were immunised i.p. twice as described in Figure 4.9. 2 weeks after the last immunisation, spleens and sera from four mice in each experimental group were isolated. (A) Spleen cells (2 x 10^6 cells/ml) were cultured with FHA (0.5 or 2 μg/ml) or medium as a negative control and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). $^{**}p < 0.01$, $^{***}p < 0.001$ versus PBS; $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus Ag + c-di-GMP; $++p < 0.01$ versus Ag + LP1569 by two-way ANOVA with the Bonferroni post test. (B) FHA-specific antibody titres in sera were quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus PBS; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus Ag + c-di-GMP; $+p < 0.05$ versus Ag + LP1569 by one-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	Ag + alum	Ag + c-di-GMP + LP1569
	78.53	58.95	45.18

Figure 4.11 An experimental two-component aP vaccine adjuvanted with a TLR2-STING agonist combination is more effective than an experimental alum-adjuvanted aP vaccine at conferring protection against *B. pertussis* infection.

Mice were immunised i.p. twice (0 and 4 weeks) with PBS as a control or the antigens FHA and rPT (Ag; 0.2 µg/mouse) formulated with alum (100 µg/mouse) or a combination of c-di-GMP (10 µg/mouse) and LP1569 (50 µg/mouse). Immunised mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation and lungs were taken from 4 mice per group at intervals post infection to determine bacterial colonisation. (A) Results shown are mean ± SEM CFU counts (n=4 mice). $^{**}p < 0.01$, $^{***}p < 0.001$ versus PBS by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

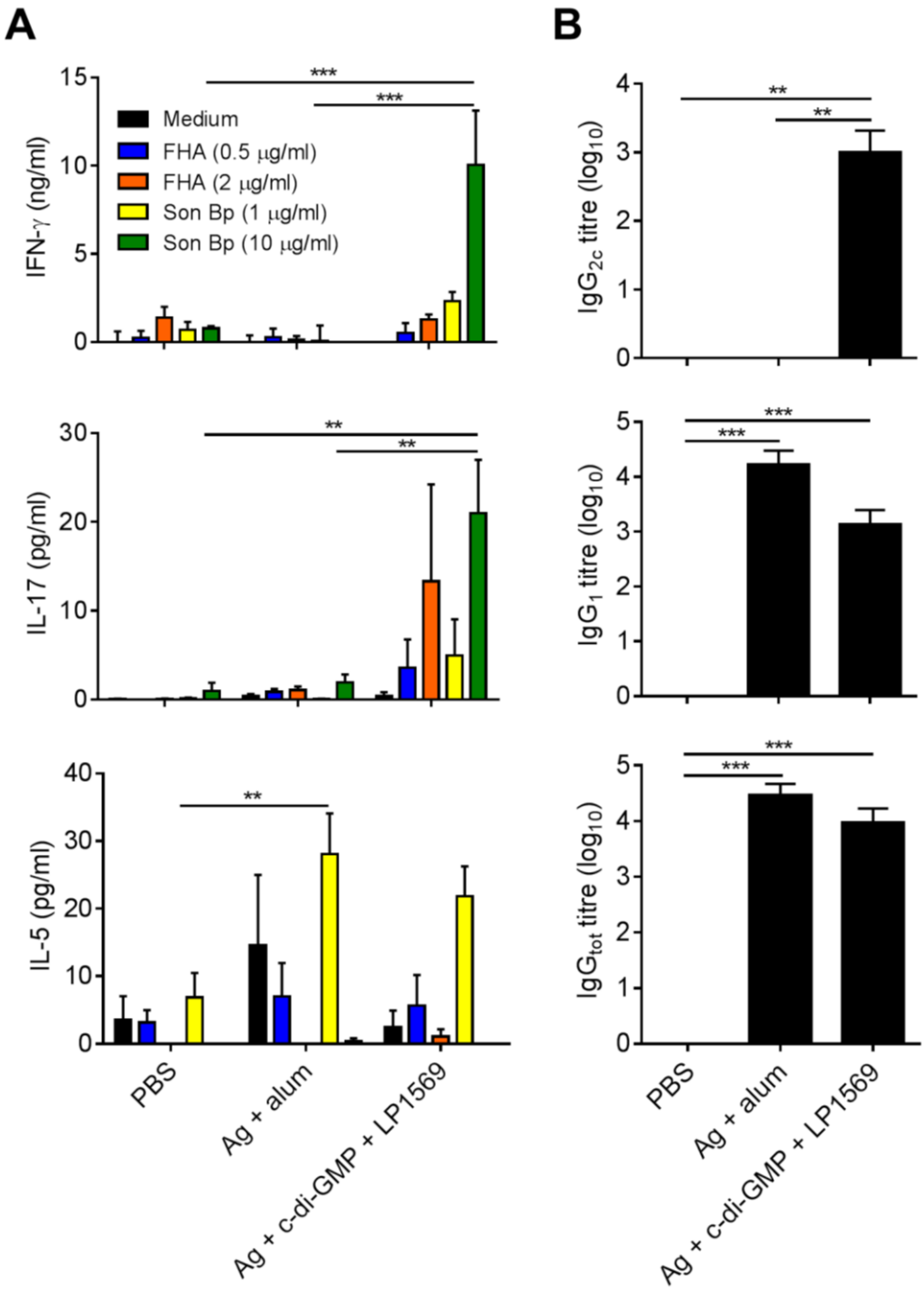


Figure 4.12 A two-component aP vaccine adjuvanted with a TLR2-STING agonist combination induces significantly greater Th1 and Th17 responses *in vivo* than a two-component alum-adjuvanted aP vaccine.

Mice were immunised i.p. twice as described in Figure 4.11. 2 weeks after the last immunisation, spleens and sera from four mice in each experimental group were isolated. (A) Spleen cells (2 x 10^6 cells/ml) were cultured with FHA (0.5 or 2 μg/ml), Son Bp (1 or 10 μg/ml) or medium as a negative control and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). $^{**}p < 0.01$, $^{***}p < 0.001$ by two-way ANOVA with the Bonferroni post test. (B) FHA-specific antibody production in sera was quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). $^{**}p < 0.01$, $^{***}p < 0.001$ by one-way ANOVA with the Bonferroni post test.

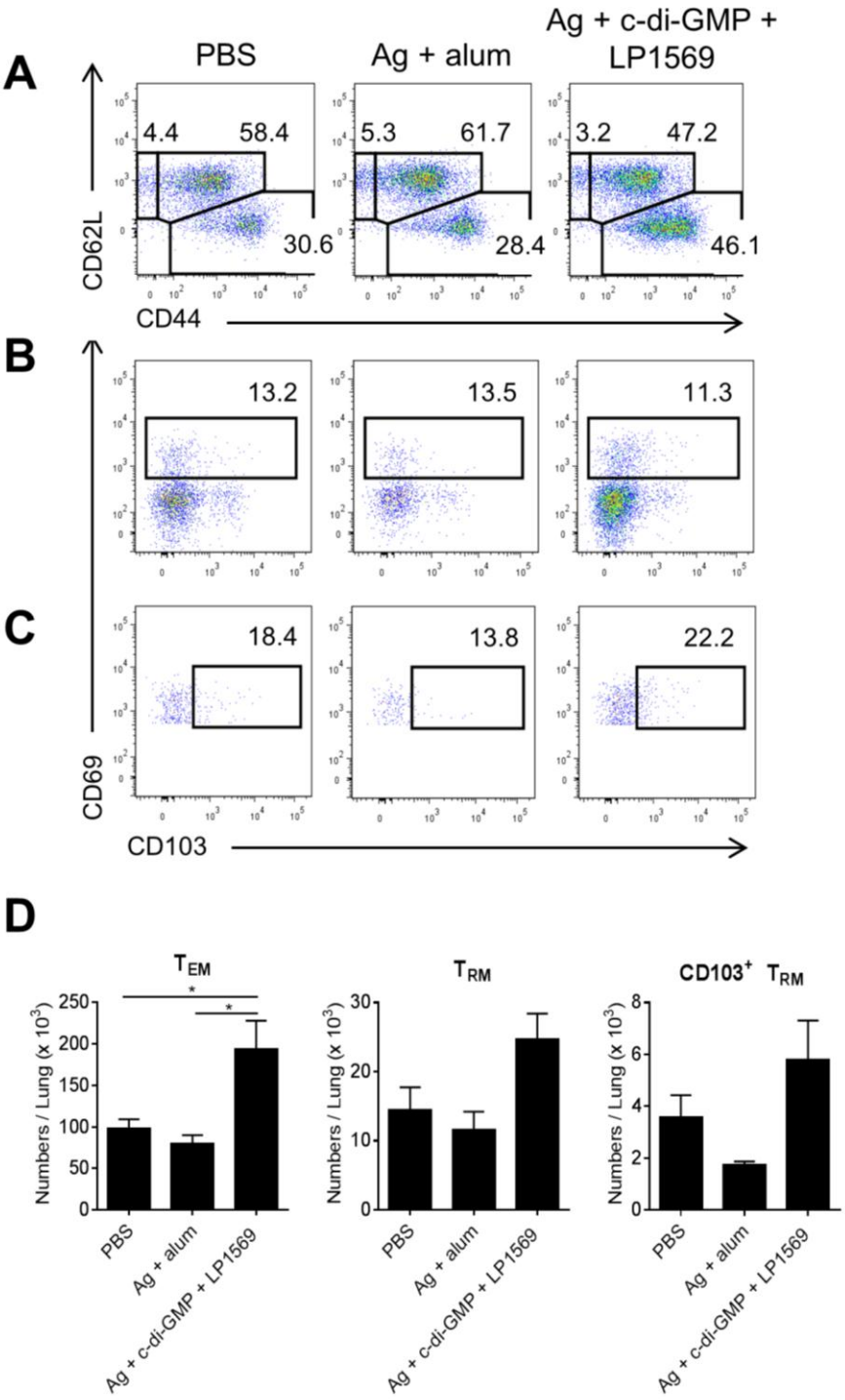


Figure 4.13 Immunisation with an experimental aP vaccine formulated with a TLR2-STING agonist combination recruits more CD4$^+$ T_{EM} and T_{RM} cells to the lungs than an alum-adjuvanted aP vaccine.

Mice were immunised i.p. twice as described in Figure 4.11. The lungs were isolated 2 weeks after the last immunisation and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing the (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). *p < 0.05 by one-way ANOVA with the Bonferroni post test.

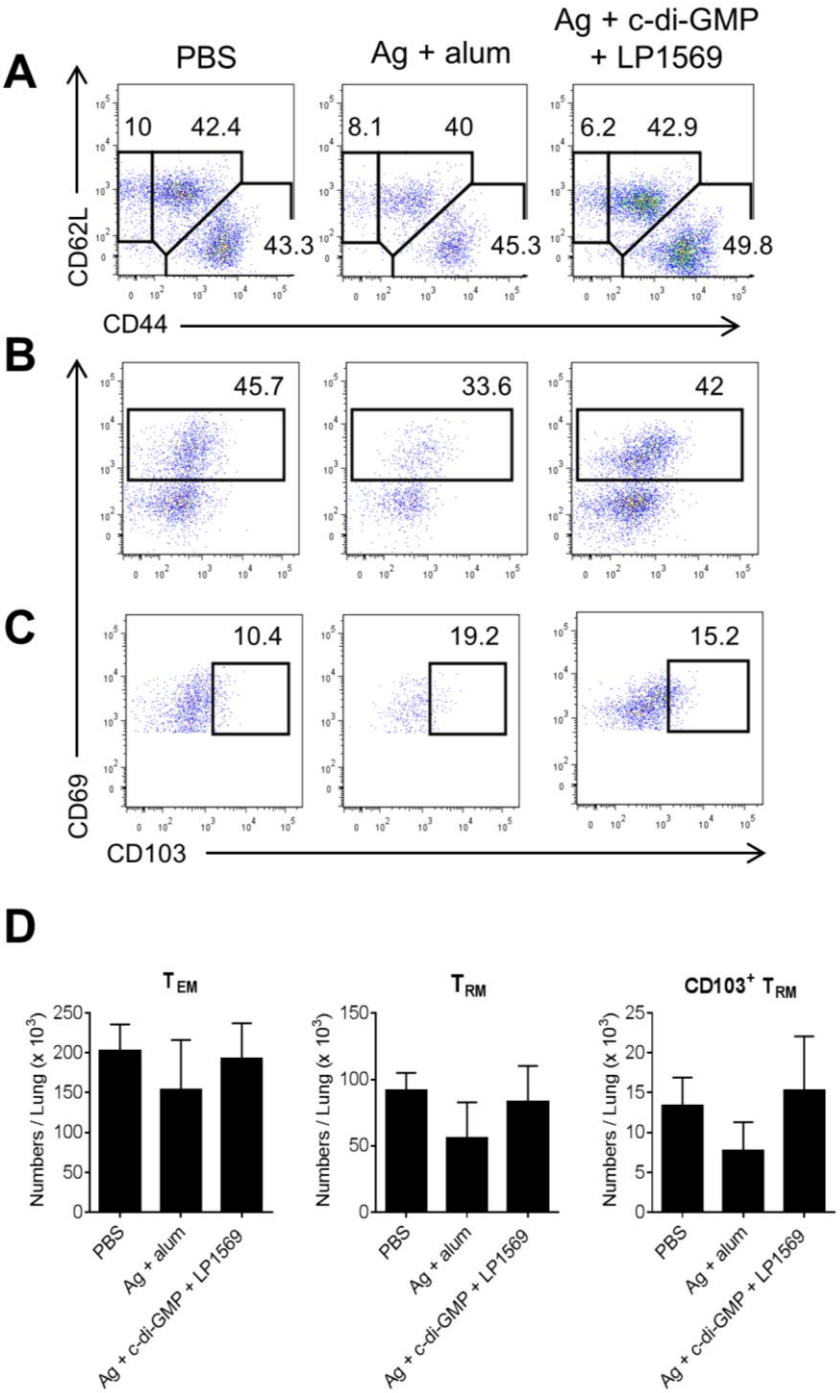
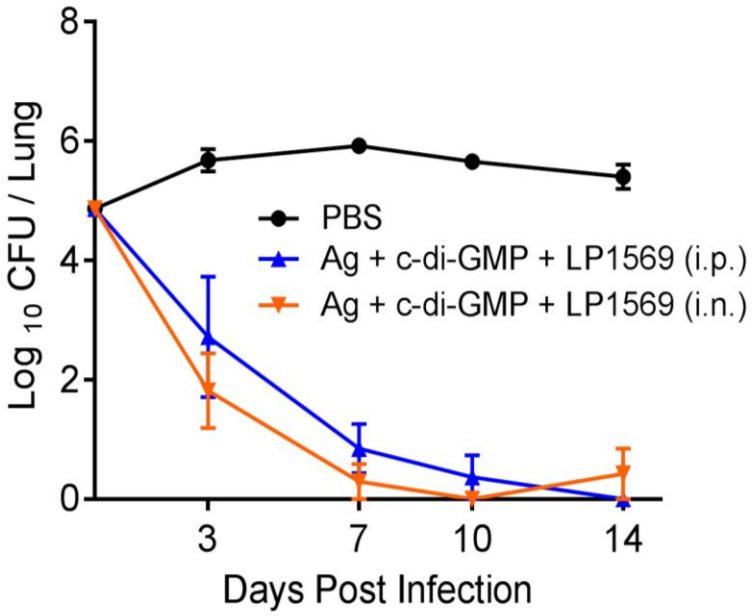


Figure 4.14 After challenge with *B. pertussis,* an experimental TLR2-STING agonist-adjuvanted aP vaccine and experimental alum-adjuvanted aP vaccine recruit similar numbers of T_{RM} cells into the lungs.

Mice were immunised i.p. twice as described in Figure 4.11 and 2 weeks after the last immunisation, mice were aerosol challenged with live *B. pertussis.* The lungs were isolated 7 days post infection and cells were stained with the markers described in Figure 4.13. FACS plots showing the (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). No significant differences between groups by one-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	Ag + c-di-GMP + LP1569 (i.p.)	Ag + c-di-GMP + LP1569 (i.n.)
	78.52	21.07	15.55

Figure 4.15 Intranasal immunisation with an experimental aP vaccine containing a TLR2-STING agonist combination is as effective at conferring protection against *B. pertussis* infection in mice, as intraperitoneal immunisation with the same vaccine.
Mice were immunised i.p. twice (0 and 4 weeks) with PBS as a control or the antigens FHA, Prn and rPT (Ag; 0.2 µg/mouse) formulated with a combination of c-di-GMP (10 µg/mouse) and LP1569 (50 µg/mouse) or were i.n. immunised twice with the Ag + c-di-GMP + LP1569 vaccine. Immunised mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation and lungs were taken from 4 mice per group at intervals post infection to determine bacterial colonisation. (A) Results shown are mean ± SEM CFU counts (n=4 mice). No significant differences between the vaccinated groups by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using Graphpad Prism.

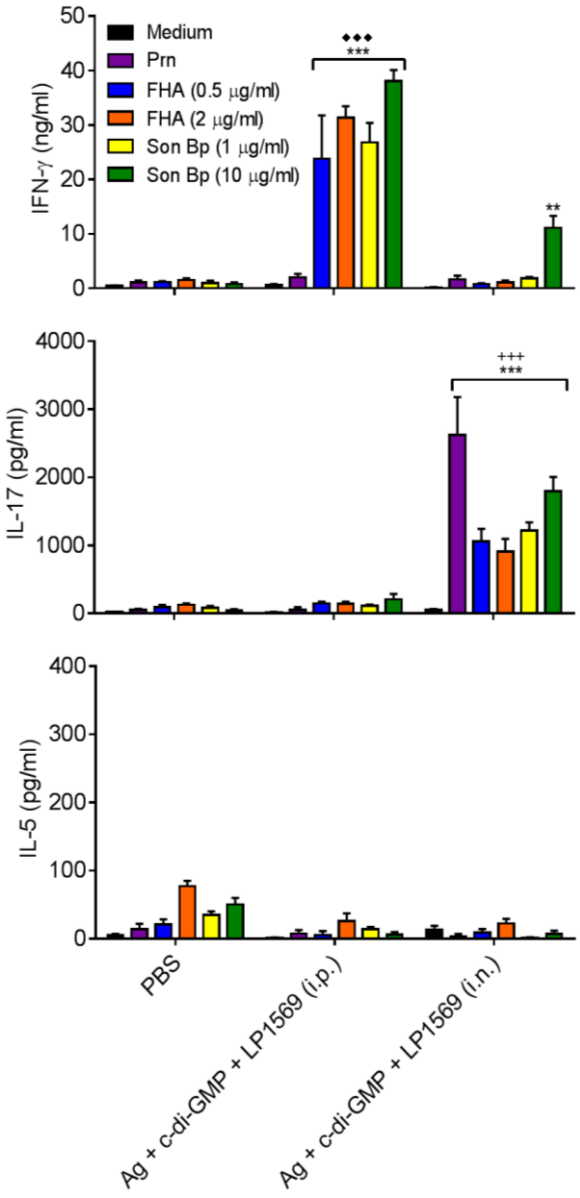


Figure 4.16 Administering an experimental aP vaccine containing a TLR2-STING agonist combination intranasally rather than intraperitoneally switches the immune profile from Th1 to Th17.

Mice were immunised as described in Figure 4.15. 2 weeks after the last immunisation, the spleens from four mice in each experimental group were isolated. Spleen cells (2 x 10^6 cells/ml) were cultured with Prn (2 µg/ml), FHA (0.5 or 2 µg/ml), Son Bp (1 or 10 µg/ml) or medium as a negative control and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). **$p < 0.01$, ***$p < 0.001$ versus PBS; +++$p < 0.001$ versus Ag + c-di-GMP + LP1569 (i.p.); ◆◆◆ $p < 0.001$ versus Ag + c-di-GMP + LP1569 (i.n.) by two-way ANOVA with the Bonferroni post test.

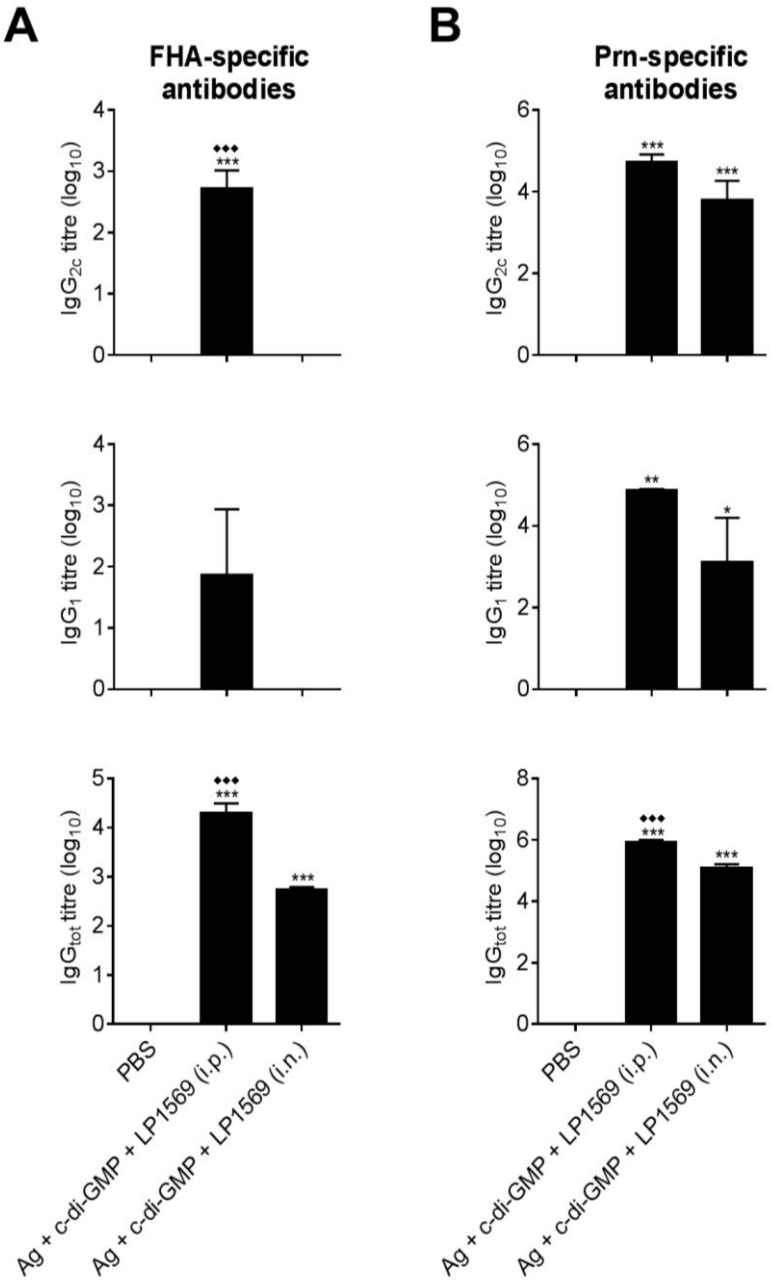


Figure 4.17 Intraperitoneal immunisation with an experimental aP vaccine containing a TLR2-STING agonist combination induces stronger circulating antibody responses than intranasal immunisation with the same vaccine.

Mice were immunised as described in Figure 4.15. 2 weeks after the last immunisation, the sera from four mice in each experimental group were collected. (A) FHA-specific antibody production and (B) Prn-specific antibody production in sera was quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus PBS; ◆◆◆$p < 0.001$ versus Ag + c-di-GMP + LP1569 (i.n.) by one-way ANOVA with the Bonferroni post test.

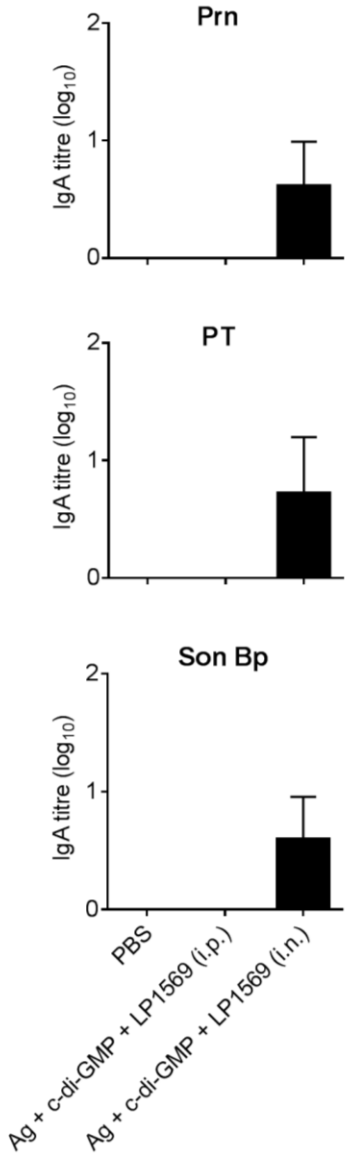


Figure 4.18 Intranasal immunisation with an aP vaccine containing a TLR2-STING agonist combination induces a modest antigen-specific IgA response in the lung after *B. pertussis* challenge.

Mice were immunised and challenged with live *B. pertussis* as described in Figure 4.15. 3 days after aerosol challenge, the lungs from four mice in each experimental group were isolated and antigen-specific IgA antibody production was quantified by ELISA on the lung homogenates. Prn-specific, PT-specific and *B. pertussis*-specific IgA antibody production in the lungs was quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). No significant differences between the groups by one-way ANOVA with the Bonferroni post test.

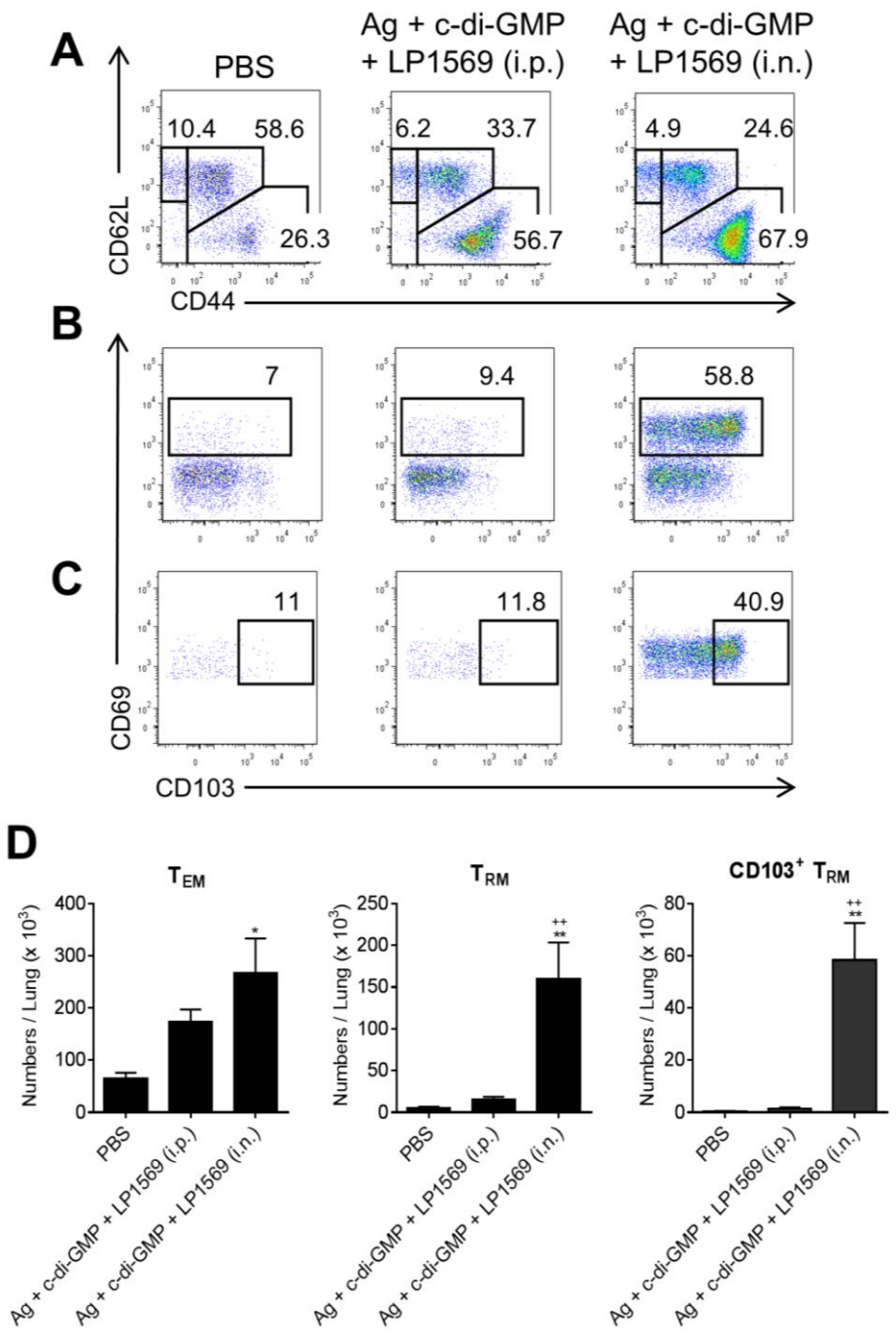


Figure 4.19 Intranasal immunisation with an experimental aP vaccine formulated with a TLR2-STING agonist combination recruits more T_{EM} and T_{RM} cells to the lungs than intraperitoneal immunisation with the same vaccine.

Mice were immunised i.p. twice as described in Figure 4.15. The lungs were isolated 2 weeks after the last immunisation and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) cell recruitment to the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). *$p < 0.05$, **$p < 0.01$ versus PBS; ++$p < 0.01$ versus Ag + c-di-GMP + LP1569 (i.p.) by one-way ANOVA with the Bonferroni post test.

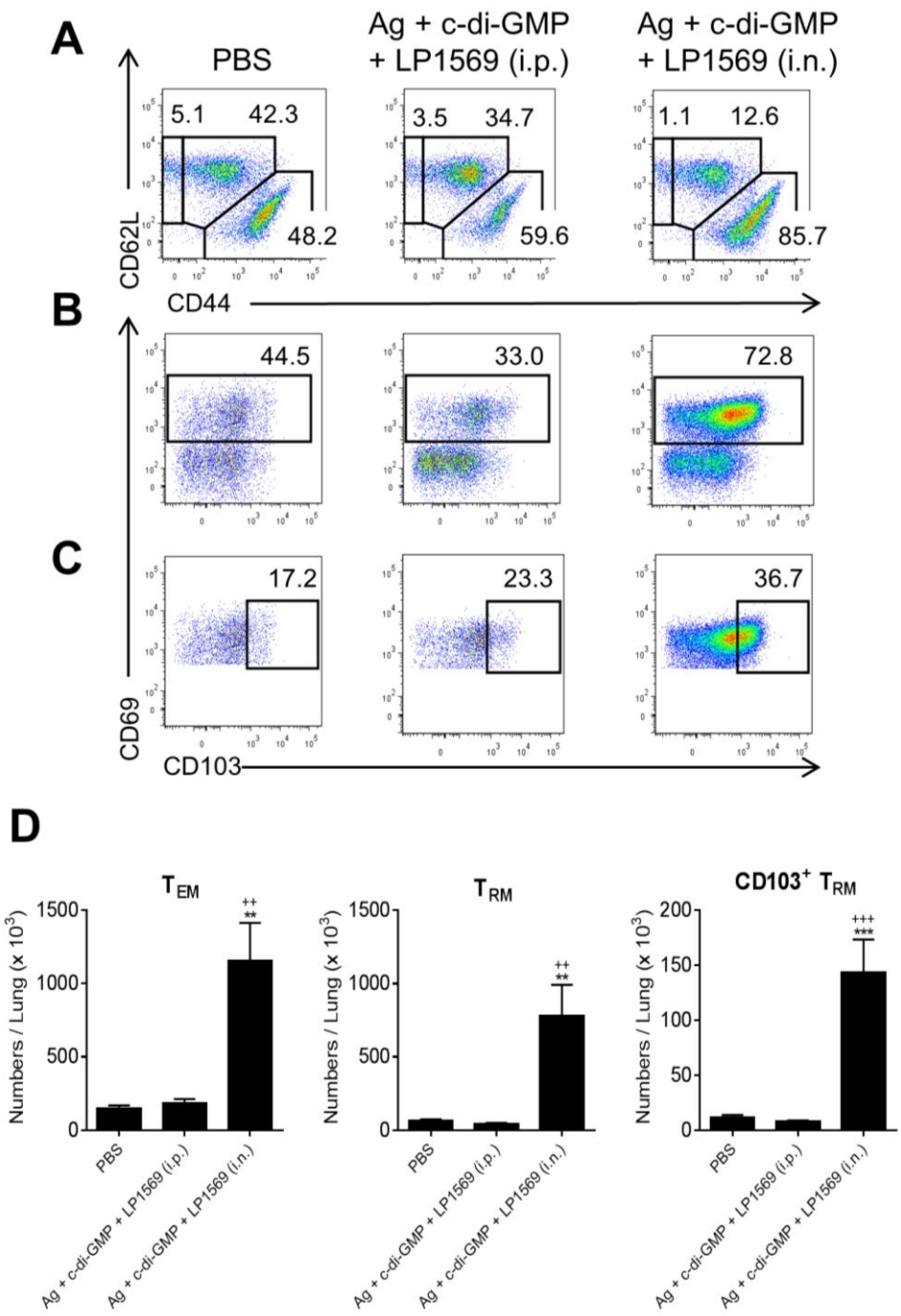


Figure 4.20 Intranasal immunisation is the most effective method of inducing potent $CD4^+$ T_{EM} and T_{RM} recruitment to the lungs after aerosol challenge with *B. pertussis.*

Mice were immunised i.p. twice as described in Figure 4.15. 2 weeks after the last immunisation, mice were aerosol challenged with live *B. pertussis.* The lungs were isolated 7 days post infection and cells were stained with the markers described in Figure 4.19. FACS plots showing (A T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). $^{**}p < 0.01$, $^{***}p < 0.001$ versus PBS; $^{++}p < 0.01$, $^{+++}p < 0.001$, versus Ag + c-di-GMP + LP1569 (i.p.) by one-way ANOVA with the Bonferroni post test.

Chapter 5:

An adjuvant combination that enhances cell-mediated immunity and memory T cell responses to *B. pertussis*

Chapter 5: An adjuvant combination that enhances cell-mediated immunity and memory T cell responses to *B. pertussis*

5.1 Introduction

The results from Chapters 3 and 4 demonstrated that novel adjuvants were capable of inducing greater cell-mediated immunity and memory responses than alum when formulated with aP vaccines. The results in Chapter 3 showed that addition of a TLR agonist to the commercial aP vaccine enhanced antigen-specific IFN-γ and IgG2c responses *in vivo.* However, as the paediatric combination vaccine contains a number of other components in addition to pertussis, such as diphtheria and tetanus, simply adding a TLR agonist or another adjuvant may not be a feasible option. Providing evidence that protective immunity to the other components of the vaccine is enhanced or at least not compromised would be logistically very difficult. A simpler and more achievable goal would be to keep the current commercial alum-adjuvanted aP vaccine in place, and develop a booster aP vaccine formulated with a Th1-inducing adjuvant, for administration to pre-school children, adolescents and maybe adults. However, one important question that has not been previously addressed is whether an aP vaccine formulated with a Th1/Th17-promoting adjuvant, or adjuvant combination, can shift the immune response from Th2 to the more protective Th1, in children who have previously been given a complete course of the existing alum-adjuvanted aP vaccines.

Another alternative option for consideration would be to introduce an aP vaccine containing a novel Th1-promoting adjuvant for administration to infants before their currently scheduled DTaP vaccinations. Although redesigning an aP vaccine containing a novel adjuvant, or adjuvant combination, for neonatal immunisation would face significant challenges, this might be the most effective method of inducing potent Th1 responses in vulnerable infants. Indeed, there is evidence that priming with a wP vaccine induces a Th1-dominated response, whereas priming with an aP vaccine induces predominantly a Th2-type response [396]. Furthermore, higher rates of pertussis have been documented in individuals primed and boosted with aP vaccines, than those primed with wP and boosted with aP vaccines [397]. A live attenuated *B. pertussis* vaccine has recently been developed which has delivered promising results in a mouse model when administered prior to immunisation with alum-adjuvanted aP vaccines; neonatal mice i.n. immunised with a single dose of BPZE1 prior to two i.p. immunisations with a commercial alum-adjuvanted aP vaccines maintained a Th1 and Th17-biased response [307].

These findings indicate that priming with an aP vaccine containing a Th1-promoting adjuvant may be an efficient way to induce potent cellular immunity.

The aim of this study was to investigate if it is possible to redirect immune responses from Th2 to Th1 by boosting with an experimental aP vaccine containing a Th1-promoting adjuvant, after initial priming with an experimental alum-adjuvanted aP vaccine. The hypothesis being tested was that boosting with an experimental aP vaccine containing a potent Th1/Th17-promoting adjuvant could enhance Th1 responses and $CD4^+$ T_{RM} cell recruitment to the lungs, and therefore augment protection conferred against *B. pertussis* by a Th2-inducing alum-adjuvanted aP vaccine. There is also evidence that priming with a wP or live attenuated *B. pertussis* vaccine induces a potent Th1 response that persists despite subsequent boosting with alum-adjuvanted aP vaccines. Therefore, the ability to set a Th1 response by priming with an experimental aP vaccine formulated with a TLR2-STING agonist combination before boosting with an experimental alum-adjuvanted aP vaccine was also investigated. It was postulated that this may be an effective method of inducing a long-lasting Th1 response.

5.2 Results

5.2.1 Priming and boosting mice with an experimental aP vaccine formulated with LP1569 is more effective than boosting only for inducing protective cellular immunity.

As LP1569 was shown to be a more potent inducer of Th1 and Th17 cells than CpG in Chapter 3 (Figure 3.20A), it was selected as the adjuvant for the initial investigation into the plasticity of the immune response after primary vaccination. Mice were immunised i.p. twice at 0 and 4 weeks with PBS, an experimental aP vaccine formulated with alum (Ag + alum) or LP1569 (Ag + LP1569), or were primed with the experimental alum-adjuvanted aP vaccine and boosted with the experimental LP1569-adjuvanted aP vaccine. Mice were aerosol challenged with live *B. pertussis* 2 weeks after the last immunisation. Recent evidence in a baboon model of infection has suggested that while the aP vaccine protects against severe disease, it cannot prevent transmission of *B. pertussis* to other susceptible individuals [228]. Warfel *et al.* showed that aP-immunised baboons were more heavily colonised than those immunised with a wP vaccine and could transmit *B. pertussis* to their vulnerable, unimmunised cage-mates [228]. To more extensively examine bacterial colonisation in the mouse model of *B. pertussis* infection, the CFU counts in the trachea, as well as in the lungs, were examined at intervals post infection.

Mice immunised with Ag + LP1569 had considerably lower CFU counts in the lungs post challenge than mice immunised with Ag + alum, or mice primed with Ag + alum and boosted with Ag + LP1569, and this reached statistical significance on days 10 and 14 after aerosol challenge (Figure 5.1A). The CFU counts in the trachea supported the data seen in the lungs; mice immunised with Ag + LP1569 twice had lower bacterial colonisation in the trachea than mice immunised with Ag + alum, or mice primed with Ag + alum and boosted with Ag + LP1569, with significantly lower CFU counts on days 7 and 10 post infection (Figure 5.1B). Bacterial burden in the lungs and tracheas post challenge was similar for mice immunised with Ag + alum twice and mice primed with Ag + alum and boosted with Ag + LP1569. Analysis of the areas under the curves (Figure 5.1C) confirmed the efficacy of immunising with Ag + LP1569 twice (Lungs: 33.65; Trachea: 5.854), compared with immunising with Ag + alum twice (Lungs: 48.72; Trachea: 22.23) or priming with Ag + alum and boosting with Ag + LP1569 (Lungs: 51.69; Trachea: 15.53).

Spleens and sera were taken from four mice in each experimental group 1 day prior to aerosol challenge to examine the antigen-specific T cell and antibody responses induced by the various experimental vaccines. Spleen cells from mice immunised with Ag + LP1569 twice produced significantly more antigen-specific IL-17 and IL-5 when compared with spleen cells from mice

immunised with Ag + alum twice, or mice primed with Ag + alum and boosted with Ag + LP1569 (Figure 5.2A). While the spleen cells from mice immunised with Ag + LP1569 twice produced significantly more antigen-specific IFN-γ than cells from mice immunised with Ag + alum twice, the increase was not statistically significant when compared with the spleen cells from mice primed with Ag + alum and boosted with Ag + LP1569.

Antigen-specific IgG2c antibody titres in the sera of mice immunised with Ag + LP1569 twice were greater than those seen in the sera of mice immunised with Ag + alum twice, or mice primed with Ag + alum and boosted with Ag + LP1569, but were only statistically significant compared with mice immunised with Ag + alum twice (Figure 5.2B). Antigen-specific IgG1 and total IgG antibody titres in sera were similar between the three groups of immunised mice.

The findings of this study indicate that boosting mice with an experimental aP vaccine adjuvanted with LP1569, after priming them with an experimental alum-adjuvanted aP vaccine, only marginally enhances antigen-specific IFN-γ production and serum IgG2c levels, but isn't sufficient to enhance protection against *B. pertussis* infection. Priming and boosting with an experimental LP1569-adjuvanted aP vaccine is the most effective way of inducing protective Th1, Th17 and IgG2c responses.

5.2.2 An experimental aP vaccine formulated with a TLR2-STING agonist combination induces potent Th1, Th17, T_{RM} and IgG2c responses, but not in Th2-primed mice.

Preliminary evidence indicated that protection conferred against *B. pertussis* infection could not be enhanced and that the immune response could not be redirected towards Th1 after primary immunisation with an aP vaccine containing a Th2-promoting adjuvant (Figure 5.1 and 5.2). However, it was hypothesised that perhaps LP1569, the included adjuvant, was not strong enough on its own to skew the immune response away from Th2 and towards Th1/Th17. It was postulated that boosting with a vaccine containing a stronger Th1/Th17-promoting adjuvant, such as the TLR2-STING agonist combination examined in Chapter 4, could enhance the Th1 response after initial priming with an experimental alum-adjuvanted aP vaccine.

Mice were immunised twice at 0 and 4 weeks with an experimental aP vaccine composed of three *B. pertussis* antigens (Ag; FHA, Prn, rPT), formulated with alum (Ag + alum), c-di-GMP and LP1569 (Ag + c-di-GMP + LP1569), or were primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569, prior to aerosol challenge with live *B. pertussis*. All immunised mice had considerably less CFU counts in the lungs than the PBS-immunised control mice, indicating

they were significantly more protected from infection (Figure 5.3A). Protection conferred with the three experimental vaccines was similar and it was difficult to distinguish between them. Analysis of the areas under the curves (Figure 5.3B) showed that mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569 were the most protected from *B. pertussis* infection (16.70), then mice immunised with Ag + alum (17.71) or mice immunised with Ag + c-di-GMP + LP1569 (21.07). The only significant difference in clearance was between mice immunised with Ag + c-di-GMP + LP1569 and mice immunised with Ag + alum on day 3 post aerosol challenge. All the immunised mice cleared the infection by day 14.

Mice immunised with an experimental alum-adjuvanted aP vaccine were shown to lack the protective Th1/Th17 response necessary to protect against *B. pertussis* infection in Chapter 4 (Figure 4.12A and 4.16). To investigate if boosting mice with an efficacious experimental aP vaccine formulated with c-di-GMP and LP1569 could enhance the Th1/Th17 response after initial priming with an experimental alum-adjuvanted aP vaccine, the spleens from four mice in each experimental group were isolated 1 day before aerosol challenge and their antigen-specific T cell responses were examined.

Spleen cells from mice immunised with Ag + c-di-GMP + LP1569 twice produced significantly more FHA and *B. pertussis*-specific IFN-γ and IL-17 than spleen cells from mice immunised with Ag + alum twice, or spleen cells from mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569 (Figure 5.4). Spleen cells from mice immunised with Ag + alum twice produced significantly more Prn, FHA and *B. pertussis*-specific IL-5 than spleen cells from mice immunised with Ag + c-di-GMP + LP1569 twice, or spleen cells from mice that were primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569. Spleen cells from mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569 did not produce any substantial antigen-specific IFN-γ, IL-17 or IL-5.

To examine if administering an experimental aP vaccine adjuvanted with a TLR2-STING agonist combination to mice after a primary dose of an experimental alum-adjuvanted aP vaccine enhanced antigen-specific IgG2c production, the sera were collected from four mice in each experimental group 1 day prior to aerosol challenge. Mice that were immunised with Ag + c-di-GMP + LP1569 twice had the highest FHA and Prn-specific IgG2c titres in sera, and while they appeared to have lower levels of FHA-specific IgG1 antibodies in sera compared with the other two immunised groups, their Prn-specific IgG1 antibody titres were as robust (Figure 5.5A and 5.5B). In contrast, there were no significant differences in FHA or Prn-specific serum IgG2c antibodies in mice that were immunised with Ag + alum twice or mice that were primed with

Ag + alum and boosted with Ag + c-di-GMP + LP1569. Total FHA and Prn-specific IgG levels were similar between the immunised groups of mice.

The results from this study indicate that boosting mice with an experimental aP vaccine formulated with c-di-GMP and LP1569 after initial priming with an experimental alum-adjuvanted aP vaccine does not enhance a Th1, Th17 or IgG2c immune response, or improve clearance of *B. pertussis* infection over that seen in mice immunised twice with an experimental alum-adjuvanted aP vaccine.

In Chapter 4, it was shown that immunising mice with an experimental aP vaccine adjuvanted with c-di-GMP and LP1569 recruited more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells to the lungs than immunising with an experimental alum-adjuvanted aP vaccine before and after *B. pertussis* challenge (Figure 4.13 and 4.14). To investigate if boosting mice with an experimental aP vaccine formulated with a TLR2-STING agonist combination after initial priming with an experimental alum-adjuvanted aP vaccine would enhance memory T cell recruitment to the lungs, lungs were isolated in addition to spleens and sera 1 day before aerosol challenge and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for flow cytometry analysis.

Mice immunised twice with Ag + c-di-GMP + LP1569 had considerably higher percentages of $CD4^+$ T_{EM} cells recruited to the lungs than mice immunised with Ag + alum twice or mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569 (Figure 5.6A). However, the percentages of $CD4^+$ T_{RM} and $CD103^+$ T_{RM} cells recruited to the lungs were similar between the three immunised groups of mice (Figure 5.6B and 5.6C). Calculation of the absolute numbers revealed that mice immunised with Ag + alum, and mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569, had similar numbers of $CD4^+$ T_{EM} cells recruited to the lungs (Figure 5.6D). These numbers were substantially lower than those recruited to the lungs of mice immunised twice with Ag + c-di-GMP + LP1569, but only the difference between mice immunised twice with Ag + alum or twice with Ag + c-di-GMP + LP1569 reached statistical significance. Furthermore, mice immunised with Ag + c-di-GMP + LP1569 twice appeared to have the highest numbers of $CD4^+$ T_{RM} and $CD103^+$ T_{RM} cells recruited to the lungs compared with mice immunised twice with Ag + alum, or mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569, although this did not reach statistical significance. These results indicated that boosting mice with an experimental aP vaccine formulated with c-di-GMP and LP1569 after priming with an experimental alum-adjuvanted aP vaccine does not enhance

recruitment of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells to the lungs over that seen in mice immunised with an experimental alum-adjuvanted aP vaccine twice.

To investigate if the numbers of memory T cells recruited to the lungs of mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569 were enhanced after infection, mice were aerosol challenged with *B. pertussis* 2 weeks after the second immunisation. 7 days after challenge, lung cells were stained with the same markers as described earlier. After *B. pertussis* challenge, mice immunised with Ag + c-di-GMP + LP1569 twice appeared to have the greatest percentage of $CD4^+$ T_{EM} cells in the lungs (Figure 5.7A). The percentages of T_{RM} and $CD103^+$ T_{RM} cells in the lungs were substantially lower in mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569 than the other two immunised groups of mice (Figure 5.7B and 5.7C). However, the control mice appeared to have the highest percentage of $CD4^+$ T_{RM} cell in the lungs overall and the absolute numbers reflected this (Figure 5.7D). Mice immunised with Ag + c-di-GMP + LP1569 twice had slightly elevated numbers of $CD4^+$ T_{EM} cells than mice immunised with Ag + alum twice, however, the numbers of $CD4^+$ T_{RM} cells were comparable. Mice primed with Ag + alum and boosted with Ag + c-di-GMP + LP1569 appeared to have the lowest numbers of $CD4^+$ $T_{EM,}$ T_{RM} and $CD103^+$ T_{RM} cells in the lungs after *B. pertussis* challenge. These data suggest that mice boosted with an experimental aP vaccine formulated with c-di-GMP and LP1569 after being primed with an experimental alum-adjuvanted aP vaccine do not have as many $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs as mice immunised with the same experimental aP vaccine twice, even after *B. pertussis* challenge.

Overall, the results of this study indicate that boosting mice with an experimental aP vaccine formulated with c-di-GMP and LP1569 after initial priming with an experimental alum-adjuvanted aP vaccine does not improve clearance of *B. pertussis* infection over that seen in mice immunised twice with an experimental alum-adjuvanted aP vaccine. Furthermore, boosting with an aP vaccine containing a Th1/Th17-promoting adjuvant after priming with an aP vaccine formulated with a Th2-promoting adjuvant does not shift the immune responses to Th1, Th17 or IgG2c, or restore numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells trafficking to the lungs to the levels observed in mice immunised twice with an experimental aP vaccine formulated with c-di-GMP and LP1569.

5.2.3 Priming and boosting with an experimental aP vaccine containing a TLR2-STING agonist combination is essential for $CD4^+$ T_{RM} cell recruitment to the lungs, and robust cellular and humoral immunity.

It was shown that boosting with an experimental aP vaccine containing a potent Th1/Th17-promoting adjuvant after priming with an experimental aP vaccine containing a Th2-promoting adjuvant did not significantly enhance the rate of *B. pertussis* clearance from the lungs (Figure 5.3). This was postulated to be due to the inability of the boosting vaccine to generate a potent Th1 response, possibly due to the immune response being set by the initial Th2-promoting vaccine (Figure 5.4). Therefore, it was investigated if priming with an experimental aP vaccine containing a Th1-promoting adjuvant would set the immune response at Th1 before secondary immunisation with an experimental aP vaccine containing a Th2-promoting adjuvant, and thus enhance protection against *B. pertussis* infection. The premise here was that introducing a novel aP vaccine containing a Th1-promoting adjuvant to give to infants prior to their DTaP vaccinations in the first few months of life, would set their immune profiles to Th1 before boosting with the Th2-promoting alum-adjuvanted DTaP vaccine.

Mice were immunised twice at 0 and 4 weeks with an experimental aP vaccine composed of three *B. pertussis* antigens (Ag; FHA, Prn, rPT), formulated with alum (Ag + alum) or c-di-GMP and LP1569 (Ag + c-di-GMP + LP1569), or mice were immunised with Ag + c-di-GMP + LP1569 as the primary dose and Ag + alum as the secondary dose and 2 weeks later were challenged with an aerosol of live *B. pertussis*. All the immunised mice had significantly lower CFU counts in the lungs post challenge than the PBS-immunised control mice, indicating they were more protected from *B. pertussis* infection (Figure 5.8A). Analysis of the areas under the curves (Figure 5.8B) showed that mice immunised with Ag + alum were the most protected from *B. pertussis* infection (17.66), followed by Ag + c-di-GMP + LP1569-immunised mice (18.93) and finally the mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum (20.52). There were no significant differences between any of the immunised groups of mice on any days examined.

To investigate if priming with an experimental aP vaccine formulated with c-di-GMP and LP1569 could set the immune response at Th1 despite a following secondary dose with an experimental alum-adjuvanted aP vaccine, spleens were isolated from four mice in each experimental group 1 day before aerosol challenge and the antigen-specific T cell responses were examined. Spleen cells from mice immunised with Ag + c-di-GMP + LP1569 produced robust FHA and *B. pertussis*-specific IFN-γ as well as modest amounts of IL-17 (Figure 5.9). In

contrast, spleen cells from mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum produced no antigen-specific IFN-γ, with levels similar to those seen in the PBS-immunised control mice. However, spleen cells from these mice produced significantly higher antigen-specific IL-17 than spleen cells from mice immunised twice with Ag + c-di-GMP + LP1569 or Ag + alum, and also had enhanced antigen-specific IL-5 production. Spleen cells from mice immunised with Ag + alum produced no antigen-specific IFN-γ, low levels of IL-17 and modest amounts of IL-5.

Given that boosting mice with an experimental aP vaccine formulated with c-di-GMP and LP1569 could not enhance the antigen-specific serum IgG2c response due to initial Th2 priming with an experimental alum-adjuvanted aP vaccine (Figure 5.5), it was hypothesised that priming with this experimental aP vaccine containing a TLR2-STING agonist combination would set the Th1-associated IgG2c response prior to boosting with an experimental alum-adjuvanted aP vaccine. The sera from four mice in each experimental group were collected 1 day prior to aerosol challenge to quantify the antigen-specific antibody responses. While mice immunised twice with Ag + c-di-GMP + LP1569 had substantial amounts of FHA-specific antibodies in sera, particularly of the IgG2c isotype, the Prn-specific antibody responses were considerably more robust (Figure 5.10A and 5.10B). Mice immunised with Ag + alum twice, or primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum, had similar levels of FHA-specific IgG1 and total IgG in sera. However, mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum had enhanced Prn-specific IgG1 and total IgG titres in sera when compared with mice that were immunised with Ag + alum twice, and these levels were similar to mice that were immunised with Ag + c-di-GMP + LP1569 twice. While mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum had no detectable FHA-specific IgG2c in sera, the Prn-specific IgG2c response was robust, with similar levels to mice that were immunised with Ag + c-di-GMP + LP1569 twice detected. The results so far suggest that two immunisations with an experimental aP vaccine containing c-di-GMP and LP1569 are necessary to induce potent Th1 and FHA-specific IgG2c responses *in vivo.*

The findings from the previous study showed that boosting with an experimental TLR2-STING agonist-adjuvanted aP vaccine after priming with an experimental alum-adjuvanted aP vaccine does not enhance recruitment or expansion of memory T cells in the lungs (Figure 5.6 and 5.7). Therefore, it was investigated if initial priming with an experimental aP vaccine containing a Th1/Th17-promoting adjuvant, before boosting with an experimental aP vaccine formulated with a Th2-promoting adjuvant would induce memory T cell infiltration into the lungs. The lungs were isolated in addition to spleens and sera 1 day before aerosol challenge, and lung

cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for flow cytometry analysis.

Mice immunised twice with Ag + c-di-GMP + LP1569 twice had substantially higher percentages of $CD4^+$ T_{EM} cells recruited to the lungs than the mice immunised with Ag + alum twice, or mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum (Figure 5.11A). In contrast, mice immunised with Ag + alum twice appeared to have the greatest percentages of $CD4^+$ T_{RM} and $CD103^+$ T_{RM} cells in the lungs compared with the other two groups of immunised mice (Figure 5.11B and 5.11C). However, calculation of the absolute numbers revealed that mice immunised twice with Ag + c-di-GMP + LP1569 had the highest numbers of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells infiltrating into the lungs following immunisation, although this did not reach statistical significance. In contrast, mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum had similar numbers to mice immunised twice with Ag + alum, indicating that priming with an experimental aP vaccine containing a Th1/Th17-promoting adjuvant before boosting with an experimental aP vaccine formulated with a Th2-promoting adjuvant does not enhance memory T cell recruitment to the lungs.

To investigate if the numbers of memory T cells in the lungs of mice primed with an experimental aP vaccine formulated with c-di-GMP and LP1679, and boosted with an experimental alum-adjuvanted aP vaccine were enhanced after infection, mice were aerosol challenged with *B. pertussis* 2 weeks after the second immunisation. 7 days after challenge, lung cells were stained with the same markers as described earlier, to examine the memory responses induced after infection. Mice immunised twice with Ag + c-di-GMP + LP1569 had the highest percentages of $CD4^+$ T_{EM} and T_{RM} cells in the lungs after *B. pertussis* challenge (Figure 5.12A and 5.12B). Furthermore, the infected, PBS-immunised control mice had elevated percentages of these memory T cell subtypes in the lungs compared with mice immunised with Ag + alum twice, or mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum. The percentages of $CD103^+$ T_{RM} cells in the lungs was similar between mice immunised with Ag + c-di-GMP + LP1569 twice and mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum, and the PBS-immunised control mice (Figure 5.12C). The absolute numbers were more revealing, indicating that mice immunised twice with Ag + c-di-GMP + LP1569 had significantly more $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs than the other three groups of mice (Figure 5.12D). Interestingly, the numbers of $CD4^+$ T_{EM} and T_{RM} cells in the lungs of the infected, PBS-immunised control mice was greater than that seen in mice immunised with Ag + alum twice, or mice primed with Ag + c-di-GMP + LP1569 and boosted with Ag + alum.

Overall, the data from this study indicate that priming and boosting with an experimental aP vaccine formulated with c-di-GMP and LP1569 is essential to induce robust antigen-specific IFN-γ and FHA-specific IgG2c production *in vivo*. Furthermore, it seems that immunising mice twice with an experimental aP vaccine containing this TLR2-STING agonist combination is the most effective method of inducing recruitment or expansion of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells in the lungs after *B. pertussis* challenge. Moreover, immunising mice twice with Ag + alum, or priming with Ag + c-di-GMP + LP1569 and boosting with Ag + alum appears to impair influx of these memory T cells, as the infected PBS-immunised control mice had greater numbers in the lung.

5.3 Discussion

Currently, infants are immunised with a Th2-biased combined paediatric vaccine against pertussis in the first few months of life. Developing a booster aP vaccine containing a Th1-promoting adjuvant could be an achievable strategy for enhancing protective immunity against *B. pertussis* infection, however, it has not been determined if the Th2/Th17 response is set from primary immunisation, or if boosting with a vaccine containing a novel adjuvant can redirect the immune response to Th1/Th17.

In this study, it was found that mice primed with an experimental alum-adjuvanted aP vaccine and boosted with an experimental LP1569-adjuvanted aP vaccine had slightly enhanced antigen-specific IFN-γ and IL-17 production, and higher IgG2c levels in sera when compared with mice immunised with an experimental aP vaccine formulated with alum only. However, upon examination of the CFU counts in the lungs post challenge, it was observed that they were no more protected from *B. pertussis* infection than the mice immunised twice with the experimental alum-adjuvanted aP vaccine. This suggested that even though the Th1 response was marginally enhanced by boosting with an experimental LP1569-adjuvanted aP vaccine, the protection conferred against *B. pertussis* infection was not superior to immunising twice with an experimental alum-adjuvanted aP vaccine. Moreover, it was shown that priming and boosting with the LP1569-adjuvanted aP vaccine was the most effective method of inducing potent antigen-specific IFN-γ, IL-17 and IgG2c responses *in vivo* and protecting against *B. pertussis* infection.

Recent evidence from a baboon model has suggested that although the commercial aP vaccine is capable of preventing severe disease, it is not capable of preventing *B. pertussis* colonisation of the respiratory tract or transmission to unvaccinated individuals. Warfel *et al.* demonstrated in a non-human primate model of infection that baboons vaccinated with the aP vaccine, although protected from severe disease, were still heavily colonised by the bacteria and did not clear *B. pertussis* from the airways any faster than the unvaccinated baboons [228]. In contrast, wP-immunised baboons were not colonised as heavily and cleared the bacteria faster. This group also demonstrated that baboons immunised with an aP vaccine were still capable of transmitting *B. pertussis* to unvaccinated, susceptible cage-mates. Further evidence of the inability of the aP vaccine to prevent transmission was provided by a group in Pennsylvania State University. Smallridge and colleagues examined *Bordetella bronchiseptica* infection in a newly developed mouse transmission model of infection [398]. Their results demonstrated that mice immunised with an aP vaccine (Adacel) shed similar numbers of bacteria from the nares as infected control mice. In contrast, mice immunised with a wP

vaccine shed 90% less bacteria than the control mice on day 8 post challenge, despite bacterial colonisation of the nares being similar between the two groups. Consistent with Warfel's observations [228], the results from the present study showed that tracheal colonisation was higher in the mice immunised with an experimental alum-adjuvanted aP vaccine compared with an experimental LP1569-adjuvanted aP vaccine, reinforcing the evidence that mice immunised with the aP vaccine formulated with LP1569 were more protected from airway colonisation. This suggested that although alum-adjuvanted aP vaccines prevent severe disease in immunised individuals, they are still capable of being colonised by *B. pertussis* and could potentially act as a reservoir, transmitting bacteria to those susceptible within the population. To more extensively investigate *B. pertussis* transmission by mice immunised with an experimental aP vaccine formulated with LP1569, colonisation and shedding from the nares of infected mice should be examined.

Preliminary experiments in the present study indicated that the Th1 response cannot be significantly enhanced after primary immunisation with a Th2-promoting experimental alum-adjuvanted aP vaccine, and that clearance of *B. pertussis* from the lungs cannot be accelerated by boosting with an experimental aP vaccine containing a Th1-promoting adjuvant. However, it was shown in Chapter 4 that the combination of c-di-GMP and LP1569 was more effective at inducing potent Th1 and Th17 cell responses than LP1569 alone. It was hypothesised that LP1569 may not be powerful enough on its own to overcome the Th2 response induced with an experimental alum-adjuvanted aP vaccine, but that the use of a TLR2-STING agonist combination as an adjuvant for an experimental aP vaccine could skew the immune response induced towards Th1 and Th17, after priming with an experimental aP vaccine containing a Th2-promoting adjuvant.

In this study, it was found that boosting mice with an experimental aP vaccine formulated with c-di-GMP and LP1569 after priming with an experimental alum-adjuvanted aP vaccine did not enhance Th1 or Th17 cell responses, or antigen-specific IgG2c production. This indicated that the immune response is fixed from primary immunisation and that boosting with a vaccine containing even a potent adjuvant combination, such as c-di-GMP and LP1569, is not sufficient to induce a desirable cell-mediated immune response. Therefore, it was hypothesised that priming mice with an experimental aP vaccine containing a TLR2-STING agonist combination as the adjuvant would set the immune response at Th1, before boosting with an experimental alum-adjuvanted aP vaccine. The results from this study showed that spleen cells from mice primed with an experimental aP vaccine formulated with c-di-GMP and LP1569 before boosting with an experimental alum-adjuvanted aP vaccine produced no antigen-specific IFN-γ.

These data are at variance with studies carried out by Reynolds *et al.* and Bancroft *et al.,* both of whom reported the induction of potent antigen-specific IFN-γ in individuals primed with the wP vaccine, despite subsequent boosting with the aP vaccine [396, 399]. One potential reason for this discrepancy is the vaccine used in this study is an experimental aP vaccine containing low doses of the three included antigens, whereas the wP vaccine, which contains significantly more *B. pertussis* antigens, was used in the previous investigations. With this in mind, future experiments that would be important to carry out in mice include boosting with a wP vaccine after initial priming with an aP vaccine. This would be crucial to conclusively determine if the immune response is set from initial vaccination as if it is not possible to redirect the immune response with the most potent *B. pertussis* vaccine currently available, the wP vaccine, it will not be possible to with a subunit aP vaccine.

Despite the clear evidence that priming and boosting with an experimental aP vaccine formulated with a TLR2-STING agonist combination was the most effective method of inducing desirable cellular and humoral immune responses, it was not possible to demonstrate an increase in protection due to the great efficacy of these three-component aP vaccines. One hypothesis for this was that the addition of Prn skewed the protection conferred with these experimental vaccines to an antibody-mediated mechanism. There is evidence that Prn-specific antibodies play a key role in protection against *B. pertussis* infection by facilitating antibody-mediated phagocytosis. Importantly, a study by Hellwig *et al.* showed that anti-Prn antibodies are crucial for triggering phagocyte effector functions by human leukocytes, whereas the contribution by anti-PT, anti-FIM and anti-FHA antibodies was insignificant [400]. Furthermore, a number of clinical trials have demonstrated enhanced vaccine efficacy upon inclusion of additional antigens including Prn, indicating that this virulence factor contributes to protection conferred by aP vaccines [36, 53, 54, 401]. These reports, taken together with the present demonstration of robust levels of Prn-specific antibodies induced with an experimental aP vaccine formulated with a TLR2-STING agonist combination, supports the likelihood that addition of Prn to the experimental aP vaccines promoted protective humoral, as well as cellular, responses. In order to confirm that the enhanced cellular immunity induced by priming and boosting with an experimental aP vaccine formulated with c-di-GMP and LP1569 vaccine does correlate with an increase in protection against *B. pertussis* infection, it would be important to repeat these experiments using weaker, two-component aP vaccines.

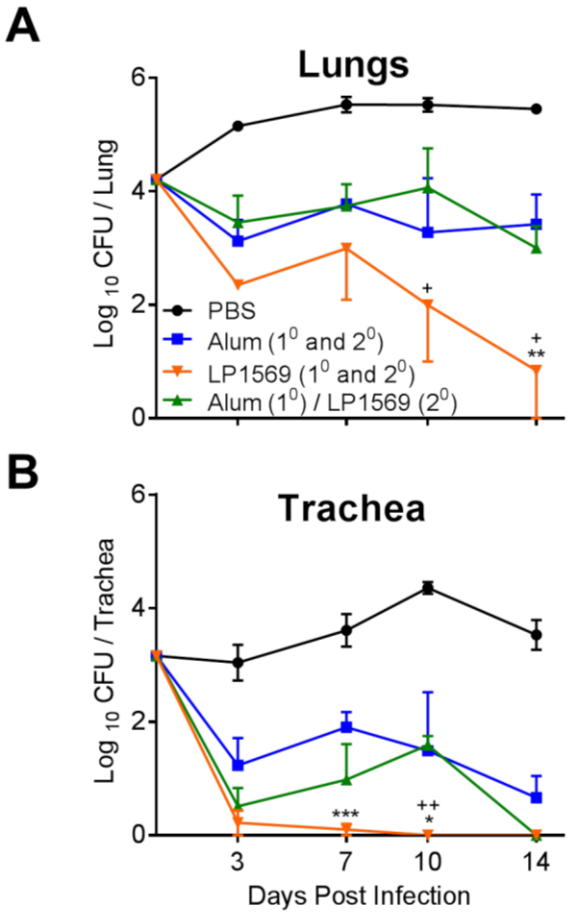
From these studies, it could be argued that priming with an experimental alum-adjuvanted aP vaccine fixes the immune response at Th2, thereby making it difficult to promote a Th1 immune response upon boosting with an experimental aP vaccine formulated with c-di-GMP

and LP1569. However, the T cell response data gave no indication that a potent Th2 response was induced in mice primed with an experimental alum-adjuvanted aP vaccine and boosted with an experimental aP vaccine formulated with a TLR2-STING agonist combination. Instead, it appears that immunising twice with an experimental aP vaccine containing the same adjuvant is necessary to induce any robust T cell responses. The second study supports this hypothesis, as priming with an experimental aP vaccine formulated with c-di-GMP and LP1569 did not induce Th1 responses. However, it is possible that the booster alum-adjuvanted aP vaccine dampened the Th1 response in this study, rendering it undetectable. Nevertheless, it is clear that priming and boosting with an experimental aP vaccine containing a TLR2-STING agonist combination is capable of inducing robust Th1 and Th17 cell responses, whereas a single immunisation appears to be sufficient to generate a potent antibody response, especially Prn-specific antibodies, which were particularly robust in these studies.

It was also found in this study that recruitment of $CD4^+$ T_{EM}, T_{RM} and $CD103^+$ T_{RM} cells to the lungs requires priming and boosting with an experimental aP vaccine containing a strong Th1/Th17-promoting adjuvant. This result reinforces the hypothesis discussed in Chapters 3 and 4, that potent Th17 immune responses are needed for the infiltration of large numbers of memory $CD4^+$ T cells to the lungs, as no robust T cell responses were generated by priming and boosting with experimental aP vaccines containing different adjuvants. While the correlation between T_{RM} cells and long-term protection has not been directly evaluated, the results indicate that an experimental aP vaccine containing a TLR2-STING agonist combination as the adjuvant, would induce longer lasting protection against *B. pertussis* infection than an experimental alum-adjuvanted aP vaccine. With this in mind, an important future experiment would be to immunise mice and then wait for a considerable period of time before challenging them with live *B. pertussis,* to evaluate the efficacy and longevity of the memory responses induced with the different experimental vaccines. Furthermore, the data indicated that mice immunised with an experimental alum-adjuvanted aP vaccine were not as effective as unimmunised mice at recruiting $CD4^+$ T_{EM} and T_{RM} cells to the lungs after *B. pertussis* challenge. While this could be representative of the difference in bacterial burden between the immunised and control mice, it was also suggested that the induction of Th2 immune responses limits the migration of $CD4^+$ T cells to the lungs post challenge with *B. pertussis*. This could be responsible for the poor memory responses and waning immunity observed after multiple aP vaccinations [231], as discussed in Chapter 3.

Overall, this study has given insight into the induction of cell-mediated immunity and memory responses following priming and boosting with vaccines containing different adjuvants. It was

found that immunising mice twice with an experimental aP vaccine formulated with a TLR2 agonist alone, or in combination with a STING agonist, induces potent Th1 and Th17 immune responses. Furthermore, it appears that it is not possible to redirect the immune response towards Th1 after priming with an experimental aP vaccine containing a Th2-promoting adjuvant. If this is the case, then designing an effective booster aP vaccine with a Th1-promoting adjuvant would be difficult unless it included new antigens, and reinforces the need for research into a new pertussis vaccine. Finally, it was found that an experimental alum-adjuvanted aP vaccine was not only incapable of preventing colonisation of the lungs, but also of the tracheas. Given the previous literature indicating that mice immunised with an aP vaccine shed significantly more *B. bronchiseptica* from the nares than mice immunised with a wP vaccine [398], it could be possible that these mice have a greater capacity to transmit *B. pertussis* than mice immunised with an LP1569-adjuvanted aP vaccine, however more extensive studies would need to be performed to confirm this.



C

AUC	PBS	Alum (1^0 and 2^0)	LP1569 (1^0 and 2^0)	Alum (1^0) / LP1569 (2^0)
Lungs	73.89	48.72	33.65	51.69
Trachea	50.32	22.23	5.854	15.53

Figure 5.1 Priming and boosting with an aP vaccine containing LP1569 is more effective at conferring protection against *B. pertussis* challenge than priming with an alum adjuvanted aP vaccine and boosting with an LP1569 containing aP vaccine.

C57BL/6 mice were immunised i.p. twice (0 and 4 weeks) with PBS as a control, an experimental aP vaccine consisting of rPT and FHA (Ag; 0.2 µg/mouse) with alum (100 µg/mouse) or LP1569 (50 µg/mouse) or were primed (1^0) with the alum-adjuvanted aP vaccine (Alum) and boosted (2^0) with the LP1569-adjuvanted aP vaccine (LP1569). Mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation. Four mice from each group were sacrificed on days 3, 7, 10 and 14 post challenge. The number of viable bacteria in the (A) lungs and (B) trachea were estimated by performing CFU counts on serially diluted lung and tracheal homogenates from individual mice. Results are shown as mean ± CFU counts (n=4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus Alum (1^0 and 2^0); +$p < 0.05$, ++$p <$ 0.01 versus Alum (1^0) / LP1569 (2^0) by two-way ANOVA with the Bonferroni post test. (C) Total AUC was calculated using GraphPad Prism.

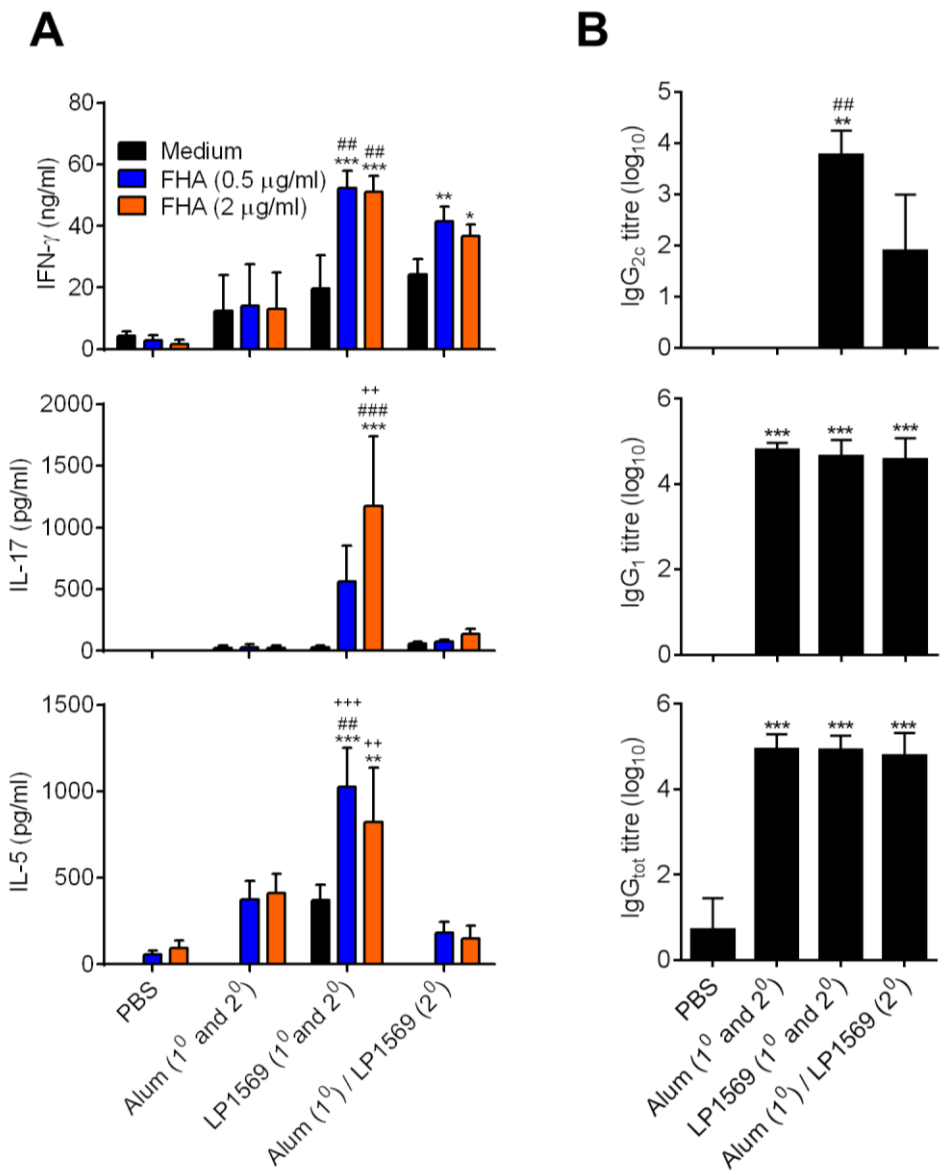
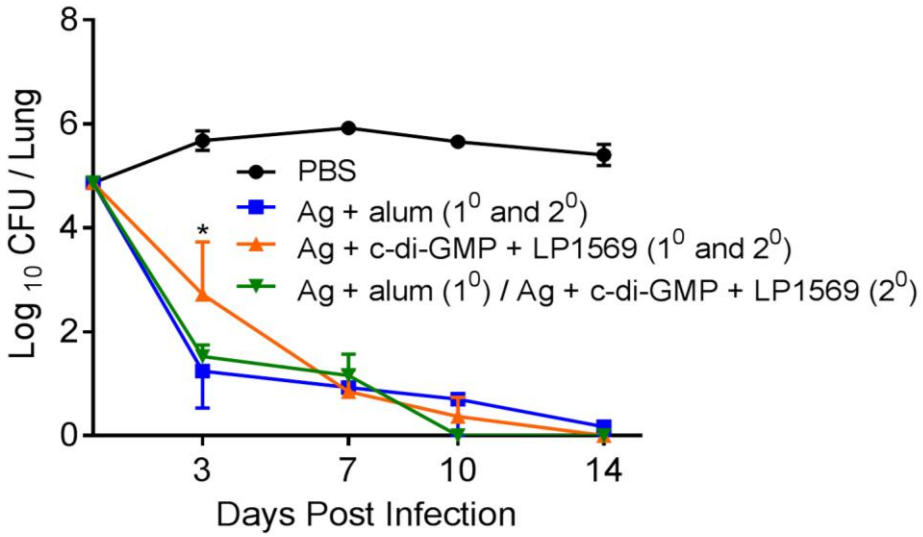


Figure 5.2 Immunising mice twice with an LP1569-adjuvanted aP vaccine is the most effective method of inducing robust antigen-specific IFN-γ, IL-17 and IgG2c production.
Mice were immunised i.p. twice as described in Figure 5.1. Spleens and sera from immunised mice were isolated 1 day prior to aerosol challenge. (A) Spleen cells (2 x 10^6 cells/ml) were cultured with medium as a control or FHA (0.5 or 2 µg/ml) and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results are shown as mean ± SEM (n=4 mice). *p < 0.05, **p < 0.01, ***p < 0.001 versus PBS; ##p < 0.01, ###p < 0.001 versus Alum (1^0 and 2^0); ++p < 0.01, +++p < 0.001 versus Alum (1^0) / LP1569 (2^0) by two-way ANOVA with the Bonferroni post test. (B) FHA-specific antibody production in serum was quantified by ELISA. Results are shown as mean ± SEM (n=4 mice). **p < 0.01, ***p < 0.001 versus PBS; ##p < 0.01 versus Alum (1^0 and 2^0) by one-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	Ag + alum	Ag + c-di-GMP + LP1569	Ag + alum (1^0) Ag + c-di-GMP + LP1569 (2^0)
	78.52	17.71	21.07	16.70

Figure 5.3 Boosting with an experimental TLR2-STING agonist adjuvanted aP vaccine after priming with an experimental alum-adjuvanted aP vaccine does not significantly accelerate clearance of *B. pertussis* infection.

Mice were immunised i.p. twice (0 and 4 weeks) with PBS as a control or the antigens FHA, Prn and rPT (Ag; 0.2 µg/mouse) formulated with alum (100 µg/mouse), a combination of c-di-GMP (10 µg/mouse) and LP1569 (50 µg/mouse) or were primed (1^0) with the Ag + alum vaccine and boosted (2^0) with the Ag + c-di-GMP + LP1569 vaccine. Immunised mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation and lungs were taken from 4 mice per group at intervals post infection to determine bacterial colonisation. (A) Results shown are mean ± SEM CFU counts (n=4 mice). *$p < 0.05$ versus Ag + alum (1^0 and 2^0) by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

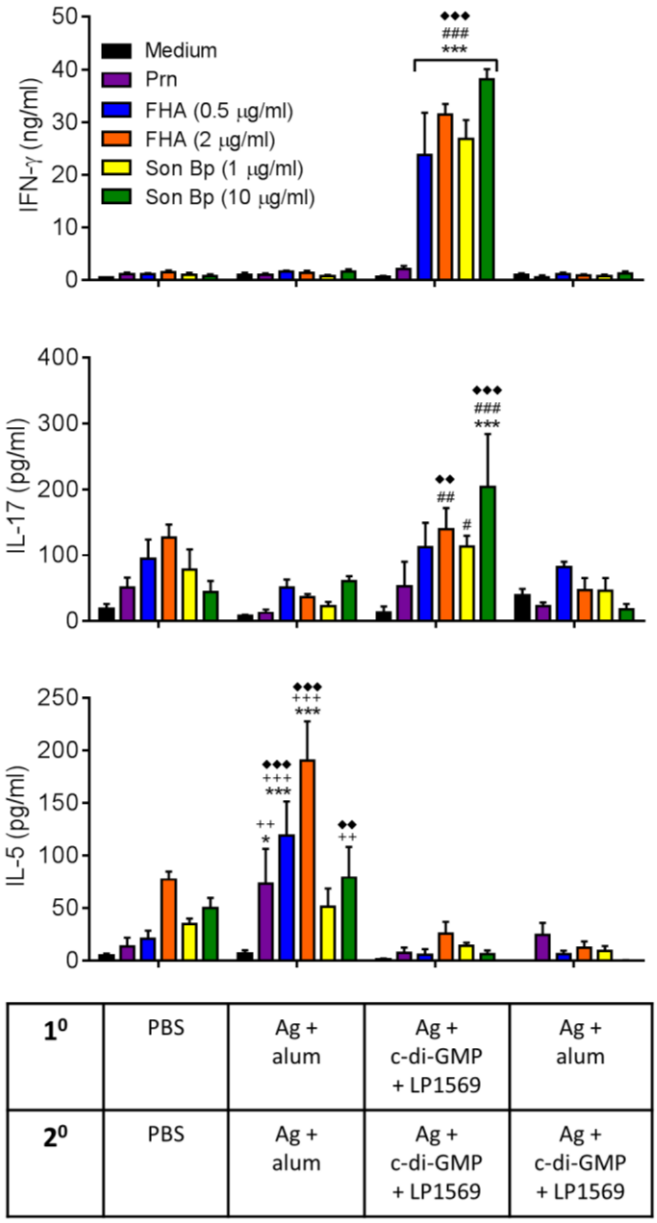


Figure 5.4 An experimental aP vaccine with a TLR2-STING agonist combination induces potent Th1 and Th17 responses, but not in Th2-primed mice.

Mice were immunised i.p. twice as described in Figure 5.3. 2 weeks after the last immunisation, the spleens from four mice in each experimental group were isolated. Spleen cells (2 x 10^6 cells/ml) were cultured with Prn (2 μg/ml), FHA (0.5 or 2 μg/ml), Son Bp (1 or 10 μg/ml) or medium as a negative control and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). *$p < 0.05$, ***$p < 0.001$ versus PBS; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ versus Ag + alum (1^0 and 2^0); ++$p < 0.01$, +++$p < 0.001$ versus Ag + c-di-GMP + LP1569 (1^0 and 2^0); ◆◆$p < 0.01$, ◆◆◆$p < 0.001$ versus (1^0) Ag + alum / (2^0) Ag + c-di-GMP + LP1569 by two-way ANOVA with the Bonferroni post test.

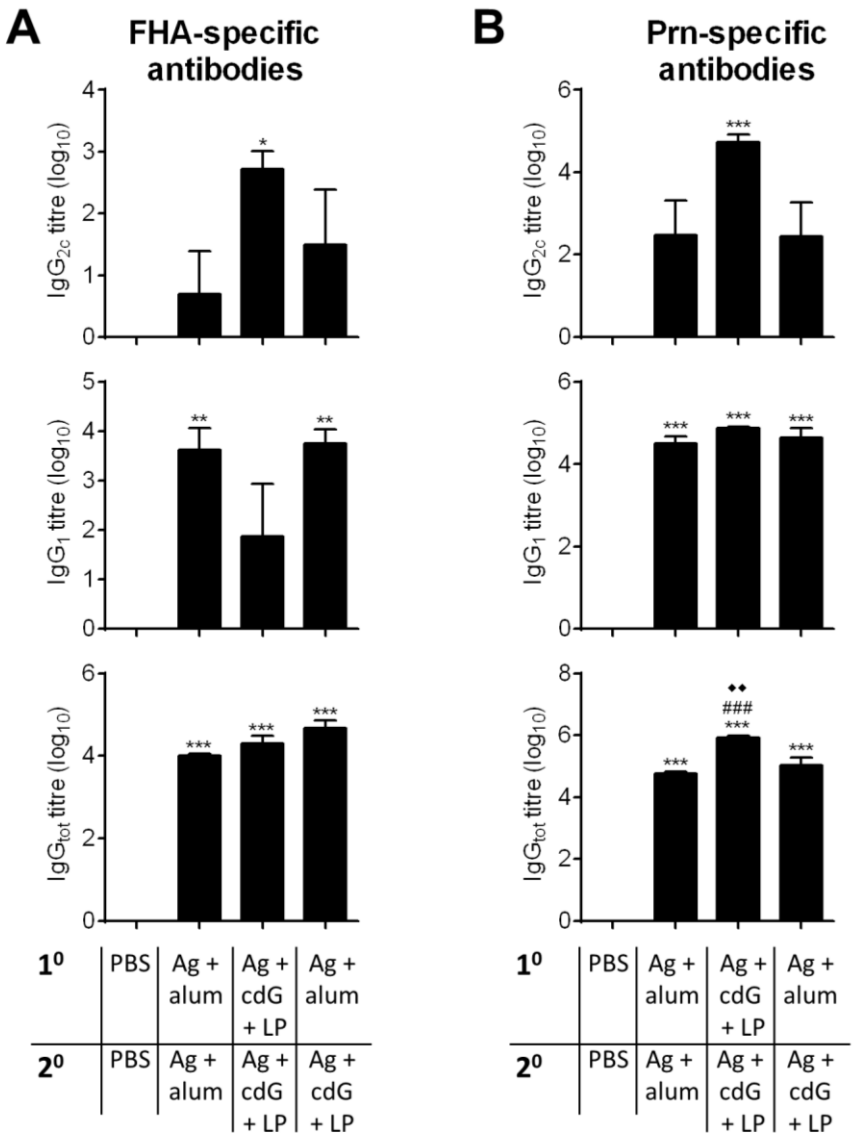


Figure 5.5 An experimental aP vaccine containing a TLR2-STING agonist combination promotes potent IgG2c antibody responses, but not in mice primed with an experimental alum-adjuvanted aP vaccine.

Mice were immunised i.p. twice with the vaccines described in Figure 5.3; PBS, Ag + alum, Ag + c-di-GMP + LP1569 (Ag + cdG + LP). 2 weeks after the last immunisation, the sera from four mice in each experimental group were collected. (A) FHA-specific antibody production and (B) Prn-specific antibody production in sera were quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus PBS; ###$p < 0.001$ versus Ag + alum (1^0 and 2^0); ◆◆$p < 0.01$ versus (1^0) Ag + alum / (2^0) Ag + cdG + LP by one-way ANOVA with the Bonferroni post test.

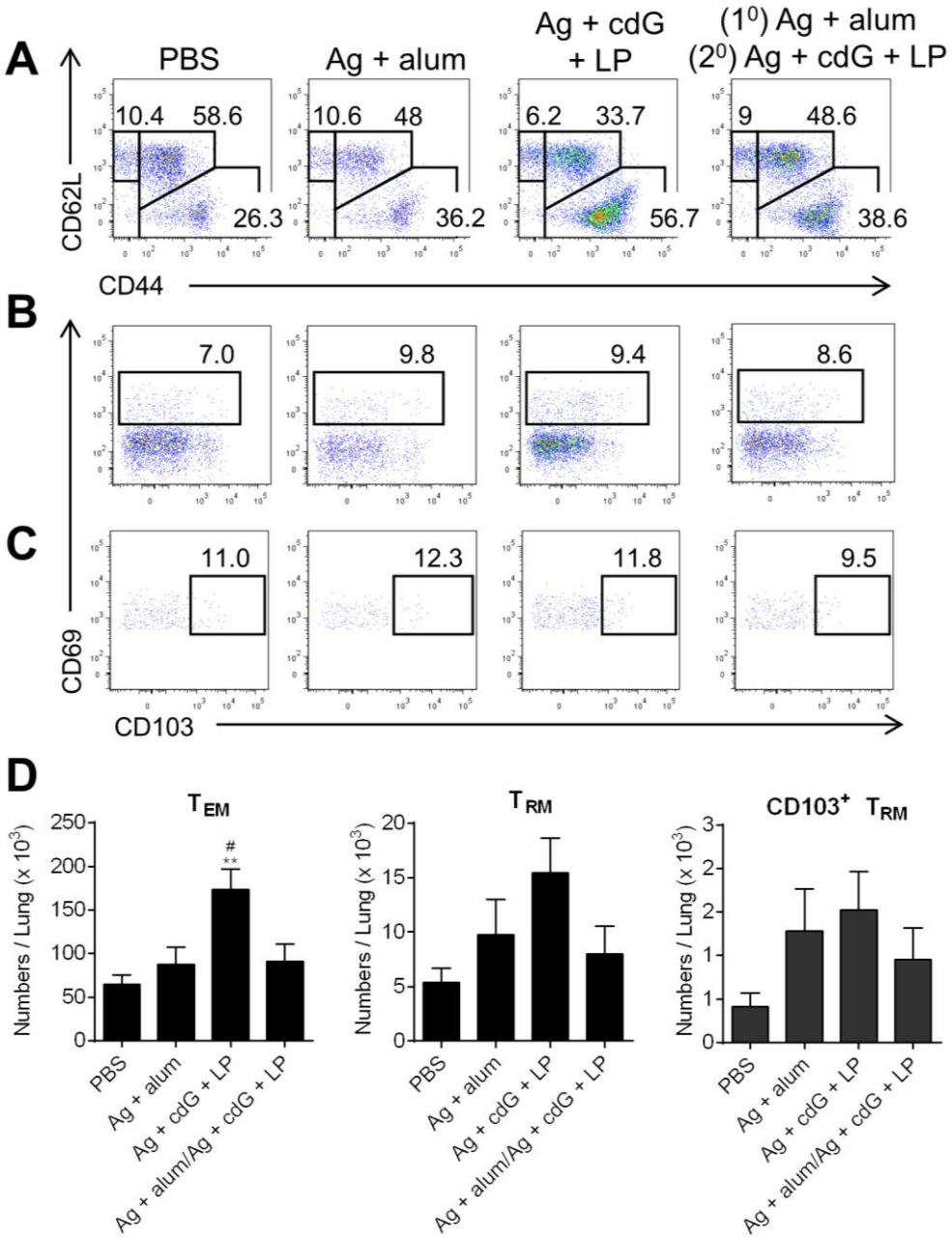


Figure 5.6 Priming and boosting mice with an experimental aP formulated with c-di-GMP and LP1569 is the most effective method of recruiting $CD4^+$ T_{RM} cells to the lungs of mice.
Mice were immunised i.p. twice with the vaccines described in Figure 5.3; PBS, Ag + alum, Ag + c-di-GMP + LP1569 (Ag + cdG + LP). The lungs were isolated 2 weeks after the last immunisation and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing the (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+CD103^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). $^{**}p < 0.01$ versus PBS; #$p < 0.05$ versus Ag + alum by one-way ANOVA with the Bonferroni post test.

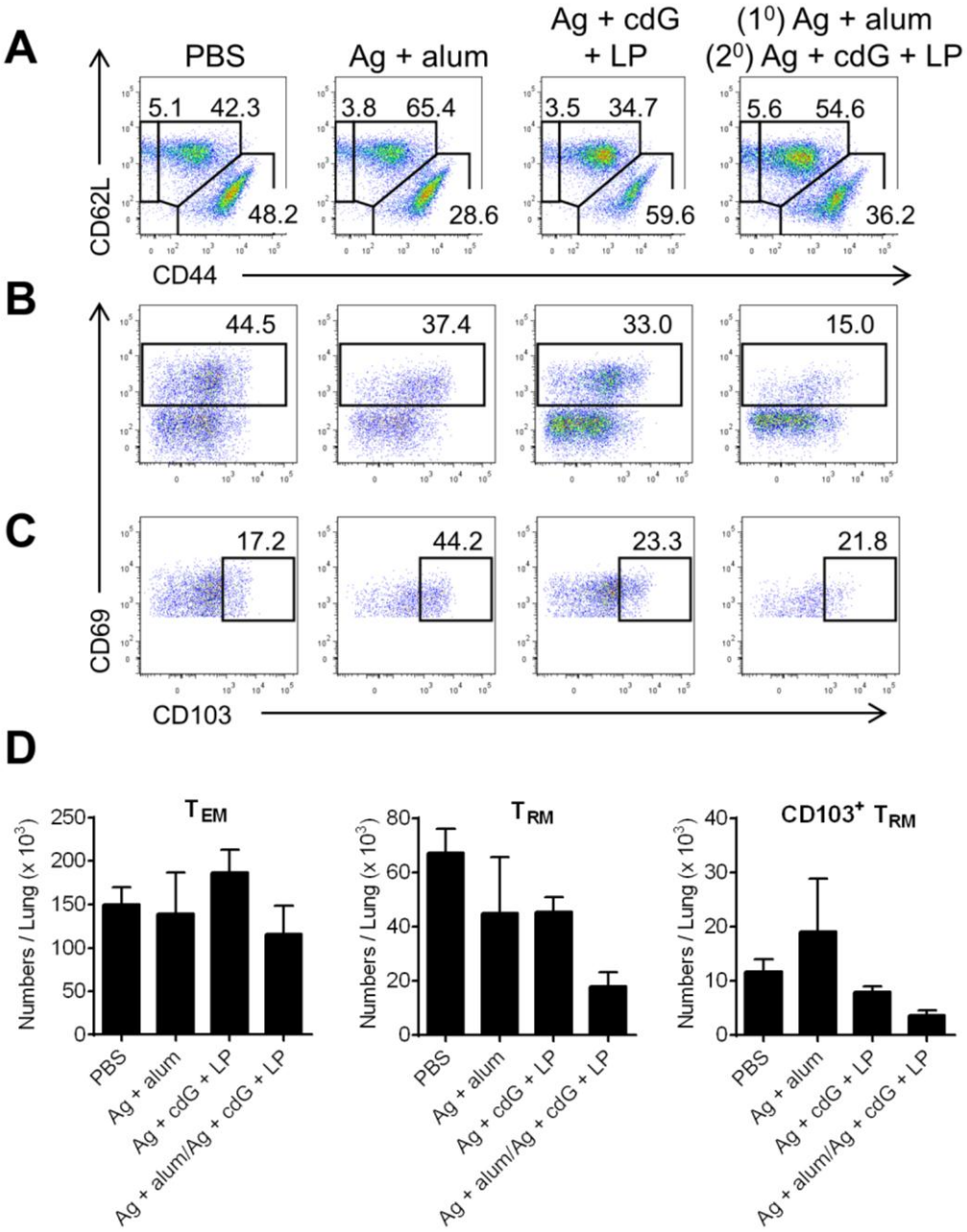
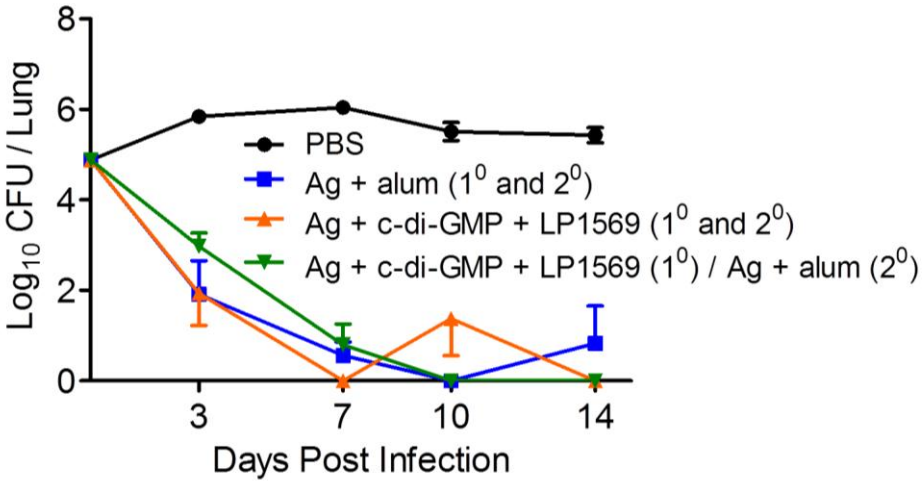


Figure 5.7 After challenge with *B. pertussis,* there is poor expansion of CD4$^+$ T_{EM} and T_{RM} cells in the lungs of mice primed with an experimental alum-adjuvanted aP vaccine and boosted with an experimental TLR2-STING agonist adjuvanted aP vaccine.

Mice were immunised i.p. twice with the vaccines described in Figure 5.3; PBS, Ag + alum, Ag + c-di-GMP + LP1569 (Ag + cdG + LP). 2 weeks after the last immunisation, mice were aerosol challenged with live *B. pertussis.* The lungs were isolated 7 days post infection and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing the (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+CD103^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). No significant differences between groups by one-way ANOVA with the Bonferroni post test.

A



B

AUC	PBS	Ag + alum	Ag + c-di-GMP + LP1569	Ag + c-di-GMP + LP1569 (1^0) Ag + alum (2^0)
	79.06	17.66	18.93	20.52

Figure 5.8 Priming with an experimental three-component aP vaccine containing a Th1-promoting adjuvant before boosting with an experimental alum-adjuvanted aP vaccine does not enhance the rate of clearance of *B. pertussis* infection.

Mice were immunised i.p. twice (0 and 4 weeks) with PBS as a control or the antigens FHA, Prn and rPT (Ag; 0.2 µg/mouse) formulated with alum (100 µg/mouse), a combination of c-di-GMP (10 µg/mouse) and LP1569 (50 µg/mouse) or were primed (1^0) with the Ag + c-di-GMP + LP1569 vaccine and boosted (2^0) with the Ag + alum vaccine. Immunised mice were challenged by exposure to live *B. pertussis* 2 weeks after the last immunisation and lungs were taken from 4 mice per group at intervals post infection to determine bacterial colonisation. (A) Results shown are mean ± SEM CFU counts (n=4 mice). No significant differences between the immunised groups by two-way ANOVA with the Bonferroni post test. (B) Total AUC was calculated using GraphPad Prism.

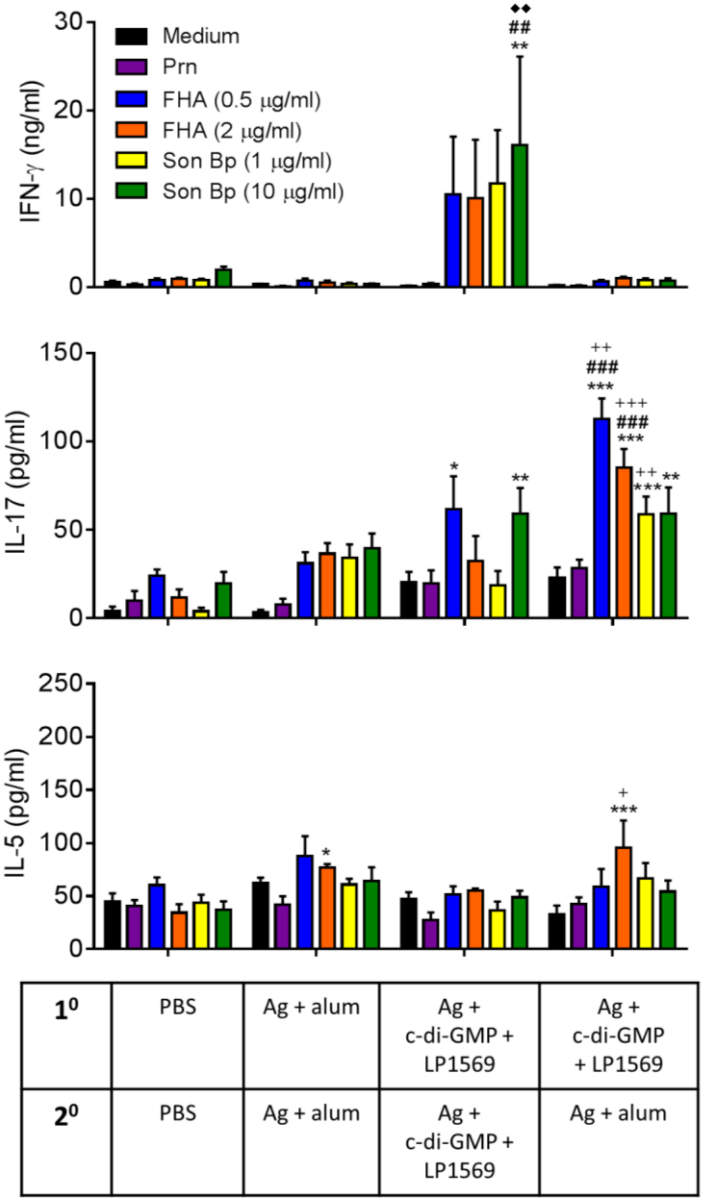


Figure 5.9 Priming and boosting with an experimental aP vaccine containing a TLR2-STING agonist combination is essential for robust antigen-specific IFN-γ production.
Mice were immunised i.p. twice as described in Figure 5.8. 2 weeks after the last immunisation, the spleens from four mice in each experimental group were isolated. Spleen cells (2 x 10^6 cells/ml) were cultured with Prn (2 μg/ml), FHA (0.5 or 2 μg/ml), Son Bp (1 or 10 μg/ml) or medium as a negative control and after 72 hours IFN-γ, IL-17 and IL-5 concentrations in supernatants were quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus PBS; ##$p < 0.01$, ###$p < 0.001$ versus Ag + alum (1^0 and 2^0); +$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$ versus Ag + c-di-GMP + LP1569 (1^0 and 2^0); ◆◆$p < 0.01$ versus (1^0) Ag + c-di-GMP + LP1569 / (2^0) Ag + alum by two-way ANOVA with the Bonferroni post test.

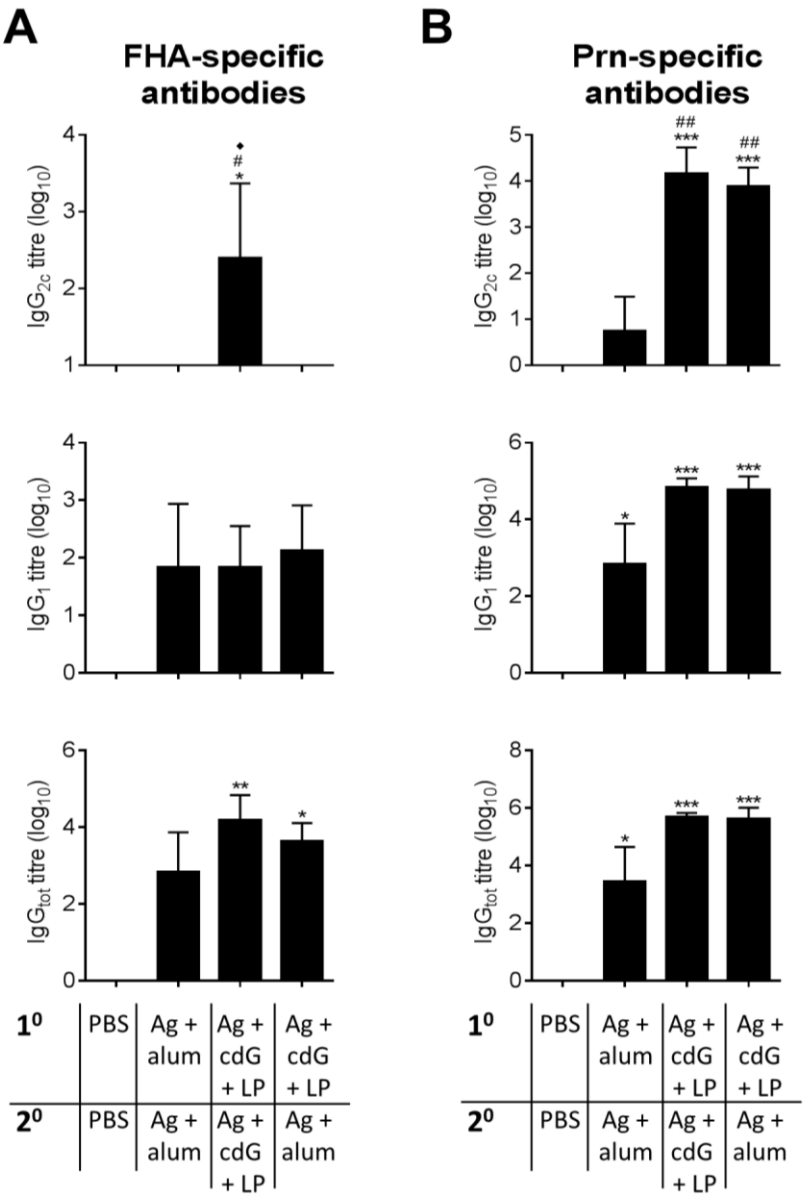


Figure 5.10 Priming and boosting with an experimental aP vaccine containing a TLR2-STING agonist combination is essential for induction of FHA-specific, but not Prn-specific, IgG2c antibodies *in vivo*.

Mice were immunised i.p. twice with the vaccines described in Figure 5.8; PBS, Ag + alum, Ag + c-di-GMP + LP1569 (Ag + cdG + LP). 2 weeks after the last immunisation, the sera from four mice in each experimental group were collected. (A) FHA-specific antibody production and (B) Prn-specific antibody production in sera was quantified by ELISA. Results shown in each panel are mean ± SEM values (n=4 mice). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus PBS; #$p < 0.05$, ##$p < 0.01$ versus Ag + alum (1^0 and 2^0); ◆$p < 0.05$ versus (1^0) Ag + cdG + LP / (2^0) Ag + alum by one-way ANOVA with the Bonferroni post test.

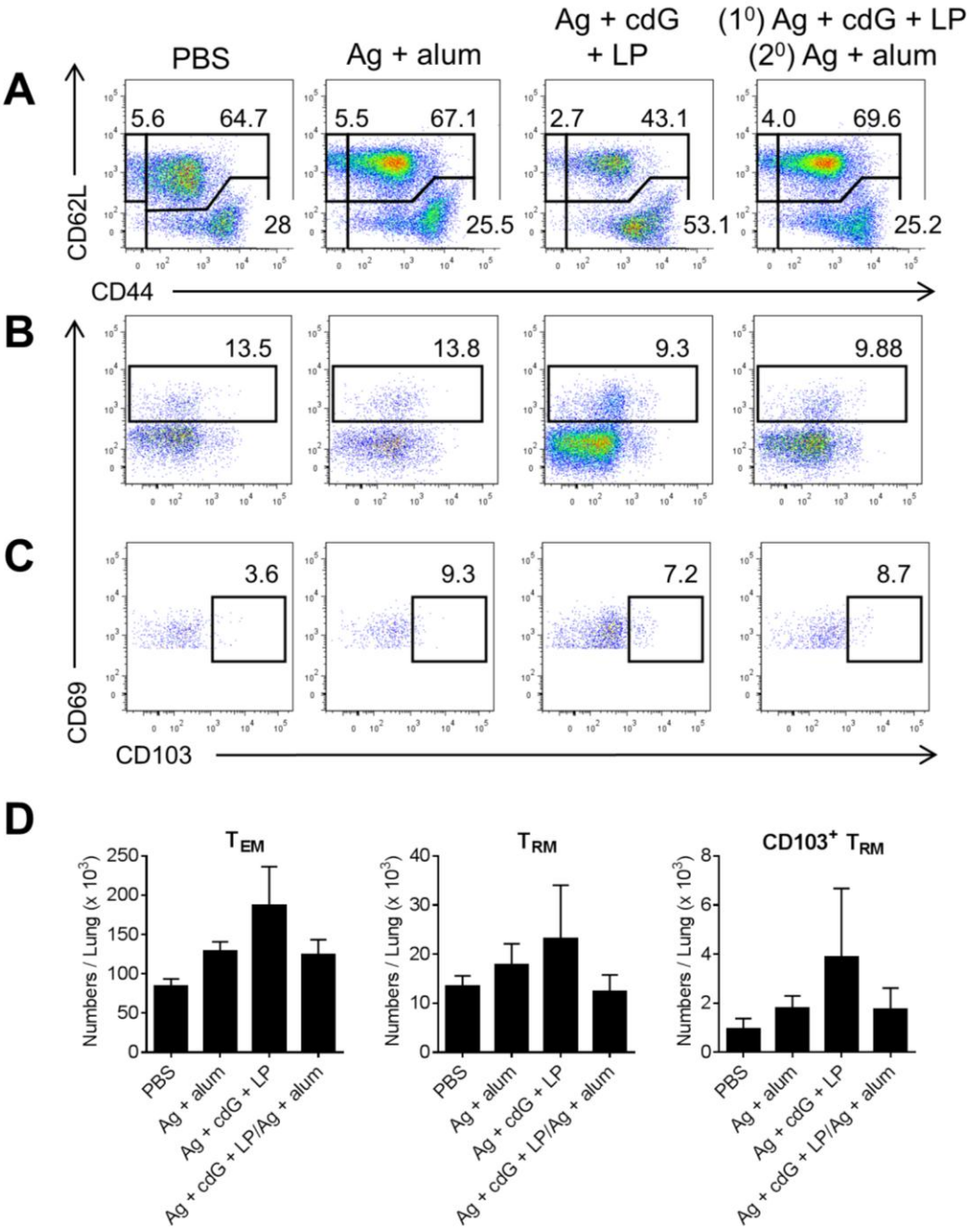


Figure 5.11 CD4$^+$ T_{EM} and T_{RM} cell recruitment to the lungs of mice primed with an aP vaccine formulated with a TLR2-STING agonist combination and boosted with an alum-adjuvanted aP vaccine is no better than in mice immunised with an alum-adjuvanted aP vaccine twice.
Mice were immunised i.p. twice with the vaccines described in Figure 5.8; PBS, Ag + alum, Ag + c-di-GMP + LP1569 (Ag + cdG + LP). The lungs were isolated 2 weeks after the last immunisation and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing the (A) T_{EM} (CD4$^+$CD8$^-$CD44$^+$CD62L$^-$), (B) T_{RM} (CD4$^+$CD8$^-$CD44$^+$CD62L$^-$CD69$^+$) and (C) CD103$^+$ T_{RM} (CD4$^+$CD8$^-$CD44$^+$CD62L$^-$CD69$^+$CD103$^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). No significant differences between groups by one-way ANOVA with the Bonferroni post test.

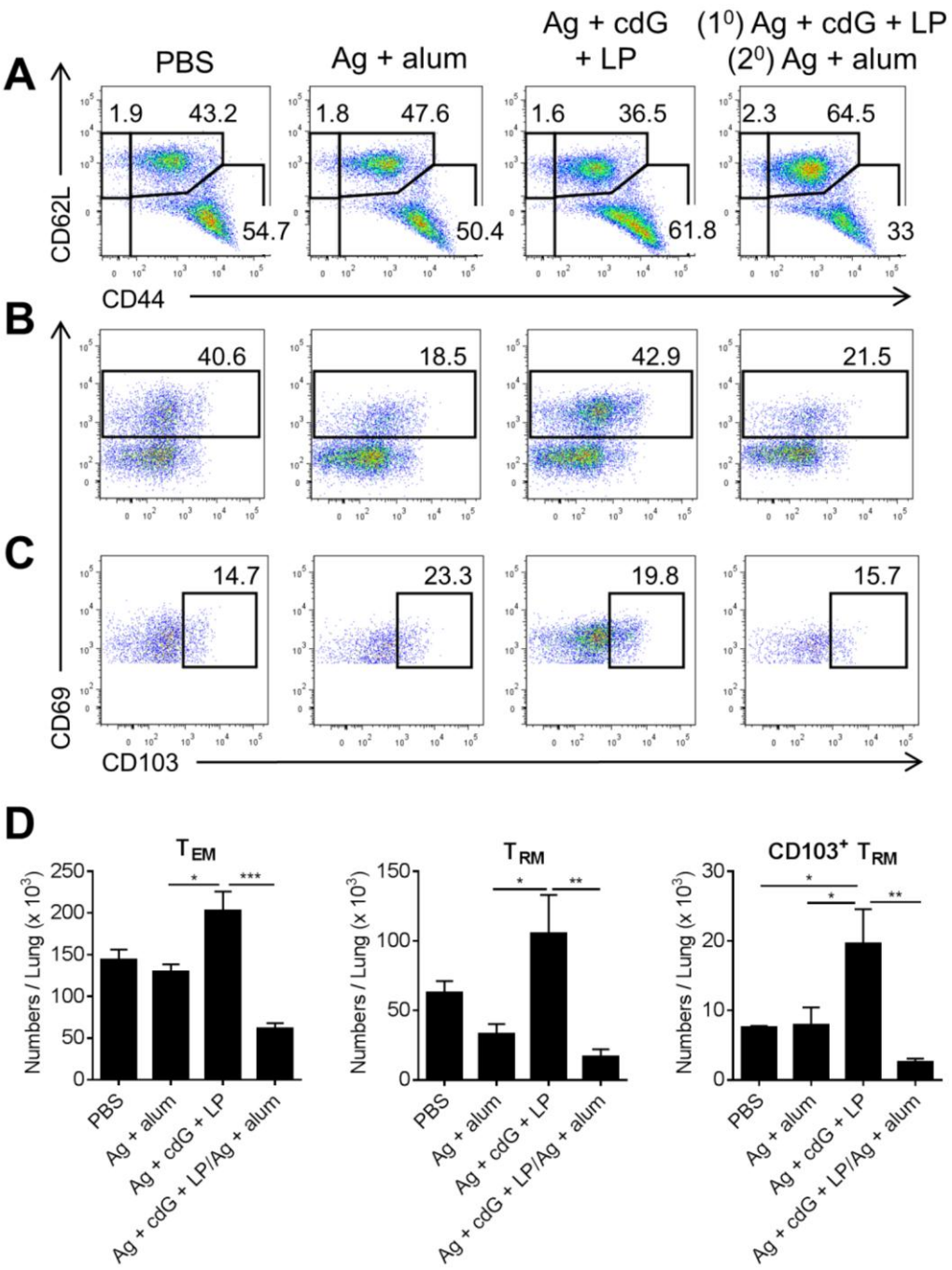


Figure 5.12 Mice immunised twice with an experimental aP vaccine formulated with c-di-GMP and LP1569 have the greatest CD4$^+$ T_{RM} cell expansion in the lungs after aerosol challenge with *B. pertussis*.

Mice were immunised i.p. twice with the vaccines described in Figure 5.8; PBS, Ag + alum, Ag + c-di-GMP + LP1569 (Ag + cdG + LP). 2 weeks after the last immunisation, mice were aerosol challenged with live *B. pertussis.* The lungs were isolated 7 days post infection and cells were stained with markers for CD4, CD3, CD8, CD44, CD62L, CD69 and CD103 for FACS analysis. FACS plots showing the (A) T_{EM} ($CD4^+CD8^-CD44^+CD62L^-$), (B) T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+$) and (C) $CD103^+$ T_{RM} ($CD4^+CD8^-CD44^+CD62L^-CD69^+CD103^+$) cell populations in the lungs. (D) Corresponding absolute numbers of memory T cells in the lungs. Results shown are mean ± SEM values (n=4 mice). *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA with the Bonferroni post test.

Chapter 6:

General Discussion

Chapter 6: General Discussion

Until recently, effective vaccines were believed to be those that induced potent humoral immune responses, with vigorous antibody production accepted as the primary biomarker of efficacy. However, it is becoming increasingly evident that robust cellular immunity is needed to protect against several intracellular pathogens. Indeed, Th1 and Th17 cell responses have been shown to be very important in the clearance of *B. pertussis* infection [64, 88, 188]. However, there is mounting evidence that the current commercial aP vaccines are incapable of inducing robust cell-mediated immunity, most likely due to the use of alum as the adjuvant [189, 243]. Alum was the first adjuvant to be licensed for human use and due to its widely accepted safety profile, it is the adjuvant of choice for most commercial vaccines. However, the current study highlighted the failure of alum-adjuvanted aP vaccines to induce potent Th1 and Th17 cell responses. While this may in part explain some of the aP vaccine problems, it also has implications for the development of vaccines against other infectious diseases, in particular those against intracellular pathogens such as HIV [402], and reinforces the need for more extensive research into novel adjuvants.

It has recently been shown that Th1 cells are important for the clearance of systemic *S. aureus* infection in mice and that this $CD4^+$ T cell subtype is expanded in the blood of human patients infected with this Gram-positive bacterium [403]. Similar to pertussis vaccine development, vaccines against *S. aureus* previously focused on the induction of potent antibody responses, and this could in part explain their failure to induce protection in several human clinical trials [404]. A study by Brown and colleagues demonstrated that an experimental vaccine containing the *S. aureus* antigen clumping factor A (ClfA) adjuvanted with CpG induced a more potent Th1-biased immune response and protected mice from *S. aureus* infection better than immunising mice with either CpG or ClfA alone [403]. Furthermore, Tougan *et al.* reported that addition of the K3-type CpG oligodeoxynucleotide to the malaria vaccine candidate SE36/AHG induced greater Th1-type cellular and humoral immunity in immunised non-human primates, than in those immunised with SE36/AHG alone [405]. The data from the present study supports these findings; it was shown that CpG was a potent Th1-inducing adjuvant and that an experimental aP vaccine with CpG conferred greater protection against *B. pertussis* infection than an experimental alum-adjuvanted aP vaccine. Together, these data indicate that CpG could be a potential adjuvant for a range of infectious disease vaccines requiring Th1-type immune responses. While licensing a new vaccine adjuvant is a long and challenging process,

CpG has already been shown to be safe for human use in a number of clinical trials which could accelerate the approval of this adjuvant [361-363].

BP1569 is a *B. pertussis* lipoprotein that was recently identified by the Mills lab and shown to be an antigenic component of *B. pertussis* [276]. LP1569 is a synthetic lipopeptide version of this lipoprotein, synthesised to carry out more extensive *in vivo* studies. The present study demonstrated that LP1569, which is a TLR2 agonist, was an effective adjuvant for an aP vaccine, inducing more robust Th1 and Th17 cell responses than an experimental CpG-adjuvanted aP vaccine. However, if LP1569 retains the same characteristics as BP1569, its efficacy in an experimental aP vaccine may be due to antigenic properties, in addition to its adjuvant activity. Therefore, LP1569 may not be as successful as an adjuvant for non-pertussis vaccines.

The STING agonist c-di-GMP has been shown to protect *in vivo* against *B. pertussis* infection when administered i.n. 1 day before infection [292]. Furthermore, several vaccine studies in well-established murine disease models, including *S. aureus* [288] and *S. pneumoniae* [375, 406], have reported that c-di-GMP has potent adjuvant activity. Immunisation of mice by the i.n or sublingual route with the H5N1 influenza vaccine NIBRG-14 formulated with c-di-GMP as an adjuvant, enhanced local and systemic antigen-specific humoral and cellular immunity, compared with when NIBRG-14 was administered alone [407]. Moreover, there is also evidence that c-di-GMP is a more potent inducer of Th1 and IgG2a responses than CpG [393]. Recent reports have also indicated that TLR and STING agonists can act synergistically to induce potent Th1-biased immune responses [380, 381] and consistent with this, the present study demonstrated that c-di-GMP and LP1569 induced robust Th1 and Th17 cell responses when formulated in an experimental aP vaccine, and were a more effective adjuvant combination than alum, or either agonist alone. The capacity of TLR and STING agonists to function synergistically has considerable potential for vaccines in general, as this combination could be more effective than individual adjuvants for inducing potent cellular immunity to infectious diseases.

The therapeutic potential of TLR and STING agonists extends past their ability to act as infectious disease vaccine adjuvants. Indeed, several studies have reported that TLR agonists are well tolerated in humans and can be exploited for cancer therapies. Indeed, CpG was shown to be safe in a phase II/III study of human melanoma [367]. Furthermore, the results of a phase I/II clinical trial showed that patients suffering from cutaneous T-cell lymphoma had a decrease in lesion severity, with some patients reporting complete lesion clearance, after

topical treatment with the TLR7/8 agonist R848 (resiquimod) [408]. Moreover, only minor adverse effects were reported in both of these trials [367, 408]. Studies in mice using STING agonists as a cancer therapy have also been promising. Indeed, addition of c-di-GMP to the peptide vaccine TriVax enhanced the therapeutic effects of this vaccine by inducing greater numbers of $CD8^+$ T cells specific for B16 melanoma cells than TriVax alone [409], and similarly, cGAMP-driven STING activation significantly augmented the anti-tumour immune responses induced by radiation [410]. Steps are currently being taken to commercialise STING agonists. Indeed, Aduro Biotech and Novartis Pharmaceuticals are currently recruiting participants for a phase I clinical trial to evaluate the safety and efficacy of the CDN MIW815 (ADU-S100) in a therapeutic cancer vaccine (NCT02675439).

One of the suggested reasons for the resurgence of *B. pertussis* is that aP vaccines induce poor memory B cell and antibody responses and indeed, there have been several reports that immunity generated by aP vaccination wanes rapidly after immunisation [230, 231]. However, few studies have examined the induction of persistent T cell memory responses following immunisation with aP vaccines. The results of the present study showed that immunisation with a wP vaccine recruited large numbers of $CD4^+$ T_{RM} cells to the lungs. In contrast, immunisation with an aP vaccine did not induce migration of T_{RM} cells, with numbers in the lungs similar to those seen in unimmunised mice. Furthermore, it was shown that substitution of alum with LP1569, or a combination of LP1569 and c-di-GMP, enhanced numbers of $CD4^+$ T_{RM} cells recruited to the lungs, indicating that a TLR agonist or TLR-STING agonist combination may be a more effective vaccine adjuvant than alum for establishing long-term memory T cell responses. While these $CD4^+$ T_{RM} cells have yet to be shown to have a role in clearance of *B. pertussis* infection, there are reports that they are sufficient to protect against infection with the respiratory pathogen *M. tuberculosis* [196].

While the mechanisms involved in the recruitment, expansion and retention of T_{RM} cells in tissues have yet to be fully elucidated, there is evidence implicating IL-17 [369]. The data in the present study is consistent with this hypothesis. An experimental aP vaccine formulated with LP1569 or c-di-GMP and LP1569, induced greater Th17 cell responses than an experimental alum-adjuvanted aP vaccine, and was more effective at recruiting T_{RM} cells to the lungs. Furthermore, i.n. immunisation with an experimental aP vaccine formulated with LP1569 and c-di-GMP induced significantly greater Th17 cell expansion than any of the experimental aP vaccines administered i.p., and consistent with this, this i.n. administered aP vaccine was by far the most effective at inducing recruitment of $CD4^+$ T_{RM} cells to the lungs. These findings demonstrate the importance of using an adjuvant which induces Th17 in addition to Th1 cells

in pertussis vaccines and furthermore, suggest that an i.n. vaccine may be more effective for establishing long-term memory to *B. pertussis*. Indeed, BPZE1, a live-attenuated strain of *B. pertussis,* is administered i.n. and has been shown to confer significantly more durable immunity than a commercial alum-adjuvanted aP vaccine [306]. Furthermore, BPZE1 has been shown to induce robust Th17 cell responses [304]. It would be of interest to investigate the induction of memory T cells and trafficking of T_{RM} cells to the lungs following immunisation with BPZE1, and if this could explain the long-term protection conferred with the attenuated vaccine.

An interesting and unexpected observation from the present study was the demonstration that mice immunised with alum-adjuvanted aP vaccines appeared to have lower numbers of T_{RM} cells in the lungs after *B. pertussis* challenge than unimmunised mice. While Th17 responses may promote the trafficking and establishment of T_{RM} cells in the lungs, the Th2 responses induced with alum-adjuvanted aP vaccines may inhibit T_{RM} cell recruitment to the lungs following *B. pertussis* infection. This suggests that immunisation with aP vaccines may impair the generation of long-term memory T cell responses to *B. pertussis* infection, and could explain why natural infection, which induces Th1 and Th17 cell responses, induces more durable immunity [187]. Indeed, this could also explain the very poor T_{RM} cell responses induced in this study when boosting with an experimental aP vaccine formulated with LP1569 and c-di-GMP after priming with an experimental alum-adjuvanted aP vaccine. A Th2 response is set by priming with an experimental alum-adjuvanted aP vaccine, and boosting with an aP vaccine containing a more appropriate adjuvant cannot redirect the immune response to Th1 and Th17, thereby limiting T_{RM} cell recruitment to the lungs.

Overall, the results from these studies suggest that switching the adjuvant in an aP vaccine from alum to a Th1/Th17-promoting adjuvant, and immunising by the i.n. route, are the most effective methods of inducing robust $CD4^+$ T_{RM} cell induction in the lungs. However, these experiments did not directly examine the function or lifespan of these memory cells. While the immunisation schedule used here is a well-established method for examining immune responses and protection induced with experimental aP vaccines in the short-term, this protocol of challenging with *B. pertussis* 2 weeks after the last immunisation does not facilitate analysis of the duration of immunity induced with these vaccines. Long-term studies were not performed here due to time constraints, however, it would be important in the future to repeat these experiments but extend the period of time between immunisation and challenge significantly, to determine how long these $CD4^+$ T_{RM} cells persist after immunisation and to

examine if their persistence correlates with the duration of protection induced with these experimental aP vaccines.

This study found that boosting with an experimental aP vaccine formulated with LP1569 and c-di-GMP after priming with an experimental alum-adjuvanted aP vaccine did not enhance *B. pertussis*-specific Th1 and Th17 responses. While this does not look promising for the development of a booster pertussis vaccine that would be administered to adolescents and adults, there may be a solution to this problem. aP vaccines consist of a small number of *B. pertussis* antigens adsorbed to the Th2-promoting adjuvant alum, however, boosting with a vaccine containing several novel antigens and a Th1/Th17-promoting adjuvant may enhance appropriate cellular immunity by inducing primary immune responses to the new antigens. One way this could be achieved may be to boost with BPZE1 or *B. pertussis* OMVs. Both of these vaccine candidates are similar to wP vaccines as they contain many antigens, in addition to those in the aP vaccines.

While developing a new pertussis vaccine containing a more appropriate adjuvant is necessary, the process of implementing a novel vaccine will face many logistic and licensing obstacles. However, whooping cough still remains a considerable threat to neonates. Currently, immunisation of pregnant women to promote the passive transfer of maternally-derived antibodies is the only method of protecting infants who are too young to be vaccinated, however, these antibodies have been shown to wane rapidly after birth [332, 411]. Maternal immunisation with TdaP is now recommended in many countries including Ireland [27], the UK [337] and the US [30] in an effort to extend the protection conferred by mothers to newborns. However, there is some evidence that maternal immunisation and the subsequent passive transfer of antibodies across the placenta impairs the infants' immune response to active immunisation with a pertussis vaccine [412-415]. Indeed, it has been reported that while children born to mothers vaccinated during pregnancy have higher antibody titres to pertussis antigens including dPT and FHA at birth, upon active immunisation with a pertussis vaccine, the antibody responses generated are weaker than in infants born to mothers who were not vaccinated during pregnancy [412]. There are a number of suggested methods for how maternal antibodies interfere with the generation of a potent immune response, including neutralisation of foreign antigen or masking of antigen epitopes by the maternal antibodies [416, 417]. The consequences of this 'blunting' effect have not yet been fully established and clinical trials are underway to further investigate this phenomenon (NCT00553228).

Studies in animal models have been employed to evaluate methods to avoid this maternal antibody interference. Indeed, Polewicz *et al.* reported that this 'blunting' effect could be overcome by reformulating the experimental vaccine administered to mothers with an adjuvant before administration to infants; piglets born to sows immunised with dPT did not experience interference from maternal antibodies when immunised with dPT and CpG [418]. Furthermore, it has also been demonstrated in a porcine model that by incorporating an experimental pertussis vaccine into microparticles, this shielded antigens from detection by maternal antibodies and thus facilitated the generation of robust anti-PT and anti-FHA antibodies by the piglets' immune system [419]. Currently though, only aP vaccines containing the same antigens and adjuvant have been licensed for maternal and infant immunisation. Although interference by these maternally-derived antibodies is a problem, maternal immunisation is a safe and simple technique for protecting neonates. Furthermore, to stop vaccinating pregnant mothers is not currently an option as it would leave vulnerable neonates at a high risk of infection.

Several studies using animal models have also investigated the possibility of introducing a neonatal vaccine to protect newborns from *B. pertussis* infection. Warfel *et al.* reported that immunisation of 2 day old baboons with aP vaccines before challenge with *B. pertussis* completely protected them from disease, however, they were still colonised for the same length of time as unvaccinated baboons, indicating vaccination did not shorten the infection period [420]. BPZE1 was also reported to be highly effective at protecting neonatal mice from *B. pertussis* infection [307], and it has been shown to be safe in phase I clinical trials in humans [309, 310]. While licensing a neonatal vaccine would be a very challenging and long process, it may be the most effective method of inducing optimal immunity against *B. pertussis* infection.

Animal models have been an invaluable asset for the study of disease pathogenesis, and for elucidating the mechanisms of natural and vaccine-mediated immunity to *B. pertussis.* Humans are the only natural host of *B. pertussis,* however, several species, including mice, baboons, pigs, rats and dogs, can be infected under experimental settings. As discussed above, the pig model of *B. pertussis* infection has been very useful for the study of maternal and neonatal immunisation. The immune system of pigs is similar to that of humans and furthermore, *B. pertussis* infection of neonatal piglets closely resembles the disease in human newborns, with piglets experiencing lymphocytosis, in addition to a number of respiratory symptoms, including coughing and nasal discharge [421].

The murine model of *B. pertussis* infection is the most commonly studied as the initial stages of *B. pertussis* infection in mice (colonisation and outgrowth), resemble that seen in humans. Furthermore, mice challenged with *B. pertussis* develop a persistent infection that is associated with PT-induced lymphocytosis, as do humans. Indeed, this model has provided great insight into the pathogenesis of this infectious disease. Studies using severe combined immunodeficient (SCID) and athymic mice first gave clear evidence that cellular, in addition to humoral immunity, was crucial in the immune response to *B. pertussis* infection [66, 67], and furthermore, experiments using mice lacking individual cytokines first highlighted the protective roles of IFN-γ and IL-17 in the clearance of natural infection [64, 88, 189].

The mouse model of *B. pertussis* infection has also been vital in elucidating the immune responses induced with pertussis vaccines. Ross *et al.* showed that wP vaccines induced Th1 and Th17 cells but that protection was predominantly mediated by Th1 cells, whereas aP vaccines predominantly induced Th2 and Th17 responses, but protection was entirely mediated by IL-17 and not IL-4 [189]. Furthermore, it was shown that the protection conferred by aP and wP vaccines in the mouse model correlated with vaccine efficacy in children [65]. These findings validated the use of the mouse model for studying pertussis vaccine efficacy and this model of infection has since been exploited to study the efficacy of novel pertussis vaccines, such as those containing alternative adjuvants to alum, including TLR agonists and cytokines [189, 276, 302].

The mouse model of *B. pertussis* infection was very useful for investigating the efficacy of the experimental aP vaccines used in this study, however, it does have several limitations. Mice don't display the typical symptoms of *B. pertussis* infection such as coughing or sneezing and therefore, they are poor models for studying the transmission of *B. pertussis.* In contrast, a non-human primate model of infection has been developed where symptoms more accurately reflect those observed in humans; infected baboons suffer from paroxysmal coughing, nasal discharge and leukocytosis [422]. Indeed, this model has already revealed that while aP vaccines can protect against severe disease, they are poor at preventing colonisation and transmission, whereas immunisation with wP vaccines prevents all three [228]. As the development of a novel pertussis vaccine which prevents transmission, colonisation and disease is paramount, the non-human primate model of *B. pertussis* infection could be considered more valuable than the mouse model. With this in mind, it would be of interest in the future to examine transmission of *B. pertussis* by baboons immunised with the experimental aP vaccines formulated with c-di-GMP and LP1569 which were used in these studies.

The various animal models of *B. pertussis* infection have individual advantages and disadvantages, which highlights the importance of having access to an array of diverse animal models. The elaborate animal studies performed to identify natural and vaccine-mediated immunity to *B. pertussis* have been crucial as they could not have been examined in humans. However, there is a drive to develop a human challenge model of *B. pertussis* infection to study the progression of disease in its natural host, and to validate the findings from animal studies. In order to effectively implement human challenge studies with *B. pertussis,* a number of ethical criteria would need to be fulfilled. Indeed, an effective rescue therapy would need to be put in place to prevent severe disease and distress in study subjects. Currently antibiotics are the only therapy available and these are only effective at lessening disease severity when administered early in infection, prior to symptom onset [12, 13]. Moreover, they do not limit the duration of disease. Furthermore, as *B. pertussis* infection is highly contagious, studies would need to be performed in a contained and controlled environment to prevent accidental transmission to susceptible individuals not participating in the trial.

One interesting finding that was highlighted by these studies is that Prn is a highly immunogenic *B. pertussis* antigen. Two-component aP vaccines containing FHA and rPT were predominantly used in the experiments here, and the rationale for this combination was that DTaP vaccines consisting of only these two antigens have previously been examined and were shown to be protective, but not as highly as those also containing Prn [401]. In the present studies, using weaker two-component aP vaccines produced clear results on which adjuvants were the most effective for experimental aP vaccines. In contrast, by adding Prn to the experimental aP vaccines too, it became impossible to distinguish the contribution of the different adjuvants to protection. Overall, the results from these studies indicate that the Prn-specific immune response is very robust, and that this antigen should be included in all commercial aP vaccines.

The incidence of pertussis worldwide has increased in the last decade and this may in part reflect the failure of current alum-adjuvanted aP vaccines to induce appropriate cellular immune responses or effective memory to *B. pertussis* infection. Novel adjuvant formulations need to be exploited in order to induce optimal immunity to this infectious pathogen, and research is ongoing globally in an attempt to inform the rational design of a new pertussis vaccine. Indeed, a European initiative called the PERISCOPE project has been launched this year, which aims to expand research into pertussis vaccines and vaccination strategies in order to accelerate the generation of a third-generation pertussis vaccine to tackle this re-emerging pathogen. The present study highlighted potential adjuvants in the form of TLR agonists or

TLR-STING agonist combinations, that when formulated in experimental aP vaccines induced greater Th1, Th17 and memory T cells, than alum-adjuvanted aP vaccines. This study also suggested that i.n. immunisation may be a more effective method of administration of the aP vaccines for the induction of local T cell, as well as antibody responses, to *B. pertussis.*

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