Improving pertussis vaccination:
central role of CD4 T cell programming

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Improving pertussis vaccination:
central role of CD4 T cell programming

Verbeteren van kinkhoest vaccinatie:
centrale rol voor de programmering van CD4 T cellen

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General Introduction

Introduction

Bordetella pertussis infection and disease

Pertussis, or whooping cough, is a highly infectious respiratory disease caused by the Gram-negative bacterium *Bordetella pertussis*. Upon *B. pertussis* infection, there is an incubation period of one or two weeks before symptoms are observed. The disease course can be divided into three stages, namely the catarrhal, paroxysmal, and convalescent stage. The one or two week lasting catarrhal stage is characterized by mild respiratory symptoms such as coughing, sneezing, and a running nose. During the subsequent paroxysmal stage classical pertussis symptoms develop, including severe coughing spells ending in a distinct high-pitched "whoop" sound as the patient gasps for breath. Moreover, these coughing spells can be accompanied by post-tussive vomiting, apnea, and gagging. The paroxysmal stage can last for months before a gradual transition towards the convalescent stage occurs. This latter stage is characterized by a decrease in frequency and severity of the paroxysms of coughing.

Complications associated with pertussis include pneumonia, seizures, encephalopathy, cerebral hypoxia leading to brain damage, pulmonary hypertension, subconjunctival hemorrhage, and rectal prolapse. These complications are mainly seen in non- or partially immunized children[1, 2]. Moreover, 90% of the fatalities due to pertussis are observed in infants less than 6 months of age[2]. Although pertussis is considered a childhood disease, the disease is also common in adolescents and adults, yet these age groups show atypical or mild symptoms[1, 2].

Pertussis epidemiology and vaccination

Before the introduction of vaccination in 1950s, pertussis was one of the most common childhood diseases worldwide. The first generation pertussis vaccines, introduced in The Netherlands in 1954, was the whole-cell pertussis (wP) vaccine that consisted of inactivated bacteria[3]. This vaccine was combined with diphtheria and tetanus toxoids and implemented in the Dutch National Immunization Program for infants. Pertussis vaccination led to a decrease in incidence and mortality of pertussis[4]. Since 1996, an increase in incidence of pertussis is observed in The Netherlands and epidemics cycles are seen every 2-3 years (Figure 1). This resurgence is observed in multiple high-income countries with high vaccination coverage[4-6]. As a response to this resurgence, several changes were made in the pertussis component of the Dutch National Immunization Program. First, the potency of the wP vaccine was elevated from 4 to 7 IU/HD in 1997. Second, the primary schedule was accelerated from 3, 4, and 5 months of age to 2, 3, and 4 months of age in 1999. Third, an additional booster dose was implemented at 4 years of age in 2001. This booster vaccination was not performed with the wP vaccine, but with a second generation acellular pertussis (aP) vaccine. The major motivation for the development and use of these second generation aP vaccines were concerns about adverse effects of wP vaccines leading to the claim that wP vaccines associated with encephalopathy, permanent brain damage, and sudden infant death syndrome. Yet, no relation between wP vaccines and these severe conditions could be

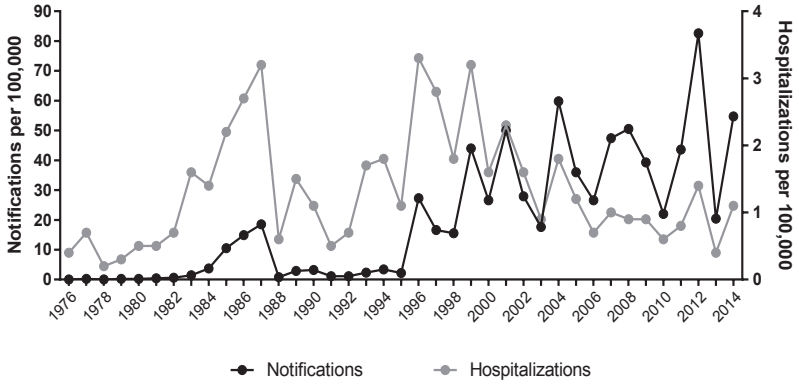

Figure 1 – Pertussis notifications and hospitalizations in The Netherlands. The pertussis notifications (left y-axis) and hospitalizations (right y-axis) per 100,000 in The Netherlands from 1976 to 2014. Note: For 2014, the hospitalization data are preliminary and incomplete. (Source: National Institute for Public Health and the Environment, Bilthoven, The Netherlands)

proven[9-12]. Minor adverse events reported after wP vaccination include fever, drowsiness, irritability, prolonged crying, vomiting, injection site discomfort, and pallor[8]. The aP vaccines are less reactogenic than the wP vaccines[8, 13]. The aP vaccines are well-defined and contain one to five purified *B. pertussis* antigens, such as pertussis toxin (Ptx), P.69 pertactin (Prn), filamentous hemagglutinin (FHA), and fimbriae type 2 and 3 (Fim 2/3). In 2005, the wP vaccines used for the primary vaccinations were replaced by aP vaccines in The Netherlands.

The measures taken in the Dutch National Immunization Program seemed to lower the incidence of hospitalizations, however they did not interrupt the pertussis resurgence (Figure 1). After the introduction of the booster vaccination at 4 years of age and aP vaccines, the peak incidence of pertussis shifted from 4-5 year old children to older individuals, including adolescents and adults[14, 15]. Seroprevalence studies in The Netherlands revealed an increase in infection frequency in the population above 9 years of age from 4% in 1995-1996 to 9.3% in 2006-2007[6].

B. pertussis infection-induced immune responses

Several explanations for the resurgence of pertussis have been suggested, such as strain adaptation and induction of a suboptimal type of immune response by the current aP vaccines. Infection with *B. pertussis* has been shown to induce the most durable protection against subsequent infection[17]. Therefore, lessons may be learned from infection-induced immunity in improving pertussis vaccination.

In the upper respiratory tract, *B. pertussis* attaches to ciliated epithelial cells and will most likely encounter airway mucosal dendritic cells and alveolar macrophages[18-22]. These cells provide the first innate line of defense against *B. pertussis* infection[21]. These antigen-presenting cells (APCs) express pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), that can sense pathogen-associated molecular patterns (PAMPs).

B. pertussis contains several PAMPs, such as lipoprotein BP1569[23] and lipooligosaccharide (LOS)[24] that respectively signal via TLR2 and TLR4. TLR4 signaling was found to be important in the protection against *B. pertussis* infection, since reduced bacterial clearance was observed from the lungs of challenged TLR4-deficient compared to WT mice[24-26]. Signaling via PRRs leads to activation of the innate cells and subsequent production of cytokines and chemokines required for licensing of antigen-specific responses by B and T cells, the adaptive line of defense. Also, other innate immune cells, such as γδ T cells[27], neutrophils[22], and NK cells[28], are recruited by these chemokines, which contribute to the clearance of the bacteria.

Influx of B cells in the lungs is also observed during the course of infection, suggesting a role of these cells in the immunity against *B. pertussis*. Both immunoglobulin (Ig)-producing plasma cells and memory B cells are induced by *B. pertussis* infection[29, 30]. These induced B cells produce both IgA and IgG antibodies[29, 31, 32]. Remarkably, the highest level of *B. pertussis*-specific IgG antibodies in serum is reached when the bacteria are almost cleared from the lungs[32, 33]. This might suggest a redundant role of B cells during primary infection, but they may be more important for protective memory immunity against subsequent infection. In addition, Leef *et al.* showed that B cells may contribute to protective immunity via antibody-independent mechanisms[34], which might include induction or re-activation of memory CD4⁺ T cells by acting as APCs[35].

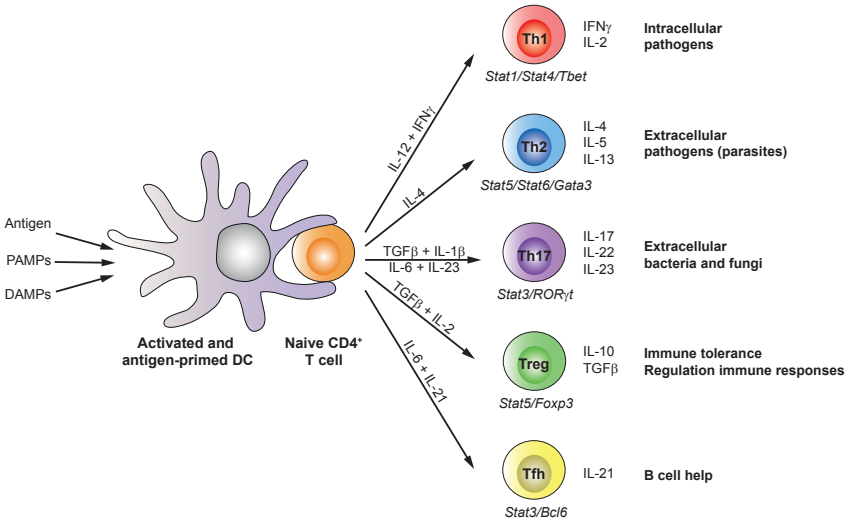

Figure 2 – CD4⁺ T cell activation and differentiation. Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are sensed by pattern-recognition receptors (PRRs) on antigen-presenting cells, such as dendritic cells (DCs), which are subsequently activated. Digestion of endocytosed antigen leads to the presentation of specific epitopes on cell surface MHC class II molecules for recognition by T cell receptors (TCRs)x on naive T cells. Depending on which PRRs are triggered and on the local inflammatory milieu, the DCs will start producing Th differentiating cytokines and will express co-stimulatory molecules. The strength of TCR signaling and co-stimulation and the type of cytokine environment will determine if, and into which Th subset the naive CD4⁺ T cell will differentiate. Each subset can be characterized by the expression of distinct transcription factors and secretion of T cell cytokines.

A study investigating the role of $CD8^+$ T cells in the protection against *B. pertussis* infection using $CD8^+$ T cell knock-out mice revealed that these cells are not critical in the protective immunity[34]. In contrast, a prominent role of $CD4^+$ T cells in the immunity against *B. pertussis* infection was shown in mice. Adoptive transfer of $CD4^+$ T cells to nude mice led, in contrast to control nude mice, to *B. pertussis* clearance after challenge[32]. Moreover, $CD4^+$ T cell knock-out mice revealed the importance of these cells in the protective immunity against *B. pertussis* infection[34]. Interestingly, the $CD4^+$ T cells provide significant functions other than induction of *B. pertussis*-specific antibody production by B cells in the protective immunity.

$CD4^+$ T cell differentiation

Specific $CD4^+$ T cells need licensing by activated APCs, such as dendritic cells (DCs), expressing antigen-specific epitope-loaded MHC class II molecules, which are recognized by clonally expressed T cell receptors (TCRs), as well as accessory molecules and soluble factors for co-stimulation and differentiation. Distinct T helper (Th) subsets, such as Th1, Th2, Th17, follicular T helper cells (Tfh), and regulatory T cells (Treg), can emerge with their own characteristics and functions[36] (Figure 2). Th1 cells secrete IFNγ and are important in the clearance of intracellular pathogens, while Th2 cells produce IL-4, IL-5, and IL-13 and are involved in the immune response against extracellular pathogens. Th17 cells produce, among others, IL-17A and are crucial for immunity against extracellular bacteria and fungi. Tfh can reside within B cell follicles, produce IL-21, and provide B cell help. Treg are characterized by the secretion of IL-10 and TGFβ and these cells prevent tissue-damaging inflammatory host responses. More recently, IL-9-producing Th9 and IL-22-producing Th22 cells are described[37, 38], yet their functions require further investigation.

Differentiation of $CD4^+$ T cells towards the Th subsets depends on the strength of TCR signaling and the cytokine environment during $CD4^+$ T cell activation. The amount of TCR signaling is influenced by antigen dose, possibly by increasing the number of antigen-specific epitope-loaded MHC class II molecules on APCs[39]. Low antigen dose favors Th2 and Tfh differentiation, intermediate dose Th1 differentiation, and high dose Th2 differentiation[39]. Various cytokines involved in $CD4^+$ T cell differentiation can be produced by activated epithelial and innate immune cells, including APCs. Depending on signaling pathway of the activated PRRs on the involved APCs, specific T cell-differentiating cytokines may subsequently be produced, e.g. IL-12 (Figure 2). Binding of these differentiating cytokines to their cognate receptors on $CD4^+$ T cells, results in the activation of receptor associated Janus kinases that will activate signal transducer and activator of transcription (Stat) proteins[36]. These Stat proteins induce the expression of genes involved in the initial differentiation of each Th subset, including master transcription factors. Therefore, each Th subset can be defined by the expression of these Stat proteins and master transcription factors, namely Stat4/Stat1/Tbet (Th1), Stat5/Stat6/Gata3 (Th2), Stat3/Rorγt (Th17), Stat5/FoxP3 (Treg), and Stat3/Bcl6 (Tfh)[36, 40, 41] (Figure 2). These master transcription factors induce expression of the characteristic cytokines of each Th subset.

Co-culture of $CD4^+$ T cells with *B. pertussis*-incubated monocyte-derived DCs revealed the

induction of Th1 and Th17 cells[24]. Notably, since *B. pertussis* can survive both extracellular and intracellular in epithelial cells and macrophages[42-44], a mixed Th1 and Th17 response would be optimal for bacterial clearance, since these subsets are important in clearing intracellular and extracellular pathogens, respectively. In mice, *B. pertussis*-specific Th1 cells, producing IFNγ, were indeed identified as the Th subset that contributed to the protective immunity to *B. pertussis* infection[34, 45-47]. In addition, with the identification of IL-17A-producing Th17 cells, also their role in the protection against *B. pertussis* infection in mice became clear[47, 48]. The induction of Th1 and Th17 $CD4^+$ T cells after *B. pertussis* infection has also been shown in the baboon model[49] and in human[50, 51].

Shortcomings of aP vaccine-induced immune responses

Currently used aP vaccines do provide protection against severe disease, however, children that received aP vaccines during their primary series have an increased risk of pertussis as adolescents than those that received wP vaccines during their primary series[52-55]. This indicates that aP vaccines induce a less durable protective immune response compared to wP vaccines. Moreover, these aP vaccines have been shown in a baboon model not to protect against colonization and transmission of *B. pertussis*[56]. The lower effectiveness of aP vaccines may be due to the induction of a suboptimal immune response, for various reasons.

The currently used aP vaccines consist of one to five purified immunogenic *B. pertussis* antigens, making the vaccine more defined and less reactogenic than the wP vaccines[13]. However, as a consequence, the aP vaccine-induced immune response has a narrow specificity. This could lead to strain adaptation of *B. pertussis* due to vaccination pressure. Although variations in *B. pertussis* antigens have also been observed before the introduction of aP vaccines[57], there is evidence of ongoing variation in vaccine antigens in the aP vaccination era, as has been shown for Ptx, Prn, and Fim2/3[58-60]. Moreover, Prn-deficient strains have emerged following introduction of aP vaccines in Europe[61-64], Australia[65], Japan[66], and the USA[67-69]. Therefore, it is possible that the immune responses induced by the one to five antigens from the single isolate used to manufacture the aP vaccine do not optimally protect against at least some of the current circulating strains.

In addition, the aP vaccine-induced $CD4^+$ T cell response has a distinct functional programming compared to that induced by wP vaccination and *B. pertussis* infection. Vaccination with the aP vaccine leads to a Th2-dominated $CD4^+$ T cell response. However, this Th2 component appears to be redundant in the protective mechanisms to a *B. pertussis* challenge in mice[47]. wP vaccination and *B. pertussis* infection induce Th1 and Th17 cells and confer enhanced protective immunity compared to aP vaccination[34, 45-47, 56].

Finally, there is evidence that aP vaccine-induced $CD4^+$ T cell responses are more end-stage differentiated than infection- and wP vaccine-induced responses. The proportion of *B. pertussis*-specific terminally differentiated T cells was higher in aP- compared to wP-vaccinated children[70], while central memory T cells (T_{CM}) are preferred for long-term

immunity[71]. This effect may be attributed to the high antigen dose in the aP compared to wP vaccines, since high antigen and adjuvant dose induce vigorous short-term immunity, but can lead to exhaustion of the immune system and impede long-term immunity[39, 72, 73]. A detailed comparison of the literature describing the differences in immune responses induced by aP vaccination, wP vaccination, and infection is given in **Chapter 2**.

Strategies to improve pertussis vaccination

The lessons from studies on *B. pertussis*-specific T cells in mice, baboons, and humans have suggested that next generation pertussis vaccines should induce broad immune response, including $CD4^+$ T cells with a T_{CM} phenotype and a mixed Th1 and Th17 profile, like those induced by infection or immunization with wP vaccines. Different approaches to improve pertussis vaccination have been proposed and studied by various research groups.

One approach could be replacing or complementing alum, the adjuvant used in the current vaccine, with PAMPs known to induce Th1 or Th17 responses. Indeed, several studies using TLR2[23], TLR4[74], and TLR9[47, 75, 76] ligands show that these agonists can steer the aP vaccine-induced $CD4^+$ T cell response towards a more favorable Th1 and Th17 programming. Therefore, pertussis vaccine formulations containing experimentally selected adjuvants could be considered as more effective alternatives for current aP vaccines (**Chapter 2**).

Another promising third generation vaccine approach is the outer-membrane vesicle (OMV) pertussis vaccine concept. OMVs are commonly shed by Gram-negative bacteria, including *B. pertussis*, and these vesicle contain proteins and molecules present in the outer-membrane and periplasmic space[77, 78]. Moreover, cytoplasmic proteins of bacteria can be detected in their OMVs[78]. Consequently, vaccines based on OMVs have a broad antigen composition and can induce the favorable Th1 and Th17 response, due to the presence of PAMPs like the TLR4 ligating lipooligosaccharide. In mice, OMV vaccines evoke high serum IgG levels specific for a broad range of antigens[77]. Moreover, both intraperitoneal and intranasal administration of this type of vaccine provides protection against subsequent *B. pertussis* challenge in mice[79, 80].

To further improve pertussis vaccination, a change in administration route might be an option. *B. pertussis* is a respiratory pathogen and infection induces a mucosal immune response. This type of response is associated with the presence of IgA antibodies and tissue-resident T cells (T_{RM}) in the mucosa[81, 82]. Direct immunization at the respiratory mucosa might induce a comparable type of immune response as that induced by infection.

Notwithstanding the fact that the shortcomings of current aP vaccines are starting to become more understood, providing leads for new vaccine and vaccination concepts, there are still many gaps in our knowledge in the field of vaccination against pertussis that need to be addressed.

Thesis scope and outline

There is evidence that current aP vaccines are less effective in providing long-term protection against *B. pertussis* infection compared to previous infection and wP vaccines, indicating the need for improvement of pertussis vaccination. Induction of a suboptimal type of immune response for proving protection against pertussis by these vaccines may underlie this phenomenon. The main objective of this thesis is to determine characteristics of the optimal protective immune response against *B. pertussis* infection and to apply this knowledge to improve pertussis vaccination, with the main focus on $CD4^+$ T cell programming. In this thesis, several approaches to improve current pertussis vaccination are evaluated using innovative and systems based technology.

In **Chapter 2**, the immune responses induced by *B. pertussis* infection, wP vaccination, and aP vaccination are reviewed. Moreover, the shortcomings of the current aP vaccines are addressed. The lessons that can be learned from the immune responses induced by infection and wP vaccines in the development of third generation pertussis vaccines are highlighted in this review.

Chapter 3 describes the molecular signatures of the immune response after *B. pertussis* infection in mice. The sequence of molecular and cellular responses after intranasal *B. pertussis* infection was determined using a systems biology approach, which can provide a benchmark for optimal immunity since natural infection induce a more durable protective immune response than current vaccination.

In **Chapter 4**, a study investigating TLR2 and TLR4 signaling by clinical isolates of *B. pertussis* is described. The capacity of these strains to signal via TLR2 and/or TLR4 was evaluated using TLR2- or TLR4-expressing cell lines. Moreover, induction of human monocyte-derived DC maturation by these strains was the determined.

In **Chapter 5**, the effect of addition of a TLR4 ligand to an aP vaccine on the $CD4^+$ T cell response is evaluated. In this study, we determined whether the aP vaccine-induced Th2 dominated response in mice could be steered towards the favorable Th1/Th17 response by adding TLR4 ligand LpxL1, a meningococcal LPS derivative, to the vaccine. Moreover, the effect of the additional adjuvant on the memory phenotype of the specific $CD4^+$ T cells was determined using a newly developed MHC class II tetramer.

Chapter 6 presents a study that unraveled the molecular mechanism underlying the skewing of the aP vaccine-induced $CD4^+$ T cell response towards a Th1 and Th17 response by the addition of the TLR4 ligand LpxL1 to the aP vaccine in mice. The transcriptomes of the aP vaccine antigen-specific $CD4^+$ T cells was determined using microarray analysis.

In **Chapter 7**, we described a study investigating the immune response at the molecular and systems level after immunization with a new candidate vaccine, namely a *B. pertussis* OMV vaccine. In addition, mice were immunized pulmonary or subcutaneously to determine the effect of immunization route on the OMV vaccine-induced protection against *B. pertussis* challenge and OMV vaccine-specific mucosal and systemic immune responses.

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Chapter 2

Roads to the development of improved pertussis vaccines paved by immunology

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Abstract

Current acellular pertussis vaccines have various shortcomings, which may contribute to their suboptimal efficacy and waning immunity in vaccinated populations. This calls for the development of new pertussis vaccines capable of inducing long-lived protective immunity. Immunization with whole-cell pertussis vaccines and natural infection with *Bordetella pertussis* induce distinct and more protective immune responses when compared with immunization with acellular pertussis vaccines. Therefore, the immune responses induced with whole-cell vaccine or after infection can be used as a benchmark for the development of third generation vaccines against pertussis. Here, we review the literature on the immunology of *B. pertussis* infection and vaccination and discuss the lessons learned that will help in the design of improved pertussis vaccines.

Introduction

Bordetella pertussis is a respiratory tract pathogen that causes the severe disease whooping cough (pertussis) in infants and young children, yet it can also infect adolescents and adults. The disease was largely controlled in developed countries following the introduction of whole-cell pertussis (wP) vaccines in the 1940s/1950s. However, pertussis still kills over 200,000 infants yearly worldwide, mostly in developing countries, but the incidence of disease is also increasing in many developed countries[1]. This has been attributed to the introduction of acellular pertussis (aP) vaccines in the 1990s, which were developed in response to concerns around the safety of wP vaccines. Unlike wP vaccines, which are composed of killed bacteria with a wide range of antigens and pathogen-associated molecular patterns (PAMPs) that bind to pattern-recognition receptors (PRRs) and activate innate immune cells, aP vaccines are composed of three to five *B. pertussis* antigens absorbed to alum as the adjuvant. A monocomponent vaccine based on hydrogen peroxide-detoxified pertussis toxin (Ptx) was also developed[2], but had lower efficacy than the three and five component aP vaccines. Although initial reports from phase 3 clinical trials carried out in the 1990s suggested that aP vaccines were at least as effective as wP vaccines[3, 4], recent studies have demonstrated a rapid and alarming drop in protection over time, suggesting a failure to sustain immunity[5].

There have been a number of explanations put forward to account for recent epidemics of pertussis in highly vaccinated populations. First, there is evidence of antigen variation in some of the key protective antigens and components of aP vaccines, namely pertussis toxin (Ptx), pertactin (Prn), and fimbriae type 2 and 3 (Fim2/3)[6-8]. Moreover, Prn-deficient strains have emerged following introduction of aP vaccination in Europe[9-12], Australia[13], Japan[14] and the USA[15-17]. Therefore, it is possible that the immune responses induced by three to five antigens from the single isolate used to manufacture the aP vaccine do not protect against at least some of the current circulating strains. Secondly, the waning protective immunity observed following immunization with aP vaccines suggests that these vaccines fail to generate effective immunological memory[5, 18-21].

Finally, there is convincing evidence that the aP vaccines do not induce the optimum profile of immune responses required for protection against infection with *B. pertussis* and that these differ significantly from those induced by natural infection or immunization with wP vaccines. The consensus view from studies in the mouse model is that wP vaccines and previous infection confer better protective immunity than aP vaccines because they induce Th1 cells and associated opsonizing antibodies, with a minor contribution by Th17 cells (Table 1). In contrast, the less effective aP vaccines induce a mixed Th2 and Th17 response[22], but the Th2 component appears to be redundant to protection and, together with associated IgE antibodies[23], may even be the culprits in rare type hypersensitivity reactions seen in children after a fourth or fifth dose of the aP vaccines[24]. This pattern of immune responses induced by the pertussis vaccines is generally similar in humans and mice. Recent evidence from the new animal model of experimental pertussis in baboons

has uncovered a major deficit in protective immunity induced by aP vaccination. While the immune responses induced by immunization with wP vaccines were able to prevent disease and enhanced clearance of the infection (though not as effectively as previous infection), immunization with the aP vaccine prevented disease, but did not prevent infection or transmission of *B. pertussis* to naive baboons[25]. Whilst it has not been conclusively proven in the baboon, it appears that the failure of aP vaccines to prevent infection reflects its failure to induce appropriate cellular immune responses, especially Th1 cells. In this minireview, immunological evidence from infection and vaccination studies will be discussed in order to guide the development of improved pertussis vaccines.

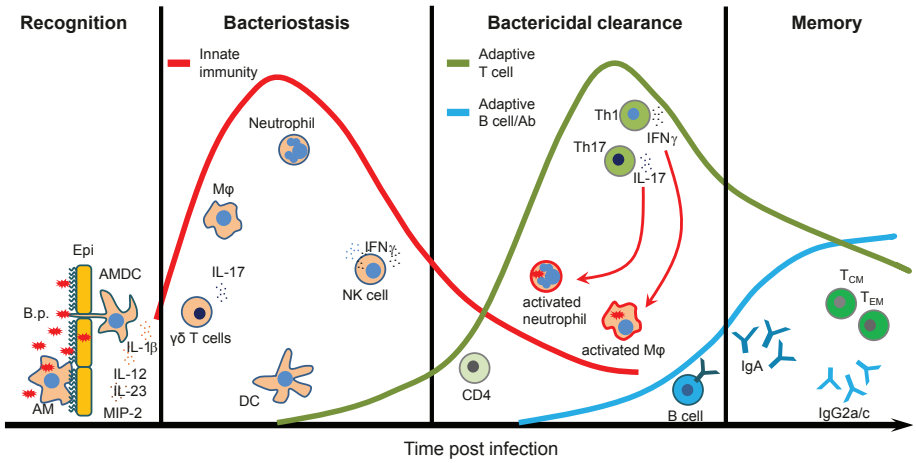

Figure 1 - Relative contribution of the cell subtypes to the induction of immune response to *B. pertussis*. The immune response to *B. pertussis* is a complex process that involves activation and recruitment of immune cells to the respiratory tract and generation of long-lasting adaptive immunity. Attachment of *B. pertussis* to ciliated epithelial cells and recognition by AMDCs and AMs provide a first line of immediate defense against *B. pertussis* infection. Secretion of cytokines and chemokines promotes recruitment of innate immune cells. Innate IL-17 together with CXCL2 (or MIP-2) secreted by activated macrophages and epithelial cells promote neutrophils recruitment. NK cells play a protective role through the secretion of IFNγ, which enhances the antimicrobial activity of macrophages as well as induces Th1 cells. Activated neutrophils and macrophages participate in an antibody-mediated phagocytosis and intracellular killing of *B. pertussis*. DCs migrate to the lymph nodes to present the antigen to the naive T cells. Primed T cells proliferate and differentiate into Th1 and Th17 cells that migrate to the lungs to further activate neutrophils and macrophages by production of IFNγ and IL-17, respectively. Activated B cells differentiate into plasma cells that produce *B. pertussis*-specific IgA or IgG2a/c antibodies (mouse). Finally, a small fraction of T and B cells become memory cells providing an effective protection after reinfection. B.p., *B. pertussis*; Epi, epithelium; AMs, alveolar macrophages; AMDCs, airway mucosal dendritic cells; DCs, dendritic cells; Mφ, macrophages; Neu, neutrophils; Th, T helper cells; T_{CM}, central memory T cells; T_{EM}, effector memory T cells.

Current state of the art on the mechanisms of natural immunity to *B. pertussis*

The innate immune response plays a crucial role in controlling the initial stages of *B. pertussis* infection and helps to shape the subsequent adaptive immune responses. *B. pertussis* attaches to ciliated epithelial cells on the upper respiratory tract[26, 27]. These cells may also provide a first barrier, by preventing pathogen penetration and through production of mucine by secretory goblet cells which can protect the lungs from pathogens that are inhaled into the respiratory track. Two resident innate immune cell types, airway mucosal dendritic cells (AMDCs) and alveolar macrophages (AMs), which are in close contact with the lung epithelium, mediate the first cellular response to *B. pertussis*. AMDCs are strategically positioned between epithelial cells to take up antigen directly from the airway lumen and prime T cells after migration to the lymph node[28, 29], whereas AMs reside within the mucous layer and appear to be important for phagocytosis and killing of *B. pertussis*[30]. These lung-resident innate immune cells provide a first line of immediate immune defense against *B. pertussis* infection. Additionally, they initiate and orchestrate complex and tightly regulated processes that involve activation and recruitment of other immune cells and generation of long-lasting adaptive immunity (Figure 1 and Table 1).

B. pertussis was classically considered to be an extracellular pathogen that infects the upper respiratory tract, but it can also penetrate the lungs and has been found inside ciliated respiratory epithelial cells and in lung macrophages[31-33]. The recognition of *B. pertussis* PAMPs and virulence factors including lipooligosaccharide (LOS), adenylate cyclase toxin (ACT), filamentous hemagglutinin (FHA), and TLR2 lipoproteins by PPRs expressed by macrophages and DCs (and other cells of the innate immune system) leads to their maturation and production of cytokines and chemokines that mediate and regulate immune responses to the bacteria. It was also shown that Ptx can act as PAMP, triggering the TLR4 signaling pathway[34, 35] and enhancing IL-12 production, thereby acting as an adjuvant to promote Th1 responses[36-38]. Binding of LOS to TLR4 results in DC maturation and production of IL-12 and IFNγ, cytokines required for development of Th1 responses[39]. TLR4 activation on DCs by LOS can also induce IL-10 production, consequently generating IL-10-secreting type-1 regulatory T cells (Tr1) that may dampen Th1 responses and limit inflammatory pathology in *B. pertussis*-infected lungs[39]. DCs can also be activated by intrinsic recognition of other *B. pertussis* virulence factors and toxins. It was shown that ACT activates the NLRP3 inflammasome and caspase-1, leading to production of mature bioactive IL-1β. Active IL-1β together with IL-23 promotes expansion of murine Th17 cells, which help to recruit neutrophils that promote killing of *B. pertussis*[40]. Furthermore, studies with human DCs have demonstrated that ACT enhances Th17 responses but suppresses Th1 responses by inhibiting IL-12p70 production[41]. ACT also modulates TLR-induced signaling to upregulate IL-10 production and promotes the development of Treg cells[42].

It was shown that TLR4-deficient C3H/HeJ mice had a more severe course of *B. pertussis* infection when compared with TLR4-sufficient C3H/HeN mice[43-45]. TLR4 expressed by DCs

and macrophages plays a central role in protective immunity to this pathogen. Furthermore, an early burst of pro-inflammatory cytokines and chemokines produced by AMs soon after infection depends on TLR4 signaling pathway. Mice that lack MyD88 adaptor-like protein (Mal; also known as TIRAP) were unable to control infection[46]. The bacteria disseminated from the lungs causing lethality in nearly 50% of $MAL^{-/-}$ mice. Additionally, AMs were completely depleted from the lungs of $Mal^{-/-}$ mice early after infection, suggesting that these cells are critical for mediating protection to *B. pertussis*[46].

The initial recruitment of DCs and macrophages into the lungs of *B. pertussis*-infected mice is followed by recruitment or expansion of γδ T cells and infiltration of neutrophils and NK cells. It is thought that γδ T cells orchestrate the early cell trafficking into the lungs by producing IL-17[47]. This cytokine together with CXCL2 (MIP-2) secreted by activated macrophages and epithelial cells is required for neutrophil recruitment as well as induction of Th1 and Th17 responses to *B. pertussis*.

A primary role of neutrophils during the microbial infection is antibody-mediated phagocytosis and intracellular killing of pathogenic bacteria and formation of neutrophil extracellular traps[48, 49]. It was shown that ACT can inhibit neutrophil function but does not impact on protective immunity to *B. pertussis*. It was suggested that neutrophils are important, but not essential, in the controlling of bacteriostasis of *B. pertussis*, but are critical for clearance of *B. bronchiseptica*; infection of neutrophil-deficient mice with *B. bronchiseptica* had a lethal outcome[50]. It was proposed that *B. bronchiseptica* can kill resident and recruited phagocytes in the lungs or that AMs are sufficient to control *B. pertussis* infection[50].

NK cells play a protective role in immunity to *B. pertussis* through secretion of IFNγ, which enhances the antimicrobial activity of macrophages. Production of IFNγ by NK cells is dependent on IL-12 from *B. pertussis*-activated DCs. Depletion of NK cells from *B. pertussis*-infected mice resulted in the dissemination of the bacteria from the respiratory tract to the liver and this was associated with reduced IFNγ production and Th1 responses[51]. Thus, NK cells play a critical role in early innate immune control of infection and in shaping the adaptive immune response to *B. pertussis* (Figure 1).

The lungs of naive mice contain very low numbers of T cells. However, during the course of infection with *B. pertussis*, the adaptive immune response slowly develops and T cells are recruited to or expanded in the respiratory tract and mediate subsequent bacterial clearance of *B. pertussis* from the respiratory tract. Human infants without fully developed adaptive immunity are particularly susceptible to the severe symptoms of whooping cough, which can be lethal. Studies in mice have demonstrated that $CD4^+$ T cells play a key role in the clearance of *B. pertussis* infection. Primary infection of athymic *nu/nu* mice, which lack all T cells, results in a persistent or even lethal infection with *B. pertussis*[52]. Adoptive transfer of immune splenic T cells into athymic mice reversed chronic infection and resulted in bacterial clearance within 14 to 21 days post challenge. Similarly, transfer

of *B. pertussis*-specific $CD4^+$ T cells into immunosuppressed sublethally irradiated naive BALB/c mice before infection resulted in clearance of the pathogen. In contrast, sublethally irradiated mice were not able to clear primary infection after transfer of *B. pertussis*-specific $CD8^+$ cells[52]. In addition, antigen-specific $CD4^+$ T cells from infected mice produce IFNγ and/or IL-17, but not Th2 type cytokines, suggesting that Th1 and Th17 cells are involved in protective immunity to *B. pertussis* infection. Indeed, $IFN\gamma^{-/-}$ mice exhibit impaired ability to clear the pathogen from the lungs[53], whereas $IFN\gamma R^{-/-}$ mice develop a lethal disseminated infection. Interestingly, $IFN\gamma R^{-/-}$ mice that survive the initial infection develop neither Th1 nor Th2 response, suggesting that Th17 cells might be involved in developing protective immunity to *B. pertussis*[54]. Indeed, it has been demonstrated that $IL\text{-}17^{-/-}$ mice infected with *B. pertussis* have a significantly greater bacterial load in comparison to wildtype (WT) mice[22]. This impaired ability of $IL\text{-}17^{-/-}$ mice to clear the bacteria correlated with a significant decreased frequency of neutrophils in the lungs during the course on infection[22]. Taken together, the published studies demonstrate that Th1 and Th17 cells both contribute to the protective immunity induced by primary *B. pertussis* infection in mice (Figure 1).

Humoral immunity may play an auxiliary role in the clearance of infection from the lungs and contribute to a development of the long-lasting, cell-mediated immunity to *B. pertussis*. The number of B cells significantly increases in the lungs during the course of infection reaching the highest number when the infection is almost cleared. Similarly, *B. pertussis*-specific IgG antibodies are only detectable in the serum at significant levels when the pathogen is almost cleared from the lungs[52, 55]. However, IgA antibodies were detected in the lungs 2 weeks after challenge[52]. This suggests that IgG antibodies do not play a major role in the clearance of a primary natural infection, but may be more important in adaptive immunity induced by previous infection or vaccination. Leef *et al.* showed that B cells may contribute to the protective immunity by different mechanisms than specific antibody production[56]. Although, *B. pertussis*-infected $Ig^{-/-}$ mice developed a chronic infection and could not clear the infection, these mice also lack mature B cells and fail to generate an effective T cell response to *B. pertussis*[54]. It is also possible that B cells may contribute to the induction of memory $CD4^+$ T cells by acting as the antigen-presenting cells[56, 57].

Convalescent mice are able to clear the pathogen from the lungs as soon as 3-7 days after reinfection[52]. Baboons recovered from a *B. pertussis* infection are protected from disease and do not transmit the pathogen upon subsequent challenge and this was associated with a long-lived mixed Th1/Th17cell response[25, 58]. Hence, natural infection induces strong and persistent immune protection against reinfection with *B. pertussis*, although it does not result in life-long immunity.

Table 1 - The putative roles of different immune cells in protective immunity to *B. pertussis*

Cell type	Infection[1]	wP	aP	Species	Function	Reference
Th1 cell	+++	++	+/-	Man	IFNγ production, macrophage activation, opsonizing Ab, prevents dissemination of B.p.	66-70, 114
	+++	+	+	Baboon		58
	+++	++	+/-	Mouse		22, 52, 64, 108
Th2 cell	+/-	+/-	+++	Man	No identified role in mice; unknown role in baboons and humans	66-70, 114 van Twillert, unpublished
	-	-	+++	Baboon		58
	-	-	+++	Mouse		22, 52, 64, 108
Th17 cell	+++	++	+/-	Man	IL-17 production, neutrophil recruitment and activation	69 van Twillert, unpublished
	+++	++	-	Baboon		25
	+++	++	+/-	Mouse		22, 64, 108
T_{RM} cell	+++	++	-	Mouse	Sustains local cellular immunity in the respiratory tract?	Wilk and Mills, unpublished
Tfh cell	+++	++	+	Mouse	Activates Ab production and memory B cells?	Wilk, Allen, and Mills, unpublished
T_{CM} cell	?	+	+	Man & Mouse	Maintains long-term immunity	64, 74
T_{EM} cell	?	+	++	Man & Mouse	Immediate effector function	64, 74
T_{TD} cell	?	+	++	Man	Waning immunity?	74
γδ T cell	++	?	?	Mouse	Immune regulation early in infection	47
B cell	+	++	+++	Mouse	Ab production	54, 56, 62
AM	+++	+	-	Mouse	Early response to infection, phagocytosis and killing of B.p.	46
DC	+++	?	?	Mouse	Activation of naive T cells in lymph nodes	115
NK cell	+++	?	?	Mouse	Early IFNγ, prevents dissemination of B.p.	51
Neutro	+++	++	-	Mouse	Ab-dependant phagocytosis and killing of opsonized B.p.	22, 48, 49

[1]Responses denoted as +++, ++, +, +/-, -, and ? equate to strong, medium, weak, weak/inconsistent, undetectable responses or not tested respectively; B.p., *B. pertussis*; Ab, antibody; Th, T helper cells; T_{RM}, tissue-resident memory T cell; Tfh, follicular T helper cell; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{TD}, terminally differentiated T cell; AM, alveolar macrophage; DC, dendritic cell; Neutro, neutrophil

Mechanisms of vaccine-induced immunity and limitations of current aP vaccines

Due to their high reactogenicity, wP vaccines were replaced in the 1990s in most developed countries by aP vaccines containing a high dose of three to five immunogenic *B. pertussis* antigens, namely Ptx, Prn, FHA, Fim2/3. Both vaccine types provide protection against severe disease, however, with important underlying differences. aP vaccine-induced protective immune responses are less durable, and in contrast to wP vaccine-induced immunity only protect against clinical symptoms of pertussis but not against colonization and transmission of *B. pertussis*[25].

Besides its narrow specificity, various other features of aP vaccine-induced immunity may account for these differences. First, aP vaccine-induced T cell responses have a distinct functional programming, compared to those induced by wP vaccines, as evidenced by both humoral and cell-mediated responses. While the two vaccine types readily induce significant levels of *B. pertussis* antigen-specific IgG antibodies, as reported in humans[59, 60] and in mice[61-63], there is a difference in class switching patterns. In mice, aP vaccines induce predominantly antibodies of the IgG1 subclass[62, 64], reflecting the induction of a Th2 response, whereas wP vaccines induce predominantly IgG2a/b, as well as IgG1, and IgG3[63], which is consistent with the strong induction of Th1 cells. A comparison of the antibody isotype in cohorts of children that had been primed with either wP or aP vaccines, and followed between 3 and 9 years of age, showed that IgG1 was the predominant IgG subclass induced by both vaccines[60]. In humans, however, this complement activating and opsonizing IgG subclass is thought to be driven by Th1 cells[65]. Only low levels of the IgG2 subclass were observed in both wP- and aP-primed children[60]. The Th2-associated *B. pertussis*-specific IgG4 subclass was detected only after aP vaccination, with higher concentration after a greater number of doses[60]. Moreover, these IgG4 antibodies strongly correlate with the total IgE production in these aP-vaccinated children[60], indicating that Th2-skewed immune responses are induced by aP vaccination in humans as well as in mice.

Further evidence for different programming of immune responses induced with aP and wP vaccines comes from studies focused on $CD4^+$ T cell responses and assessing the importance of their secreted cytokine profiles in the context of protective immunity in different species (Table 1). In mice, aP vaccination is associated with induction of $CD4^+$ T cells that produce IL-4, IL-5 and IL-17, but relatively lower concentrations of IFNγ[22, 64], representing a mixed Th2/Th17 response, while wP vaccines induce a mixed IFNγ/IL17A (Th1/Th17) response[22]. Studies using cytokine-defective mice have shown that protection against *B. pertussis* induced by immunization with an aP vaccine is as effective in IL-$4^{-/-}$ or IFNγ$^{-/-}$ mice as in WT mice, whereas protection is significantly diminished in IL-$17A^{-/-}$ mice[22]. This indicates that Th17 cells play an essential role in the protective immune response induced by the aP vaccine, while the Th2 cells do not contribute to protection. In contrast, Th1 are required for protection induced with wP vaccines[22].

In the baboon model, immunization with an aP vaccine, which conferred protection against disease but not infection or transmission, induces a mixed Th1 and Th2 response. In contrast, wP vaccination, providing protection against colonization and host-to-host transmission, induced a mixed Th1/Th17 memory response[25], suggesting that both Th1 and Th17 cells may be important in prevention of *B. pertussi*s colonization in this model.

In humans, aP vaccination has been shown to induce a Th2-dominated, yet mixed Th2/Th1/Th17 response in children[23, 66-69]. In contrast, wP vaccines induce a mostly mixed Th1/Th17 response, similar to natural infection[22, 66, 68, 70-72]. Notably, some of the Th1 responses in certain aP-vaccinated children may be attributed to exposure to or subclinical infections with *B. pertussis*[66, 67, 69, 70]. In general, the studies in humans are consistent with the data from animal models, i.e. indicating Th2 dominance associated with aP vaccination and a mixed Th1/Th17 profile after wP vaccination, implying that aP vaccines may have a poorly protective functional T cell profile in humans.

A second feature of the immune response that might contribute to the difference in long-term effectiveness of aP and wP vaccines has recently emerged from the series of head-to-head comparisons of the cellular immunity in aP- and wP-primed cohorts of children by Buisman and coworkers. It was found that PBMCs from aP-primed 4-year-old children, three years after their primary pertussis vaccine series, produced higher specific levels of $CD4^+$ T cell cytokines than those from wP-primed children; however, this response was not boosted after a fifth dose of aP vaccine, in contrast to the response in wP-primed children[69]. Yet, at the age of 6, two years after this booster dose, PBMCs from aP-primed children produced lower levels *B. pertussis*-specific IL-17 when compared with PBMCs from wP-primed children[73]. Furthermore, assessment of CCR7 and CD45RA expression, which allowed $CD4^+$ T cells to be categorized as naive T cells (T_N), effector memory T cells (T_{EM}), central memory T cells (T_{CM}) or terminally differentiated T cells (T_{TD}), showed that the proportion of *B. pertussis* antigen-specific T_{TD} cells was higher in the aP-primed cohort[74]. Together, these data suggest that aP-primed $CD4^+$ T cell responses might be more end-stage differentiated than those induced by wP vaccines. The high antigen dose in aP vaccines compared to wP vaccines may contribute to this phenomenon. High antigen and adjuvant dose may induce vigorous short-term immunity, but can also exhaust the immune system and impede long-term immunity[75-78]. For example, in the Leishmania model, high dose vaccination with a Leishmania protein-expressing adenoviral vector in mice elicited higher numbers of total IFNγ-producing $CD4^+$ T cells compared to low dose vaccination; however, high dose vaccination resulted in poor protective immunity[75].

Thus, aP vaccine-induced immunity has various shortcomings, including immune responses of narrow specificity and with the wrong type of Th subsets and end-stage differentiated memory T cells which may collectively contribute to its suboptimal efficacy.

Approaches to improve efficacy of current aP vaccination by TLR ligation

The lesson from studies on T cells in mouse models and humans to date have suggested that next generation aP vaccines should induce $CD4^+$ T cells with a T_{CM} cell phenotype a mixed Th1/Th17 profile, like those induced by infection or immunization with wP vaccine. An important factor in natural infection with *B. pertussis* and immunization with wP vaccines that steers protective adaptive immune responses is the presence of PAMPs, including LOS that binds to TLR4 and lipoproteins that bind to TLR2[79]. These PAMPs activate innate cells via PRRs and thereby promote DC maturation and production of pro-inflammatory cytokines that direct the induction of Th1 and Th17 cells[80]. Current aP vaccines do not contain classic PAMPs. Although active Ptx has adjuvant properties and might be considered to be a PAMP, the Ptx preparations in aP vaccines are aldehyde treated which destroys immunostimulatory activity[36]. Overall, Ptx-containing aP vaccine induces a Th2-dominated response, likely due to the Th2-promoting properties of the adjuvant alum. One approach to enhance the efficacy of aP vaccines could be to address this shortfall by replacing or complementing alum with PAMPs, which are known to induce Th1 responses. Indeed, there is evidence from mouse studies that several TLR agonists may be effective Th1 promoting adjuvants for experimental aP vaccines.

A natural TLR2 ligand of *B. pertussis*, BP1569, was recently identified[79]. Immunization of mice with an experimental aP vaccine containing a corresponding synthetic lipopeptide LP1569 induced a higher level of protection against aerosol *B. pertussis* challenge when compared with the same vaccine formulated with alum. Furthermore, when compared with an alum-adjuvanted vaccine, the same vaccine formulated with the TLR2 lipoprotein was found to induce significantly higher IgG2a antibodies and promote higher IL-17 and IFNγ, and lower IL-5 production by spleen cells after antigen stimulation *ex vivo*.

The TLR4 agonist LOS is an important PAMP in wP vaccines enhancing Th1 responsiveness, but was also the main mediator of their high reactogenicity[81]. Although mice are less sensitive to endotoxin than humans[82], LOS from *B. pertussis* is less effective in activating human macrophages and DCs than LPS from other bacteria[80]. Therefore, well-defined detoxified LPS derivatives from other Gram-negative bacteria with Th1-promoting capacities have been tested as adjuvant for an experimental aP vaccine. Immunization of mice with an aP vaccine adjuvanted with monophosphoryl lipid A (MPL), the hydrophobic biologically active lipid A part of *Salmonella minnesota* LPS, was more effective in protecting against *B. pertussis* challenge than the aP vaccine adjuvanted with alum[83]. Furthermore, *B. pertussis*-specific IL-5 production by spleen cells was lower following immunization with MPL-adjuvanted vaccine, indicating that this adjuvant can skew the immune response away from a Th2 response. Substantiating these data, the authors recently found, at the single T cell level, that addition of the water-soluble genetically engineered derivative from *Neisseria meningitidis* LPS, LpxL1[84], to the aP vaccine increased the percentage of specific IFNγ and IL-17-producing $CD4^+$ T cells and diminished the percentage of IL-5-producing $CD4^+$ T cells[64]. Combining direct MHC class II tetramer staining of Prn-specific $CD4^+$ T cells

with the memory markers CD44 and CD62L in this model indicated that the ratio of T_{CM} and T_{EM} was not altered by addition of the adjuvant; however, there was a net benefit in the number of T_{CM}, since higher numbers of *B. pertussis*-specific memory $CD4^+$ T cells were detected after vaccination with the LpxL1-containing aP vaccine[64]. Hence, this analysis confirmed that there was no trade-off for the altered cytokine profile after TLR4 engaging aP vaccination by a reduction of induced T_{CM} cells. Since there are species differences in specificity of ligands for TLR4[85, 86], caution should be exercised in translating studies on LPS-based adjuvants from mice to humans.

TLR9 agonists, including CpG oligonucleotides from bacterial DNA, can also induce Th1 and Th17 responses and have been used as adjuvants for experimental aP vaccines tested in mice. The complementation or replacement of alum in an aP vaccine by CpG increased the anti-PtxIgG2a titers, and the IgG2a/IgG1 ratio, providing indirect evidence for induction of Th1 responses[87]. In addition, *B. pertussis*-specific IFNγ production by spleen cells was found to increase when Ptx, FHA and Prn were administered intranasally with both CpG and alum, but not with CpG alone[88]. Finally, in another report substituting alum with CpG in an experimental parenterally delivered aP vaccine enhanced its efficacy against aerosol challenge with *B. pertussis* and was associated with stronger *B. pertussis*-specific IFNγ and IL-17, but lower IL-4 and IL-13 production by spleen cells, and higher IgG2a concentrations in sera[22]. The failure of the study by Asokanathan *et al.* to detect IFNγ following immunization with pertussis antigens and CpG (without alum) may be due to the route of immunization (i.n.) and the lower dose of CpG (30 μg) employed[88]. Together, these studies demonstrate that TLR agonists such as TLR2, TLR4 and TLR9 ligands can steer the aP vaccine-induced $CD4^+$ T cell response towards a more favorable protective Th1/Th17 response, and that pertussis vaccine formulations containing experimentally selected adjuvants could be considered as more effective alternatives for current aP vaccines. In addition to the type of adjuvant, the antigen composition and dose of both adjuvant and antigen may influence the induction of T cell response with aP vaccines. Therefore, careful evaluation of choice of adjuvant and antigen and their dose will be required to ensure that a vaccine induces the appropriate functional Th subset and profile of memory $CD4^+$ T cells required for complete and sustained protective immunity against infection (Figure 2).

Several pertussis vaccine candidates in development address the absence of functional PRR triggering by current aP vaccines by expressing endogenous PAMPs. BPZE1, a live-attenuated *B. pertussis* vaccine candidate has been shown to induce FHA- and Ptx-specific Th1 responses in immunized neonatal mice and confer protection against challenge with virulent *B. pertussis*[89]. Furthermore, BPZE1 has been shown to induce maturation of human DCs and subsequent induction of IFNγ- and IL-17-producing T cells, indicating that BPZE1 should be capable of inducing the favorable Th1/Th17 response in humans[90]. This vaccine is currently being tested in the baboon model and is in clinical development[91]. Another vaccine concept based on *B. pertussis* outer-membrane vesicles also has been shown to induce a mixed Th1/Th17 response in mice and to induce protection against *B. pertussis* challenge[92] [Raeven and Brummelman *et al.* manuscript in preparation].

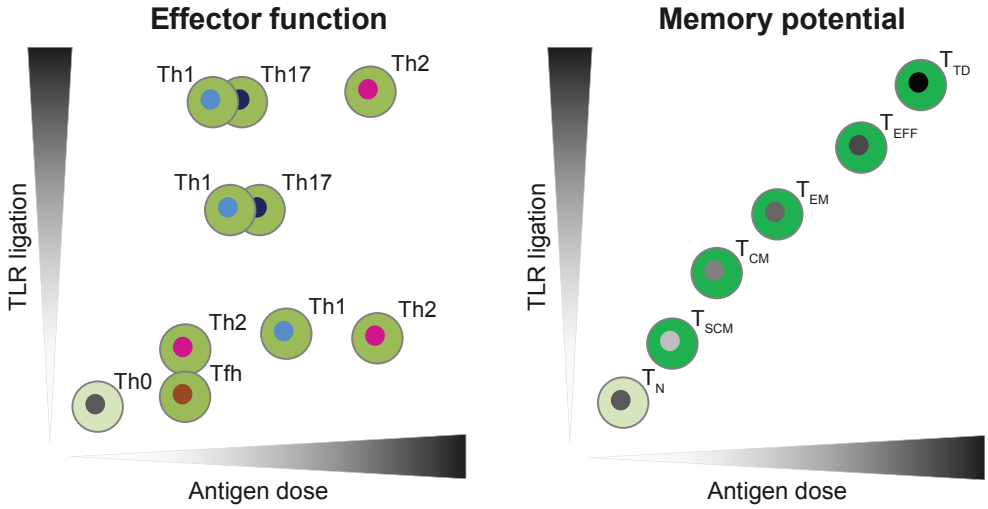

Figure 2 - Models for the influence of antigen dose and the strength of TLR ligation on CD4⁺ T cell differentiation. Vaccine-induced CD4⁺ T cell responses can be evaluated based on effector function i.e. Th type (left panel) and memory potential i.e. memory differentiation stage (right panel). Antigen dose can affect TCR signal strength, thereby influencing Th type and memory T cell differentiation[77, 78]. Inflammatory signals, such as induced by TLR ligation, during T cell priming can also influence the Th type and memory potential of antigen-specific T cells via innate mechanisms[76, 78]. The x-axes depict the range of antigen dose and the y-axes show the amount of TLR ligation present during vaccination or infection. *In vivo*, antigen and TLR ligand dose may form a gradient of local concentrations, possibly inducing an array of differentiation states. In the presence of low dose TLR ligands, a low antigen dose will steer towards Th2 or Tfh cells, while an intermediate dose promotes Th1 cells, and a high dose Th2 cells. Increasing TLR ligation induces Th17 and Th1 cells, yet high exposure to both TLR ligation as antigen dose will promote Th2 cells[78]. In parallel, both low and high antigen dose and TLR ligation strength may also induce regulatory functions in cell populations (not shown). The memory differentiation stage is correlated with both the antigen dose and TLR ligation, meaning that the memory potential of the cells decreases when the cells are exposed to increasing TLR ligation and antigen dose[76, 77]. The color gradient of memory phenotypes from light to dark indicates loss of long-term memory potential. TLR, Toll-like receptor; T_N, naive T cell; T_{SCM}, stem-cell memory T cell; T_{CM}, central memory T cell; T_{EM}, effector memory T cell; T_{EFF}, effector T cell; T_{TD}, terminally differentiated T cell.

These vaccine candidates also address other limitations of aP vaccines, by expressing 'natural' (low) doses antigen (preventing an exhausted immune response) and a broader panel of antigens, important to ensure maintenance of long-lasting protective immunity against circulating *B. pertussis* strains with altered antigenic make up.

Immunological evaluation of new pertussis vaccines in (pre-)clinical development

The development of new pertussis vaccines requires immunological tools to determine the functional differentiation, memory potential, breadth, and protective capacity of the induced immune responses in reliable preclinical animal models, important to predict efficacy in humans. Mouse and more recently baboon models have been used to evaluate candidate

pertussis vaccines. One of the problems with these models is that the level of protection is usually assessed shortly after vaccination[93]. Furthermore, mice are not the natural host of *B. pertussis* and relatively high vaccine doses are often used in these challenge models to induce measureable immune responses; thus, the results may be difficult to translate to the humans. Future studies in these models should address the issue of waning immunity by assessing the long-term protection of experimental aP vaccines, and the protection induced by lower doses. While it is possible to do medium-term (6-12 month) studies in the mouse model, studies of greater than 1 year will require experiments in the recently developed baboon model. Warfel and Merkel have used species-specific tools to assess waning of the vaccine induced $CD4^+$ T cell response up to 15-24 month after *B. pertussis* infection in baboons[58]. However, cost and animal availability will be significant limitations to extensive use of this model. In both mice and baboons, identifying early biomarkers that predict long-term immunity would be valuable.

One such biomarker of long-term immunity in pre-clinical and clinical vaccination settings is the memory phenotype of the induced specific $CD4^+$ T cells, since T_{CM} are important for long-term immunity[94-97] (Figure 2). Furthermore, there is emerging evidence from other infectious diseases that follicular helper T (Tfh) cells[98, 99] and tissue-resident memory (T_{RM})[100, 101] may be key to long-term immunity and these have yet to be addressed in the field of pertussis vaccines. Memory phenotypes can be determined in the early stage after vaccination. Recently, we showed that MHC class II-tetramers specific for the immunodominant Prn epitope can be used to evaluate the $CD4^+$ T cell memory phenotype on a single cell base in both mouse and humans[64, 102]. The knowledge on human pertussis MHC class II T cell epitopes is increasing by studies using prediction methods, and advanced immunoproteomics analysis[103, 104], however, is still very limited[105-107]. However, routine assessment of memory T cells induced by pertussis vaccine will require optimization and standardization of techniques. The availability of MHC class II tetramer with peptides from *B. pertussis* antigens together with single cell analysis using intracellular cytokine staining is one approach that may facilitate accurate quantification of *B. pertussis*-specific memory T cells induced with pertussis vaccines.

Furthermore, systems biology approaches could aid in identifying novel early biomarkers that predict the outcome of the adaptive immune response. Using this approach, a recent study revealed molecular and cellular immunological processes in response to *B. pertussis* infection and may provide guidance in evaluating new candidate vaccines[108]. State-of-the art tools, such as MHC class II tetramers and systems biology, are needed for mouse, baboon and humans to evaluate the quality of the immune response induced by current and potential new pertussis vaccines.

Conclusions and future perspectives

The potential shortcomings of current aP vaccines are still a subject of debate. While the switch from wP to less effective aP vaccines is one explanation for the resurgence of pertussis, it has also been argued that recent epidemics may be part of natural cycles of disease that have always been with us, even in the wP vaccine era[109]. Nevertheless, the fact remains that the incidence of pertussis is higher than of any other vaccine-preventable infectious disease. There is now growing acceptance that we do have a problem and must come up with short- and long-term solutions. While a rationally designed third generation pertussis vaccine would appear to be the ideal solution to improve the aP vaccine in use today, the development and especially the clinical evaluation, regulation, licensing and implementation of new aP vaccines into pediatric immunization programs will face many logistic as well as scientific hurdles.

In the interim, there are a number of short-term measures that are being introduced or considered. These include (1) maternal immunization to protect disease in the newborn by passive transfer of antibody, which has proved very effective in prevent infant death in certain countries (e.g. UK); (2) additional booster vaccination with current aP vaccines in adolescents, to tackle the problem of waning immunity; (3) immunization of adults with a vaccine that prevented infection as well as diseases to reduce the carriage and spread of the bacteria; and (4) cocooning strategies to contain local outbreaks of disease

Before considering a new pertussis vaccine for infants, the first step may be to develop an alternative aP formulation that includes a more powerful new generation adjuvant, such as TLR agonists, instead of or in addition to alum, and to evaluate it as part of a pertussis only vaccine for boosting adolescents and maybe adults. While this will still require clear evidence of improved immunogenicity over the existing vaccine and stringent safety data before being licensed, it would be logistically less difficult than introducing a new pertussis vaccine for pediatric use. However, one scientific issue that may mitigate against success of this approach is that there is evidence that the immune responses is 'set' after the initial immunization and attempts to induce *B. pertussis*-specific Th1 responses in individuals that have received a full course of aP vaccination may be difficult even through the use of Th1-inducing adjuvants. So switching from Th2 to Th1 responses by changing the adjuvants may not be so straightforward in a primed population, but should be less of an issue in naive individuals. Although it has been argued that the immune responses is Th2 skewed in neonates[110], it has been demonstrated that infection with *B. pertussis* or immunization with wP vaccines can induce potent Th1-skewed $CD4^+$ T cell responses in human infants[23, 66, 68, 71], suggesting that it should be possible to induce Th1 responses in infants with an aP vaccine formulated with the appropriate Th1-promoting adjuvant.

In the longer term, a new pertussis vaccine will be required for primary and booster vaccination of infants and children. There is now convincing evidence from clinical studies that the current aP vaccine does not induce sustained protective immunity[5], and

that *B. pertussis* is evolving to escape immune responses that the vaccines generate[111]. Furthermore, studies in mice complemented by those in humans have indicated that the aP vaccine fails to induce protective Th1 responses[22, 66]. The development of a new aP vaccine should address these limitations and should be capable of inducing Th1 responses and sustained immunity mediated by effective T and B cell memory. Preliminary studies have suggested that current aP vaccines are less effective than wP vaccines in inducing Tfh and T_{RM} cells [Wilk and Mills, unpublished data]. Tfh play a crucial role in T cell help for antibody production and in memory B cell formation, whereas T_{RM} appear to be critical for sustained cellular immunity at mucosal surfaces. Therefore, new generation vaccines also need to consider the induction of these T cell types. The inclusion of additional antigen(s), such as ACT [112] or the autotransporter BrkA[113], could help to broaden the immune responses and reduce the problem associated with immune escape but will add further complexity, especially for manufacturer and regulatory agency approval.

The clinical evaluation and regulatory approval of a new pertussis vaccine will not be straightforward. At this stage, the criteria for testing and licensing of a new vaccine are not clear. Large-scale phase 3 clinical trials, such as those carried out in Sweden and Italy in the 1990s, to estimate the efficacy of current aP vaccines[3, 4] will not be possible, because there is no equivalent cohort of non-vaccinated individuals, such as those available in Sweden and Italy in the 1990s when these countries had stopped using wP vaccines. Evidence of immunogenicity and safety in smaller clinical trials combined with estimates of efficacy from an animal model is one approach that may be considered. Alternatively, human challenge studies are being considered and have the obvious advantage over mice and baboons of being in the target species, albeit in adults rather than in children. However, most, if not all, adult volunteers, even if they lack circulating anti-*B. pertussis* antibodies, are likely to have memory T and B cells specific for *B. pertussis* antigens induced either by previous immunization or exposure to live *B. pertussis* through clinical or subclinical infections. Therefore, while human challenge may be useful for comparing immune responses and protection against infection induced by new compared with existing aP vaccines in primed individuals, they are unlikely to tell us what type of immune responses these vaccines will induce in unprimed individuals or whether they will protect naive individuals and more importantly naive children. Regardless of the complexity of the task, the obstacles to development, assessment and regulation of new pertussis vaccines must be overcome using lessons from immunology in order to tackle the growing threat from this vaccine-preventable infectious disease.

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Chapter 3

Molecular signatures of the evolving immune response in mice following a *Bordetella pertussis* infection

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Abstract

Worldwide resurgence of pertussis necessitates the need for improvement of pertussis vaccines and vaccination strategies. Since natural infections induce a longer-lasting immunity than vaccinations, detailed knowledge of the immune responses following natural infection can provide important clues for such improvement. The purpose was to elucidate the kinetics of the protective immune response evolving after experimental *Bordetella pertussis* infection in mice. Data were collected from (i) individual analyses, i.e. microarray, flow cytometry, multiplex immunoassays, and bacterial clearance; (ii) twelve time points during the infection, and (iii) different tissues involved in the immune responses, i.e. lungs, spleen and blood. Combined data revealed detailed insight in molecular and cellular sequence of events connecting different phases (innate, bridging, and adaptive) of the immune response following the infection. We detected a prolonged acute phase response, broad pathogen recognition, and early gene signatures of subsequent T cell recruitment in the lungs. Activation of particular transcription factors and specific cell markers provided insight into the time course of the transition from innate towards adaptive immune responses, which resulted in a broad spectrum of systemic antibody subclasses and splenic Th1/Th17 memory cells against *B. pertussis*. In addition, signatures preceding the local generation of Th1 and Th17 cells as well as IgA in the lungs, considered key elements in protection against *B. pertussis*, were established. In conclusion, molecular and cellular immunological processes in response to live *B. pertussis* infection were unraveled, which may provide guidance in selecting new vaccine candidates that should evoke local and prolonged protective immune responses.

Introduction

The Gram-negative bacterium *Bordetella pertussis*, the causative agent of whooping cough (pertussis), accounted for high mortality rates among infants in the pre-vaccine era. Whole-cell (wP) and acellular pertussis (aP) vaccines have drastically decreased the number of cases[1]. However, recently resurgence of pertussis in the vaccinated population has been observed in the USA[2, 3] and in European countries[4, 5]. Currently pertussis is still endemic, ranking in the top-ten of vaccine preventable diseases worldwide, according to the WHO. Next to improved diagnostics and public awareness, also strain adaptation and waning immunity are thought to be responsible for this increase in disease cases[6]. Thus, a call for vaccines with improved efficacy is evident.

As a main feature of the innate immune response upon *B. pertussis* infection in mice the recognition of lipooligosaccharide (LOS) by TLR4 has been acknowledged[7, 8]. This activation of TLR4 by *B. pertussis* leads to upregulation of cytokine gene expression and recruitment of neutrophils into the lungs[9, 10]. It was found in animal models that *B. pertussis* infection results in formation of T helper (Th) 1 and Th17 cells[11-13]. Since immunity induced by natural infections provides faster clearance upon reinfection and is longer lasting compared to both wP and aP vaccination[14, 15], immune mechanisms induced upon infection or vaccination have been compared. In human and murine studies, immunization with wP or aP vaccines results predominantly in a Th1 or a Th2 response, respectively[11, 16]. In addition, in both the intramuscular (human) or subcutaneous (mice) administered acellular and wP vaccines, the humoral response is characterized by systemic IgG antibodies[17, 18], while mucosal immune responses seem absent. Despite the absence of direct evidence for correlates of protection against *B. pertussis*, both Th1 and Th17 type of $CD4^+$ T cells as well as IgA-producing B cells seem to play an important role in a protective immune response against *B. pertussis*[12, 15, 19-22]. Since these responses are not induced by the currently available vaccines[23], but more resemble responses induced by infection, a better understanding of the immune response induced in the host following *B. pertussis* infection is needed. Despite knowledge on particular elements of the immune response generated by a *B. pertussis* infection, little is known about the kinetics and sequential relation of these elements. For this, systems biology can be an important tool, as was shown for tuberculosis and influenza infection[24-26].

Here, systems biology was applied to elucidate molecular and cellular events in the different phases of the immune response after primary *B. pertussis* infection in a murine model. To this end, innate and adaptive immune responses were investigated over a period of 66 days post infection. Gene expression profiles in spleen and lungs, cytokine profiles in sera, and cellular composition of the spleen were determined at twelve time points. Furthermore, cellular and antibody mediated immune responses against *B. pertussis* were investigated. Herewith, we revealed a chronological cascade of immunological processes consisting of recognition, processing, presentation and clearance of *B. pertussis*. The extensive insights into the immune response upon *B. pertussis* infection generated in this study may serve as a solid base for future research on pertussis vaccines and vaccination strategies.

Materials and methods

Ethics Statement

The independent ethical committee for animal experimentations 'Dierexperiment Commissie (DEC)' of the National Institute for Public Health and the Environment (RIVM) reviewed the animal experiments in this study according to the guidelines provided by the Dutch Animal Protection Act. The committee approved documents with the identification numbers 'DPA201100230' and 'DPA201100348'.

Materials

B. pertussis strain B1917[27] was isolated in The Netherlands in 2000 and was kindly provided by Frits Mooi. Bordet-Gengou agar plates with 15% sheep blood were purchased from BD (BD, The Netherlands). Verweij medium was obtained from Bilthoven Biologicals (BBio, Bilthoven, The Netherlands). PBS (pH 7.2) and Roswell Park Memorial Institute Media 1640 (RPMI) were purchased from Invitrogen (Gibco, Invitrogen). All RPMI media used in this experiment were supplemented with 10% fetal calf serum (FCS, Hyclone, New Zealand), 100 units penicillin, 100 units streptomycin, and 2.92 mg/ml L-glutamine (p/s/g; Invitrogen). Shockbuffer for splenocyte isolation consisted of 8.3 g/L NH_4CL, 1 g/L $NaHCO_3$, and 5000 IE/L Heparine dissolved in dH_2O (pH 7.4; ice-cold) and was filtered (0.22 µm) before use. FACS buffer consisted of PBS pH 7.2 supplemented with 0.5% BSA (Sigma Aldrich, Germany) and 0.5 mM EDTA (ICN Biomedicals). Horseradish peroxidase (HRP) labelled goat-anti-mouse IgG and R-Phycoerythrin (RPE)-conjugated goat-anti-mouse IgM, IgA, IgG, IgG1, IgG2a, IgG2b, and IgG3 were obtained from Southern Biotech (Southern Biotech, United States). Fluorescently labeled antibodies for flow cytometry, anti-CD3-Pacific blue (17A2), anti-CD4-Pacific blue (GK1.5), anti-IA-IE-PerCP-Cy5.5 (M5/114.15.2), anti-33D1-APC (33D1), and anti-IL-5-APC were purchased from Biolegend (ITK, The Netherlands). Fluorescently labeled antibodies, anti-CD4-APC (RM4-5), anti-CD8a-PerCP (53 6.7), anti- CD69-FITC (H1.2 F3), anti-Gr-1-PE (RB6-8C5), anti-CD19- FITC (1D3), anti-CD14-PE (rmC5-3), anti-DX5-APC (DX5), anti-CD11b-PE (M1/70), anti-CD40-FITC (HM40-3), anti-CD44-FITC (IM7), and anti-IFNγ-PE were purchased from BD (BD Biosciences, The Netherlands). Fluorescently labeled antibodies anti-CD11c-PE-Cy7 (N418) and anti-IL-17A-PerCP-Cy5.5 were obtained from eBioscience (eBioscience, Austria). Live/dead staining was purchased from Invitrogen (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Invitrogen). Anti-CD16/CD32 was purchased from BD (BD, The Netherlands).

Cultivation of *B. pertussis*

B. pertussis strain B1917 was grown on Bordet-Gengou agar plates for 4 days at 35°C. Subsequently, multiple colonies were transferred onto new plates and grown for 24 hours at 35°C, which was repeated 3 times. Colonies on each plate were harvested in 1.5 ml Verweij medium[28] and the suspensions were pooled. After centrifugation (30 min, 1932 g), cells were washed with 15 ml Verweij medium. Subsequently, bacterial suspensions of approximately $2x10^{10}$ cfu/ml were resuspended in Verweij medium containing 15% glycerol (v/v) and aliquots of 0.25 ml were flash frozen in ethanol and dry ice and stored at -80°C.

Challenge culture

A stock of *B. pertussis* B1917 was diluted with Verweij medium to a final concentration of $5x10^6$ cfu/ml. The number of cfu was confirmed by plating 100 µl of the infection suspension (1:2000 in Verweij medium) on Bordet-Gengou agar plates. Plates were incubated for 4 days at 35°C and cfu were counted by using a colony counter (ProtoCOL, Synbiosis, Cambridge, United Kingdom).

Animal experiment

Female BALB/c mice (Harlan, The Netherlands), 8-weeks-old, were divided in groups of three animals and housed in cages (macrolon III including filter top). Three healthy mice were euthanized at day 0 and considered as naive group. Other mice were intranasally infected under anesthesia (isoflurane/oxygen), with $2x10^5$ cfu *B. pertussis* B1917 in 40 µl Verweij medium at day 0. Groups of animals (n=3) were euthanized after 2, 4, 6 hours, and after 1, 2, 4, 7, 10, 14, 21, and 28 days of infection, respectively. At 56 days p.i. five groups of primary infected animals and five groups of naive animals (n=3) were infected as previously described and euthanized after 4 hours, and after 2, 7, 10, and 14 days of infection. In addition, six infected and six healthy mice were euthanized at day 21 and day 66 for the analysis of $CD4^+$ T cell responses. Mice were bled, under anesthesia (isoflurane/oxygen), by orbital bleeding and sacrificed by cervical dislocation. Whole blood from each mouse was collected in a blood collection tube (MiniCollect 0.8 ml Z Serum Sep GOLD, Greiner Bio-One, Austria). After coagulation (10 min at room temperature), sera were taken after centrifugation (10 min, 3000 g) and stored at -80°C. Lung lobes were excised. The right lobe was placed in 900 µl Verweij medium and kept at room temperature. The left lobe was placed in 1 ml RNAlater (Qiagen), incubated overnight at 4°C and stored at -80°C. The spleen was excised and divided in two equal parts. One piece was placed in 5 ml of RPMI medium and kept on ice. The other piece was placed in 1 ml RNAlater (Qiagen), incubated overnight at 4°C and stored at -80°C. For the analysis of $CD4^+$ T cell responses, whole spleen was collected and placed in 5 ml of RPMI medium and kept on ice.

Colonization assay

Lung tissue in 900 µl Verweij medium was homogenized for 15 seconds using a Labgen 7 Homogenizer (Cole Palmer, Schiedam, The Netherlands). Homogenizer was sanitized between samples with ethanol (80%) and water. Lung homogenates were serially diluted (undiluted, 1:10, 1:100 or 1:1000) in Verweij medium depending on the expected number of cfu. Suspensions of 100 µl were plated on Bordet-Gengou agar plates and incubated for 4 days at 35°C. The numbers of colonies were counted by using a colony counter (ProtoCOL, Synbiosis, Cambridge, United Kingdom) and calculated as cfu per mouse.

RNA isolation

Lung and spleen tissues were homogenized by using a Labgen 7 Homogenizer (Cole Palmer, Schiedam, The Netherlands) in 700 µl and 2100 µl Qiazol (Qiagen Benelux, Venlo, The Netherlands), respectively. RNA isolation was performed using a miRNeasy Mini Kit with DNAse treatment (Qiagen Benelux, Venlo, The Netherlands) according to the manufacturer's

protocol. RNA concentrations were determined by UV spectroscopy (Tech3 module, Synergy Mx, BioTek, Winooski, United States). RNA quality was determined by using electrophoresis (RNA nano 6000 kit, 2100 Bioanalyzer, Agilent Technologies, Amstelveen, The Netherlands). Results were given as RNA integrity numbers (RIN) between 1 and 10 (manual Bioanalyzer). Samples with a RIN of 7 or higher were used for microarray analysis. For microarray analysis of lung tissue, RNA concentrates of individual mice were analyzed for the following time points: naive, 4 hours, 2 days, 4 days, 7 days, 10 days, 14 days, 21 days, and 28 days post infection (p.i.). For spleen tissue, samples of individual mice were analyzed for the following time points: naive, 4 hours, 1 day, 2 days, 4 days, 7 days, 10 days, 14 days, 21 days, and 28 days p.i.

Microarray analysis

Amplification, labeling and hybridization of RNA samples to microarray chips was carried out at the Microarray Department of the University of Amsterdam, The Netherlands, as described previously[29]. Briefly, 500 ng total RNA of each sample was amplified (aRNA) according to the Agilent QuickAmp kit manual (Agilent technologies). A common reference sample for either lung or spleen tissue was made by pooling equimolar amounts of ARNA from individual samples of the respective tissues. Cy3 and Cy5 monoreactive dyes (GE Healthcare) were used to label individual samples and the common reference sample, respectively. Labeling was performed with 10 ml of CyDye solution and incubated for 1 hour before the reaction was quenched by adding 5 ml 4 M hydroxylamine (Sigma-Aldrich). Purification was performed by using a clean-up kit (E.Z.N.A. MicroElute RNA Clean Up Kit, Omega Bio-Tek, Norcross, United States). The yields of amplified RNA and incorporation of CyDye were determined by using a spectrophotometer (NanoDrop ND-1000, NanoDrop products, Wilmington, United States). Hybridization mixture was prepared by adding a dried mixture (1:1) of sample (Cy3) and common reference (Cy5) together with sample tracking control (STC, Roche NimbleGen) and hybridization cocktail (NimbleGen Arrays User's Guide - Gene Expression Arrays Version 5.0, Roche NimbleGen). Samples were incubated successively for 5 min at 95°C and 5 min at 42°C. Hybridization was performed by loading a sample onto a microarray (NimbleGen 126135 k Mus musculus, Roche, Germany) containing probes for 44,170 genes with 3 spots per target probe. Hybridization was performed with a NimbleGen Hybridization System 4 (Roche NimbleGen) for 20 hours at 42°C. After washing, slides were scanned in an ozone-free room with a microarray scanner (Agilent DNA microarray scanner G2565CA, Agilent Technologies). Each microarray corresponded to labeled RNA from specific tissue (lung or spleen) from one individual mouse. Feature extraction was performed with NimbleScan v2.5 (Roche NimbleGen) resulting in a table containing individual probe signal intensities for both dyes. Complete raw and normalized microarray data and their MIAME compliant metadata from this publication have been submitted to the GEO database (www.ncbi.nlm.nih.gov/geo) and assigned the identifier GSE53294.

Data analysis of gene expression

Quality control was performed on raw data by means of Cy3-Cy5 scatter plots, and by comparing signal average and distribution across slides. All slides passed quality control.

Raw microarray data for gene-coding probes were normalized in R (www.r-project.org), by using a four step approach[29]: (1) natural log-transformation, (2) quantile normalization of all scans, (3) correcting the sample spot signal for the corresponding reference spot signal and (4) averaging data from replicate probe spots. Further analysis of normalized data of 44,170 probes was performed in R and Microsoft Excel.

Genes differentially expressed between experimental groups (naive and various time points p.i.) were identified by using ANOVA. Fold ratio induction or repression of individual genes was calculated by comparing mean gene expression levels of infected groups to the group with naive mice. Data are presented as the average normalized gene expression levels of three mice per group. Probes were considered differentially expressed if they met the following two criteria: (i) a p-value≤0.001 (ANOVA), which corresponds to a Benjamini-Hochberg False discovery rate (FDR)[30] of <5%; and (ii) an absolute fold ratio ≥1.5 (infected compared to naive mice) for at least one time point. If multiple probes corresponding to the same gene were significant, their data were averaged to remove redundancy for further analysis. Gene expression of each group was compared with the group of naive mice. Differences in gene expression were visualized in heat maps (GeneMaths XT, Applied Maths, St-Martens-Latem, Belgium). Genes were arranged according to similar expression patterns in time at which genes exceeded the fold ratio cut-off of 1.5. To facilitate visual interpretation of heat maps, only induction (red) and repression (green) of gene expression levels with fold ratios ≥1.5 are visualized, therefore presenting fold ratios <1.5 as naive level (black).

Functional enrichment with an over-representation analysis (ORA) was carried out by using DAVID[31] based on Gene Ontology Biological Processes (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Cell-specific or tissue-specific gene sets were obtained by using data from BioGPS[32, 33]. For each cell type or tissue, gene expression data were compared to the average of all tissues included in the data set. The top 100 genes, which had the highest tissue-specific expression and were included on the NimbleGen array, were selected. If the BioGPS dataset included multiple entries for the same cell type (e.g., $CD4^+$ and $CD8^+$ T cells), the resulting sets were combined in our subsequent analysis.

Whole-cell *B. pertussis* ELISA

Immunoglobulin G responses against *B. pertussis* were determined in sera by an ELISA. Therefore, *B. pertussis* B1917 was heat-inactivated for 45 min at 56°C, after which ELISA plates (Immunolon 2HB, Thermo Scientific) were coated overnight at room temperature with 100 µl/well whole-cell *B. pertussis* (OD590nm = 0.1 in PBS). Plates were washed with 0.03% (v/v) Tween-80 in water. Then, plates were incubated for 1 hour at 37°C with 100 µl/well serial dilutions of serum samples in PBS, pH 7.2 (Gibco) with 0.1% (v/v) Tween-80. Plates were washed twice and incubated (1 hour, 37°C) with 100 µl/well of HRP-conjugated goat-anti-mouse IgG (1:5000) in PBS with 0.1% (v/v) Tween-80, and 0.5% (w/v) Protifar (Nutricia). After washing twice, 100 µl/well peroxidase substrate (4.2 mM Tetra Methyl Benzidine and 0.012% H_2O_2 in 0.11 M sodium acetate buffer, pH 5.5.) was

added, plates were incubated at room temperature and the reaction terminated after 10 min by adding 100 µl/well, 2 M H_2SO_4 (BBio, Bilthoven, The Netherlands). Finally, the absorbance was recorded at 450nm with a plate reader (BioTek reader EL808, Bio-Tek, USA) and antibodies against whole-cell *B. pertussis* are presented in OD450 signal, because a reference is not available.

Multiplex immunoassay (MIA) for antibody response

Responses of IgM, IgA, total IgG, and the IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) against *B. pertussis* antigens P.69 pertactin (P.69 Prn), filamentous hemagglutinin (FHA), pertussis toxin (Ptx), combined fimbria type 2 and 3 antigens (Fim2/3), and outer membrane vesicles B1917 (OMV B1917) were determined in sera by using a MIA. Outer membrane vesicles from *B. pertussis* B1917 were produced as previously described[34] with additional changes. Conjugation of antigens and OMVs to beads was performed as described previously[35]. Serum was diluted 1:100 in PBS containing 0.1% Tween-20 and 3% bovine serum albumin and mixed 1:1 with conjugated beads. After incubation with R-Phycoerythrin (RPE)-conjugated anti-mouse IgM (1:100), IgA (1:100), IgG (1:200), IgG1 (1:200), IgG2a (1:40), IgG2b (1:200), and IgG3 (1:200), samples were analyzed by using a Bio-Plex system (Bio-Plex 200, BioRad). Total IgG titers (U/ml) against *B. pertussis* antigens were calculated from mean fluorescence intensity (MFI) by using a reference from the National Institute for Biological Standards and Control (Code 99/520, NIBSC, England) in the Bio-Plex Manager software (Bio-Rad Laboratories). Results for IgM, total IgG, IgG subclasses, and IgA antibodies against OMV B1917 are presented in fluorescence intensity (FI), because a reference is not available.

Lung lysate preparation and IgA antibody assay

The lung lysates used for the colonization assay were centrifuged (10 min, 2000 g) and filtered (0.22 µm Millex-GV, Merck KGaA, Darmstadt, Germany) to remove cell debris and bacteria. Filtrates were stored at -80°C. Prior to analysis, lung lysates were diluted 1:10 in PBS containing 0.1% Tween-20 and 3% bovine serum albumin. Analysis of IgA antibodies in the lung lysate was performed against *B. pertussis* antigens (Ptx, Prn, FHA, and Fim2/3) and OMV B1917 as described in the section multiplex immunoassay (MIA) for antibody response.

Splenocyte isolation

Spleen tissue was forced through a 70 µm cell strainer (BD Falcon, BD Biosciences, USA) and washed two times with 5 ml medium (RPMI, FCS, p/s/g) to obtain a single cell suspension. After centrifugation (5 min, 550 g), 1 ml shock buffer was added to cell pellet to lyse erythrocytes. After one minute, shock incubation was stopped by adding 25 ml medium. For cell count (CASEY model TT, Innovatis, Roche, Germany), cells were centrifuged and resuspended in 1 ml medium.

Cellular composition of spleen

Splenocytes were transfered to each well of a V-bottom 96-wells plate. After centrifugation (3 min, 400 g), pellets were washed with 100 µl FACS buffer. Fc-specific receptors on

cells were blocked with anti-CD16/CD32 in FACS buffer (2%, v/v) by 5 min incubation on ice to prevent aspecific binding of labeled antibodies to cells. Cells were stained after centrifugation by using a mixture (100 µl) of labeled antibodies (30 min, 4°C). To determine the number and identity of cells, three different panels of labeled antibodies were used to prevent overlapping fluorescence spectra. Panels consisted of 1) total T cell population (CD3), CD4 T cell (CD4), CD8 T cell (CD8), early activated T cell (CD69), and neutrophil (Gr-1); 2) T cell (CD3), B cell (CD19), monocyte (CD14), and natural killer cell (DX5); 3) T cell (CD3), macrophage (CD11b), DC (33D1, CD11c), MHC class II (IA/IE), and activation marker (CD40). Data acquisition was performed with a FACSCanto II (BD Biosciences, USA) and data analysis was done by using FlowJo software (Tree Star Inc., USA). Debris and dead cells were excluded by using forward/side scatter characteristics and live/dead staining, respectively.

Stimulation of splenocytes

Splenocytes were resuspended in the culture medium IMDM (Gibco) + 8% FCS+p/s/g + 20 mM β-mercaptoethanol (Sigma) and cultured (24-wells plate; $6x10^6$ cells/well) for 7 days at 37°C in a humidified atmosphere containing 5% CO_2. Culturing was in the presence of either the culture medium, or the culture medium supplemented with Prn P.69 (in house expressed and purified as described previously[36]), heat-inactivated (15 min at 95°C) Ptx (Kaketsuken, Japan), or FHA (Kaketsuken, Japan), at a final concentration of 1 µg/ml. Supernatants were collected after 7 days for cytokine analysis. Subsequently, cells were transferred to U-bottom 96-wells plates ($5x10^6$ cells/well) and stimulated overnight by using the same antigens and conditions and used for intracellular cytokine staining.

Intracellular cytokine staining

The intracellular cytokine staining was performed on 8-days stimulated splenocytes by using a Cytofix/Cytoperm Fixation/ Permeabilization Solution Kit (BD Biosciences, USA). Briefly, cells were incubated during the last 5 hours of stimulation with 10 µg/ml Golgiplug (BD Biosciences), 1 µg/ml anti-CD28 (BD Pharmingen), and 1 µg/ml anti-CD49d (BD Pharmingen). Cells were stained after washing with FACS buffer with anti-CD4-Pacific blue, anti-CD44-FITC, and live/dead staining. Subsequently, the cells were fixed, permeabilized, and stained with anti-IFNγ-PE, anti-IL-5-APC and anti-IL-17A-PerCP-Cy5.5. Data were acquired on FACS Canto II (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc., USA). CD44 was used to distinguish between naive and antigen-experienced T cells. IL-5 was used as a Th2 cytokine, IFNγ as a Th1 cytokine, and IL-17A as a Th17 cytokine.

Multiplex immunoassay (MIA) for cytokines

Cytokine (IL-4, IL-5, IL-10, IL-13, IL-17A, TNFα, and IFNγ) concentrations (pg/ml) were determined in culture supernatants of 7-days stimulated splenocytes of individual mice by using a MIA (Milliplex mouse cytokine 7-plex luminex kit; Merck KGaA, Darmstadt, Germany). Cytokine (Eotaxin, G-CSF, GM-CSF, IFNγ, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, VEGF) concentrations (pg/ml) present in serum were determined by using a MIA (Milliplex MAP Mouse Cytokine/ Chemokine - Premixed

32 Plex; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, serum/supernatant (25 µl) was diluted in supplied assay buffer (1/1; v/v), mixed with 25 µl conjugated beads and incubated overnight at 4°C on a plate shaker. Subsequently, beads were washed twice with washing buffer by using a Bio-Plex handheld magnetic washer (BioRad, USA) and incubated with 25 µl detection antibodies (1 hour, 20-25°C) followed by incubation with 25 µl streptavidin-phycoerythrin (30 min, 20-25°C). After washing twice with 200 µl washing buffer, beads were resuspended in 150 µl PBS. Samples were analyzed using a multiplex system (Bio-Plex 200, BioRad, USA). In total, 50 beads per region were analyzed in 100 µl sample volume. Mean fluorescence intensity (MFI) of each cytokine was converted into pg/ml by using a dilution series of a standard (3.2-10,000 pg/ml) and Bio-Plex Manager software 5.0 (Bio-Rad, USA).

MIP-3α (CCL20) cytokine ELISA

Serum concentration of Macrophage Inflammatory Protein 3α (MIP3α/CCL20) was determined by ELISA (Mouse MIP-3α ELISA kit, RAB0061, Sigma-Aldrich, Germany) according to the manufacturer's manual. In short, serum (50 µl) was diluted in assay buffer A (1/1; v/v) and incubated in an antibody-coated ELISA plate (overnight, 4°C, gentle shaking). After washing four times with 300 µl wash buffer, wells were incubated with 100 µl biotinylated antibody (1 hour, room temperature, gentle shaking). After washing four times with 300 µl wash buffer, wells were incubated with 100 µl streptavidin solution (45 min, room temperature, gentle shaking). After washing four times, wells were incubated with 100 µl TMB one-step substrate reagent (30 min, room temperature) and reaction stopped with 50 µl stop solution. Finally, the absorbance was recorded at 450nm with a plate reader (BioTek reader EL808, Bio-Tek) and converted to MIP-3α concentration by using a dilution series of a standard (2.06–1,500 pg/ml) and Gentech 5 software (BioTek).

Statistical analysis

Cellular composition of spleen, antibody titers and cytokines levels were analyzed by using a one-way ANOVA with multiple comparisons of all time points post infection vs. naive mice followed by a Dunnett t-test. ICS data was analyzed by using a Mann-Whitney t-test. For cytokine profiling after re-stimulation of splenocytes, MIA data was first log-transformed before t-test analysis. In all corresponding figures, p-values are represented as *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$.

Results

Lung clearance of infected mice

The presence of *B. pertussis* in lungs of mice was examined during a period of 28 days post infection (p.i.), providing the benchmark for this study (Figure 1A). Therefore, mice were intranasally infected with *B. pertussis* using a dose of 10^5 colony forming units (cfu). Approximately 13% of the intranasal dose was traceable in the lungs of mice 2 hours p.i. The number of bacteria remained fairly constant for one day, and increased from the second day to a maximum 7 days p.i. (10^7 cfu). Subsequently, a decrease in the number of bacteria was observed and complete clearance in 2 out of 3 mice was achieved 28 days p.i. To determine whether single intranasal infection with *B. pertussis* leads to protection, mice were reinfected 56 days after primary infection (Figure 1B). A similar number of viable bacteria was detected 4 hours p.i. in lungs of both reinfected and naive mice. Reinfected mice were able to clear *B. pertussis* from the lungs within 2 days p.i., whereas naive mice showed a similar pattern as observed before. In conclusion, naive mice can clear *B. pertussis* from the lungs in about 28 days. Furthermore, mice previously infected with *B. pertussis* had developed sterilizing immunity, which clears the lungs in two days.

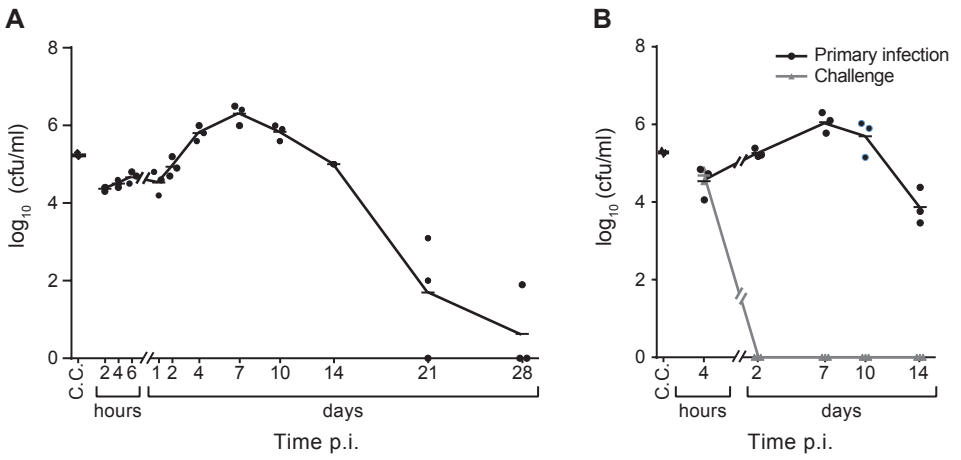

Figure 1 - Lung clearance of naive and reinfected mice after *B. pertussis* infection. (A) Number of colony forming units (cfu) in challenge culture (C.C.) was confirmed before challenge. All other cfu were determined in lung of challenged mice (mean n=3). A large fraction of the original infection dose was traceable in the lungs of mice 2 hours p.i. Bacteria were able to colonize and multiply approximately 100-fold at 7 days p.i. After one week, the mice were able to clear bacteria, which resulted in cleared lungs (2 out of 3 mice) at the last time point (day 28 p.i.). (B) Reinfection was performed 56 days after primary infection and the number of cfu were counted 4 hours p.i. While reinfected mice were able to clear *B. pertussis* from the lungs within 2 days p.i., naive mice showed a similar pattern as observed in Figure 1A.

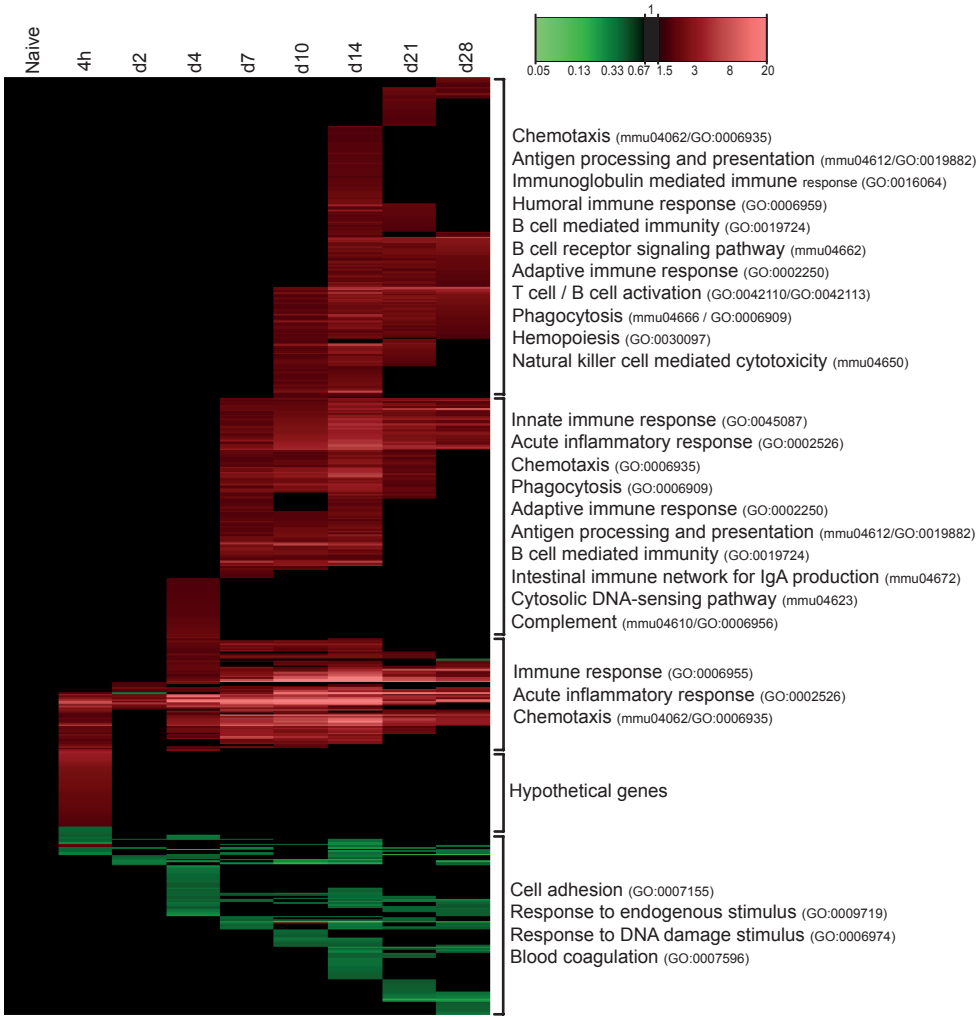

Figure 2 - Pulmonary gene expression profiles as function of time after *B. pertussis* infection. Fold changes in expression were calculated compared to naive mice and significant gene expression results (p-value≤0.001, FR≥1.5) are visualized as heatmap (mean of n=3). In total, 558 genes were found to be differentially regulated, divided in 446 upregulated genes (red) and 112 downregulated genes (green). Genes not exceeding a fold change of 1.5 are depicted as basal level (black). Results are divided in 5 clusters representing up- or downregulation and time of involvement including corresponding GO-BP terms and KEGG pathways.

Gene expression in lung tissue

The gene expression in lung tissue of infected mice was monitored over a period of 28 days. In total, 558 genes of the genome were differentially regulated (p-value≤0.001, fold ratio (FR)≥1.5): 446 genes were upregulated and 112 genes were downregulated (Figure 2). A complete list of gene names is given in Table S1. Gene expression profiles changed already

4 hours p.i. with largest differences at 14 days p.i. (377 responsive genes). The number of genes with an altered expression had declined 21 and 28 days p.i. However, the expression of many genes had not yet returned to naive basal levels. Principal component analysis confirmed the largest differences at 14 days p.i. and the decline at 21 and 28 days p.i. (Figure 3). Interestingly, the time point with the least changes in gene expression (2 days p.i.) preceded the increase in cfu in the lungs (Figure 1).

To determine which biological processes were involved, functional analysis of all 558 genes was performed using DAVID. Over-representation analysis (ORA) (Benjamini-corrected p-value≤0.05) in GO-BP and KEGG databases resulted in respectively 122 terms and 20 pathways significantly enriched. A selection of these terms is presented in Figure 4. The selection is based on immunological relevance and by exclusion of overlapping terms. Acute phase response and chemotaxis were observed right after infection (4 hours p.i.) (Figure 2). Subsequently, innate immune response, phagocytosis, antigen processing and antigen presentation were initiated (7 days p.i.). Involvement of B cell mediated immunity in this stage is mainly demonstrated by the expression of complement related genes. Eventually, the gene expression showed initiation of the adaptive immune response, such as activation of B cells and T cells, B cell receptor (BCR) signaling, humoral immune response (complement activation) and immunoglobulin-mediated immunity from 10 until 28 days p.i.

The gene expression data was compared with the BioGPS database to identify the influx, presence or activation of particular immunological cells in the lungs (Figure S1). 61 genes, which are predominantly expressed in macrophages, suggest two events: (i) triggering of alveolar macrophages 4 hours p.i. and (ii) recruitment of macrophages in the lungs 7 days p.i. The influx of macrophages was observed on cellular level using fluorescence microscopy [37]. In addition, the increased expression of 29 genes was attributed to the presence of neutrophils in the lungs 4 days p.i. Moreover, data suggests altered expression of 32, 48, and 17 genes of B cells, dendritic cells (DCs) and mast cells, respectively, 7 days p.i., and 19 T cell genes 14 days p.i.

Differentially regulated genes in lung tissue after the infection with *B. pertussis* were classified according to function, pathway and cell type. For eleven different groups details are described below.

(1) Cytokines. Twenty-one cytokine-encoding genes were differentially expressed in the lungs during *B. pertussis* infection (Figure 5). Sixteen cytokines are associated with a chemotactic function. The other five genes (*Flt3l*, *Ifng*, *Il17a*, *Il17f*, *Il16*) reflect cytokines involved in the activation of T cells, such as Th1 and Th17 cells.

(2) Membrane receptors. Forty-six membrane receptors were identified with altered gene expression (Figure 5). The receptors were divided in eight groups: cytokine receptors, C-type lectins (CLRs)[38], Fc-receptors, epithelial cell specific receptors, including the IgA transporter (*Pigr*), myeloid-associated immunoglobulin-like receptors, semaphorins[39], lymphocyte receptors (e.g., *Pdl1*)[40, 41] and the mucosal-homing receptor (*Itgb7*). Other membrane

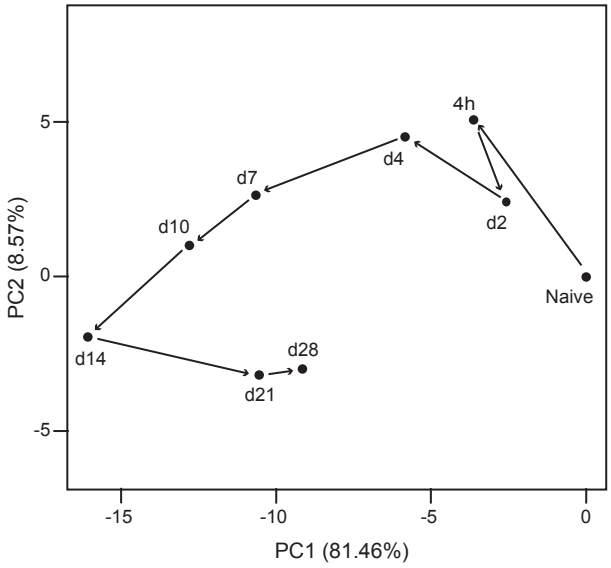

Figure 3 - Principal component analysis of pulmonary gene expression. An evolving gene expression profile was indicated after *B. pertussis* infection of naive mice (mean of n=3), illustrated as principal component analysis (PCA). PCA is a mathematical algorithm[117], which describes data based on (dis)similarity. Therefore, a greater distance between points in the plot corresponds to a greater dissimilarity. In this figure, the similarity of the 10 time points are compared based on the expression profiles of the 558 differentially expressed genes. Results indicate that 81% and 9% of the variance between time points could be addressed by principal component 1 (PC1) and PC2, respectively. The next principle components contribute for the residual variances. The plot revealed a notable change in gene expression at 2 days p.i. compared to 4 hours p.i., and largest differences at 14 days p.i. The gene expression at 28 days p.i. was still far from the naive state.

receptors are collected in one group (Figure 5). Gene expression profiles of both cytokines and cytokine receptors enabled early prediction of immune cell recruitment towards the lungs on transcriptomic level in this study. Increased expression of *Cxcl13* and *Ccl20* was followed by the upregulation of receptors *Cxcr5* and *Ccr6*. These chemokines lead to the recruitment of CXCR5$^+$ B cells, CXCR5$^+$ T cells, CCR6$^+$ DCs and CCR6$^+$ Th17 cells into the lungs[42-44]. Likewise, the expression of *Cxcl9*, *Cxcl10* and *Cxcl11*, induced by interferon gamma (IFNγ), is associated with recruitment of CXCR3$^+$ T cells or CXCR3$^+$ pDCs[45].

(3) Antimicrobial peptides (AMPs). AMPs have defensive properties against bacterial infections, recruit immune cells and model subsequent immune responses[46]. Gene expression of four AMPs was elevated for 21 days p.i. (Figure 6). These four AMPs recruit neutrophils or protect against respiratory pathogens[47-50]. Interestingly, the chemokine *Cxcl17*, primarily expressed in the lungs, was described as an antimicrobial mucosal chemokine that recruits DCs and CD14$^+$ monocytes and protects against pathogenic bacteria[51, 52].

(4) Acute phase response. Primary reaction to an infection is the acute phase response, which leads to the release of pro-inflammatory cytokines and activation of inflammatory cells. Strong upregulation of 19 genes involved in the acute phase

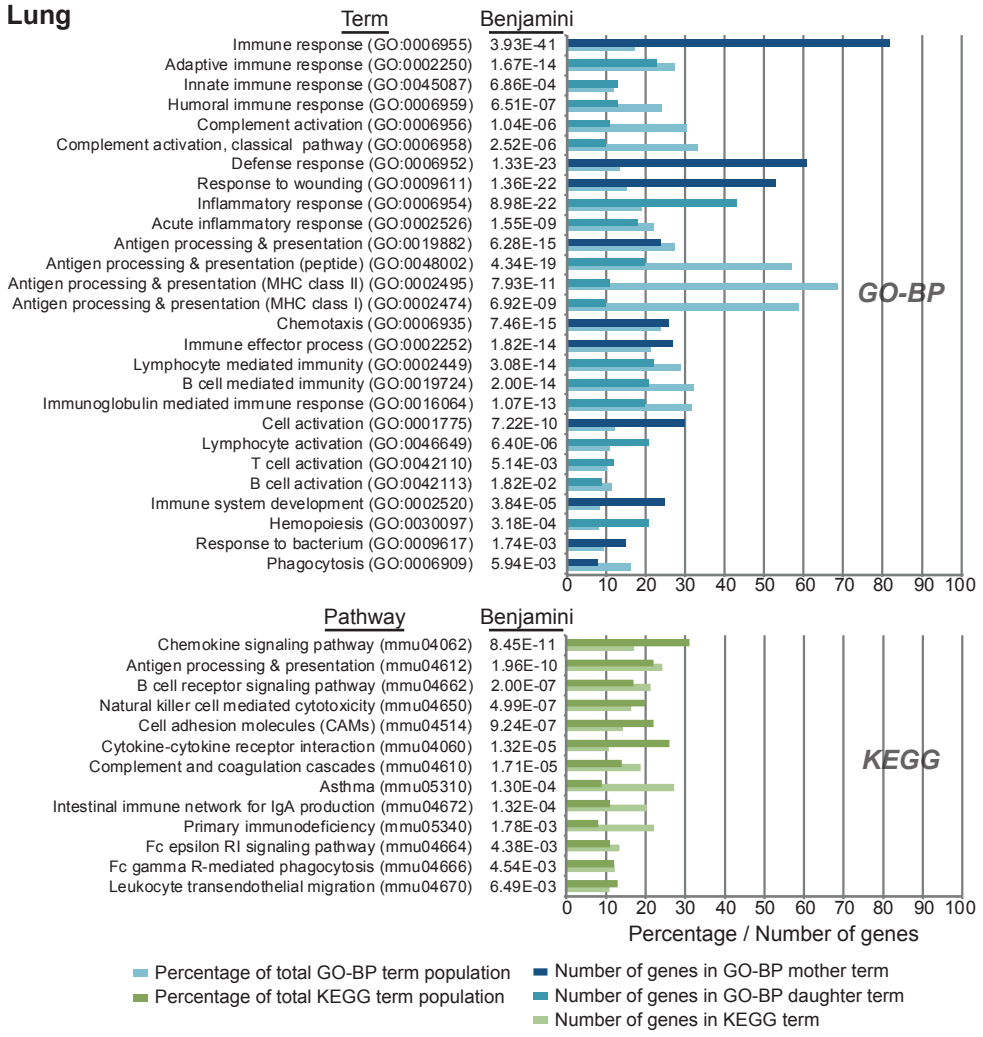

Figure 4 - Functional annotation of pulmonary gene expression data after *B. pertussis* infection. Over-representation analysis (Benjamini- corrected p-value≤0.05) in GO-BP and KEGG databases of pulmonary gene expression data resulted in respectively 122 terms and 20 pathways significantly enriched. A selection of terms and pathways, based on immunological relevance and exclusion of overlapping terms, is given in this figure. GO-BP terms were divided in mother terms (dark blue) and corresponding daughter terms (light blue). For each term the amount of genes, the percentage of the total term population and the Benjamini-corrected p-value are shown.

response was detected at 4 hours p.i. Remarkably, the gene expression of the acute phase response remained highly expressed at least until day 28 p.i. (Figure 6).

(5) Pathogen recognition. Enhanced gene expression suggested activation of TLR4, TLR9, DAI and CLR signaling pathways, implying pathogen recognition by several ligands of *B. pertussis* (Figure 6). The induction of *Md-1* and transcription factor *Irf5* genes suggested TLR4 signaling upon sensing of LOS[53]. Upregulation of *Ctsk* and the downstream transcription

factor *Irf7* assumes activation of TLR9 by sensing of unmethylated CpG sequences in bacterial DNA[54]. In addition, the cytosolic DNA sensor (DAI) was induced[55]. Elevated gene expression of several CLRs was noticed, such as Mgl1 and the mouse DC-SIGN-related proteins *Signr7* and *Signr8*[56]. Mgl1 recognizes N-acetylgalactosamine (GalNAc), which is present in *B. pertussis*. Mgl1 has recently been linked to *B. pertussis* mast cell interaction[57].

(6) Transcription factors. Ten transcription factors were differentially expressed in the lungs (Figure 6). These include genes of factors *Pu.1*, *Shp-1*, *Nfil3*, *Syk*, *Irf5*, *Irf7*, and *Irf8*. All of these transcription factors have been designated as regulators of immunological processes, thereby defining the direction of the host immune response upon bacterial infection[58-64].

(7) Phagocytosis. Six genes representing subunits of NADPH oxidase (NOX) were activated between 4 hours and 28 days p.i. (Figure 6). The activation suggests the production of reactive oxygen species, which are involved in a phagocyte respiratory burst to assist in the killing of bacteria during phagocytosis[65].

(8) Antigen presentation. 38 genes representing antigen presentation were upregulated between 7 and 28 days p.i. (Figure 6). Results indicate that both antigen presentation via MHC class I and class II were included. This suggests antigen presentation to both $CD4^+$ T cells and cytotoxic $CD8^+$ T cells (CTLs). In line with these findings, the upregulation of *Cd4* and *Ctsw* gene expression in lung tissue was observed, which may indicate presence of $CD4^+$ T cells[66] and CTLs[67], respectively (Figure S1).

(9) Complement activation. 12 genes of the complement system were more abundantly expressed between 7 and 28 days p.i. (Figure 6). Increased gene expression was observed for the complement components C1 and C4, which belong to the classical pathway. In contrast, reduced gene expression of the C1-inhibitor (*Serping1*) was observed day 4 p.i. Furthermore, strong upregulation of *Cfb* suggests activation of the alternative complement pathway.

(10) BCR signaling. 15 genes of the BCR signaling pathway were upregulated between 7 and 28 days p.i. (Figure 6). BCR activation was detected by elevated gene expression of eight downstream proteins (*Blnk*, *Card11*, *Grb2*, *Pik3cd*, *Plcg2*, *Prkcb*, *Rac2*, and *Vav1*). Furthermore, the gene expression of the BCR signaling pathways indicates a balanced B cell response because of an interplay of stimulation by *Cd79a* and *Ifitm1*, and inhibition by *FcgrIIb*, *Lilrb3* (*Pirb*), *Cd22*, *Cd72*, and *Shp-1*. These co-inhibitors prevent overstimulation of B cells[68].

(11) Immunoglobulins. Gene expression associated with antibody formation was observed after 4 days p.i. and was increased up to 28 days p.i. (Figure 6). The microarray data revealed gene expression of seven kappa (κ) light chain genes, heavy chain Ig alpha chain C (*Ihg-vj558*), *Ighm,* and *Ighg*, indicating the formation of IgA, IgM, and IgG antibodies.

In summary, approximately 40% of the genes expressed in the lungs could be annotated according to function. The chronology of immunological processes comprises broad pathogen recognition, prediction of cell recruitment by cytokine and receptor expression, MHC class I and II antigen presentation, and markers for both antibody and cell-mediated immunity.

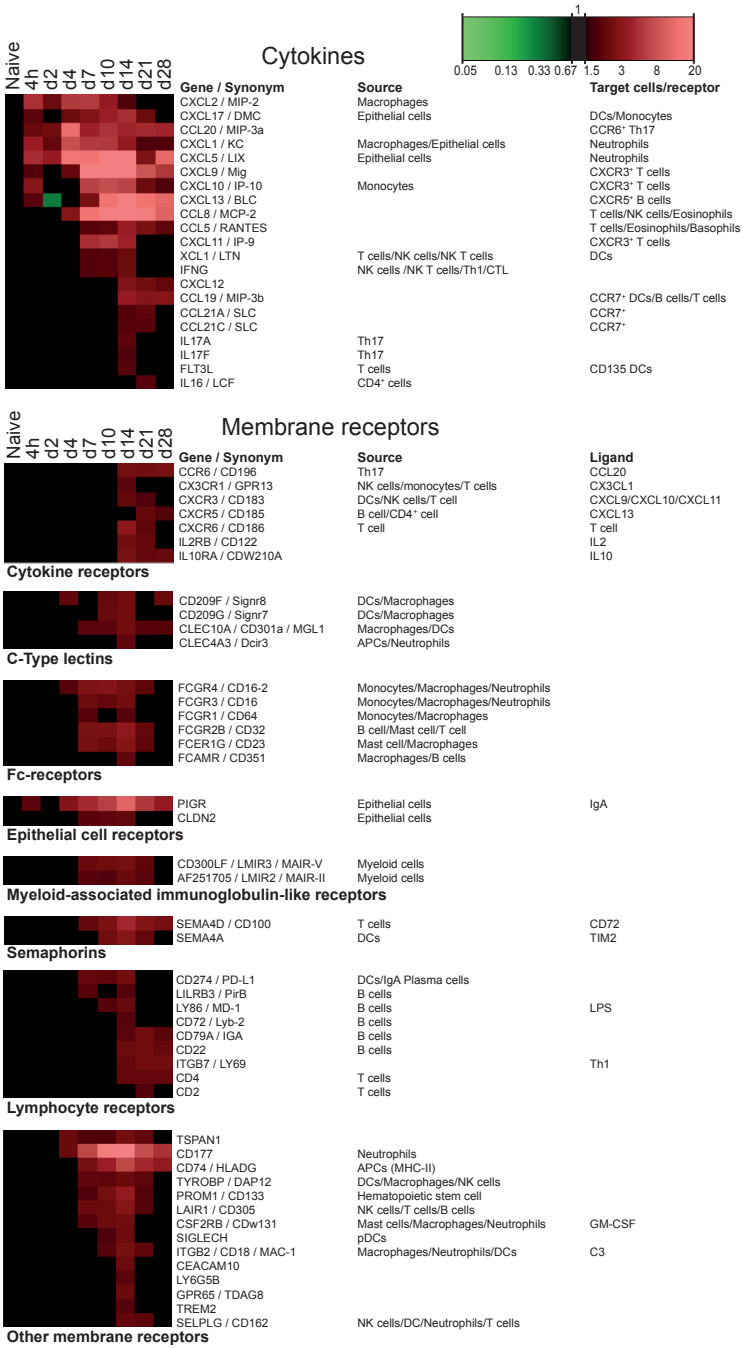

Figure 5 - Gene expression profiles of pulmonary cytokines and membrane receptors after *B. pertussis* infection. Results (mean of n=3) for cytokines and membrane receptors include expected source and known corresponding receptors, target cells and receptor ligands. Membrane receptors are divided in eight different groups according to function.

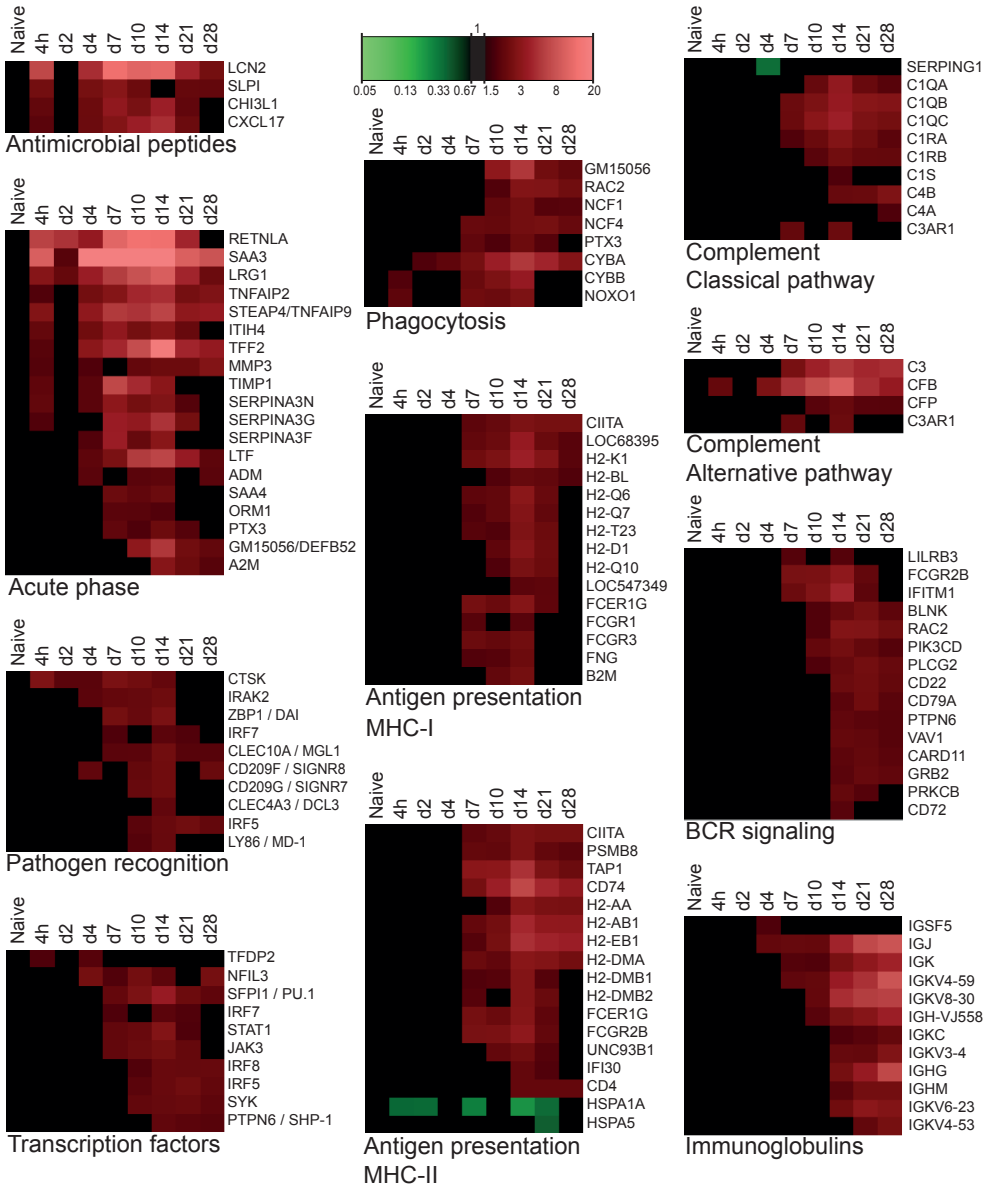

Figure 6 - Pulmonary gene expression representing immunological pathways as function of time after *B. pertussis* infection. Details on pulmonary gene expression profiles (mean of n=3) indicated expression of anti-microbial peptides, acute phase proteins, pathogen recognition, phagocytosis, both classical and alternative pathways of complement cascade, transcription factors, antigen presentation via both MHC-I and MHC-II, B-cell receptor (BCR) signaling, and immunoglobulin related genes.

Cytokine profiles in mouse sera

The concentrations of 33 cytokines were determined in mouse sera by ELISA and multiplex immunoassay (MIA). *B. pertussis* infection induced nine cytokines: CCL11, CCL20, CXCL9, CXCL10, G-CSF, IL-1α, IL-6, IL-9, and IL-17A (Figure 7). The pro-inflammatory cytokines, IL-1α and IL-6, are both involved in the acute phase response. The concentration of IL-1α was significantly increased 1 day p.i. Enhanced concentrations of IL-6 were detected at three different time points: 2 hours (not significant), 4 days, and 7 days p.i. (Figure 7A). Remarkably, IL-6 concentrations returned to basal level 6 hours p.i. Production of IL-1α by epithelial cells was most likely a result of LOS or tracheal cytotoxin (TCT) exposure[69]. The enhanced concentration of IL-6 observed 2 hours p.i. was most likely caused by lung-resident immune cells, such as macrophages and mast cells. These cells are the first to recognize bacterial infection[13]. Elevated concentrations of chemokines CXCL9 (MIG) and CXCL10 (IP-10) were measured between 14-28 days p.i., which suggests a Th1 response[70]. CCL11 (Eotaxin) was enhanced at 14 days p.i. (Figure 7E). Eotaxin is involved in allergic inflammation and is chemoattractive for eosinophils[71] and CCR3$^+$ Th2 cells[72]. IL-9 was increased 7 days p.i. (Figure 7F). IL-9 is mainly produced by T cells and originally associated with Th2 responses[73]. However, there is evidence that IL-9 is produced by a specific Th subset: Th9 cells[74] and Th17 cells[75]. A Th17 response was identified by markers, such as IL-17A, CCL20 (MIP-3a) and granulocyte colony-stimulating factor (G-CSF)[44]. Enhanced concentrations of IL-17A and CCL20 were determined in mouse sera 14 days p.i. (Figure 7G–H). CCL20 binds exclusively to CCR6, which is highly expressed on Th17 cells[42, 43]. G-CSF was produced between 4 and 28 days p.i. and is snown to be important for recruitment, differentiation and proliferation of neutrophils[76] and is linked to a Th17 response[77]. No significant changes in concentrations were detected for the other 24 cytokines. In conclusion, serum cytokine profiles indicate the induction of an acute phase response (IL-1α and IL-6) followed by a multi-flavored immune response with Th1 (CXCL9 and CXCL10), Th2 (CCL11 and IL-9) and Th17 signatures (G-CSF, CCL20, IL-9 and IL-17A).

Cellular composition of the spleen

The cellular composition of the spleen was examined (Figure 8A and S2) by flowcytometry with a panel of fluorescently labeled monoclonal antibodies binding to cell-specific membrane markers (Figure S3 and S4). Results indicate that in the naive situation, the majority of splenocytes consist of B cells and T cells (approximately 40% each). Upon *B. pertussis* infection, the ratio between B cells and T cells changed, since an increased percentage of T cells and decreased percentage of B cells was observed on day 2-4 p.i. However, this ratio was restored on day 7 p.i. The distribution of minor cell populations in the spleen, such as DCs, neutrophils and CD14$^+$ cells, changed over time upon *B. pertussis* infection. Here, we observed an early increased percentage of CD14$^+$ cells (day 1 p.i.) and DCs (day 1 and day 7-14 p.i.). Furthermore, a gradual increased percentage of neutrophils from day 4 until day 21 p.i. was observed. Importantly, enlargement of the spleen was observed during the course of infection (not quantified), suggesting influx or proliferation of cells. This might explain the observed changes in cellular distribution. In conclusion, cellular composition of the spleen indicates fluctuations over time upon *B. pertussis* infection.

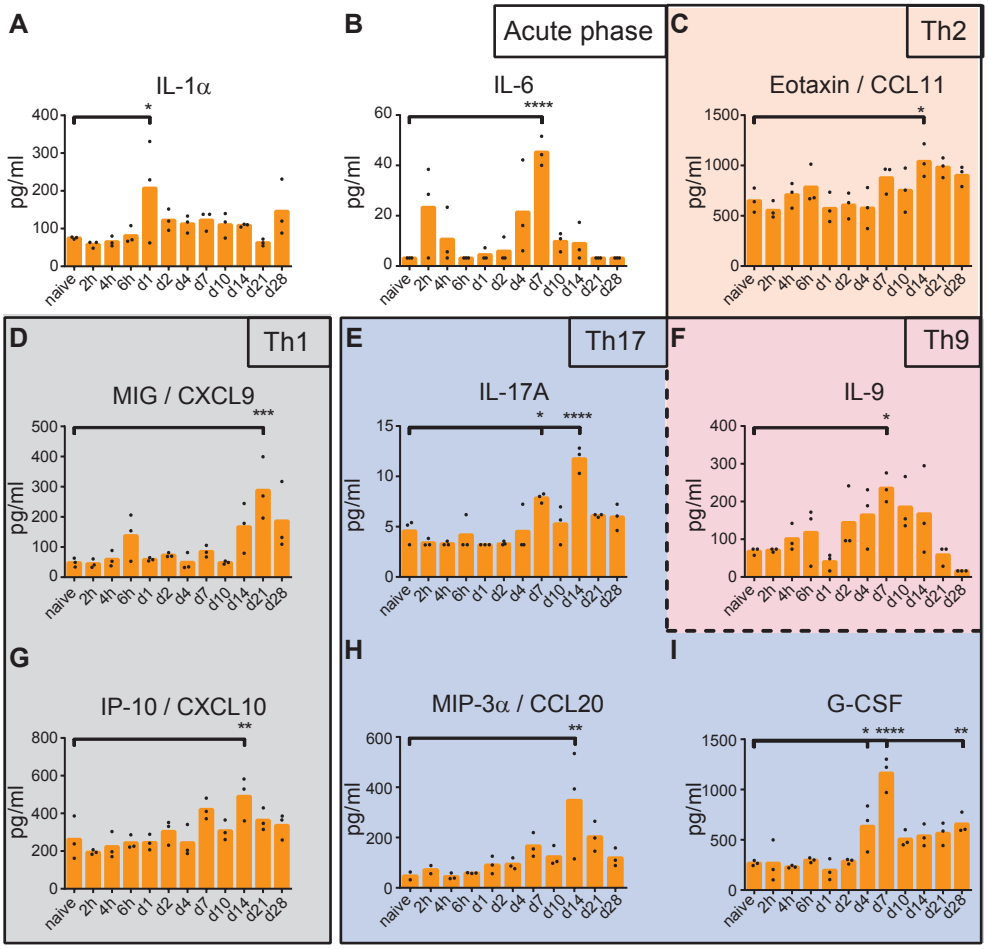

Figure 7 - Serum cytokine concentrations as function of time after *B. pertussis* infection. The concentrations of (A) IL-1α, (B) IL-6, (C) CCL11, (D) CXCL9, (E) IL-17A, (F) IL-9, (G) CXCL10, (H) CCL20, and (I) G-CSF in serum were analyzed over time. Results indicated that after the acute phase response (IL-1α and IL-6) a multi-flavored immune response with Th1 (CXCL9 and CXCL10), Th2 (CCL11 and IL-9), and Th17 (G-CSF, CCL20, IL-9, and IL-17A) was induced. Data represented as mean concentrations including individual values (n=3). p-values were determined by one-way ANOVA with multiple comparison compared to naive mice, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

Gene expression in the spleen

Gene expression in the spleen of infected mice was determined by microarray analysis. The study identified 798 genes of the whole genome that were differentially regulated (p-value≤0.001, FR≥1.5), of which 342 genes were upregulated, 425 genes were downregulated, and 31 genes that were initially downregulated and subsequently upregulated (Figure 8B). All involved genes are listed in Table S2. A limited set of genes showed altered gene expression between 4 hours and 7 days p.i. However, large changes in gene expression were observed 21 days p.i., while the gene expression was almost back

to the original values 28 days p.i. Only eight genes still showed altered expression. ORA of the 798 differentially expressed genes in the spleen revealed 66 GO-BP terms and two KEGG pathways. A selection of 16 terms and 2 pathways is presented in Figure 9, which are involved in transcription, cell cycle, chromatin assembly and a number of immunological processes. The corresponding proteins are involved in proliferation, differentiation or activation of immune cells in the spleen. Downregulated genes encode proteins that are involved in transcription, adaptive immune responses, antigen processing, antigen presentation and chemokine signaling (Figure 8B and 10). Downregulation of these genes suggests suppression of the immune response or migration of immune cells.

(1) Immune response. 41 immune response genes were identified (Figure 10), 13 of which are associated with the adaptive immune response. Interestingly, downregulation of genes involved in T cell activation, B cell activation, antigen processing and presentation, and the subsequent BCR signaling pathway were observed 21 days p.i. (Figure 10). Upregulation of genes encoding immunoglobulin heavy chains *Ighg*, *Igha*, and *Igh-vj558* indicate production of immunoglobulins IgG and IgA by B cells (Figure 10). Downregulation of the *Ighm* gene suggests a suppressed IgM production or a class-switch to IgG or IgA.

(2) Membrane receptors. Upregulation of genes encoding myeloid cell membrane markers, Fc-receptors and membrane attack complex (MAC) inhibitory proteins (*Cd59a*/*Cd59b*) suggests increased presence of myeloid cells, such as neutrophils, between 10 and 21 days p.i. (Figure 10). The induction of the MAC-inhibitory genes suggests inhibition of MAC formation, which is part of the complement cascade. In addition, BioGPS analysis (Figure S5) revealed the presence of myeloid cell markers that are expressed on neutrophils (*Trem3* and *Clec4e*) or macrophages (*Clec4e*). Downregulated gene expression of membrane markers of B cells (*Cd22*, *Cd72*, *Cd74*, and *Siglecg*) and T cells (*Sema4d*, *Tcra*) implies decreased presence of lymphocytes 21 days p.i. (Figure 10).

(3) Transcription. 95 genes that were differentially regulated in the spleen turned out to be involved in transcription (Figure 9), of which 70 genes were downregulated (data not shown).

(4) Cell cycle. Most of the genes involved in the cell cycle process (31 out of 45) were upregulated (Figure 10) which suggest increased cell replication between 10 and 21 days p.i. in the spleen.

(5) Regulation of apoptosis. Apoptosis was characterized by upregulation of 12 genes (between 7 and 21 days p.i.) and downregulation of 24 genes (21 days p.i.) (Figure 10). The role of apoptosis is especially interesting because of the recently described link betweenapoptotic markers and influenza vaccine responsiveness[78]. However, no overlap was found between the markers from the study of Furman et al. and the current study.

In summary, most prominent differences in gene expression in the spleen were found around 21 days p.i. These differences point to downregulation of immunological processes. After 28 days p.i. the expression profiles returned to basal level, at the same moment that the bacteria were almost cleared from the lungs. Interestingly, when combining cellular composition (Figure 8A) with gene expression profiles (Figure 8B and S5), the increase in neutrophils (10-21 days p.i.) was observed with both techniques. However, changes in the

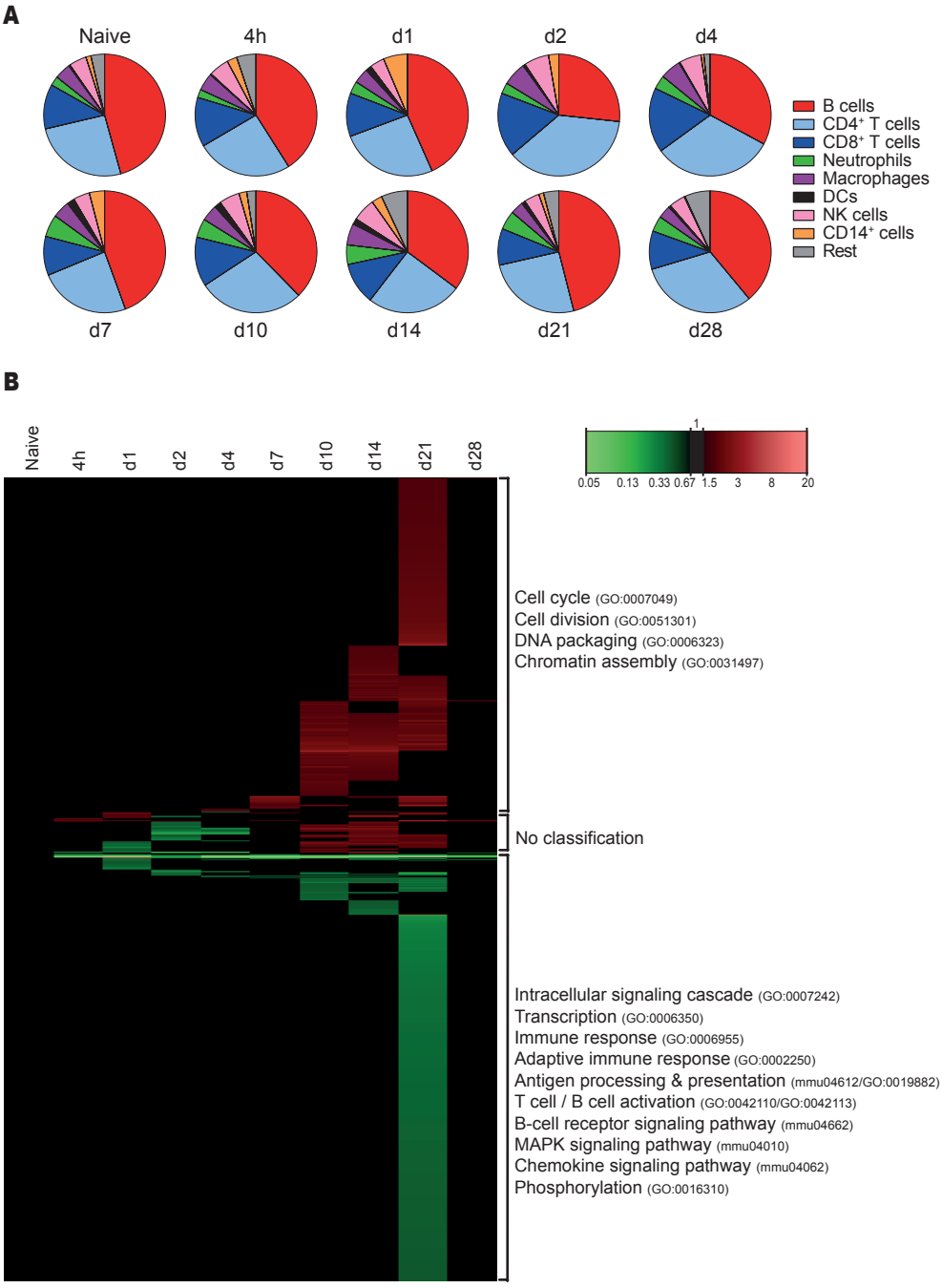
A
Naive
4h
d1
d2
d4
d7
d10
d14
d21
d28
B cells
CD4+ T cells
CD8+ T cells
Neutrophils
Macrophages
DCs
NK cells
CD14+ cells
Rest
B
Naive
4h
d1
d2
d4
d7
d10
d14
d21
d28
1
0.05
0.13
0.33
0.67
1.5
3
8
20
Cell cycle (GO:0007049)
Cell division (GO:0051301)
DNA packaging (GO:0006323)
Chromatin assembly (GO:0031497)
No classification
Intracellular signaling cascade (GO:0007242)
Transcription (GO:0006350)
Immune response (GO:0006955)
Adaptive immune response (GO:0002250)
Antigen processing & presentation (mmu04612/GO:0019882)
T cell / B cell activation (GO:0042110/GO:0042113)
B-cell receptor signaling pathway (mmu04662)
MAPK signaling pathway (mmu04010)
Chemokine signaling pathway (mmu04062)
Phosphorylation (GO:0016310)

Figure 8 - Splenic cellular composition and gene expression profiles as function of time after *B. pertussis* infection. (A) The distribution of the splenic cellular composition illustrated as pie charts at a selection of time points during the *B. pertussis* infection. FACS was used to determine the percentage (mean of n=3) of B cells ($CD19^+$), $CD4^+$ T cells, $CD8^+$ T cells, neutrophils ($Gr\text{-}1^+$), macrophages ($CD11b^+$), dendritic cells ($33D1^+$), NK cells ($DX5^+$), and $CD14^+$ cells in splenocytes. (B) Fold changes of expression were calculated compared to naive mice and significant gene expression results (p-value≤0.001, FR≥1.5) were visualized as a heatmap (mean of n=3). This test was performed for each gene at each time point. In total, 798 genes were differentially regulated (p-value≤0.001, FR≥1.5) compared to naive mice, divided in 342 upregulated (red) and 425 down-regulated genes (green). Additionally, a distinct group of 31 genes showed downregulation between 4 hours and 4 days p.i., but were upregulated between 10 and 21 days p.i. Genes not exceeding a fold change of 1.5 are depicted as basal level (black) at this time point. Results are divided in 3 clusters representing up/down-regulation and time of involvement and included corresponding GO-BP terms and KEGG pathways.

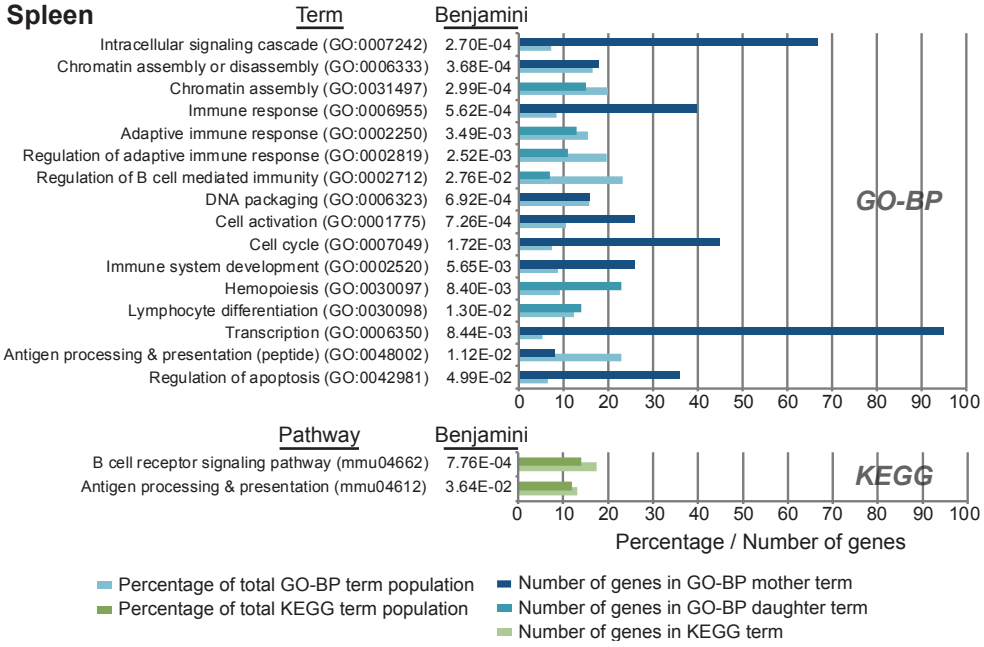

Figure 9 - Functional annotation of splenic gene expression data after *B. pertussis* infection. Over-representation analysis (Benjamini-corrected p-value≤0.05) of all 798 differentially expressed genes in the spleen in GO-BP and KEGG databases, This resulted in respectively 66 terms and 2 pathways significantly enriched of which a selection, based on immunological relevance and exclusion of overlapping terms, of 16 terms and 2 pathways are presented. GO-BP terms were divided in mother terms (dark blue) and corresponding daughter terms (light blue). For each term the amount of genes, the percentage of the total term population and the Benjamini-corrected p-value are shown.

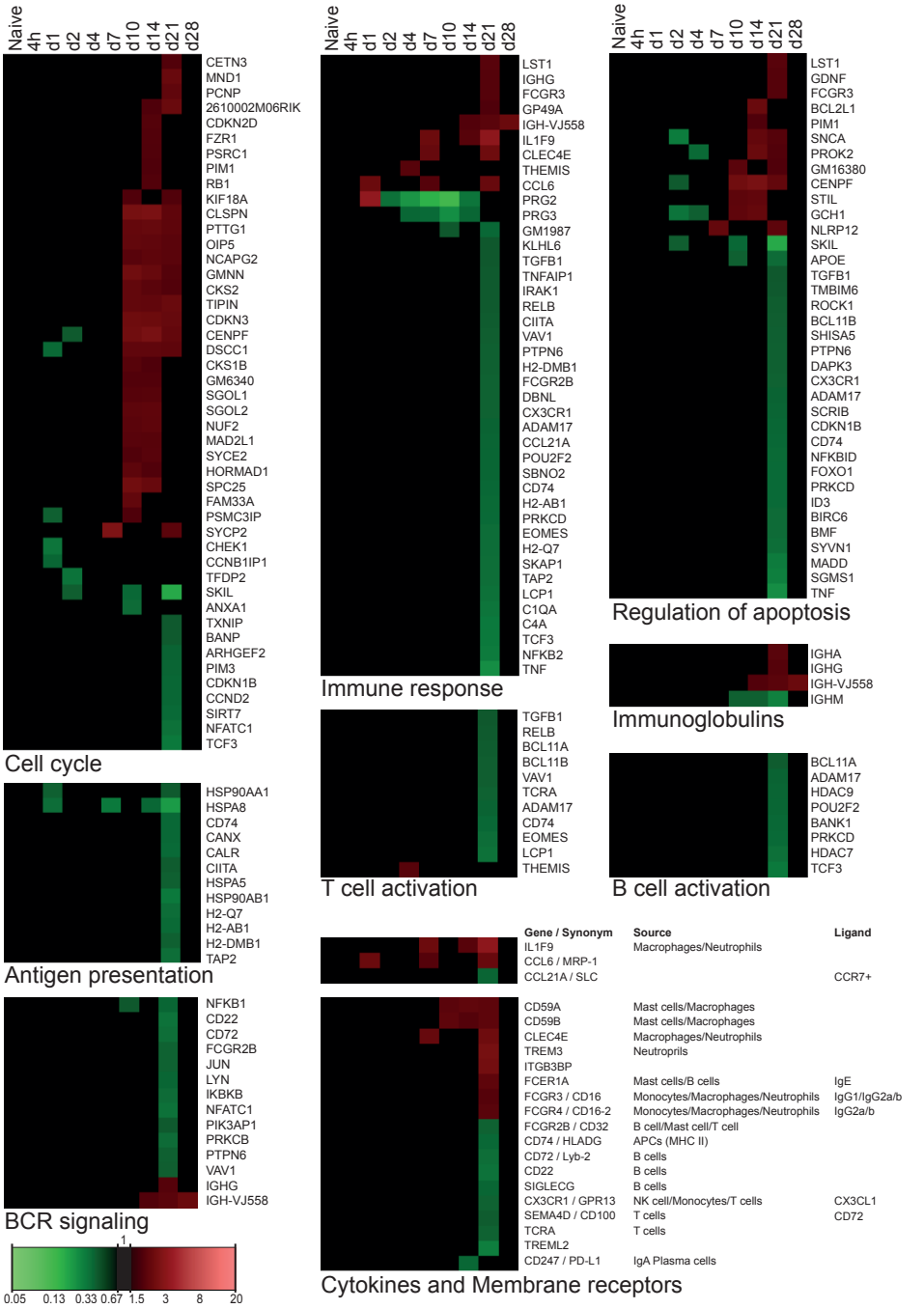

Figure 10 - Splenic gene expression profiles of specific processes as function of time after *B. pertussis* infection. Expression profiles (mean of n=3) are summarized for immune response, cell cycle, regulation of apoptosis, T cell activation, B cell activation, immunoglobulin, B cell receptor (BCR) signaling, antigen processing, cytokines and membrane receptors in spleen.

distribution of B cells and T cells on day 2 and 4 p.i. were not detected on gene expression level. Overall, these findings suggest that gene expression profiles cannot directly be translated in cellular fluctuations in the spleen.

Overlap of transcriptomic responses in lungs and spleen

A comparison of transcriptomic datasets was made to determine the related biological processes that occurred in both spleen and lungs (Figure S6). 72 genes were differentially expressed in spleen and lungs, which were divided in four groups: (i) 20 genes that were upregulated in both tissues, (ii) 42 genes that were upregulated in lungs and downregulated in the spleen, (iii) 2 genes that were downregulated in lungs and upregulated in the spleen, and (iv) 8 genes that were downregulated in both tissues. Fifteen genes of the second group were involved in the immune response, such as leukocyte activation, BCR signaling pathway, antigen processing and presentation, and chemotaxis. Remarkably, the immunoglobulin-mediated immune response was the only process upregulated in both tissues, suggesting local and systemic antibody production. In summary, both tissues showed activation of antibody-mediated immunity upon a *B. pertussis* infection. In addition, most differentially expressed genes representing immunological processes were observed upregulated in the lungs and downregulated in the spleen.

B. pertussis-specific IgM, IgG and IgA antibody responses

Antibody isotypes, IgM, IgG, and IgA antibodies specific for *B. pertussis* were analyzed for a period of 28 days p.i. The formation of IgM against *B. pertussis* antigens present in outer-membrane vesicles (OMV) was not observed during the course of infection (Figure 11A). Contrarily, anti-*B. pertussis* IgG in mouse sera was detectable from 10 days p.i. and the responses were progressively increased until 28 days p.i. (Figure 11B). Notably, no IgG titers were detectable against the individual purified *B. pertussis* antigens pertactin (Prn), pertussis toxin (Ptx), filamentous hemagglutinin (FHA), or fimbria type 2 and 3 (Fim2/3) (Figure S7). However, anti-OMV IgG was significantly increased at 14, 21, and 28 days p.i. (Figure 11C) and consisted of IgG1, IgG2a, and IgG2b subclasses, but not IgG3 (Figure 11D-G). Remarkably, IgG1 levels dropped significantly at 28 days p.i. compared to 21 days p.i. In addition, significantly increased anti-OMV IgA responses were measured in sera (Figure 11H) as well as in lung lysates (Figure 11I) 28 days p.i. In conclusion, intranasal infection leads to a broad spectrum of systemic antibody subclasses against outer membrane proteins of *B. pertussis* within 14 days p.i., but responses against purified virulent antigens Ptx, Prn, FHA and Fim2/3 remain undetectable until 28 days p.i. Moreover, *B. pertussis*-specific anti-OMV IgA antibodies were secreted in the lungs.

$CD4^+$ T cell responses after *B. pertussis* infection

Cytokine profiles of antigen-specific memory $CD4^+$ T cells in the spleen were determined 66 days p.i. Splenocytes of naive and infected mice were stimulated for 8 days with Ptx, FHA or Prn, important virulence factors present in the acellular pertussis vaccine. Cytokine profiling on single cell level was established by using intracellular cytokine straining (ICS), while cytokine concentrations in splenic culture supernatants were determined by using a MIA.

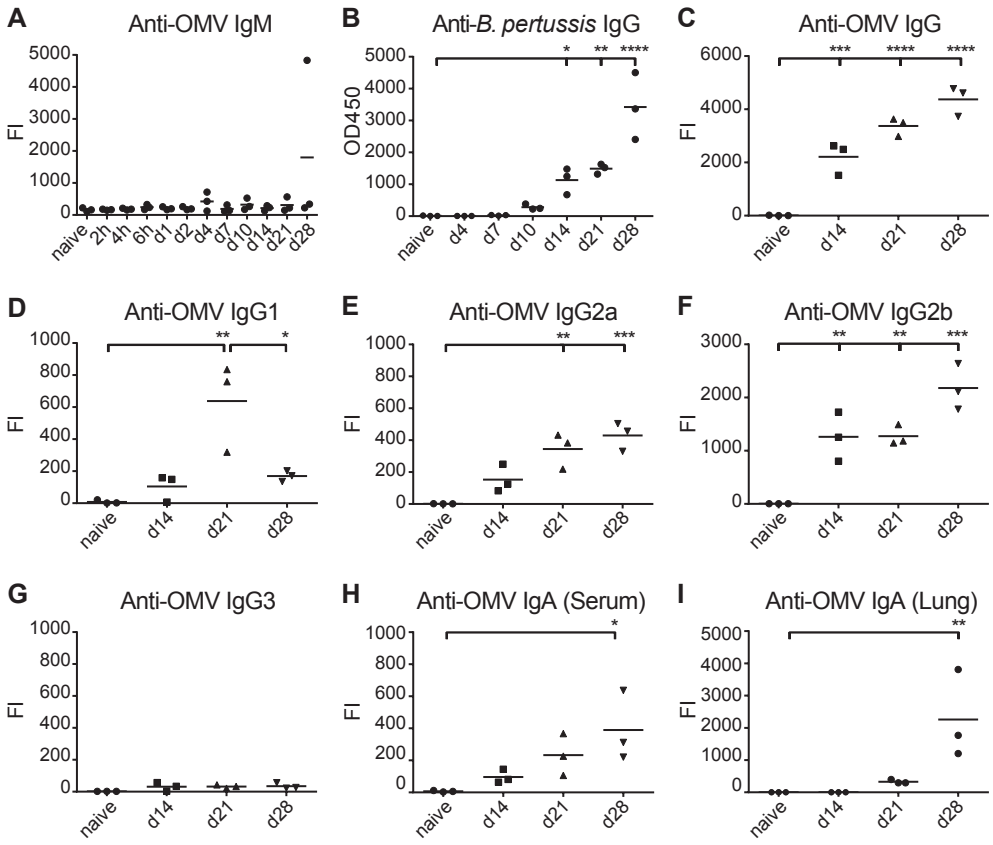

Figure 11 - Antibody profiling after *B. pertussis* infection. Antibody responses were determined in mouse sera after intranasal infection by using a whole-cell *B. pertussis* ELISA or MIA and results were expressed as OD450 or fluorescence intensity (FI), respectively (mean of n=3). (A) IgM antibodies against OMV B1917 were absent. (B) Whole-cell *B. pertussis* ELISA indicated IgG antibody formation 10-14 days p.i., which increased until day 28 days p.i. (C-G) Levels of total IgG antibodies and individual subtypes (IgG1, IgG2a, IgG2b, and IgG3) against OMV B1917 indicated presence of multiple subclasses. (H) Anti-OMV IgA antibodies in serum were induced after 14-28 days p.i. (I) In lung lysates, anti-OMV IgA antibodies were detected 14 and 28 days p.i. p-values were determined by one-way ANOVA with multiple comparison compared to naive mice. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

CD44 expression was used to focus on activated $CD4^+$ T cells. Results of ICS indicate a significant increase in IFNγ- and IL-17A-producing Prn-, FHA-, and Ptx-specific $CD4^+CD44^+$ T cells after infection (Figure 12A and B). Except for FHA-stimulation, no evidence was found for specific IL-5-producing $CD4^+CD44^+$ T cells (Figure 12C). Cytokine profiles in culture supernatant revealed a significant increase in TNFα production by splenocytes of infected mice after FHA stimulation and IL-17A production after Prn, Ptx, or FHA stimulation (Figure 12D-F). No changes were observed in IFNγ production in culture supernatants (Figure 12D–F). Notably, FHA stimulation induced besides Th1 and Th17 cytokines an increase in IL-5, IL-13, IL-4, and IL-10 production by splenocytes of infected mice (Figure 12E). IL-5 and IL-13 production occurred also after Prn stimulation (Figure 12D).

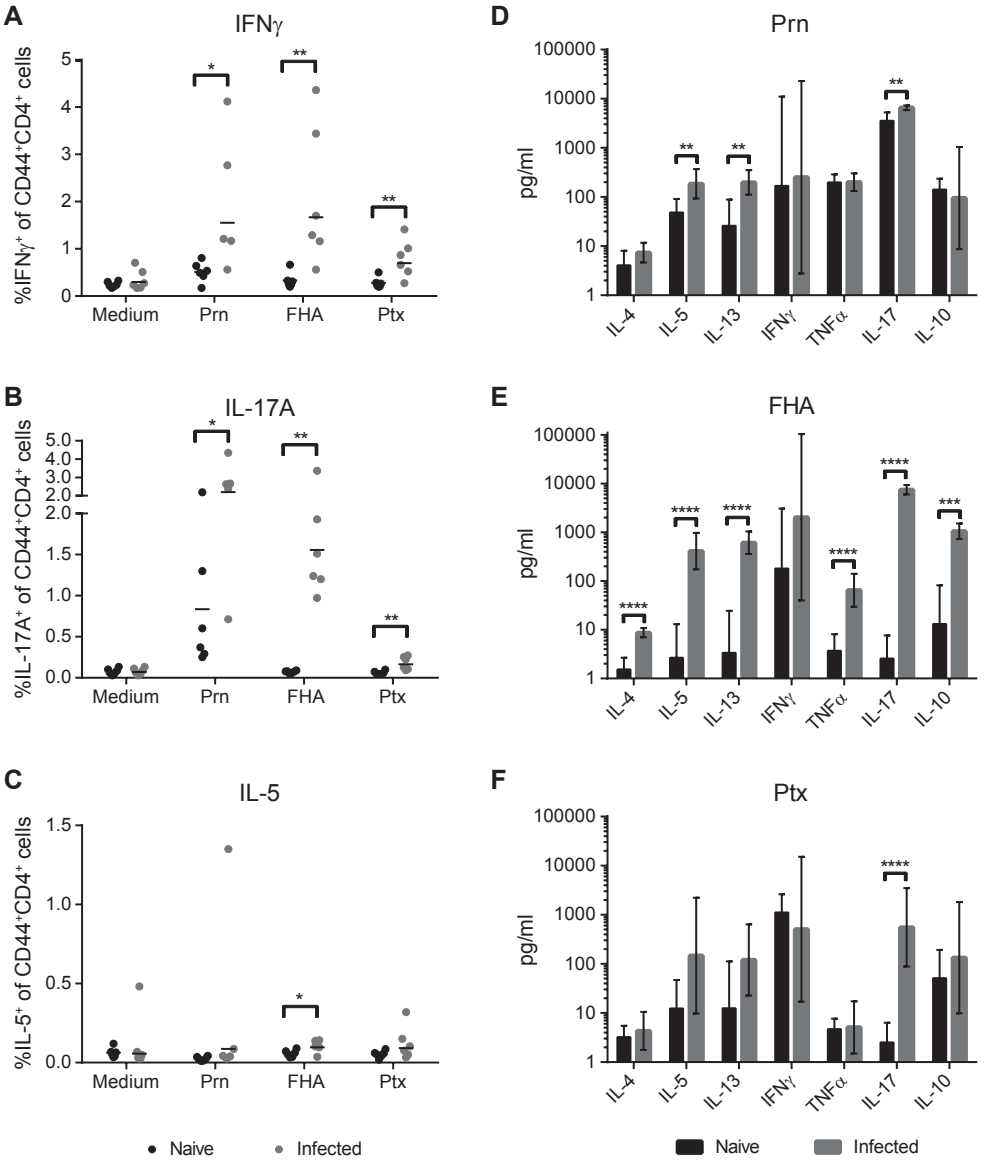

Figure 12 - Systemic memory Th cytokine profiles. (A-C) Splenocytes of naive and infected mice were collected 66 days p.i. and *in vitro* restimulated with Prn, FHA, or Ptx for 8 days. The percentages of IFNγ-, IL-17A-, and IL-5-producing $CD4^+CD44^+$ T cells were determined using ICS (mean of n=6). (D-F) Cytokine levels in supernatant after 7 days of stimulation were determined by using a MIA. Results (mean of n=6) are corrected for the background level in the presence of medium as control. Statistical differences between the groups were detected for the ICS with a non-parametric Mann-Whitney test and for the MIA with a Student t-test on the log-transformed data. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

This might indicate that these three *B. pertussis* antigens induce a mixed Th response, of which Th1 and Th17 are the major components. While ICS specifically focuses on $CD4^+CD44^+$ T cells, multiple cell types can produce the accumulated cytokines present in the splenic culture supernatants after stimulation. The production of IL-17A is exclusive for Th17 cells[79]. Besides Th1 cells, IFNγ could also be produced by CTLs and NK cells. The high levels of IFNγ produced by splenocytes of naive mice after Ptx stimulation may be explained by TLR4 activation on innate cells by Ptx[80]. A high background of Prn-responding $IL\text{-}17A^+CD4^+CD44^+$ T cells and IL-17A in supernatant was seen in two out of six naive mice, indicating Prn-activated IL-17A production in unprimed splenocytes. In summary, $CD4^+$ memory T cell analysis revealed that infection with *B. pertussis* primarily generates specific Th1 and Th17 responses, regarded essential in protection against a *B. pertussis* infection[12, 20, 21].

Discussion

An integrated systems biology approach was applied to investigate the evolving immune response of mice following a primary intranasal *B. pertussis* infection, considered capable of inducing sterilizing immunity. Data sets from individual analytical platforms, such as microarray, flow cytometry, multiplex immunoassays, and colony counting, addressing different biological samples taken at multiple time points during the immune response were merged. Altogether, a comprehensive overview of immunological processes occurring upon primary infection of mice with *B. pertussis* was constructed in three consecutive phases, as shown in Figure 13: (i) the innate phase, (ii) the bridging phase, and (iii) the adaptive phase.

Highlights featured in this study are: (i) a protracted acute phase response, as part of the innate phase, lasting for at least 28 days, which co-occurred with prolonged antigen exposure; (ii) the recognition of several *B. pertussis* pathogen-associated molecular patterns (PAMPs), i.e. LOS, DNA, and GalNac, (iii) early signatures for recruitment of adaptive immune cells e.g. the cytokine CCL20 that attracts $CCR6^+$ Th17 cells, (iv) expression of specific membrane markers (e.g. DAP12) and transcription factors that determine the strength and direction of the adaptive immune response. Furthermore, features of adaptive immunity consisted of: (v) IgA secretion in the lungs; (vi) a broad systemic antibody response; (vii) a Th1- and Th17-mediated response in the lungs and systemic formation of specific memory Th1 and Th17 cells.

Various signatures, associated with the induced protective immune response as presented in Figure 13, are discussed in detail hereafter. The innate phase was characterized by the presence of AMPs, acute phase proteins and chemotactic cytokines as detected by microarray analysis and MIA (Figure 5-7). Expression of the involved genes is most likely initiated by activation of TLR2 and TLR4 by LOS of *B. pertussis*[8, 10]. Upregulated gene expression of four AMPs (*Chi3l1*, *Cxcl17*, *Lcn2,* and *Spli*) was found in the lungs during the whole course of infection. These AMPs contribute to innate protection as was shown for other respiratory pathogens[48-50, 52]. In addition, these AMPs have the capacity to recruit immune cells, such as neutrophils, DCs, and monocytes[47, 51] and are therefore important for the induction of subsequent immune responses[46].

Furthermore, the expression of particular cytokines on gene and protein level in the innate phase (Figure 5 and 7), in combination with increased gene expression of cytokine receptors and the change in cellular composition of the spleen (Figure 5 and 8A), gave detailed insight into the immunological processes during the course of infection, as depicted in Figure 13. For example, the gene expression levels of the cytokine *Cxcl1*, *Cxcl2*, *Cxcl5*, and *Cxcl17* was in agreement with the recruitment of neutrophils and DCs towards the site of infection[51, 81, 82]. Furthermore, the gene expression of the cytokines *Cxcl9*, *Cxcl10*, *Cxcl11,* and *Ccl20* was increased early after infection. CXCL9, CXCL10, and CXCL11 are known to be involved in the recruitment of $CXCR3^+$ Th1 cells, and CCL20 in the recruitment of $CCR6^+$ Th17 cells[42, 43, 70]. The upregulation of *Ccr6* and *Cxcr3* genes was observed at day 14 p.i. in the lung (Figure 13).

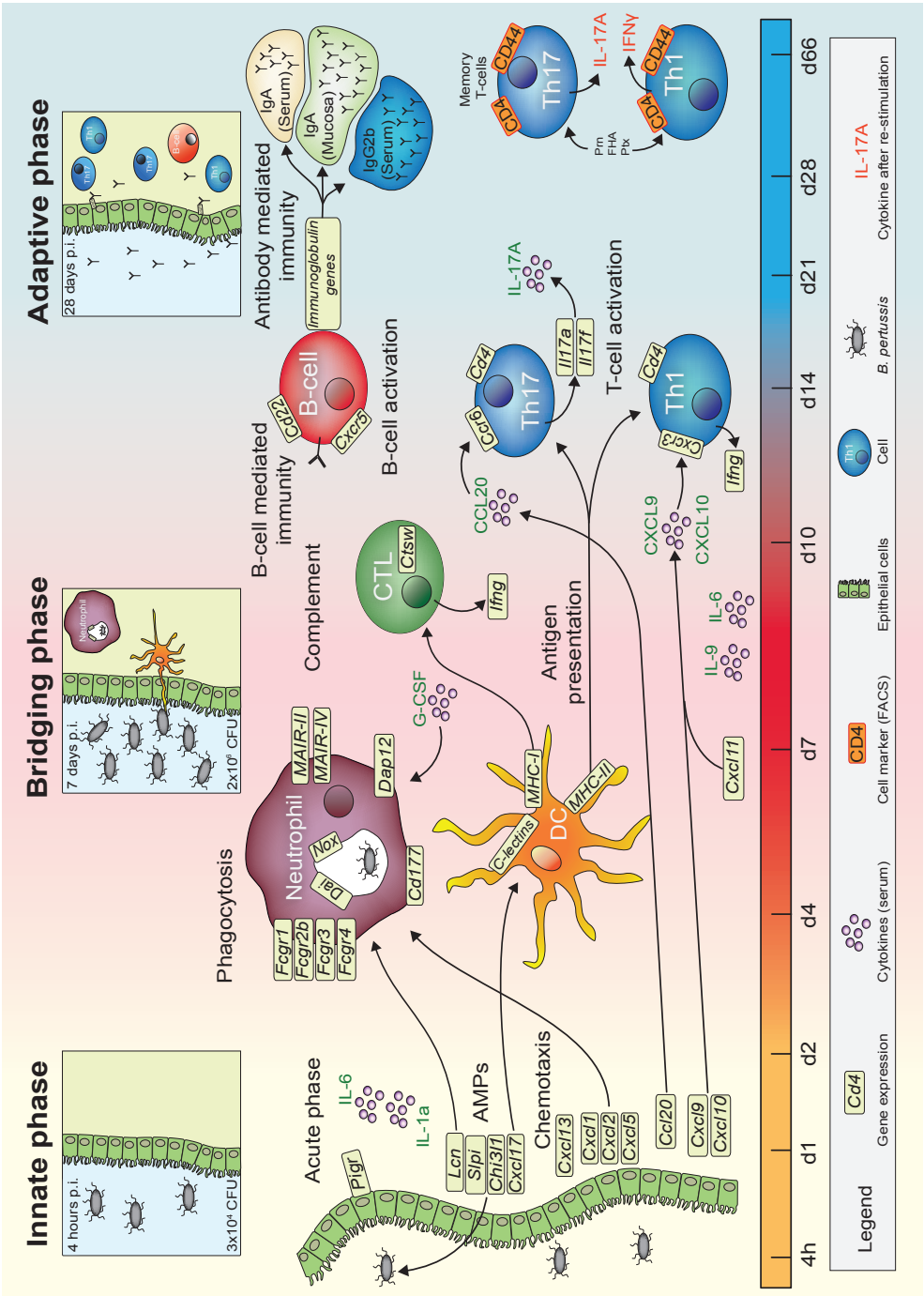
Innate phase
Bridging phase
Adaptive phase
4 hours p.i.
3x10⁴ CFU
7 days p.i.
2x10⁷ CFU
28 days p.i.
Neutrophil
Th1
Th17
B-cell
Acute phase
IL-6
IL-1a
Pigr
Lcn
Slpi
Chi3l1
Cxcl17
AMPs
Chemotaxis
Cxcl13
Cxcl1
Cxcl2
Cxcl5
Ccl20
Cxcl9
Cxcl10
Phagocytosis
Fcgr1
Fcgr2b
Fcgr3
Fcgr4
Neutrophil
MAIR-II
MAIR-IV
Dap12
Dal
Nox
Cd177
G-CSF
C-lectins
MHC-I
DC
MHC-II
Cxcl11
IL-9
IL-6
CTL
Ctsw
Ifng
Complement
Antigen presentation
B-cell mediated immunity
Ccr2
B-cell
Cxcr5
B-cell activation
Antibody mediated immunity
Immunoglobulin genes
IgA (Serum)
IgA (Mucosa)
IgG2b (Serum)
CCL20
Ccr6
Cd4
Th17
Il17a
Il17f
IL-17A
T-cell activation
CXCL9
CXCL10
Cxcr3
Th1
Ifng
Memory T-cells
CD4
CD44
Th17
IL-17A
Pm
FHA
Ptx
Th1
IFNγ
4h
d1
d2
d4
d7
d10
d14
d21
d28
d66
Legend
Gene expression
Cytokines (serum)
Cell marker (FACS)
Epithelial cells
Cell
B. pertussis
IL-17A
Cytokine after re-stimulation

Figure 13 - Overview of immune response evolving after *B. pertussis* infection. Merging data from a wide array of functional analyses reveals a comprehensive overview of the immune response of mice upon *B. pertussis* infection. (i) Innate phase, (ii) bridging phase and (iii) adaptive phase are indicated in the response facing variable levels of *B. pertussis* present in the initial inoculum, the exponential growth phase, and in the clearance phase, respectively. Highlights of the study are given, based on lung clearance, relative gene expression levels (lungs), cytokine profiles (serum), cellular response (spleen) and *B. pertussis*-specific responses in serum and spleen as depicted in the figure legend.

Remarkably, gene expression of cytokines, acute phase proteins, and AMPs was temporary suppressed at 2 days p.i. Simultaneously, the proliferation of bacteria started in the lungs. These results indicate temporary suppression of the immune system by *B. pertussis*. Several immune evasion strategies of *B. pertussis* have been described in literature[83-87]. The evasion is attributed to virulence factors, such as adenylate cyclase toxin (ACT), Ptx, TCT, and FHA. These investigators demonstrated enhanced production of IL-10 and regulatory T cells, delay of neutrophil recruitment, and inhibition of early chemokine production in mice in relation to secretion of *B. pertussis* virulence factors. In the present study, these immunosuppressive properties of *B. pertussis* might explain the suppression of the differential gene expression observed 2 days p.i. In addition, secretion of OMVs occurs during bacterial colonization[88]. These OMVs are thought to serve as decoy for the host immune response and their immunomodulatory properties support survival of the bacteria[89, 90].

During the bridging phase, the number of bacteria in the lungs increased (Figure 1). The highest number of bacteria in the lungs was found at 7 days p.i. followed by a decrease, suggesting that day 7 is the turning point in the progress of the infection. Simultaneously, microarray analysis suggests the presence of neutrophils, macrophages, and DCs in the lungs based on BioGPS analysis (Figure S1). These cells are likely responsible for (i) phagocytosis, (ii) pathogen recognition, (iii) antigen presentation, and (iv) differentiation by expression of cell receptors in the period between 4–10 days p.i. (Figure 13).

The innate immune cells appeared to cause the initial decrease in numbers of bacteria in the lungs from 7 days p.i., because a strong antibody response (IgM, IgG, IgA) (Figure 11) and $CD4^+$ T cell response were not yet present. Based on gene expression of neutrophil markers, the neutrophil invasion in the lungs probably occurred from 4 days p.i., which is observed on cellular level by McGuirk *et al.*[91]. This influx of cells is most likely orchestrated by IL-17A and G-CSF[76, 77, 79]. In the present study, both cytokines were enhanced in the sera of infected mice. Furthermore, genes involved in phagocytosis were found to be upregulated (Figure 6) which is important for protection against *B. pertussis*[65, 92].

This study demonstrated also that *B. pertussis* infection activates different pattern-recognition receptor (PRR) pathways, such as TLR4, TLR9, DAI, and several CLRs (Figure 6). Bacterial ligands, e.g. LOS, DNA, and GalNAc trigger these signaling pathways[8, 55, 57]. Upregulation of genes involved in TLR4-signalling (*Md-1*, *Irf5*) is probably caused by detection of bacterial LOS[8, 10, 53]. Bacterial DNA recognition occurred through the TLR9 pathway (*Ctsk*, *Irf7*) and the cytosolic DNA sensor (*Dai*). The receptor DAI recognizes both host DNA, upon tissue

damage, and bacterial DNA, which leads to an innate immune response[55]. Furthermore, increased gene expression of the CLRs, *Signr7*, *Signr8*, and *Mgl1* was observed, which are predominantly expressed on DCs[38, 93]. The enhanced expression of mouse DC-SIGN-related genes *Signr7* and *Signr8* during the *B. pertussis* infection is a novel finding. Despite indications of sensing of 6-sulfo-sialyl Lewis(x) oligosaccharide by Signr7, the exact ligands and role of these receptors remains to be discovered[56]. Recognition of GalNAc via Mgl1 has recently been associated with mast cell activation by *B. pertussis*[57]. An important conclusion is that ligands of *B. pertussis* activate multiple PPRs during the infection. This may be important for the induction of a broad adaptive immune response, including Th1 as well as Th17 cells, mucosal IgA and systemic IgG.

During the bridging phase, upregulation of MHC class I and II genes were measured, enabling antigen presentation to both $CD4^+$ Th cells and cytotoxic $CD8^+$ T cells (CTLs). Several studies focused on the role of Th cells because of their important contribution to protect against *B. pertussis*[12, 20, 21]. Nevertheless, the CTLs seem also important for protection against *B. pertussis*, indicated by CTL infiltration, based on upregulation of *Ctsw,* in lungs in our study by formation of FHA-specific IFNγ-producing CTLs upon *B. pertussis* infection in other studies in mice and humans[91, 94].

In addition, the bridging phase was characterized by simultaneous gene expression of several receptors on innate immune cells in the lungs and spleen. Interestingly, this included MAIRs, TREMs, semaphorins, and DAP12, which is the first evidence that these receptors might play a role in *B. pertussis* infection. Especially DAP12, which is abundantly expressed in lungs, interacts with other membrane receptors and influences cytokine expression[95]. Coupled to MAIR-II, DAP12 is an inhibitor of B cell responses[96]. Together with TREM2, DAP12 downregulates TLR and FcR expression[97]. Overall, these former receptors function as an important bridge between innate and adaptive immune responses, determining the strength and direction of the adaptive immune response by the successive activation of lymphocytes, such as CTLs, Th cells, and B cells[39, 98-101]. Since the exact role of these receptors in the protection against *B. pertussis* remains unresolved, additional research is needed to find whether targeting of these receptors might be a viable strategy to elicit desired immune responses. In summary, the bridging phase suggests the presence of APCs and myeloid cells, such as DCs and neutrophils based on gene expression of membrane markers. However, this was not validated by histological data.

Finally, the adaptive phase was recognized by the formation of T and B cell responses and a broad antibody response (Figure 13). In this same period, the naive mice were able to clear *B. pertussis* from their lungs. Gene expression, cytokine secretion and cellular analysis revealed presence of activated $CD4^+$ T cells. The highest number of $CD4^+$ T cells was detected at 14 days p.i., which is in agreement with a study showing the largest influx of Th17 cells in the lungs after *B. pertussis* infection at 14 days p.i.[12]. Phenotyping of $CD4^+$ T cells, based on cytokine profiles in serum (protein) and lung (genes) and expression of specific transcription factors or membrane markers, suggests a broad Th response in the present

study. The Th1 response in the lungs was characterized by *Ifng* and *Stat1* expression[102]. Gene expression of the mucosal homing receptor *Ccr6*, *Card11*, *Ccl19*, *Ccl21a*, *Ccl21c*, and signature cytokines *Il17a* and *Il17f* might indicate Th17 cells in the lungs[42, 79, 103-106]. Upon re-stimulation of splenocytes with purified *B. pertussis* antigens, Prn- and FHA-specific Th1 and Th17 type $CD4^+$ T cells could be detected in the memory phase (66 days p.i.) (Figure 12). In addition, enhanced IL-9 levels in serum suggest activation of Th9 cells and Th17 cells as these cells produce large amounts of IL-9[74, 75]. However, Tregs, NKT cells and mast cells are also sources of IL-9. In summary, the different phases of T cell development were mapped; the expansion phase of naive T cells in the spleen (2 days p.i.), the effector phase in the lungs (14 days p.i.), contraction phase in the spleen (21 days p.i.) and finally the specific memory T cells (66 days p.i.) (Figures 5, 8A, 10 and 12). Data from this study suggests generation of local specific Th1/Th17 cells in the lungs. Th1 cells, which produce IFNγ are important for clearance of *B. pertussis*. Postponed clearance was shown in IFNγ-depleted mice[107]. Th17 cells have been indicated as key cells to control bacterial infections at mucosal sites[108]. Recent studies revealed that Th17 cells increase the clearance of *B. pertussis* after an intranasal infection in animals[12, 13, 109].

Infection with *B. pertussis* induces also B cell and antibody mediated immunity. Microarray analysis showed increased gene expression of the BCR in the lungs. In addition, gene expression of IgM, IgG, and IgA was observed in lungs and spleen. Whereas, most genes for antibody isotypes were found upregulated in both tissues, IgM was downregulated in the spleen (Figure 6 and 10). This was confirmed by the detection of IgA and IgG (IgG1, IgG2a, and IgG2b) antibodies after 14 days p.i. However, IgM antibodies were not found systemically (Figure 11). In mice, IgG1 is associated with a Th2-like response, while IgG2a (in Balb/c mice) and IgG2b suggest induction of a Th1 response[110]. IgG2b is also linked to Th17 lymphocytes[111]. All antibody responses in the present study were directed against whole-cell *B. pertussis* and outer membrane vesicles, but not against the *B. pertussis* antigens that are typically present in acellular vaccines: Ptx, Prn, FHA, and Fim2/3. However, memory Th1 and Th17 cells were detected upon re-stimulation with Prn and FHA (Figure 12). The polymeric immunoglobulin receptor (pIgR) was highly expressed during the whole course of *B. pertussis* infection in the lungs and pIgR is essential for the transport of IgA into the mucus and is seen as a bridge between innate and adaptive mucosal responses[112, 113]. The expression of pIgR was drastically enhanced 14 days p.i., leading to secretion of mucosal IgA in the lungs (Figure 5 and 11). Interestingly, the increased pIgR expression coincided with IL-17A production in the lungs and sera (Figure 5 and 7), which is in agreement with the finding that Th17-mediated responses influence the local humoral response by inducing pIgR expression and elevating secretory IgA levels[114]. Therefore, the transport of IgA to the mucosa, which is orchestrated by IL-17A via the induction of pIgR, supports an important role for local immunity. In summary, *B. pertussis* infection induces a broad humoral response, which was observed by gene and protein expression.

Our study also contains several conceptual points for discussion. First, altered gene expression in the spleen caused by *B. pertussis* infection was modest, except for 21 days

p.i. Two possible reasons can be given: (i) the infection initiated a local immune response in the lungs and (ii) gene expression is required to be rather strong to exceed the significance threshold. After the lungs and the draining lymph nodes, the spleen is the third organ in line involved in the generation of the immune response. Therefore, gene expression effects could be less abundant. Furthermore, the spleen consists of many cell types with different gene expression profiles, so changes in expression levels in a single cell type might not have been detected. Cell sorting of individual cell types and subsequent gene expression profiling could overcome this problem. Second, specific antibody and T cell responses were only measured for a limited set of available purified antigens. The inclusion of other antigens, such as outer membrane proteins, would perhaps have allowed a more detailed analysis. Third, in this study we propose murine infection-induced immune signatures as a benchmark to be used in the development of improved human pertussis vaccines. Since *B. pertussis* is not a natural pathogen for mice, translation of the results obtained in this study to the human situation remains to be interpreted with caution. Nevertheless, using a (naive) murine model has several advantages. It is regarded an important small animal model for pertussis vaccine development[115] and enables to dissect the local and systemic immune response in great detail, as shown in this study, rather than using blood or nasal washes from humans undergoing a *B. pertussis* infection. Furthermore, the role of individual gene signatures and products could be assessed in future research in mice by using knock-in or knock-out strains, or by functional interventions. Encouraging is that many observations made in this study, such as the rapid clearance upon reinfection and involvement of Th17 and Th1 responses, are in line with recent studies on *B. pertussis* infection in baboons, which resemble the human situation more closely[13, 15]. Translation of biomarkers from mouse to man however should be performed with care.

Overall, our study describes the molecular and cellular sequence of events that eventually lead to the induction of protective immunity to *B. pertussis*, including local immunity in the lungs, which is not induced by current vaccines. Especially the inductions of Th1 and Th17 cells and IgA antibodies in the lungs are regarded key elements in superior immunity. Our data can be used to select vaccine concepts that resemble infection, with respect to e.g. administration route and prolonged antigen exposure, and immune signatures elicited. Local delivery of vaccines may be an important advancement for improved protection as was previously demonstrated with a live attenuated pertussis vaccine[116]. Also adjuvants could be selected and optimized that can steer host immunity of inactivated vaccines towards an infection-like immune response with minimal adverse effects. The molecular and cellular fingerprint of the local immune response discovered in *B. pertussis* infected mice thus can provide a strong guidance for developing improved pertussis vaccines.

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Supplementary data

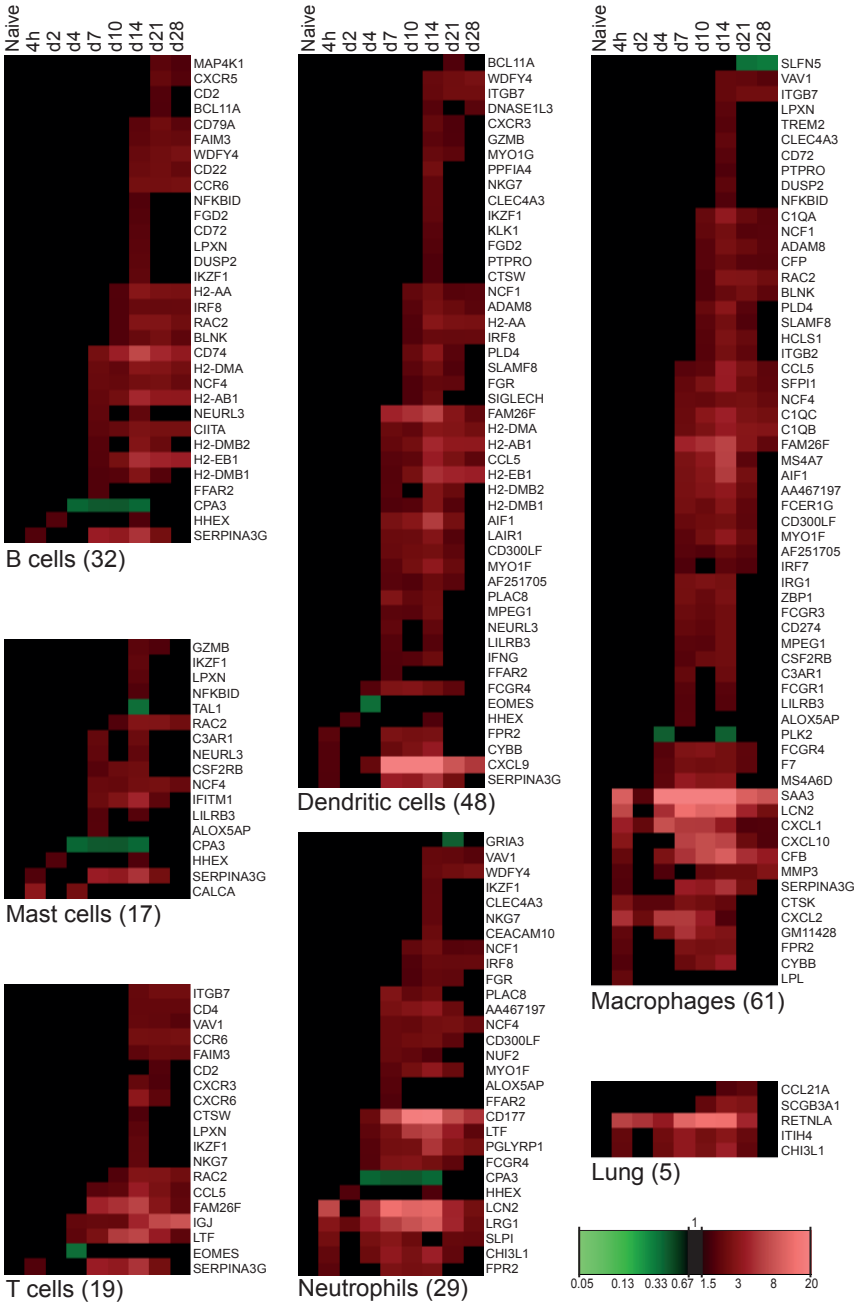

Figure S1 - Cell type comparison analysis for lung gene expression. Data (mean of n=3) represent gene profiles in the lung per cell type or tissue extracted from BioGPS. Results from B cells, mast cells, T cells, dendritic cells, neutrophils, and lung-specific genes are depicted.

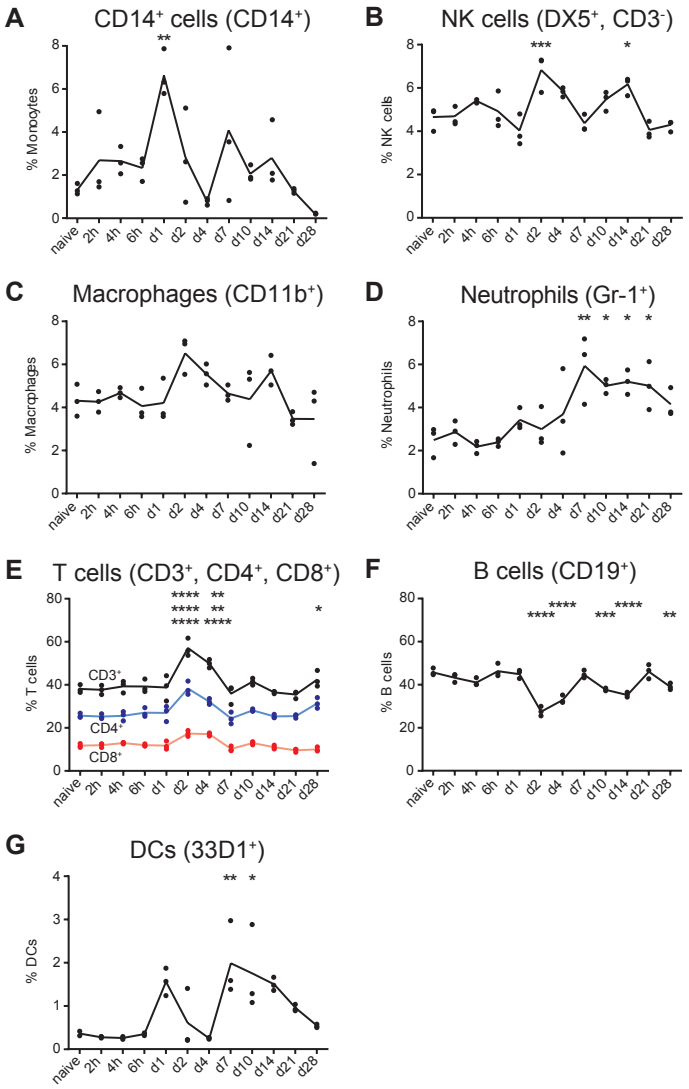

Figure S2 - Cellular composition of the spleen as function of time after *B. pertussis* infection. The percentage of (A) $CD14^+$ cells ($CD14^+$), (B) NK cells ($DX5^+$), (C) macrophages ($CD11b^+$), (D) neutrophils ($Gr\text{-}1^+$), (E) T cells ($CD3^+$, $CD4^+CD8^-$, and $CD8^+CD4^-$), (F) B cells ($CD19^+$), and (G) dendritic cells ($33D1^+$) in splenocytes were analyzed over time. p-values were determined by one-way ANOVA with multiple comparison compared to naive mice. *p<0.05, **p<0.01, ***p< 0.001, and ****p<0.0001 (mean of n=3). An increased percentage of $CD14^+$ cells (monocytes, DCs and macrophages) was found 1 day p.i., followed by natural killer (NK) cells and macrophages at 2 days p.i. There was a gradual increase in the percentage of neutrophils in the spleen until 7 days p.i. Furthermore, an increased percentage of T cells, distinguished by the CD3 marker, was found 2 and 4 days p.i. A similar increase was observed for both $CD4^+CD8^-$ and $CD8^+CD4^-$ T cell subsets, indicating that this increase was not specific for either T helper or cytotoxic T cells. The $CD4^+$ cells increased significantly 21 days p.i. and remained constant until at least 28 days p.i. B cells decreased during two periods: 2–4 and 10–14 days p.i. Finally, a significant increase in percentage of DCs ($33D1^+$) was detected 7 days p.i.

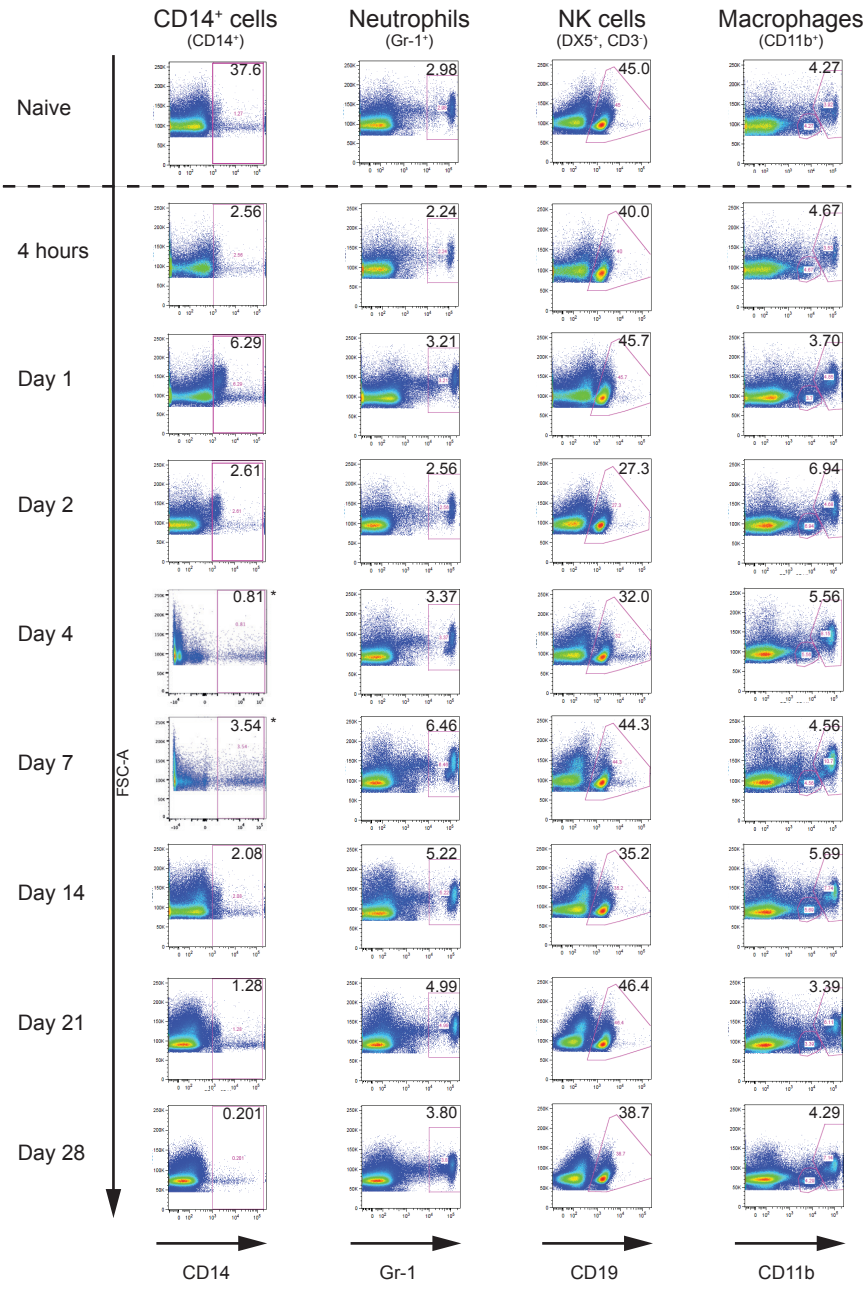

Figure S3 - Flow cytometric-gating strategy for CD14⁺, Gr-1⁺, CD19⁺, and CD11b⁺ cell population analysis in splenocytes. Each FACS plot shows results from one mouse of the group. Per cell type, the change in numbers of cells is visible over time for CD14⁺, Gr-1⁺, CD19⁺, and CD11b⁺ cell populations. Time points 2 hours, 6 hours and 10 days p.i. were excluded in this figure. *FACS plot for CD14⁺ cells at day 4 and day 7 p.i. were manually adapted by BiExponential transformation in FlowJo for better visualization without influencing the gating.

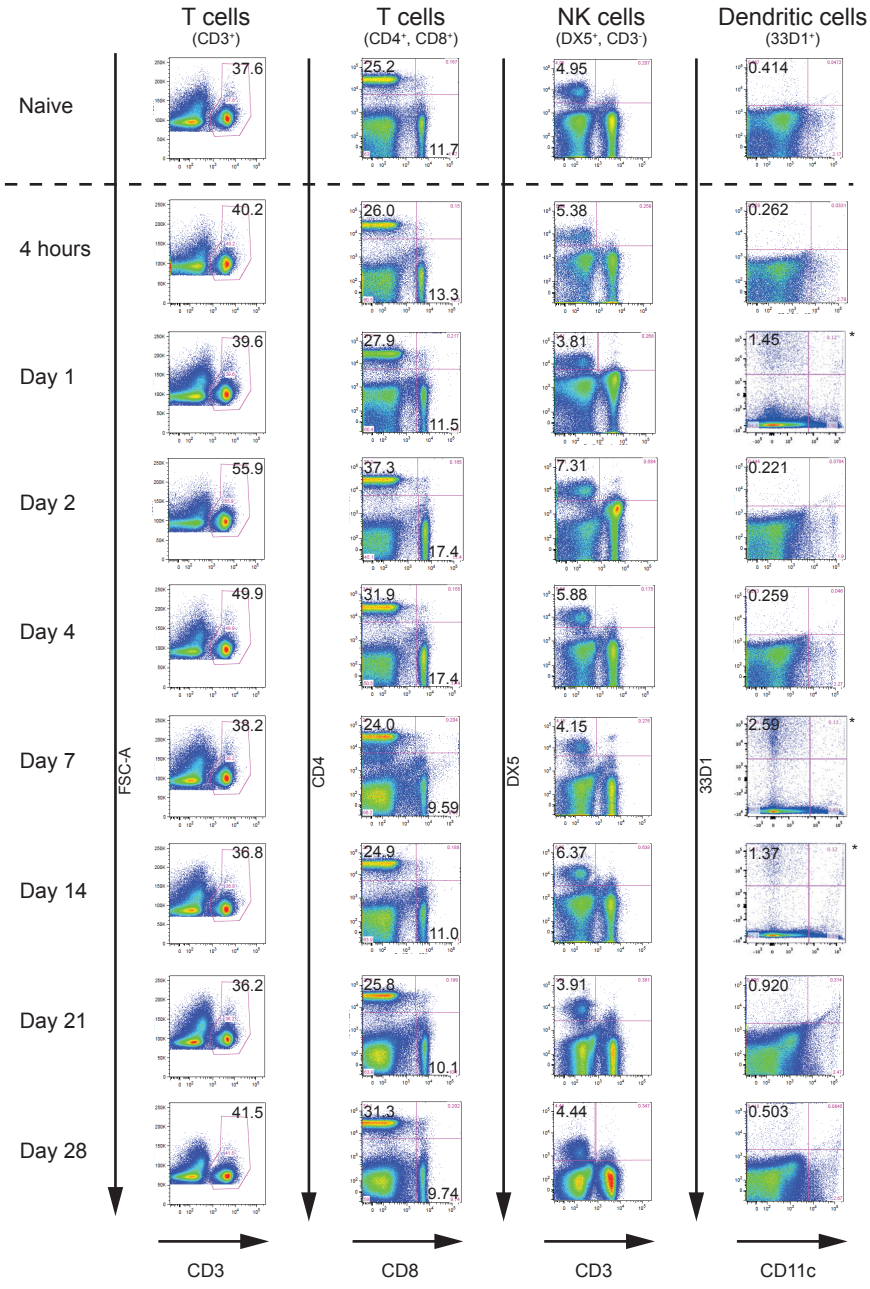

Figure S4 - Flow cytometric-gating strategy for CD3A$^+$, CD4$^+$, CD8$^+$, DX5$^+$ and 33D1$^+$ cell population analysis in splenocytes. Each FACS plot shows results from one mouse of the group. Per cell type, the change in numbers of cells is visible over time for CD3$^+$, CD4$^+$, CD8$^+$, DX5$^+$, and 33D1$^+$ cell populations. Time points 2 hours, 6 hours, and 10 days p.i. were excluded in this figure. *FACS plot for 33D1$^+$ cells at day 1, day 7, and day 14 p.i. were manually adapted by BiExponential transformation in FlowJo for better visualization without influencing the gating.

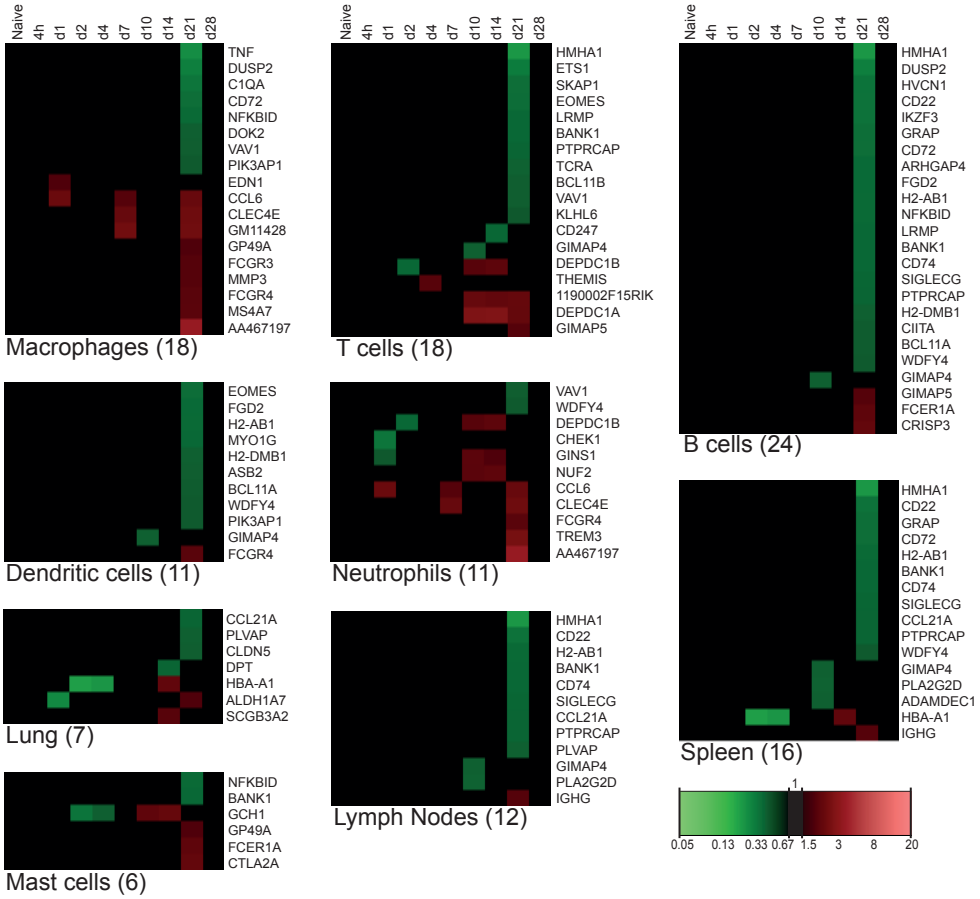

Figure S5 - Splenic gene expression profiles of cell types and tissues extracted from BioGPS databases. Data (mean of n=3) represent gene profiles of the following cell types or tissues: B cells, neutrophils, mast cells, T cells, macrophages, dendritic cells (DCs), lung, lymph nodes, and spleen.

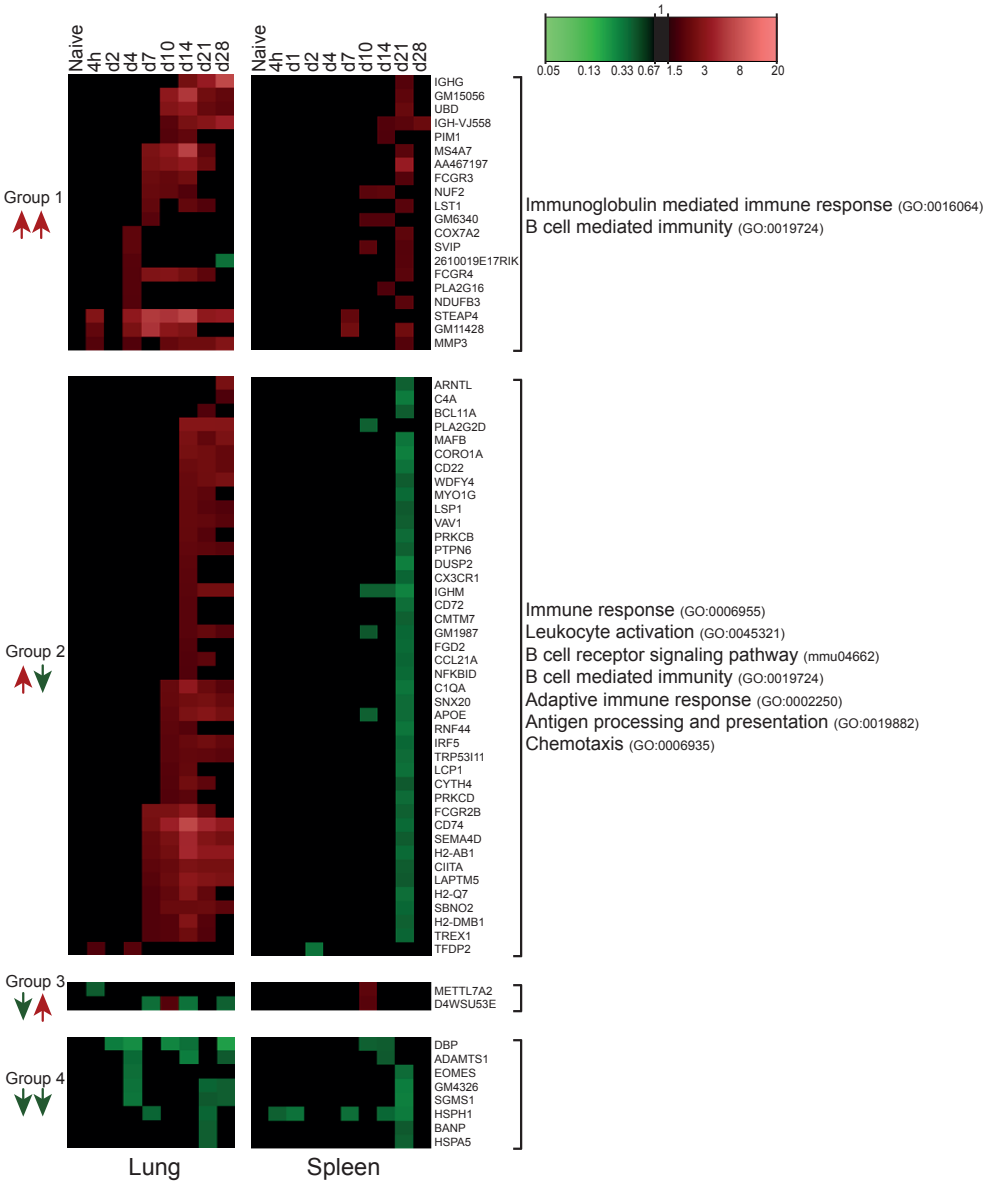

Figure S6 - Genes differentially regulated in both lung and spleen. Comparison of transcriptomic data in lung and spleen (mean of n=3) revealed an overlap of 72 genes differentially regulated in both tissues. Genes divided in 4 groups based on expression pattern. Functional annotation showed that genes in group 2 were mostly involved in immunological processes.

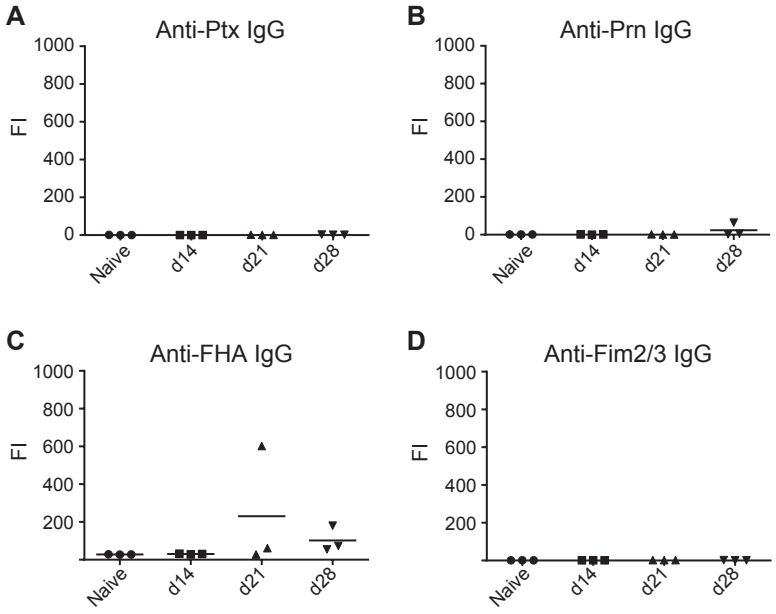

Figure S7 - IgG antibody response in serum against Ptx, Prn, FHA, and Fim2/3 after *B. pertussis* infection. IgG antibody levels specific for purified *B. pertussis* antigens; (A) pertussis toxin (Ptx), (B) pertactin (Prn), (C) filamentous hemagglutinin (FHA), and (D) fimbriae type 2 and 3 (Fim2/3) were determined using a multiplex immunoassay (mean of n=3). The antibody levels given as fluorescence intensities (FI) were determined in mouse sera 14, 21, and 28 days after an intranasal infection with *B. pertussis*. No antibodies were detected for Ptx, Prn, and Fim2/3. For FHA, 1 out of 3 mice showed induced titers antibody titers at 21 days p.i.

Table S1 - Full list of genes expressed in the lungs. This supplementary table can be found in the online version at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0104548**.** This table contains the full list of significantly expressed genes obtained by microarray analysis of the lungs, which can be used for additional in-depth information for each gene. In total 558 genes were significantly altered compared to naive mice. The expression pattern for each gene is given including the fold changes at each time point. In addition to the official gene symbol (obtained from the Nimblegen array), the full gene name, synonyms and gene ID are given. Synonyms are not given for all genes but only for genes that were included in detail in the manuscript. Gene ID is a linked to gene information on the NCBI website. Subsequently, there are three columns with gene information obtained in databases; (i) BioGPS, (ii) Gene Ontology - Biological Processes (GO-BP), (iii) KEGG pathways. Pull-down menus can be used to search for specific information in the full gene list. For BioGPS, information about cell and tissue specific gene expression is given for the following databases; B cells (B), dendritic cells (DC), lung (Lu), lymph node (LN), mast cells (MC), macrophages (MF), neutrophils (N), spleen (Sp) and T cells (T). For GO-BP the following biological processes were included; 'acute inflammatory response', 'acute-phase response', 'antigen processing and presentation', 'cell activation', 'chemotaxis', 'defense response', 'immune response', 'inflammatory response', 'intracellular signaling cascade', 'phagocytosis', 'regulation of apoptosis', 'response to bacterium', and 'transcription'. For KEGG the following pathways were included; 'Antigen processing and presentation', 'Apoptosis', 'B cell receptor signaling pathway', 'Cell adhesion molecules (CAMs)', 'Chemokine signaling pathway', 'Complement and coagulation cascades', 'Cytokine-cytokine receptor interaction', 'Endocytosis', 'Leukocyte transendothelial migration', 'MAPK signaling pathway', 'NOD-like receptor signaling pathway', and 'Toll-like receptor signaling pathway'.

Table S2 - Full list of genes expressed in the spleen. This supplementary table can be found in the online version at http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0104548**.** This table contains the full list of significantly expressed genes obtained by microarray analysis of the spleen, which can be used for additional in-depth information for each gene. In total, 798 genes were significantly altered compared to naive mice. The expression pattern for each gene is given including the fold changes at each time point. In addition to the official gene symbol (obtained from the Nimblegen array), the full gene name, synonyms and gene ID are given. Synonyms are not given for all genes but only for genes that were included in detail in the manuscript. Gene ID is a linked to gene information on the NCBI website. Subsequently, there are three columns with gene information obtained in databases; (i) BioGPS, (ii) Gene Ontology – Biological Processes (GO-BP), (iii) KEGG pathways. Pull-down menus can be used to search for specific information in the full gene list. For BioGPS, information about cell and tissue specific gene expression is given for the following databases; B cells (B), dendritic cells (DC), lung (Lu), lymph node (LN), mast cells (MC), macrophages (MF), neutrophils (N), spleen (Sp) and T cells (T). For GO-BP the following biological processes were included; 'acute inflammatory response', 'antigen processing and presentation', 'cell activation', 'chemotaxis', 'chromatin assembly', 'defense response', 'hemopoiesis', 'immune response', 'inflammatory response', 'intracellular signaling cascade', 'regulation of apoptosis', 'response to bacterium' and 'transcription'. For KEGG the following pathways were included; 'Antigen processing and presentation', 'Apoptosis', 'B cell receptor signaling pathway', 'Cell adhesion molecules (CAMs)', 'Chemokine signaling pathway', 'Complement and coagulation cascades', 'Cytokine-cytokine receptor interaction', 'Endocytosis', 'Leukocyte transendothelial migration', 'MAPK signaling pathway', 'NOD-like receptor signaling pathway' and 'Toll-like receptor signaling pathway'.

Chapter 4

Bordetella pertussis naturally occurring isolates with altered lipooligosaccharide structure fail to fully mature human dendritic cells

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Abstract

Bordetella pertussis is a Gram-negative bacterium and the causative agent of whooping cough. Despite high vaccination coverage, outbreaks are being increasingly reported worldwide. Possible explanations include adaptation of this pathogen, which may interfere with recognition by the innate immune system. Here, we describe innate immune recognition and responses to different *B. pertussis* clinical isolates. By using HEK-Blue cells transfected with different pattern recognition receptors, we found that 3 out of 19 clinical isolates failed to activate Toll-like receptor 4 (TLR4). These findings were confirmed by using the monocytic MM6 cell line. Although incubation with high concentrations of these 3 strains resulted in significant activation of the MM6 cells, it was found to occur mainly through interaction with TLR2 and not through TLR4. When using live bacteria, these 3 strains also failed to activate TLR4 on HEK-Blue cells, and activation of MM6 cells or human monocyte-derived dendritic cells was significantly lower than activation induced by the other 16 strains. Mass spectrum analysis of the Lipid A moieties from these 3 strains indicated an altered structure of this molecule. Gene sequence analysis revealed mutations in genes involved in Lipid A synthesis. Findings from this study indicate that *B. pertussis* isolates that do not activate TLR4 occur naturally and that this phenotype may give this bacterium an advantage in tempering the innate immune response and establishing infection. Knowledge on the strategies used by this pathogen in evading the host immune response is essential for the improvement of current vaccines or for the development of new ones.

Introduction

The innate immune system is the first line of defense against invading pathogens. In order to recognize these microorganisms, innate immune cells express multiple pathogen recognition receptors (PRR) consisting of various receptor families, including the most-studied Toll-like receptors (TLRs)[1]. Evasion of this first recognition can be critical for the pathogen establishing infection. Additionally, the innate immune system is essential for the induction and regulation of the adaptive response. For example, dendritic cells (DCs) can produce interleukin-12p70 (IL-12p70), which is associated with the differentiation of T helper 1 (Th1) cells or cytotoxic T cells[2, 3] and IL-1β, IL-6, and IL-23, which are required for differentiation and survival of Th17 cells[4, 5]. Pathogen adaptation, through changes in the structure or expression of molecules which interact with the host, might allow the pathogen to escape the host immune responses.

Pertussis, also referred to as whooping cough, is a human-specific respiratory disease caused by the Gram-negative bacterium *Bordetella pertussis*. It has been shown that *B. pertussis* is able to activate both TLR2 and TLR4 signaling on innate immune cells, which is essential for inducing protective immunity against this bacterium[6-9]. A well-defined ligand for TLR4 is lipopolysaccharide (LPS), which is produced by Gram-negative bacteria. *B. pertussis* also produces LPS; however, it lacks the O-chain structure and is therefore referred to as lipooligosaccharide (LOS)[10]. TLR4 signaling leads to the induction of pro-inflammatory cytokines or type I interferon[11]. TLR2 recognizes a broad range of ligands, including lipoproteins and peptidoglycans, and induces the production of pro-inflammatory cytokines[12]. Since 1990, outbreaks of pertussis in developed countries have been increasingly reported despite high vaccination coverage[13-16]. Several explanations have been proposed for the upsurge of pertussis, including waning immunity and adaptation of the pathogen due to selective pressure induced by the vaccine[16-20].

Indeed, variations in the structure or expression of virulence-associated proteins of *B. pertussis* have been observed[21]. Based on the allelic variation in the genes of immunodominant pertussis toxin subunit A (*ptxA*), pertactin (*prn*), promoter for Ptx (*ptxP*), and fimbriae3 (*fim3*), seven allele types (I to VII) have been defined[21]. In the past decades, circulating strains have shifted from *ptxP1* to *ptxP3*, the latter of which is associated with increased Ptx production *in vitro*[22]. Furthermore, an increase in *B. pertussis* strains lacking Prn has been recently observed in several countries[23-27]. Whether naturally occurring gene variations in *B. pertussis* strains have an impact in the recognition of the innate immune system remains unknown.

Here, we aimed to characterize the interaction between different *B. pertussis* clinical isolates and the innate receptors TLR2 and TLR4, as well as the consequences for DC activation. For this purpose, we screened a selection of 19 *B. pertussis* strains representing multiple *B. pertussis* allele types, including the laboratory Tohama I strain and the 18-323 strain commonly used in experimental models to evaluate pertussis vaccines[28], using HEK-Blue

cells expressing human TLR2 or TLR4. To verify the findings, we used a monocytic cell line expressing these and other PRR and a physiologically relevant innate immune cell type, namely, dendritic cells. Results indicated that *B. pertussis* mutant strains that do not activate TLR4 occur naturally. The lack of TLR4 activation could be one of the strategies used by this bacterium to evade and modulate the host immune response and establish infection.

Materials and Methods

Ethics statement

This study was conducted according to the principles of Good Clinical Practice. All blood donors provided written informed consent for the collection of samples and subsequent analysis, and the blood samples were processed anonymously.

Reagents

Ultrapure lipopolysaccharide from *Escherichia coli* K-12 (LPS-EK), ultrapure lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS), and PAM3CSK4 were all purchased from InvivoGen. Blocking anti-TLR2 antibodies were obtained from R&D Systems. Granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech, and recombinant human IL-4 was purchased from Sanquin.

Cell lines

Human NF-κB/SEAP reporter HEK293 cells (HEK-Blue) transfected either with human TLR2 (HEK-Blue-hTLR2) or human TLR4 (HEK-Blue-hTRL4) in combination with MD-2 and CD14, as well as untransfected (HEK-Blue-Null1) cells, were purchased from InvivoGen. All these cell lines contain an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. TLR signaling leads to the expression of SEAP, which can be detected in culture supernatants after adding the substrate Quanti-Blue (InvivoGen). The cells were grown in complete Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Thermo Scientific), 50 U/ml penicillin (Gibco), 50 µg/ml streptomycin (Gibco), 1x HEK-Blue selection (InvivoGen), and 100 µg/ml Normocin (InvivoGen); we refer to this medium here as HEK-Blue culture medium. The HEK-Blue-Null1 cell line was cultured in the HEK-Blue culture medium in the presence of 100 µg/ml Zeocin (InvivoGen) instead of 1x HEK-Blue selection. The human monocytic cell line MonoMac6 (MM6; DSMZ) was grown in Iscove's modified Dulbecco's medium (IMDM; Gibco) enriched with 10% heat-inactivated FCS, 50 U/ml penicillin, and 50 µg/ml streptomycin; we refer to this medium here as MM6 culture medium. All cell lines were cultured at 37°C in a 5% saturated CO_2 atmosphere.

Bacterial strains, growth conditions, and sequencing

The selected 19 strains represented multiple *B. pertussis* allele types based upon the DNA sequence of the *ptxP*, *ptx*, *prn*, and fimbriae genes, which have been shown to change over time[16]. The selected strains included laboratory strains Tohama I derivative (B0213; streptomycin and naladixic acid resistant) and 18-323 (B1121). The clinical isolates represented ptxP1, ptxP2, ptxP3, and ptxP4 strains and included vaccine antigen-deficient strains, lacking the expression of one of the vaccine antigens[21]. A detailed overview of the selected *B. pertussis* strains is given in Table 1. The DNA sequences of alleles for *ptxP*, *ptxA*, and *prn* were determined previously[21, 29, 30]. The sequences of genes involved in LOS synthesis (Table 2) were based on the genome sequences of strains B1121/18-323[31], B0442, and B1120[32]. The 19 strains were grown at 35°C on Bordet-Gengou (BG) plates containing

glycerol and 15% defibrinated sheep blood (BD Biosciences). After 3 days of culture, photographs using ProtoCOL (Synbiosis) were made to determine whether the strains induced hemolysis. The bacteria were collected in phosphate-buffered saline (PBS), and the optical density (OD) was measured at 600 nm. Bvg^{+33} and Bvg^{-34} strains were used as positive and negative controls, respectively. Heat-inactivated (1 hour at 56°C) or live bacteria were used in the experiments.

TLR2 and TLR4 signaling in HEK-Blue cell lines

In a 96-well plate, $2.5x10^4$ HEK-Blue-hTLR2, HEK-Blue-hTLR4, or HEK-Blue-Null1 cells were incubated in HEK-Blue culture medium in the absence or presence of PAM3CSK4 as the TLR2 ligand or LPS-EK as the TLR4 ligand. In addition, these cells were incubated with heat-inactivated bacteria (OD 0.25 to $2.6x10^{-8}$; 5-fold dilutions) or live bacteria (OD 0.01). After 22 hours of incubation at 37°C, supernatants were collected and the Quanti-Blue substrate was added. After 2 to 4 hours of incubation with the substrate, the OD values, indicating SEAP activity, were measured using an enzyme-linked immunosorbent assay (ELISA) reader

Table 1 - *Bordetella pertussis* strains

Designation	ptxP allele	ptxA allele	Remarks	Year of isolation
B0213 (Tohama I)	*ptxP1*	*ptxA-1*		1953
B0572	*ptxP1*	*ptxA-2*		1952
B0576	*ptxP1*	*ptxA-2*		1967
B0582	*ptxP1*	*ptxA-1*		1972
B1920	*ptxP1*	*ptxA-1*		2000
B3214	*ptxP1*	*ptxA-1*		2008
B0496	*ptxP2*	*ptxA-4*		1950
B1193	*ptxP2*	*ptxA-4*		1950
B0644	*ptxP3*	*ptxA-1*		1996
B1917	*ptxP3*	*ptxA-1*		2000
B2584	*ptxP3*	*ptxA-1*		2003
B3034	*ptxP3*	*ptxA-1*		2005
B3640	*ptxP3*	*ptxA-1*	Prn[1]	2010
B3645	*ptxP3*	*ptxA-1*	Prn[1]	2007
B3585	*ptxP3*	*ptxA-1*	FHA[2]	2009
B3582	*ptxP3*	*ptxA-1*	Prn[1], FHA[1]	2009
B0442	*ptxP4*	*ptxA-5*		1954
B1120	*ptxP4*	*ptxA-5*		1993
B1121 (18-323)	*ptxP4*	*ptxA-5*		1947

[1]Indicated protein is not expressed (natural mutants), [2]Indicated protein is low expressed

(OD649 nm). In order to determine whether *B. pertussis* strains can act as TLR4 antagonists, HEK-Blue-hTLR4 cells were first pre-incubated at 37°C with the Tohama I, B0442, B1120, and B1121 strains (OD 0.1, 0.01, and 0.001) or LPS-RS (10, 1, and 0.1 µg/ml) for 3 hours, after which LPS-EK (0.0002 µg/ml) was added and the cultures were incubated for 22 hours. This suboptimal LPS concentration was chosen from a titration experiment (Figure S4A) that was used to be able to detect either an increase or decrease of TLR4 activation after incubation of HEK-Blue-hTLR4 with the *B. pertussis* strains and LPS-EK.

MM6 cell stimulation

MM6 cells were seeded in a 96-well plate at $1.5x10^5$ cells per well in MM6 culture medium. In the blocking experiments, the cells were pre-incubated with MM6 culture medium or MM6 culture medium supplemented with 1 µg/ml LPS-RS or 0.5 µg/ml anti-TLR2 antibodies for 3 hours at 37°C. Subsequently, cells were held unstimulated or were stimulated with MM6 culture medium supplemented with LPS-EK, PAM3CSK4, heat-inactivated bacteria (OD 0.01, $2x10^{-3}$, or $4x10^{-4}$) or live bacteria (OD $2x10^{-3}$). After 22 hours at 37°C, supernatants were collected for IL-6 measurements.

LOS-A and LOS-B ELISA

For the LOS-A and LOS-B ELISAs, a flat-bottom 96-well plate (Immulon-2HB; Thermo Scientific) was coated for 2 hours at 37°C with a 3-fold-dilution in PBS of different *B. pertussis* strains (OD 0.081 to 0.0003). Strain B0134 was used as a control, since it only produces LOS-B[35]. Subsequently, the plates were washed with water containing 0.03% Tween-80 (Merck) and incubated for 1 hour at 37°C either with anti-LOS-A (88F8)[36] or anti-LOS-B (BL-8)[37] monoclonal antibodies diluted 1/100 and 1/10 in PBS containing 1% Tween-80, respectively. After washing, the plates were incubated for1 hour at 37°C with horse-radish peroxidase-labeled goat anti-mouse IgG3 antibody (Southern Biotech) for detection of LOS-A and Goat-anti-Mouse IgG (Southern Biotech) for detection of LOS-B diluted in PBS supplemented with 0.1% Tween 80 and 0.5% Protifar (Nutricia). Following the washing steps, the peroxidase substrate (0.1 mg/ml tetramethylbenzidine with 0.012% H_2O_2 in 0.11 M sodium acetate buffer (pH 5.5)) was added, and the reaction was stopped with 2M H_2SO_4 after 10 min. The OD at 450nm was determined with an ELISA reader.

Analysis of lipid A via ESI-MS

The lipid A moieties of LOS were isolated from whole bacterial cells, as described previously[38]. In short, the bacteria were washed with a chloroform-methanol solution (3:2, v/v), centrifuged (10 min, 16,000 g), and subsequently washed with a chloroform-methanol-water solution (12:8:1, v/v/v). Then, after centrifugation (110 min, 6,000 g), the pellets were resuspended in an isobutyric acid-2 M ammonium hydroxide-water solution (10:3:3, v/v/v) and heated to 100°C for 2 hours under magnetic stirring. Subsequently, it was cooled in ice water and centrifuged (10 min, 16,000 g). The supernatants were diluted in 3 ml water and freeze-dried. After freeze-drying, the pellets were washed twice with methanol and centrifuged (20 min, 4,600 g). The pellets were extracted using chloroform-methanol-water (12:6:1, v/v/v), centrifuged (20 min, 4,600 g), and the lipid A extract in the

upper phase was analyzed by electrospray ionization mass spectrometry (ESI-MS) with a Finnigan LCQ instrument in the negative-ion mode[39].

Monocyte-derived DC generation and stimulation

Peripheral blood derived from healthy donors was used for the generation of monocyte-derived DC (MDDCs). First, peripheral blood mononuclear cells (PBMCs) were obtained by gradient centrifugation at 1,000 g for 30 min on Lymphoprep (Nycomed). Second, using magnetically activated cell sorting in combination with anti-CD14 microbeads (Miltenyi Biotech), monocytes were isolated from the PBMC fraction. Monocytes were cultured in a 24-well culture plate ($0.4x10^6$ cells/well) in IMDM (Gibco) supplemented with 1% FCS, 100 units penicillin, 100 units streptomycin, and 2.92 mg/ml L-glutamine (DC culture medium) and in the presence of 500 U/ml GM-CSF and 800 U/ml IL-4 for 6 days. On day 6, the immature MDDC were kept unstimulated with DC culture medium containing 250 U/ml GM-CSF or stimulated with DC culture medium containing 250 U/ml GM-CSF and 100 ng/ml LPS-EK or live *B. pertussis* strains (OD $4x10^{-4}$). After 48 hours, supernatants were collected and stored at -80°C, and subsequently the MDDCs were stained with phycoerythrin-conjugated anti-CD40 (clone 5C3; BD Biosciences), allophycocyanin-conjugated anti-CD80 (clone 2D10; BioLegend), fluorescein isothiocyanate-conjugated anti-CD83 (clone HB15e; BD Biosciences), Pacific Blue-conjugated anti-CD86 (clone IT2.2; BioLegend), or with the LIVE/DEAD fixable Aqua dead cell stain kit (Invitrogen) for 30 min at 4°C. The MDDCs were washed twice and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS [pH 7.2], 0.5% bovine serum albumin, 0.5 mM EDTA). Data were acquired on a FACS Canto II apparatus (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Cytokine analysis

In supernatants from the MM6 cultures, IL-6 was measured using an IL-6 ELISA kit (Sanquin) and transforming growth factor β (TGFβ) was measured in MDDC culture supernatants by using a TGFβ ELISA kit (BioLegend). The ELISAs were performed according to the manufacturer's instructions. TGFβ was measured in samples that were either untreated or treated with an acidification and neutralization solution to activate latent TGFβ. The concentrations of various other cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70, IL-23, and tumor necrosis factor alpha (TNFα)) were determined in supernatants of the MDDC cultures by using a human ProcartaPlex multiplex kit (eBioscience) according to the manufacturer's protocol. Measurements and data analysis were performed with Bio-Plex 200, using Bio-Plex Manager software (version 5.0; Bio-Rad Laboratories). Results were calculated as mean cytokine levels relative to the cytokine levels induced by the Tohama I strain (set as 100).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 6.04. Statistical significance was determined by using a 2-way analysis of variance followed by a Bonferroni post hoc test for the experiments with the HEK-Blue cell lines and by using an unpaired t test for the experiments with the MM6 cell line and the MDDCs. p values <0.05 were considered statistically significant.

Results

Human TLR2 and TLR4 activation by different *B. pertussis* isolates

To establish whether the 19 selected *B. pertussis* isolates induced human TLR2 and TLR4 signaling, HEK-Blue cells, expressing either of these TLRs, were used. Findings indicated that all of these *B. pertussis* clinical isolates activated, in a dose-dependent manner, TLR2, as indicated by increased SEAP activity (Figure 1A). When tested on the HEK-Blue-hTLR4 cell line, 16 out of the 19 heat-inactivated strains clearly induced SEAP activity (Figure 1B). However, isolates B0442, B1120, and B1121 were not able to induce TLR4 signaling. To exclude an effect from the heat-inactivation step, the TLR2- and TLR4-transfected HEK-Blue cells were also incubated with live bacteria (OD 0.01). Comparable results were found (Figures 1C and D), namely, that also the live B0442, B1120, and B1121 isolates failed to activate TLR4, yet the TLR2 activation was comparable to that induced by the other isolates (Figures 1C and D). In order to exclude aspecific SEAP activation due to signaling via TLR3, TLR5, and NOD1, which are expressed at low levels in untransfected HEK cells (Invivogen), we incubated these cells with the 19 isolates. No SEAP activity was found, indicating that results using the TLR2- and TLR4-transfected HEK cells were specific to these TLRs (Figure S1).

Activation of the MM6 cell line by different clinical *B. pertussis* isolates

To determine whether the *B. pertussis* isolates induced a differential effect on TLR4 activation in the presence of multiple PRR, MM6, a human monocytic cell line that expresses, among other PRR, functional TLR2, TLR4, TLR5, and TLR7/8[40, 41], was used. Strains B0442, B1120, and B1121 only induced MM6 activation when the cells were incubated with the highest bacterial concentration (OD 0.01) (Figure 2A). The other 16 strains already induced IL-6 production when the MM6 cells were incubated with the lowest bacterial concentration (OD $4x10^{-4}$). Importantly, these findings indicated that even in the presence of multiple PRR, the B0442, B1120, and B1121 isolates were less efficient in activating MM6 cells than the other 16 clinical isolates.

Effect of blocking TLR2 and TLR4 signaling on MM6 cells induced by *B. pertussis* clinical isolates

Since the B0442, B1120, and B1121 isolates failed to induce TLR4 signaling with the HEK-Blue-hTLR4 cell line, we were interested in characterizing the role of TLR4 signaling in MM6 cell activation by the different *B. pertussis* strains. For this purpose, experiments blocking TLR4 on these cells were performed. Blocking TLR4 signaling by means of adding LPS-RS, a TLR4 antagonist[42], resulted in a partial blocking of MM6 activation by most of the strains (Figure 2B). However, activation of MM6 cells induced by *B. pertussis* isolates B0442, B1120, and B1121, which was already lower than with the other 16 strains, was not affected by the TLR4 antagonist (Figure 2B). To determine the effect of TLR2 signaling on MM6 cells by these strains, this TLR was blocked by using anti-TLR2 antibodies. In contrast to the effect of blocking TLR4, activation of the MM6 cells by all 19 strains was blocked when the MM6 cells were pre-incubated with the anti-TLR2 antibodies (Figure 2C). Notably, MM6 cell activation by the B0442, B1120, and B1121 isolates was completely blocked. These results indicated that

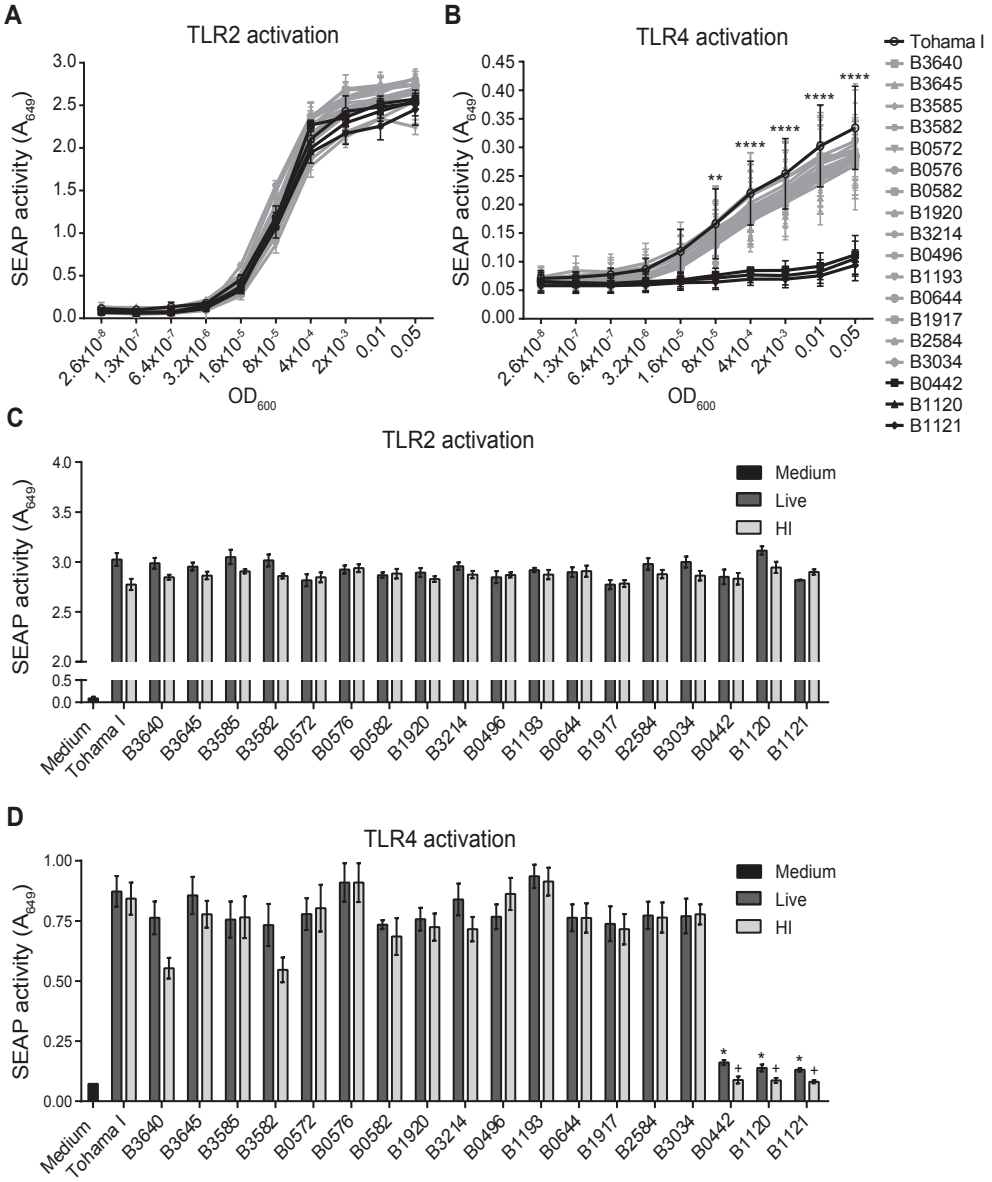

Figure 1 - Induction of TLR2 and TLR4 signaling by 19 *B. pertussis* isolates on HEK-Blue-hTLR2/hTLR4 cell lines. (A-D) Different HEK-Blue cell lines, expressing hTLR2 or hTLR4, were stimulated with several concentrations of 19 heat-inactivated (HI) or live *B. pertussis* isolates. (A-B) TLR activation was measured by SEAP activity after incubation of HEK-Blue-hTLR2 (A) or HEK-Blue-hTLR4 (B) cells with the HI *B. pertussis* strains (OD 0.05 to $2.6x10^{-8}$). **p< 0.01; ****p < 0.0001, for B1121, B1120, and B0442 strains versus the Tohama I strain. (C-D) Comparison of TLR activation after incubation of HEK-Blue-hTLR2 (C) or HEK-Blue-hTLR4 (D) cells with HI or live *B. pertussis* strains (OD 0.01). *p< 0.01 versus live Tohama I strain; +p<0.01 versus heat-inactivated Tohama I strain. Data shown are mean values of three independent experiments (A-B) or representative results of at least three independent experiments (C-D).

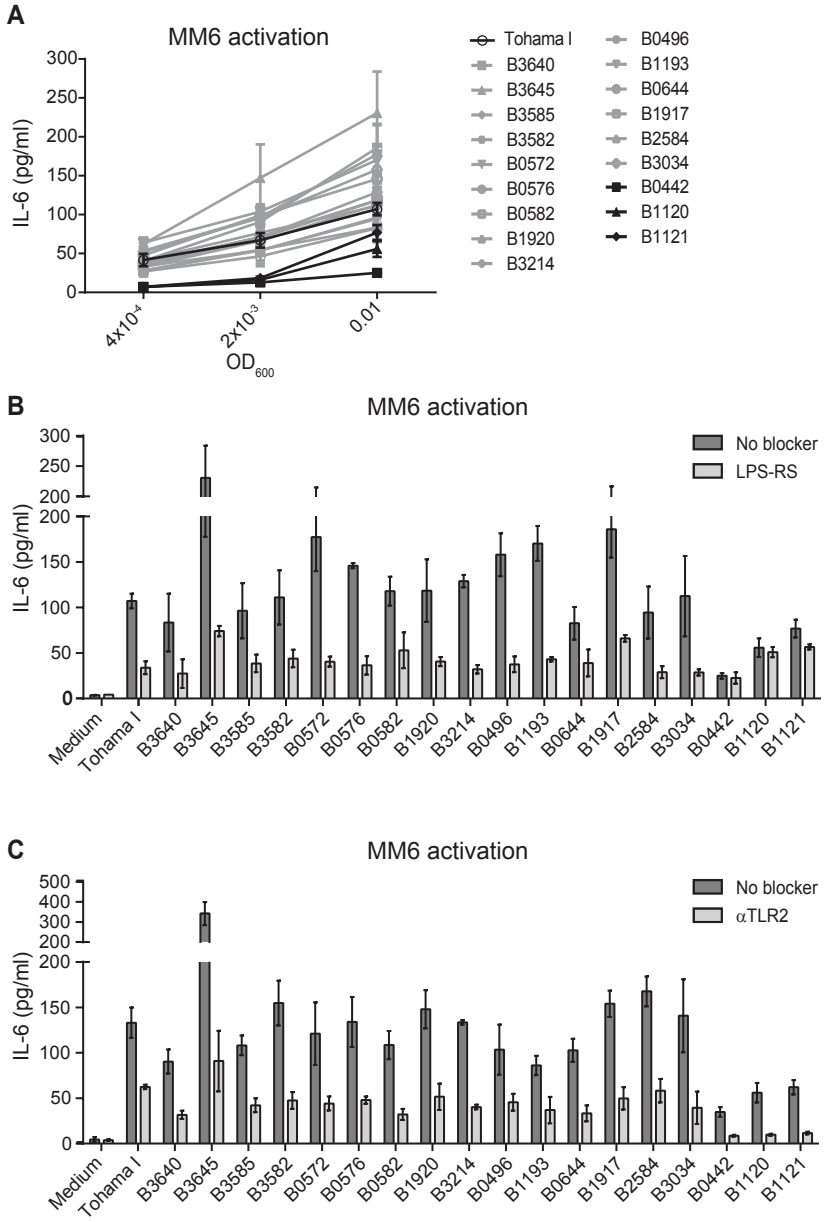

Figure 2 - Role of TLR2/TLR4 signaling in the activation of MM6 cells by 19 *B. pertussis* isolates. (A) MM6 cells were incubated with medium or heat-inactivated *B. pertussis* isolates (OD 0.01, $2x10^{-3}$, or $4x10^{-4}$) for 22 hours, after which IL-6 production was measured as a marker for cell activation. (B-C) IL-6 production by MM6 cells stimulated with heat-inactivated *B. pertussis* (OD 0.01) after 3 hours of pre-incubation with or without 1 µg/ml TLR4 antagonist LPS-RS (B) or with or without 0.5 µg/ml anti-TLR2 antibodies (C). Data shown are representative results of three independent experiments and are reported as the mean IL-6 concentration (in pg/ml) ± SD. Background IL-6 production of MM6 cells incubated with medium was 4.02 ± 1.67 pg/ml.

MM6 activation by the B0442, B1120, and B1121 isolates occurs mainly through interaction with TLR2, whereas the other strains induce both TLR4 and TLR2 signaling. To determine whether the contribution of TLR2 and TLR4 signaling in the activation of MM6 cells by heat-inactivated bacteria is similar to that of live bacteria, the MM6 cells were incubated with live Tohama I, B0442, B1120, and B1121 bacteria (OD $2x10^{-3}$). Incubation of MM6 cells with live B0442, B1120, and B1121 isolates resulted in lower IL-6 production than in cells incubated with the Tohama I strain (Figure S2). Furthermore, while MM6 activation induced by the Tohama I strain could be partially blocked by both the TLR2 and TLR4 blockers (Figure S2), IL-6 production induced by the B0442, B1120, and B1121 isolates was not influenced when cells were pre-incubated with the TLR4 blocker yet it was completely blocked when cells, were pre-incubated with the anti-TLR2 antibodies (Figure S2). Together, these results indicate that MM6 cell activation induced either by live or heat-inactivated B0442, B1120, and B1121 isolates mainly occurs via TLR2 signaling, while for the other strains it occurs via both TLR4 and TLR2 signaling.

LOS characterization

Since the lipid A component of the LOS molecule is essential for TLR4 signaling[43], we investigated whether changes in the structure of this molecule could explain the findings. For this, the structures of the lipid A molecules from Tohama I, B0442, B1120, and B1121 *B. pertussis* isolates were determined using mass spectrometry. Interestingly, strain B1121, also referred to as 18-323, has been previously described to have an altered LOS structure[44]. Results from the mass spectrometry analysis indicated a peak corresponding with the penta-acyl lipid A containing a glucosamine (GlcN) substituent (m/z 1,719.1), which could be identified in the lipid A spectrum of the Tohama I strain (Figure 3A). However, the spectra of B0442, B1120, and B1121 lacked this peak, indicating that the lipid As of these strains do not contain GlcN substituents (Figures 3B, C, and D). Furthermore, the spectra of these three isolates showed that the peak of the penta-acyl lipid A with free phosphate groups was shifted from m/z 1,558.1 toward m/z 1,502.1 and 1,530.1. These findings are in agreement with the results described by Marr et al., showing that the two peaks at m/z 1,502.1 and 1,530.1 result from a substitution of a C14-OH with either a C10-OH or a C12-OH, respectively[44]. These results indicate that the LOS structures for the B0442, B1120, and B1121 isolates are comparable and that they differ from the LOS structure of the Tohama I strain. The altered structure of the lipid A molecules of these three strains can explain the lack of TLR4 signaling observed. To exclude that the effect seen on TLR4 signaling is caused by decreased expression of LOS in B0442, B1120, and B1121, the amounts of type A and B LOS[37] were determined using an ELISA. Findings showed no significant differences in the expression of LOS among the isolates (Figure S3), indicating that the diminished TLR4 signaling by these three isolates is not due to decreased expression of the LOS molecules. Interestingly, pre-incubation of HEK-Blue-hTLR4 cells with Tohama I, which optimally activate HEK-TLR4, did not interfere with TLR4 signaling induced by LPS-EK (Figure S4C). In contrast, pre-incubation with LPS-RS, B0442, B1120, or B1121 inhibited LPS-EK-induced TLR4 signaling (Figures S4B and S4D to F). These results suggest that the LOS molecules of isolates B0442, B1120, and B1121, but not that of B0213, act as TLR4 antagonists.

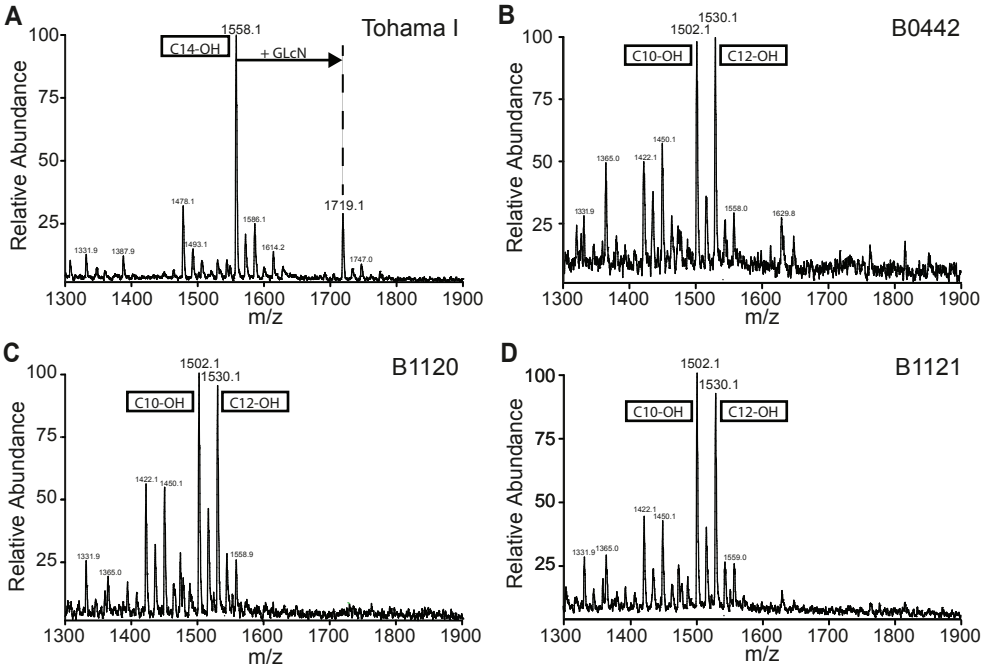

Figure 3 - Lipid A structures of Tohama I, B0442, B1120, and B1121 *B. pertussis* strains. (A to D) Structural analyses of the lipid A moieties of the *B. pertussis* isolates Tohama I (A), B0442 (B), B1120 (C), and B1121 (D) was performed by using mass spectometry. Peaks at m/z 1,719.1 represent penta-acyl lipid A with a GlcN modification at either phosphate group. Peaks at m/z 1,558.1 represent penta-acyl lipid A without GlcN modification and with a C14-OH acyl chain, and the peaks at m/z 1,502.1 and 1,530.1 represent penta-acyl lipid A without GlcN modification and with a C10-OH and C12-OH acyl chain, respectively.

Gene sequences of LOS-related genes in tested clinical isolates

To determine whether the structural changes found in the LOS of the B0442, B1120, and B1121 isolates correspond to alterations in the genes involved in LOS synthesis[45], the sequences of these genes were analyzed and compared to the gene sequence of the Tohama I strain (Table 2). Shah *et al.* described for the B1121/18-323 strain a substitution of a leucine for a serine at amino acid 173 (S173>L) in the gene encoding the LpxA protein[31]. This is the first enzyme of the LPS biosynthesis pathway, and it catalyzes acylation of the C3 position of the UDP N-acetylglucosamine backbone, forming UDP-3-O-acyl N-acetylglucosamine[46]. Results showed that this same amino acid substitution was observed for the other clinical isolates that showed no TLR4 signaling, namely, B0442 and B1120 (Table 2). Furthermore, the B0442 and B1120 isolates had a mutation in the *lpxD* gene, and all three isolates had a mutation in the *lpxL1* and *lpxL2* genes. These genes also encode acyltransferases, which can influence the length of the acyl chains. However, the alteration of the LpxA enzyme is most likely the cause of the presence of a C10-OH or C12-OH instead of C14-OH acyl chain on the C3' position of the diglucosamine backbone, as proposed by Shah *et al.*[31]. No mutations were found in the *lpxB*, *lpxC*, *lpxH*, and *pagL* genes. Shah et al. also reported a TT deletion

Table 2 – Differences in gene sequence of genes involved in LOS synthesis and modifications

Designation	*lpxA* BP1431	*lpxB* BP1432	*lpxC* BP3072	*lpxD* BP1429	*lpxH* BP1905	*lpxL1* BP3072	*lpxL2* BP3073	*PagL*	*lgmA* BP0399	*lgmB* BP0398	*lgmC* BP0397	*lgmD* BP0396
B0442	S173>L	N[a]	N[a]	R354>C	N[a]	E111>K	K292>E	N[a]	N[a]	N[a]	N[a]	N[a]
B1120	S173>L	N[a]	N[a]	R354>C	N[a]	E111>K	K292>E	N[a]	N[a]	N[a]	N[a]	N[a]
B1121	S173>L[b]	N[a]	N[a]	N[a]	N[a]	E111>K	K292>E	N[a]	N[a,b]	TT del (frameshift/ early stop codon)[b]	Absent[b]	Absent[b]

S: Serine; L: Leucine; R: Arginine; C: Cysteine; E: Glutamic Acid; K: Lysine; H: Histidine, [a] N: no difference in gene sequence compared to Tohama I, [b] Results from Shah *et al.*[31]

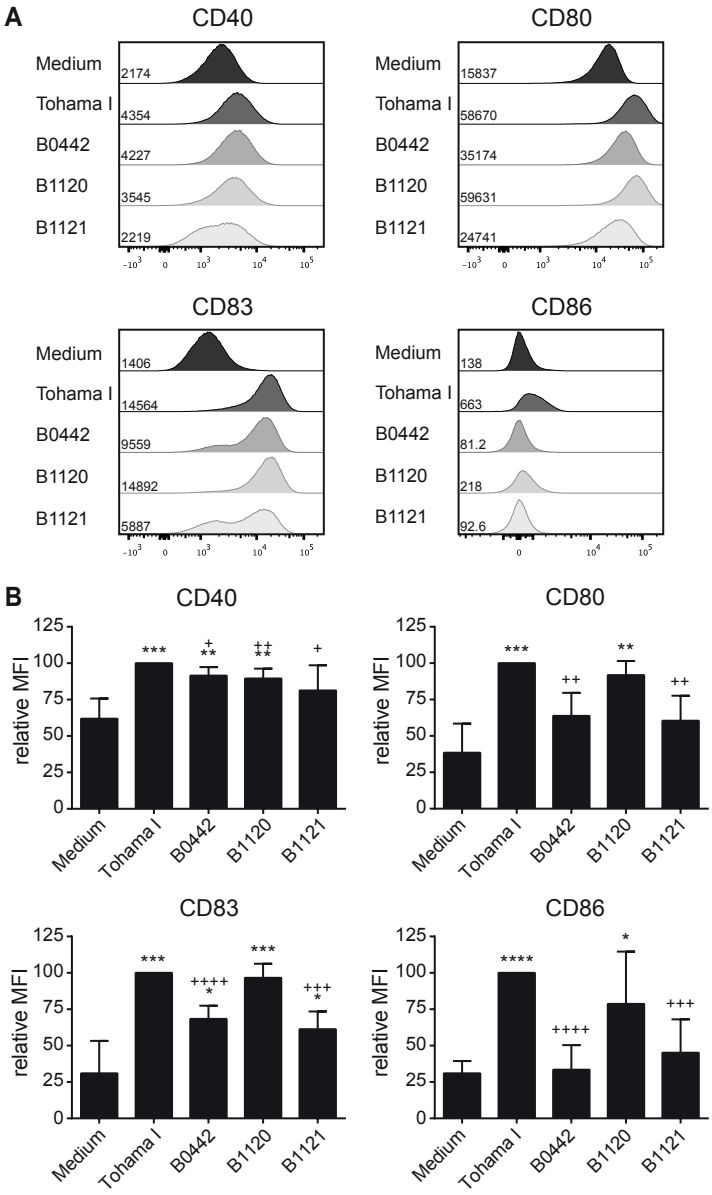

Figure 4 - MDDC maturation induced by live Tohama I, B0442, B1120, and B1121 *B. pertussis* isolates. (A-B) MDDCs, cultured from monocytes isolated from blood of healthy volunteers, were incubated for 48 hours with the live *B. pertussis* isolates (OD $4x10^{-4}$). The cells were stained for the costimulatory molecules CD40, CD80, CD83, and CD86. (A) Representative histograms of CD40, CD80, CD83, and CD86 expression with the corresponding mean fluorescence intensities (MFI). (B) MFI results relative to the MFI obtained after stimulation with the Tohama I strain (set at 100) ± the standard deviation of at least 4 independent experiments with distinct donors. *p< 0.05; **p< 0.01; ***p< 0.001; ****p< 0.0001 versus medium control. +p< 0.05; ++p< 0.01; +++p< 0.001; ++++p<0.0001 versus Tohama I.

mutation at bp 981 of *lgmB* (locus tag BP0398) in the B1121/18-323 strain, which resulted in a lack of GlcN modification of the phosphate groups. Interestingly, in the B0442 and B1120 isolates, this deletion mutation was not found. Therefore, another mutation must be involved in this process. The sequences of the genes *lgmA* (locus tag BP0399), *lgmC* (locus tag BP0397), and *lgmD* (locus tag BP0396) were therefore also analyzed for mutations. However, no mutations in these genes or in the promoter region were observed in these two isolates, suggesting that another mechanism accounts for the lack of GlcN modification. The genes involved in GlcN modification are positively regulated by the regulatory two-component system of protein virulence factors BvgAS[47, 48]. In order to determine that there was no difference in the virulence status of these *B. pertussis* isolates, the expression of ACT (adenylate cyclase toxin), a late gene regulated by BvgAS, was determined by investigating the hemolysis capacity of the bacterial isolates. Results showed that all strains tested induced hemolysis, except for the Bvg^- strain used as negative control (Figure S5), indicating that the lack of GlcN modification is not due to suppression of the BvgAS two-component system.

Dendritic cell maturation by *B. pertussis* clinical isolates

To verify the absence of TLR4 signaling by the B0442, B1120, and B1121 *B. pertussis* isolates on a physiologically innate immune cell type, human DCs, were used and their effects on DC maturation were compared to that induced by the Tohama I strain. The results indicated that, compared to the medium control, the Tohama I strain induced increased expression of all measured maturation markers on MDDCs, while the B0442 isolate induced increased expression of CD40 and CD83 but not of CD80 or CD86 (Figure 4). Notably, the expression levels of CD40 and CD83 were significantly lower than the levels induced by the Tohama I strain. After incubation with the B1120 isolate, the expression of all markers was significantly increased on the MDDCs, yet the level of CD40 expression was significantly lower than that induced by incubation with Tohama I cells (Figure 4). When the MDDCs were incubated with B1121, only the expression of CD83 was significantly increased. Notably, the expression of CD83 was significantly lower than that induced by the Tohama I strain. These results indicated that isolates B0442 and B1121, and to a lesser extent B1120, are less capable than Tohama I in activating MDDC, based on the expression of surface markers.

In addition to the expression of surface maturation markers, MDDCs secrete cytokines upon maturation, which can steer the adaptive immune response[2-5]. Therefore, the levels of cytokines secreted by the MDDCs after incubation with live bacteria were also determined. The Tohama I strain induced the production of high levels of IL-10, IL-12p70, IL-23, IL-6, and IL-8 by MDDC (Figure 5). Isolates B0442 and B1121 induced only low levels of IL-6, IL-8, and IL-10, and B1120 induced low levels of IL-6, IL-8, IL-10, and IL-23 (Figure. 5). Notably, all cytokine levels induced by these three strains were significant lower than the levels induced by the Tohama I strain. No differences were observed in the levels of TNFα. and IL-1β when MDDCs were incubated with the different isolates, and the levels of TGFβ could not be determined since they were under the detection limit (data not shown). The expression of surface maturation markers correlated with cytokine production by MDDCs,

since isolates B0442 and B1121 induced no or low expression of maturation markers and no IL-12p70 or low production of IL-10, IL-6, and IL-8. The B1120 isolate did induce modest expression of maturation markers, and although it did not induce IL-12p70 and induced only low levels of IL-10, IL-6, and IL-8, it did induce measurable levels of IL-23. The findings here cannot be explained by toxic effects of these *B. pertussis* strains on the MDDC, since no major differences were found in DC viability according to FACS analysis (data not shown). Together, these results indicated that the *B. pertussis* isolates B0442, B1120, and B1121 fail to fully mature DCs compared to Tohama I, and this is most likely due to the differences in the LOS structures.

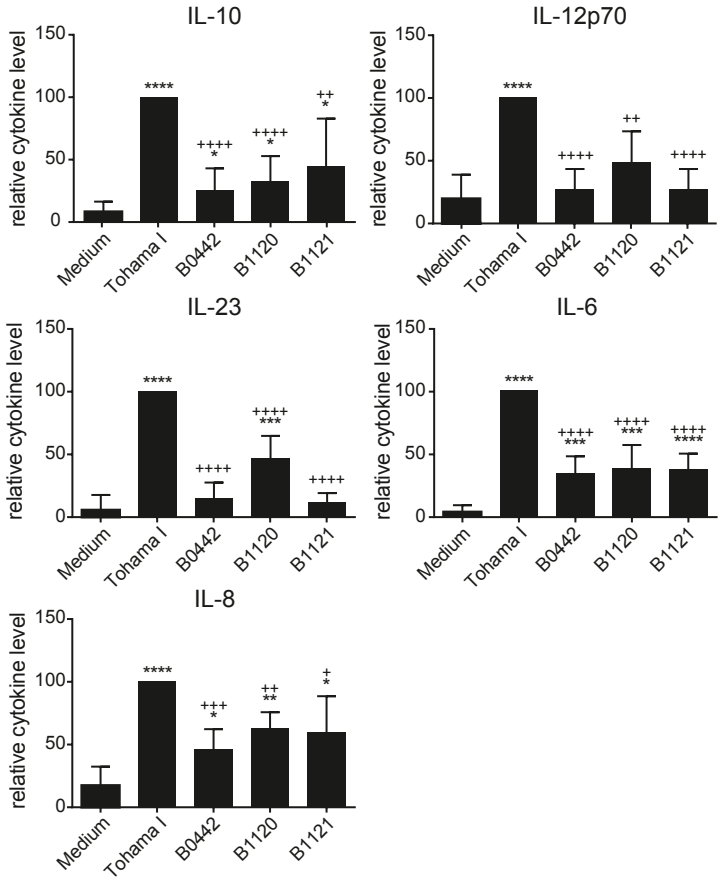

Figure 5 - MDDC cytokine production induced by live Tohama I, B0442, B1120, and B1121 *B. pertussis* isolates. MDDC were incubated for 48 hours with the live *B. pertussis* isolates, after which the levels of IL-6, IL-8, IL-10, IL-12p70, and IL-23 were measured in supernatants by using Luminex. Results shown are the mean cytokine levels relative to the cytokine levels induced by the Tohama I strain (set at 100) ± the standard deviations from at least 5 independent experiments with distinct donors. *p< 0.05; **p< 0.01; ***p< 0.001; ****p< 0.0001 versus medium control. +p< 0.05; ++p< 0.01; +++p< 0.001; ++++p< 0.0001 versus Tohama I.

Discussion

In this study, the capacities of different *B. pertussis* clinical isolates to activate the innate immune system were assessed. Nineteen *B. pertussis* isolates, representing major *B. pertussis* lineages, were compared for their abilities to induce TLR2 and TLR4 signaling[21]. The *B. pertussis* isolates taken from pertussis patients included currently emerging vaccine antigen-deficient strains as well as the laboratory-adapted Tohama I strain and the B1121/18-323 strain commonly used in experimental models to evaluate whole-cell pertussis vaccines[28]. We found, using human NF-κB/SEAP reporter HEK293 cell lines expressing human TLR4/MD-2/CD14, that isolates B0442 and B1120, similar to the already documented B1121/18-323 strain[44], did not induce TLR4 signaling. Moreover, we could establish that the low activation of a monocytic cell line by these three strains was mainly TLR2 dependent, whereas the stronger activation induced by the other strains involved both TLR2 and TLR4 signaling. Furthermore, these three strains were poor inducers of *in vitro* DC maturation. The lack of TLR4 signaling is most likely due to the observed structural alteration in the lipid A moieties of the LOS molecules derived from these three strains. Interestingly, these strains could inhibit the activation of HEK-Blue-hTLR4 cells induced by *E. coli* LPS, suggesting that the altered LOS structures act as TLR4 antagonists. These results indicated that naturally occurring *B. pertussis* strains with these alterations can infect humans and could have an effect on activating innate immune cells, which in turn could affect pertussis immunity in several ways.

First, reduced TLR4 signaling by *B. pertussis* can affect the clearance of *B. pertussis* following infection, as indicated by *in vivo* studies in which TLR4-deficient mice have been shown to be more sensitive to *B. pertussis* infection[6, 49]. The crucial role of TLR4 signaling for bacterial clearance is further supported by a study in which the TLR4 ligand LOS was co-administered intranasally with *B. pertussis* and no colonization of the bacterium in the lungs was observed[50]. This early protection against *B. pertussis* was found to be orchestrated by TLR4-dependent recruitment of neutrophils[51]. Our results showed that the B0442, B1120, and B1121 isolates do not signal through TLR4 and induce less IL-8 production by DCs than the Tohama I strain. This cytokine is important as a chemoattractant and activator of neutrophils[52]. Therefore, induction of lower IL-8 production by these strains could lead to less neutrophil recruitment and activation, which might result in delayed clearing of the bacterium. Second, reduced TLR4 signaling by *B. pertussis* can affect adaptive immunity, which is essential for complete elimination of this pathogen. Our results showed that the isolates that lacked TLR4 signaling induced less DC maturation than the Tohama I strain, as indicated by decreased expression of maturation markers as well as cytokine production. Cytokines produced by DCs are essential in steering the Th cell response, e.g., IL-12p70 for a Th1 response[2, 3] and IL-1β, IL-6, and IL-23 for a Th17 response[4, 5]. Induction of a Th1 and Th17 response is important in the protective immunity against *B. pertussis* infection[53-55]. Indeed, after incubation of DCs with the Tohama I strain, IL-12p70, IL-6, and IL-23 secretion was induced, indicating that a Th1/Th17 type of $CD4^+$ T cell response could be induced by these DCs, as previously shown by others[8]. Isolates B0442, B1120, and B1121 induced no

production of IL-12p70 and low production of IL-6 and IL-23 by MDDCs, which suggested that these isolates are less capable of inducing a Th1/Th17 response. DCs with immature characteristics have been described to exhibit tolerogenic properties[56, 57]. These tolerogenic DCs can induce the expansion of regulatory T cells either by producing large amounts of IL-10[56, 58, 59] or TGFβ[60]. It could be that strains B0442, B1120, and B1121 induce tolerogenic DCs, resulting in the expansion of regulatory T cells as a strategy to suppress the adaptive immune response against them. These strains, however, do not activate DCs to produce high levels of IL-10, and the levels of TGFβ were not measurable, which could have been due to limitations of the assay. To investigate whether these strains indeed induce tolerogenic DCs that polarize T cells to a regulatory phenotype, cytokine profiling of $CD4^+$ T cells co-cultured with these DCs is required. Third, *B. pertussis*-specific memory responses, induced by natural infection as well as by vaccination, might be suboptimally activated by strains that fail to fully induce DC maturation. Indeed, several studies have shown that DCs not fully matured can inhibit both aspecific and antigen-specific T cell activation[61, 62]. Therefore, *B. pertussis* strains that do not activate TLR4 could affect immunity against this pathogen.

Marr *et al.* previously showed that the LOS of the commonly used laboratory strain B1121/18-323 has a modified lipid A moiety, namely, C10-OH or C12-OH acyl chains instead of C14-OH at the C3' position and lack of GlcN modification[44]. Using mass spectrometry, we showed that this lipid A alteration described for the B1121/18-323 strain also occurs in the clinical *B. pertussis* isolates B1120 and B0442. Furthermore, a mutation in the *lpxLA* gene, leading to an S173>L substitution, as also described recently for *B. pertussis* 18-323 by Shah *et al.*[31], was observed in these two strains. This substitution likely explains the presence of C10-OH or C12-OH acyl chains instead of C14-OH at the C3' position of the lipid A molecule. Besides the alteration in the length of the acyl chain on the C3' position, a lack of GlcN modification was also found with all three isolates. Surprisingly, for the isolates B0442 and B1120, no mutations were found in the genes involved in GlcN modification, unlike a deletion mutation in the *lgmB* gene described for the B1121/18-323 strain[31]. In the Bvg^+ or virulent phase of the bacterium, the genes involved in the GlcN modifications are transcribed, leading to the addition of GlcN to the lipid A moieties[63]. Notably, all isolates used in this study induce hemolysis, which is mediated by ACT, a late Bvg-regulated gene[63], indicating that the isolates are in the Bvg^+ phase. Therefore, the lack of GlcN modifications is most likely not because the bacteria are in the Bvg^- phase, and identification of the genes and mechanisms involved here needs to be further investigated.

Alterations of LPS moieties by Gram-negative bacteria resulting in poor recognition by TLR4 have been described[64] and, interestingly, the type of lipid A alteration can impact the virulence of bacteria. Patients infected with Neisseria meningitidis strains with an lpxL1 mutation, which leads to penta-acylated LPS, had milder symptoms than patients infected with N. meningitidis strains with wild-type hexa-acylated LPS[65]. This lower virulence of the lpxL1 mutant strains might be caused by two mechanisms: decreased TLR4 activation or increased susceptibility to other non-TLR4-dependent host defense mechanisms, such as antimicrobial peptides[66]. The naturally occurring *B. pertussis* strains described here could

also be less virulent due to similar mechanisms. These strains do not activate TLR4 signaling and, in addition, the lack of GlcN modification of the LOS molecules leads to decreased resistance to antimicrobial peptides, as shown in recent studies using GLcN-mutants of *B. pertussis* and the closely related *B. bronchiseptica*[67, 68]. This however remains to be investigated in larger clinical cohorts.

The identified clinical *B. pertussis* isolates, B0442, B1120, and B1121, which lack the ability to induce TLR4 signaling, were all found to be members of a lineage characterized by the *ptxP4* pertussis toxin promoter allele. Whether there is an association between LPS alterations and the ptxP4 family still remains to be investigated. Additional studies screening for larger numbers of *B. pertussis* strains isolated from pertussis patients are under way. It is possible, though, that individuals infected with *B. pertussis* strains with altered lipid A structures have milder or no symptoms due to decreased virulence of the bacteria, and they do not consult a practitioner, which would result in an underestimation of the circulation frequency of these strains. Strain analysis in active surveillance programs in healthy individuals would be required to address this issue.

Taken together, we found that *B. pertussis* isolates that fail to induce TLR4 signaling due to alterations in their lipid A molecules occur naturally. This phenotype leads to reduced maturation of DCs, and as a consequence, these strains may fail to induce a protective immune response or might suboptimally activate existing pertussis memory T cells, which were induced by vaccination or natural infection. The alteration in the lipid A molecules may therefore give the bacterium an advantage in evading the host immune system and therefore establishing infection. Knowledge regarding the strategies used by this pathogen on evading the host immune response is, therefore, essential for the improvement of current vaccines or for the development of new ones.

Acknowledgments

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Supplementary data

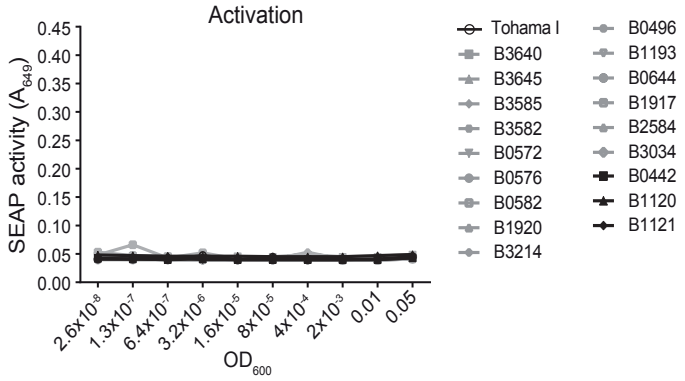

Figure S1 - Incubation of the untransfected HEK-Blue cell line with the 19 *B. pertussis* isolates. The HEK-Blue-Null1 untransfected cells were stimulated with several concentrations of the 19 heat-inactivated (HI) *B. pertussis* strains used in this study. Activation of the cells was measured by determining SEAP activity. Data shown are mean values of three independent experiments.

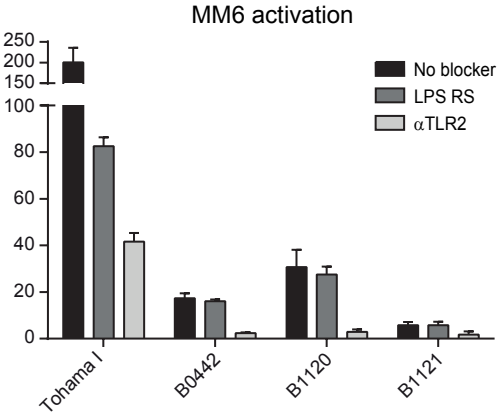

Figure S2 - Role TLR2/TLR4 signaling on the activation of MM6 cells by live *B. pertussis* isolates. MM6 cells were pre-incubated for 3 hours with or without 1 µg/ml TLR4 antagonist LPS-RS or with or without 0.5 µg/ml αTLR2 antibodies after which the cells were incubated with medium or live *B. pertussis* isolates (OD 0.01) for 22 hours. The IL-6 production was measured as a marker for cell activation. Results show representative data of one out of three experiments and is given as mean ± SD.

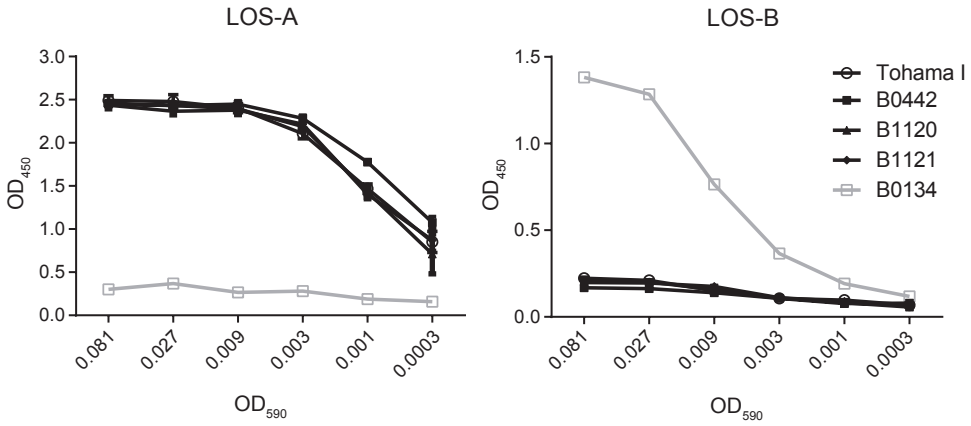

Figure S3 - Production of band A- and band B-LOS by the *B. pertussis* isolates. The LOS-A (left panel) and LOS-B (right panel) production was measured in an ELISA, where the 96-well plate was coated with heat-inactivated *B. pertussis* strains (OD 0.081 to $3x10^{-4}$; 3-fold dilutions). Strain B0134 was used as a control, since it only produces LOS-B[35]. Data shown are representative results of one out of three independent.

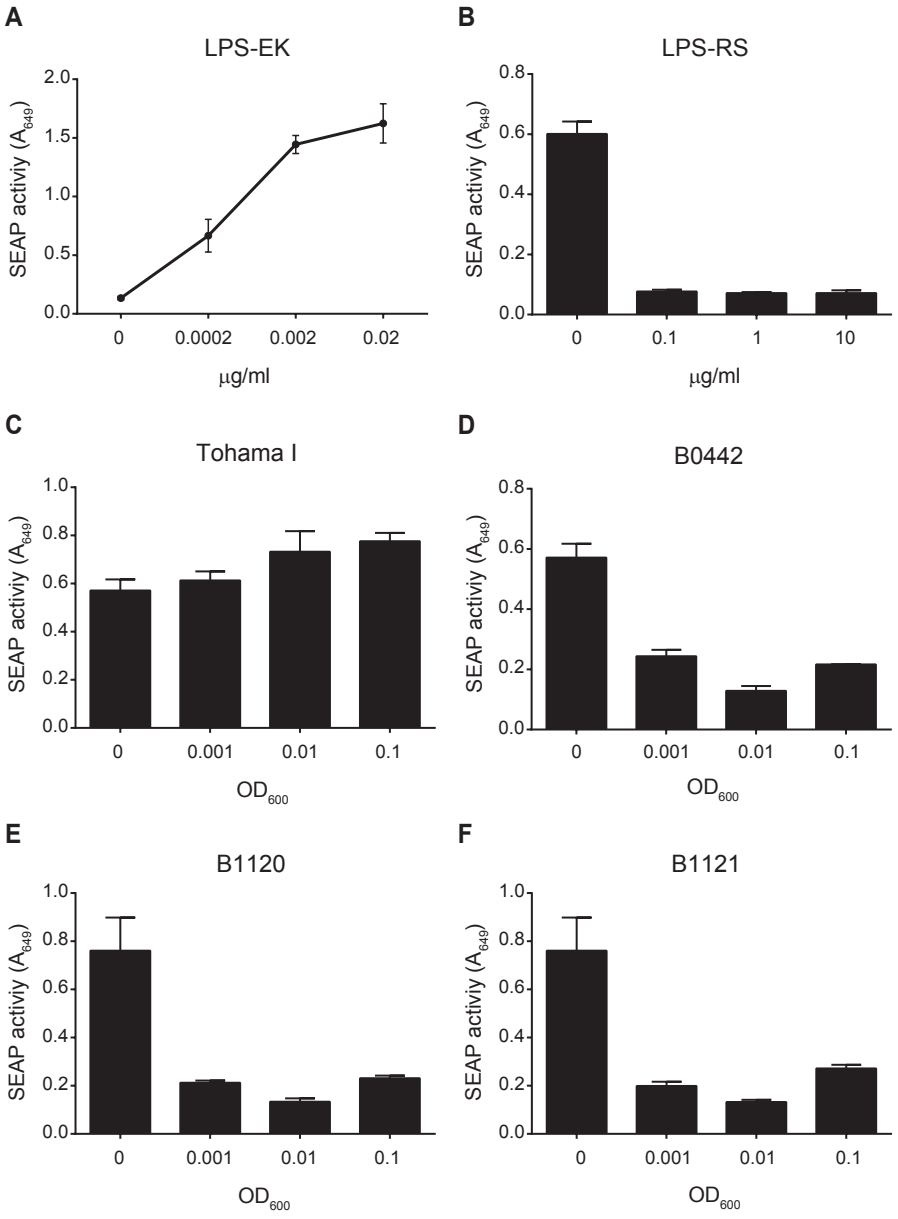

Figure S4 - Effect of Tohama I, B0442, B1120, and B1121 on the TLR4 signaling induced by LPS-EK on the HEK-Blue-hTLR4 cell line. (A) HEK-Blue-hTLR4 activation after stimulation with different concentrations of LPS-EK (0.02, 0.002, and 0.0002 μg/ml) (B-F) Activation of HEK-Blue-hTLR4 cells induced by LPS-EK (0.0002 μg/ml) after pre-incubation with LPS-RS (0.1, 1, and 0 μg/ml) (B), heat-inactivated Tohama I (C), B0442 (D), B1120 (E), or B1121 (F) strains (OD 0.1, 0.01, and 0.001).

Figure S5 - Hemolysis induced by different *B. pertussis* strains on BG plates. Different *B. pertussis* strains were grown at 35°C on BG plates containing glycerol and 15% defibrinated sheep blood for three days after which photographs were taken to evaluate the hemolysis capacity of the strains. Bvg^+ and Bvg^- *B. pertussis* strains were used as a positive and negative control, respectively. Hemolysis is indicated by lightened or transparent areas around the colonies on the BG plates.

Chapter 5

Modulation of the CD4$^+$ T cell response after acellular pertussis vaccination in the presence of TLR4 ligation

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Abstract

Whole-cell pertussis (wP) vaccines are gradually being replaced by aluminum salt-adjuvanted acellular pertussis (aP) vaccines. These promote $CD4^+$ T cell responses with a non-protective Th2 component, while protective immune mechanisms to *B. pertussis* may rather involve long-lived Th1/Th17 type of $CD4^+$ T cells. Here we asked whether addition of a non-toxic meningococcal LPS derivative, LpxL1, as adjuvant can favorably modulate the aP-induced *B. pertussis*-specific $CD4^+$ T cell response in mice. To assess the effect of TLR4 ligation, Th type, quantity, and memory potential of *B.pertussis*-specific $CD4^+$ T cells were determined at the single-cell level after aP and aP+LpxL1 vaccination using intracellular cytokine staining and MHC class II tetramers. Adding LpxL1 to the aP vaccine weakened the Th2 component and strengthened the Th1/Th17 component of the specific $CD4^+$ T cell response. Notably, LpxL1 addition also induced higher frequencies of tetramer positive $CD4^+$ T cells in draining lymph nodes or blood, depending on the phase after vaccination. Moreover, there was a net profit in the number of $CD4^+$ T cells with a central memory phenotype, preferred for long-term immunity. Thus, adding a TLR4 ligand as adjuvant to a current aP vaccine was associated with a more favorable *B. pertussis*-specific $CD4^+$ T cell response.

Introduction

The acute respiratory disease pertussis, caused by the Gram-negative bacterium *Bordetella pertussis*, is an example of a vaccine-preventable disease for which whole-cell vaccines are gradually being replaced by less toxic acellular, aluminum salt-adjuvanted vaccines. However, despite high vaccination coverage, pertussis remains endemic[1-3]. Recently, various studies reported a significantly lower incidence of pertussis among children and adolescents primed with whole-cell pertussis (wP) vaccines than among those primed with acellular pertussis (aP) vaccines[4, 5]. Improvement of current vaccine regimen includes short- and long-term strategies, such as adaption of registered vaccines or development of new vaccines, respectively.

Various studies have indicated that aP vaccines induce considerable Th2 type of $CD4^+$ T cell responses[6-9], while this Th2 compartment does not significantly contribute to the protective immune mechanism of the vaccine[10]. Th1/Th17 type of $CD4^+$ T cells rather play a crucial role in the protective immune response against *B. pertussis*[6-13]. More than aP vaccination, wP vaccination favors Th1 responses[6-9]. An important component of wP vaccines essential for this Th1 skewing is the presence of the TLR4 ligand LPS. A study observing Th1-associated IgG subclass distribution and cytokine responsiveness in Balb/c mice provided the first evidence that addition of a TLR4 ligand to an aP vaccine could steer the response towards a more favorable response[14].

A concern, however, with adding adjuvants to vaccines is that exposure to high amounts of inflammatory signals could lead to short-lived effector cells and effector memory T cells (T_{EM}), rather than central memory T cells (T_{CM}) associated with long- term immunity[15-20]. The effect of TLR4 ligation in the context of aP vaccination on the delicate ratio of $CD4^+$ T_{EM} and T_{CM} cells is currently unknown. Therefore, in this study, the impact of TLR4 ligation on the Th flavor, magnitude, and memory potential of the aP-induced specific $CD4^+$ T cell response was investigated in mice. A non-toxic penta-acylated meningococcal LPS, LpxL1, was used, lacking a 2' secondary C12 acyl chain and with Th1 promoting adjuvanticity in mice[21, 22]. aP vaccine-induced immune responses in the absence or presence of TLR4 ligation were interrogated by specific IgG (subclass) analysis of serum antibodies, intracellular cytokine staining, and multiplex analysis of cytokine production of *in vitro* restimulated splenocytes. The magnitude and memory potential of induced $CD4^+$ T cell responses were studied at the single cell level directly *ex vivo* using an I-A^d tetramer specific for the immunodominant pertactin-specific $CD4^+$ T cell epitope [23].

Materials and methods

Vaccines and antigens

A registered combined diphtheria, tetanus, and aP vaccine (Infanrix, GlaxoSmithKline, UK) was used for the immunization of mice. One human dose (HD) contains a minimum of 30 I.E. diphtheria toxoid, a minimum of 40 I.E. tetanus toxoid, 25 µg FHA, 25 µg Ptx, and 8 µg Prn P.69, all absorbed to aluminumhydroxide. The meningococcal LPS derivative LpxL1 was engineered and obtained as described elsewhere[21]. Purified FHA and Ptx were obtained from Kaketsuken (Japan), and Prn P.69 was expressed in *Escherichia coli* and purified as described elsewhere[24]. To rule out the presence of detectable impurities of *E. coli* LPS in Prn P.69 a Limulus Amebocyte Lysate (LAL) test was performed, hence endotoxin levels were <0.015 EU/ml. Ptx was heat-inactivated before use at 95°C for 15 min.

Ethics statement

This study was approved by the local Committee on Animal Experimentation under permit numbers 201100246 and 201200115. Animal handling in this study was carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation.

Mice and immunization

Adult (6-8 weeks old) Balb/c mice (Harlan, The Netherlands) or C57BL/6 mice (Charles River, Germany) were vaccinated s.c. with 1/4 HD aP vaccine or 1/4 HD of aP vaccine supplemented with 1 µg non-adsorbed LpxL1 (aP+LpxL1), or as a control with PBS, in 0.3 ml, on day 0 (right flank) and day 28 (left flank). Mice were sacrificed on day 38 or on day 52 and blood, spleen, and draining inguinal lymph nodes (dLN) were harvested from each mouse.

B. pertussis-specific antibodies

The levels of Prn-, FHA-, and Ptx-specific mouse IgG, IgG1, and IgG2a (Balb/c), or IgG2c (C57BL/6) antibodies were measured using an in-house developed mouse multiplex immunoassay, as described earlier [25]. Experimental serum samples were diluted 1:1250 and 1:12500. The used reporter antibodies were R-PE-conjugated goat-anti-mouse IgG (Southern Biotech), IgG1 (Southern Biotech), IgG2c (Jackson ImmunoResearch), or rat-anti-mouse IgG2a (Southern Biotech) antibodies. Analysis was performed with a Bio-Plex 200, using Bio-Plex Manager software (version 5.0, Bio-Rad Laboratories). MFI of ≤1 were considered as non-detectable (nd). Results indicate the averaged ^{10}Log U/ml IgG or MFI IgG subclasses per antigen per group of mice and the averaged ratios of ^{10}Log MFI IgG2a(c)/10Log MFI IgG1 (±SD) per group of mice.

Isolation and *in vitro* restimulation of splenocytes

Homogenized splenocytes were treated with erythrocyte lysis buffer and subsequently cultured in 24-well plates at 6×10^6 cells/well for 7 days at 37°C in a humidified atmosphere containing 5% CO_2 in the presence of IMDM complete medium (IMDM medium (Gibco) supplemented with 8% FCS, 100 units penicillin, 100 units streptomycin,

2.92 mg/ml L-glutamine, and 20 µM β-mercaptoethanol (Sigma)) or IMDM complete medium supplemented with 1 µg/ml Prn, 1 µg/ml Ptx, or 1 µg/ml FHA. On day 7, supernatant was collected for cytokine analysis, and subsequently cells were transferred to U-bottom 96-well plates at 5×10^5 cells/well and again restimulated overnight using the same antigen conditions.

Cytokine profiling using multiplex technology

The concentration of various cytokines (IL-4, IL-5, IL-10, IL-13, IL-17, TNFα, and IFNγ) was determined in culture supernatant, using a Milliplex mouse cytokine 7-plex Luminex kit (Millipore), according to the manufacturer's protocol. Supernatants were taken at day 7 after *in vitro* restimulation of the splenocytes, which was optimal for analysis of accumulated Th cytokines, except for the early cytokines IL-2 and IL-4 (own obervations). Measurements and data analysis were performed with Bio-Plex 200, using Bio-Plex Manager software (version 5.0, Bio-Rad Laboratories). Results were corrected for the background (IMDM complete medium as control), calculated in pg/ml per mouse per stimulation per cytokine.

Intracellular cytokine staining

Intracellular cytokine staining (ICS) was performed on the day 8 restimulated splenocytes using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), according to the manufacturer's protocol. A time curve of 3-8 days of *in vitro* splenocyte stimulation had indicated that 8 days of restimulation gave the highest sensitivity to detect antigen-specific responses in this model (own observations). Briefly, the 8 days cultured spenocytes were incubated with 10 µg/ml Golgiplug (BD Biosciences), 1 µg/ml αCD28 (BD Pharmingen), and 1 µg/ml αCD49d (BD Pharmingen) during the last 5 hours of restimulation with the *B. pertussis* antigens. Cells were then stained in FACS buffer (PBS (pH 7.2) supplemented with 0.5% BSA (Sigma Aldrich) and 0.5 mM EDTA (ICN Biomedicals)) with Pacific blue-conjugated anti-CD4 (Biolegend), FITC-conjugated anti-CD44 (BD Biosciences), and with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen). Thereafter, cells were fixed, permeabilized, and stained with PE-conjugated anti-IFNγ (BD Biosciences), APC-conjugated anti-IL-5 (Biolegend), and PerCP-Cy5.5-conjugated anti-IL-17A (eBioscience). Data were acquired on a FACS Canto II (BD Biosciences), gated (Figure S1) and analyzed using FlowJo software (Tree Star). Percentages of antigen-restimulated cytokine producing $CD4^+CD44^+$ T cells in vaccinated mice were regarded antigen-specific, both individually and at the group level, if they were at least twice above background levels in naive counterparts.

Detection and phenotyping of Prn_{10-24}-specific $CD4^+$ T cells

An APC-conjugated $I\text{-}A^d$ tetramer (TM) specific for the P.69 Prn_{10-24} epitope (NIH Tetramer Facility, Atlanta, Georgia, USA) were validated on a previously described P.69 Prn_{10-24}-specific $I\text{-}A^d$-restricted T cell hybridoma[23]. An APC-conjugated $I\text{-}A^d$ TM containing the CLIP peptide was provided as a negative control and was used to optimize staining conditions in a limited set of pilot experiments (NIH Tetramer Facility, Atlanta, Georgia, USA) (data not shown). Cells of both homogenized dLN and erythrocyte lysis buffer-treated blood were pooled per 3 mice for tetramer analysis. Cells were freshly

stained with the Prn_{10-24}/I-A^d TM or control TM in RPMI medium containing 10% FCS for 1 hour at 37°C. Subsequently, cells were stained with Pacific blue-conjugated anti-CD4 (Biolegend), FITC-conjugated anti-CD44 (BD Biosciences), PerCP-Cy5.5-conjugated anti-CD62L (Biolegend), PE-conjugated anti-CD127 (eBioscience), and LIVE/DEAD Fixable Aqua Dead Cell Stain Kit for 30 min at 4°C in FACS buffer. Data were acquired on a FACS Canto II, gated (Figure S2) and analyzed using FlowJo software. Per sample, 0.3-1.0×10^6 $CD4^+$ T cells were analyzed to allow detection of low frequent (0.01-0.1%) Prn_{10-24}/I-A^d-TM^+CD4^+ T cells at a desired level of reliability (coefficient of variation between 5-10%).

Statistics

Statistical significance of differences in responses among groups in the multiplex assay was assessed using a Student's t-test (two-tailed, unequal variance) on log-transformed data. Statistical significance differences for the TM analysis were calculated using a nonparametric Mann-Whitney test and for the ICS analysis a two-way ANOVA was used.

Results

B. pertussis antigen-specific IgG responses after aP or aP+LpxL1 vaccination

As an indirect measure of immunological programming of the CD4$^+$ T cell response, day 52 serum levels of total IgG, IgG1, and IgG2a specific for Ptx, FHA, and Prn were compared between aP and aP+LpxL1 vaccinated Balb/c mice. Both aP and aP+LpxL1 vaccination induced high antibody responses (Figure 1). Mice vaccinated with the LpxL1-adjuvanted aP vaccine had significantly higher levels of Ptx- and FHA-specific IgG than mice vaccinated with the aP vaccine alone (Figure 1, left and middle panel). Moreover, for all antigens tested, the ratio of specific IgG2a and IgG1 was increased in aP+LpxL1-vaccinated mice (Table 1). Thus, in Balb/c mice, the aP-induced *B. pertussis*-specific humoral response is influenced by the presence of LpxL1, and the increased IgG2a/IgG1 ratio suggests an underlying enhanced Th1 type of CD4$^+$ T cell response.

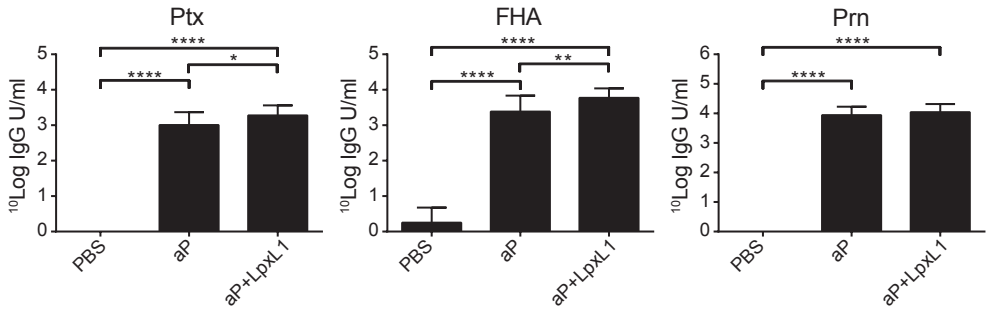

Figure 1 - Vaccine antigen-specific IgG titers in vaccinated Balb/c mice. Balb/c mice were vaccinated twice (d0 and d28) with PBS, aP, or aP+LpxL. The Ptx-, FHA-, and Prn-specific IgG levels in sera, collected on day 52, were analyzed using a multiplex immunoassay. Data are expressed as the average ^{10}LOG U/ml ± SD of 18 mice per group and are pooled data from two independent experiments. *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001.

Table 1 - aP antigen-specific IgG1 and IgG2a levels in sera of vaccinated mice on day 52

Vaccine	IgG2a[a]			IgG21[a]			IgG2a/IgG1 ratio[a]		
	Ptx	FHA	P.69 Prn	Ptx	FHA	P.69 Prn	Ptx	FHA	P.69 Prn
PBS	n.d.[c]	0.33 ±0.25	n.d.[c]	n.d.[c]	0.09 ±0.15	n.d.[c]	n.a.[d]	0.51 ±0.91	n.a.[d]
aP	1.18 ±0.82	1.00 ±0.70	0.93 ±0.41	3.76 ±0.18	3.59 ±0.27	4.07 ±0.13	0.31 ±0.21	0.27 ±0.18	0.26 ±0.10
aP+LpxL1	1.98 ±0.62	1.81 ±0.68	1.62 ±0.53	3.82 ±0.24	3.81 ±0.14	4.11 ±0.11	0.52 ±0.19*	0.47 ±0.17*	0.42 ±0.14**

[a]Represented as average ^{10}LOG MFI ± SD of 18 mice per group; [b]Represented as average ^{10}LOG MFI IgG2a/^{10}LOG MFI IgG1 ratio ± SD of individual mice.; [c]n.d., not detectable; [d]n.a., not applicable; *p < 0.05 aP+LpxL1 vaccination vs aP vaccination; **p < 0.01 aP+LpxL1 vaccination vs aP vaccination

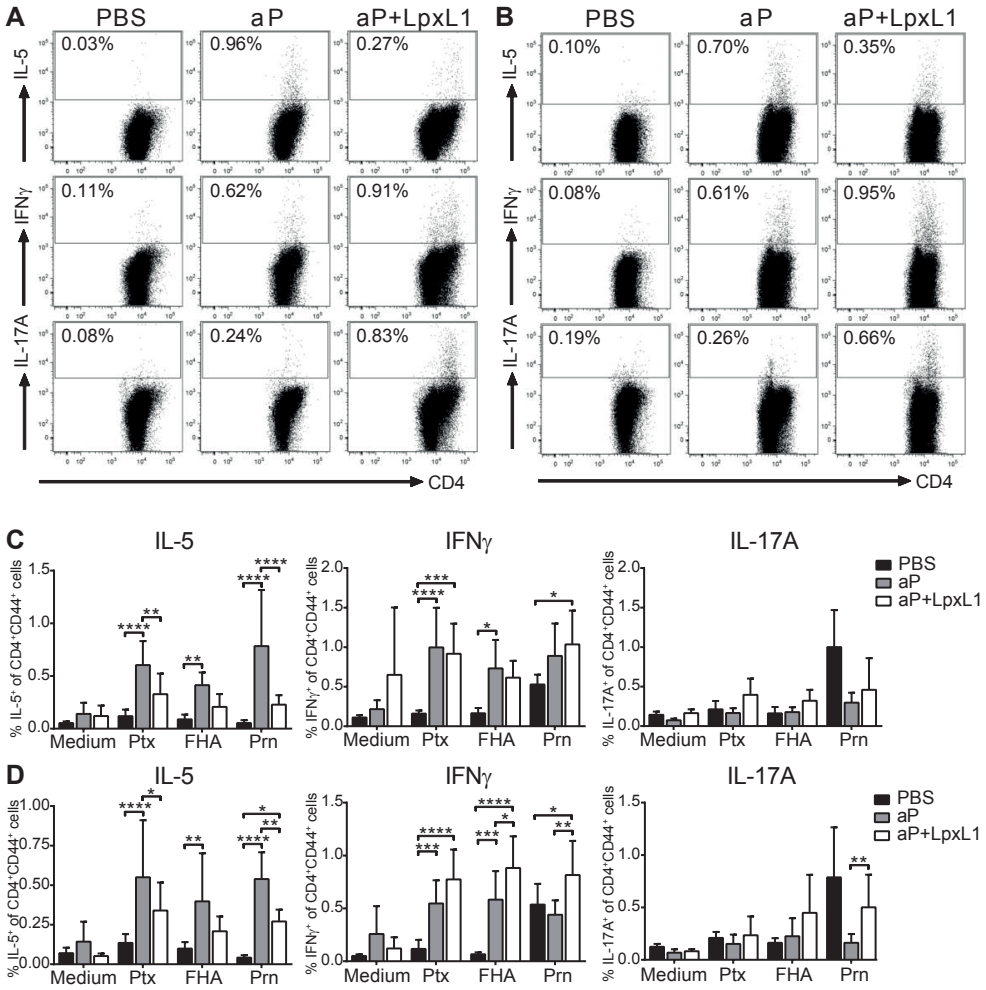

Figure 2 - Intracellular cytokine staining of vaccine antigen-stimulated splenocytes. (A-D) Balb/c mice were vaccinated twice (d0 and d28) with PBS, aP or aP+LpxL1. Splenocytes were obtained on day 38 and 52 and stimulated for 8 days with IMDM complete medium supplemented with 1 µg/ml Ptx, FHA, or Prn, or with the negative control IMDM complete medium. (A-B) Representative dotplots from splenocytes of single mice vaccinated as indicated, stimulated with Ptx, showing the percentage of cytokine-producing cells of gated $CD4^+$ $CD44^+$ T cells, are depicted for day 38 (A) and day 52 (B). (C-D) Summary of the percentage IL-5-, IFNγ-, or IL-17A-producing cells of $CD4^+CD44^+$ T cells of spleens harvested on day 38 (C) or day 52 (D) with the vaccine antigens as indicated after 8 days of *in vitro* stimulation. The results are given as mean ± SD of 9 mice per group and show data from one experiment out of two performed with similar results. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Th flavor of $CD4^+$ T cells after aP or aP+LpxL1 vaccination

The effect of adding LpxL1 to the aP vaccine on the Th flavor of *B. pertussis* antigen-specific $CD4^+$ T cells in Balb/c mice was studied at the single cell level using ICS. For Ptx stimulation, examples of the dotplots are shown in Figures 2A, 2B, and S1. After aP vaccination, IL-5-producing and IFNγ-producing $CD4^+CD44^+$ T cells, specific for Ptx, FHA, and Prn and Ptx

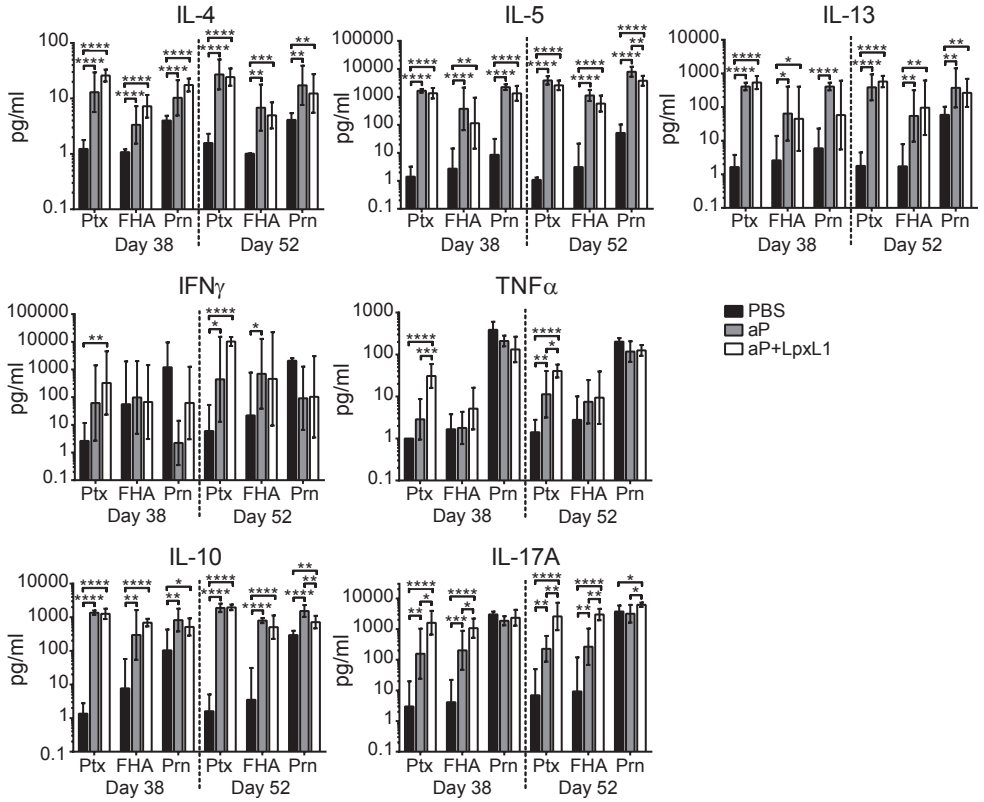

Figure 3 - Cytokine levels in supernatant of vaccine antigen-stimulated splenocytes. The presence of cytokines in day 7 culture supernatants was tested using a multiplex assay. Splenocytes collected on day 38 and day 52, from mice vaccinated as indicated, were stimulated for 7 days with either IMDM complete medium, or IMDM complete medium supplemented with 1 µg/ml Ptx, 1 µg/ml FHA, or 1 µg/ml Prn. Results in pg/ml are corrected for the background level (IMDM complete medium control) and are given as geomean ± 95% CI of 9 mice per group. Data from one experiment out of two performed with similar results is shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

and FHA, respectively, were detected on both time points (Figures 2C and D, left and middle panels). Further, no IL-17A-producing aP-antigen-specific $CD4^+CD44^+$ T cells were observed in these mice at either time point (Figures 2C and D, right panels). In contrast, after addition of LpxL1 to the aP vaccine, the percentage IL-5-producing $CD4^+CD44^+$ T cells was significantly reduced and was, except after Prn stimulation on day 52, similar to the percentage found in naive mice (Figures 2C and D, left panels). Further, aP+LpxL1 vaccination also induced IFNγ-producing $CD4^+CD44^+$ T cells, specific for Ptx and Prn on day 38 and Ptx, FHA, and Prn on day 52. Notably, vaccination with aP+LpxL1 led to an elevated percentage FHA- and Prn-specific IFNγ-producing $CD4^+CD44^+$ T cells, with the same trend for Ptx-specific cells, on day 52 when compared to percentage after aP vaccination alone (Figure 2C and D, middle panel). Although for all tested antigens aP+LpxL1 vaccination seemed to induce elevated percentage IL-17A-producing $CD4^+CD44^+$ T cells when compared to aP vaccination, at the

group level this trend was not significant, except for Prn on day 52 (Figures 2C and D, right panels). However, this latter response cannot reliably be interpreted because of the high background levels of IL-17A$^+$CD4$^+$CD44$^+$ T cells after *in vitro* Prn stimulation in naive control mice (Figures 2C and D, right panels).

To provide further insight into the capacity of the *B. pertussis*-specific CD4$^+$ T cell compartment to secrete cytokines, supernatants of 7 days-stimulated splenocytes were analyzed for accumulated cytokines using a multiplex assay. aP vaccination induced secretion of Th2 cytokines (IL-4, IL-5, and IL-13) and IL-10 by splenocytes in response to all three antigens on both time points. Further, secretion of Th1 cytokines (IFNγ and TNFα) by these splenocytes was induced after stimulation with respectively Ptx and FHA on day 52 and Ptx on both time points. IL-17A secretion was also observed after Ptx and FHA stimulation on both time points. After LpxL1-adjuvanted aP vaccination, secretion of the Th2 cytokines and IL-10 was observed in response to all three antigens, yet the levels of IL-5 and IL-10 in response to Prn were decreased compared to the levels after aP vaccination on day 52 (Figure 3). Further, aP+LpxL1 vaccination led to secretion of both measured Th1 cytokines in response to Ptx, additionally, the level of TNFα was significantly increased compared to the level secreted by splenocytes from aP vaccinated mice (Figure 3). Moreover, vaccination with aP+LpxL1 induced IL-17A secretion in response to Ptx and FHA on both time points and, notably, the levels were higher than those induced by aP vaccination (Figure 3).

It should be noted that Prn restimulation induced high background in both assays, and although the results are similar to that after Ptx and FHA restimulation, these results might be less reliable. Collectively, based on data after Ptx and FHA restimulation, the ICS and supernatant data reveal that adding LpxL1 to the aP vaccine reduced *B. pertussis*-specific Th2 type responsiveness and increased *B. pertussis*-specific Th1 and Th17 type of CD4$^+$ T cell responses in Balb/c mice.

Magnitude of the Prn$_{10\text{-}24}$-specific CD4$^+$ T cell population

In addition to Th flavor, the size and long-term circulation of memory CD4$^+$ T cell populations is important for the effectiveness of the immune response. Therefore, we developed an MHC class II TM based on the immunodominant Prn epitope in Balb/c mice, Prn$_{10\text{-}24}$/I-A^d, described by Stenger *et al.*[23]. Examples of the TM stainings of dLN cells from individual vaccinated and control mice, performed directly *ex vivo*, are depicted in Figures 4A and S2. Compared to naive mice, vaccinated mice showed a significant increase in the frequency of Prn$_{10\text{-}24}$-specific CD4$^+$ T cells in dLN and blood on both day 38 and day 52 (Figures 4A-C). Furthermore, on day 38, the frequency of Prn$_{10\text{-}24}$-specific CD4$^+$ T cells was significantly higher in the dLN of aP+LpxL1- compared to aP-vaccinated mice, yet, this effect was not seen in blood (Figures 4B and C). Notably, on day 52 a significant increase in the frequency was found in blood of aP+LpxL1-vaccinated mice, whereas in dLN, the frequencies were similarly high in both groups (Figures 4B and C). These findings indicate that addition of LpxL1 increases the size of the Prn$_{10–24}$-specific CD4$^+$ T cell population.

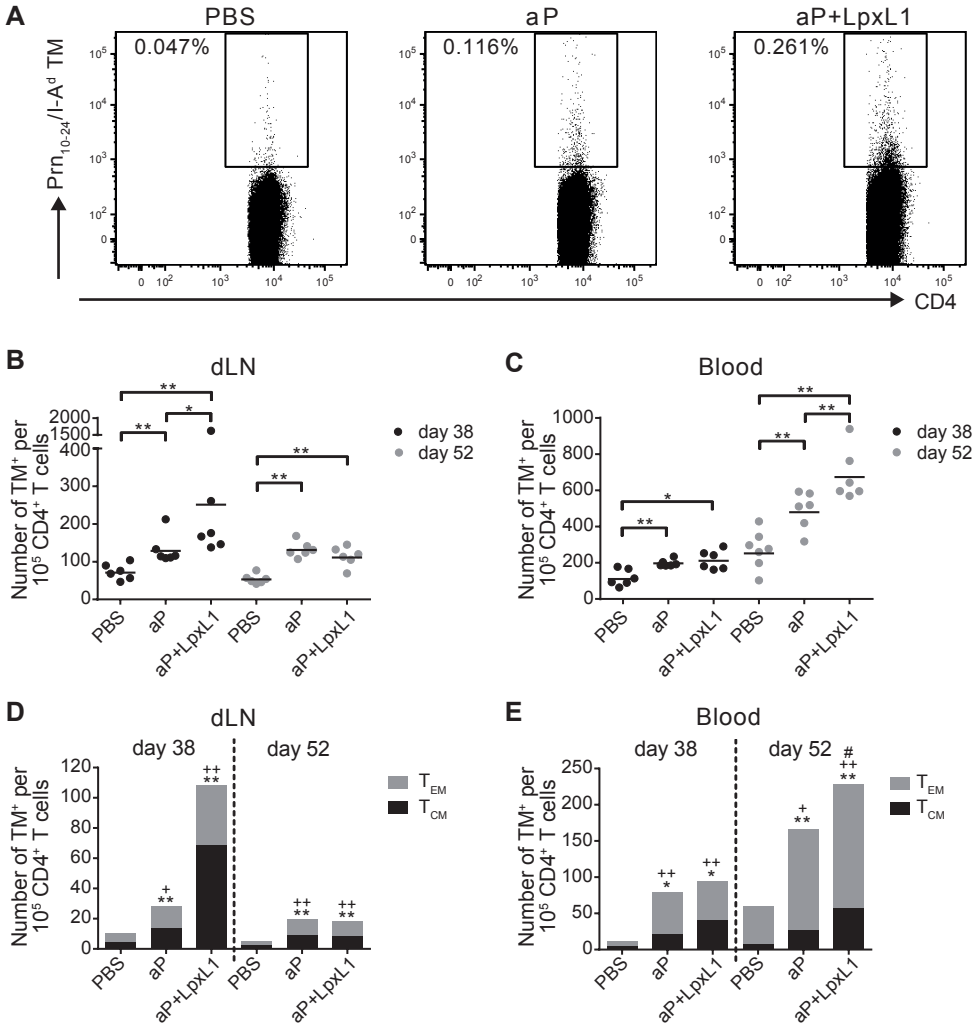

Figure 4 - Frequency and phenotype of Prn_{10-24}-specific memory $CD4^+$ T cells after vaccination. (A-G) Balb/c mice were vaccinated on day 0 and day 28 with PBS, aP, or LpxL1-adjuvanted aP vaccine. dLN and blood were collected on day 38 and day 52. (A) Representative dotplots of the Prn_{10-24}/I-A^d TM staining of dLN cells collected on day 38 from individual mice treated as indicated, showing the percentage Prn_{10-24}/I-A^d TM^+ cells of $CD4^+$ T cells. (B-C) The frequency of Prn_{10-24}/I-A^d TM^+CD4^+ T cells in the dLN (B) and in blood (C) per 10^5 $CD4^+$ T cells. *p<0.05, **p<0.01. (D-E) The number of Prn_{10-24}/I-A^d $TM^+CD4^+CD44^+CD62L^+$ cells (T_{CM}) or Prn_{10-24}/I-A^d $TM^+CD4^+CD44^+CD62L^-$ cells (T_{EM}) per 10^5 $CD4^+$ T cells in the dLN (D) and in blood (E). *p<0.05, **p<0.01 T_{EM} of aP or aP+LpxL1 versus PBS; +p<0.05, ++p<0.01 T_{CM} of aP or aP+LpxL1 versus PBS. #p<0.05 T_{CM} of aP+LpxL1 versus aP. The results are an average of 9 samples, each consisting of a pool of 3 mice and are pooled data from 2 independent experiments.

Memory phenotype of the Prn_{10-24}-specific $CD4^+$ T cell population

The homing characteristics of the memory $CD4^+$ T cells can give an indication of T cell longevity, since induction of T_{CM} cells has been correlated with long-term immunity[17-20]. Therefore, the

phenotype of Prn_{10-24}-specific $CD4^+$ T cells was investigated directly *ex vivo*, based on CD44 and CD62L expression. T_{CM} are generally characterized as $CD44^+CD62L^+$ and T_{EM} as $CD44^+CD62L^-$. In mice vaccinated with aP+LpxL1, the frequency of Prn_{10-24}-specific T_{CM} circulating in blood in the late phase after the booster vaccination was significantly higher than in mice vaccinated with the aP vaccine (57.3 ± 37.3 versus 27 ± 25.9 cells per 10^5 $CD4^+$ T cells, respectively) (Figures 4D and E). Together, the results from the TM analysis indicate that adding LpxL1 to an aP vaccine not only induces a higher frequency of Prn_{10-24}-specific $CD4^+$ T cells, but also supports the generation of a central memory phenotype, favored for providing a long term immunity.

B. pertussis antigen-specific IgG and $CD4^+$ T cell responses after aP or aP+LpxL1 vaccination in C57BL/6 mice

To investigate whether the modulating effect of LpxL1 was exclusively seen against the Th2-prone Balb/c genetic background, similar analyses were performed in the more Th1-prone C57BL/6 mouse strain. Due to the lack of available C57BL/6 epitope-specific TMs, only the Th flavor of aP antigen-specific $CD4^+$ T cells was investigated by IgG (subclass) analysis of serum antibodies, ICS, and supernatant analysis of antigen restimulated splenocytes. Mice vaccinated with aP+LpxL1 had a significantly higher level of FHA-specific serum IgG (Figure S3, middle panel). Moreover, the ratio of specific IgG2c/IgG1 in aP+LpxL1-vaccinated mice tended to be higher compared to aP-vaccinated mice (Table S1). At the single T cell level, Prn restimulated responses were assessed. After LpxL1 addition, specific $CD4^+CD44^+$ T cells producing IL-5 were significantly inhibited and those producing IFNγ significantly increased in number, while no significant effect was observed for the IL-17A response (Figure S4A). Furthermore, multiplex cytokine testing of culture supernatants of splenocytes after Prn restimulation indicated significantly reduced levels of IL-4, IL-5, and IL-10, increased levels of IL-17A, but similar levels of IFNγ and TNFα when comparing aP-vaccinated with aP+LpxL1-vaccinated mice, respectively (Figure S4B). Collectively, these data indicate that also in a Th1-prone mouse strain, aP vaccination promotes a Th2 response, and the addition of LpxL1 to the aP vaccine leads to a reduced presence of Th2 type of $CD4^+$ T cells and their cytokines, and an increased level of Th1/Th17 type of $CD4^+$ T cells and IL-17A.

Discussion

Our study asked whether adding a non-toxic TLR4 ligand to a currently used aP vaccine is a potential strategy to enhance Th flavor, magnitude, and memory potential of the *B. pertussis*-specific $CD4^+$ T cell response. Two major observations were made. First, addition of the engineered meningococcal penta-acylated LPS mutant LpxL1 to the aP vaccine shifted the *B. pertussis*-specific Th cell response from a Th2 type dominated outcome to the more favorable Th1/Th17 type response. We demonstrated this change in Th flavor indirectly by IgG subclass analysis and directly by combining single cell analysis using flowcytometry and splenocyte supernatant analysis. Notably, the effect was found not only for the Th2-prone Balb/c mouse model but also for the more Th1-prone C57BL/6 mouse. Second, by using a novel MHC class II tetramer, we showed for the first time that adding a TLR4 ligand to the aP vaccine results in a higher frequency of *B. pertussis*-specific $CD4^+$ T cells and, moreover, a more pronounced centrally homing T_{CM} phenotype, suggesting $CD4^+$ T cell self-renewal capacity and longevity.

The methods to detect cytokine profiles of the *B. pertussis*-specific $CD4^+CD44^+$ T cells might have some limitations. For detection of specific responses, a relatively long duration of *in vitro* restimulation (8 days) was required to achieve a good signal to noise ratio. This long restimulation period might have led to high background responses after Prn stimulation in naive mice, seen in both ICS and supernatant analyses. Although the results showed similar trends as after Ptx and FHA stimulation, Prn data should be interpreted with caution. Therefore, the conclusions of the Th flavor were based on the results after Ptx and FHA stimulation. This high background was mainly seen for IL-17A in the ICS analysis, since fewer *in vitro* Prn-responding IL-17A-producing $CD4^+CD44^+$ T cells were seen in vaccinated mice than in naive mice. This might be related to the presence of vaccine-primed Th1 and Th2 $CD4^+$ T cells as these cells produce IFNγ and IL-4, which may inhibit the production of IL-17A by $CD4^+$ T cells[26, 27]. Further, to some extent the alterations in cytokine responsiveness after adding LpxL1 to aP were seen in both ICS and supernatant analysis, e.g. increase in typical Th1 cytokines IFNγ (ICS) and TNFα (supernatant analysis), decrease in IL-5 production (both assays) and increase in IL-17A responsiveness (supernatant analysis). Yet the effect of adding LpxL1 to aP on the Th2 cytokines was more clearly observed in ICS than in culture supernatants, possibly due to different kinetics of the cytokines and consumption during the restimulation period. By combining both assays, a more complete overview of the cytokine responsiveness of the $CD4^+$ T cells was generated. It should be noted, that the environmental cytokine milieu such as produced in a relatively long *in vitro* culturing period, may bias the final outcome of cytokine expression patterns[28]. However, mice in the various immunization groups were all subjected to the same stimulation protocol, thus possible bias due to the long stimulation period is similar in all groups. Therefore, the results obtained with the *in vitro* restimulation protocol used in this study reflects group dependent *in vivo* T cell commitment.

The shift in Th flavor might be initiated in different manners. LPS and its derivatives are known to signal through TLR4 and MyD88, leading to IL-12p70 and IL-6 release, which are

key Th1 cell differentiation cytokines[29]. Th1 cytokines have been shown to indirectly inhibit Th2 differentiation, leading to the decrease in Th2 cells[30]. Furthermore, in LPS-primed macrophages, aluminum salts induce IL-1β release[31]. In combination with IL-6, IL-1β could promote Th17 differentiation of naive $CD4^+$ T cells[32]. Even a slight shift in Th flavor, from a Th2 dominated to a more Th1/Th17 type of $CD4^+$ T cell response, might have a large impact on vaccine outcome. Mice lacking a functional Th1 and Th17 T cell response cannot clear *B. pertussis* from the lungs[6, 10, 13], while the Th2 compartment induced by currently used aP vaccines alone does not seem to contribute to the protective immune mechanism after vaccination[10].

A concern of adding a pro-inflammatory adjuvant to a vaccine is that it leads to a short-term effector response only, due to the induction of high amounts of inflammatory signals[15, 16]. Demonstrating adjuvant-induced shifts or benefits in long-term efficacy of aP vaccination in the intranasal *B. pertussis* challenge mouse model would be difficult to achieve, since aP vaccines readily protect in this model, even when a 1/100 HD of the aP vaccine is used (data not shown). Instead, we reasoned that the generation of *B. pertussis*-specific T_{CM} cells predicts whether a long-term immunity is induced[17-20]. By directly *ex vivo* staining of splenocytes with a combination of a MHC class II-Prn_{10-24}/I-A^d tetramer and homing markers CD62L and CD44 both the magnitude and, more importantly, the memory phenotype of the *B. pertussis*-specific $CD4^+$ T cell response could be investigated. The increased Prn_{10-24}-specific $CD4^+$ T cell population in aP+LpxL1 vaccinated mice, first detectable in lymphoid tissue and subsequently in blood, might be explained by a blockade of T cell egress from the dLN early after antigen encounter, favoring an initial accumulation of specific $CD4^+$ T cells[33, 34]. After several days of activation, the T cells can migrate out of the dLN, leading to higher frequencies of specific T cells in other compartments like blood[35]. The ratio between T_{EM} and T_{CM} is not significantly affected by LpxL1 addition to the aP vaccine, consequently the higher frequencies of Prn_{10-24}/I-A^d-specific $CD4^+$ T cells leads to more cells with a T_{CM} phenotype and possible superior longevity.

MPL, an LPS derivative from *Salmonella minnesota*, and synthetic TLR4 agonist such as GLA and RC-529 are currently used in approved vaccines or under clinical evaluation, indicating that LPS derivatives as adjuvant are clinically feasible[36-38]. A downside of TLR4 agonists is that they contain only the lipid A part of the LPS, making it insoluble in water and hindering vaccine formulation. A detoxified penta-acylated LPS derivative like LpxL1 could be an alternative, since it contains the complete LPS molecule, including both the lipid A and the oligosaccharide core, making it soluble in water. For TLR4 it has been described that there is variation in ligand specificity between species[39]. Therefore, for the human situation an equivalent of LpxL1 needs to be determined. PagL LPS, a penta-acylated meningococcal LPS derivative could be a suitable candidate. This PagL LPS has the required reduced endotoxic activity compared to that of wildtype LPS[22, 40], but has retained agonistic activity for human TLR4 [van der Ley, manuscript in preparation].

Recently, other studies showed that TLR2 and TLR9 ligation can likewise induce a more

favorable Th1 response after aP vaccination[10, 41]. However, the effect of these TLR2 and TLR9 adjuvants on the T cell memory potential was not investigated. Nevertheless, the notion that that TLR4, TLR2, as well as TLR9 ligands are capable of programming aP-induced $CD4^+$ T cell responses into a more favorable cytokine pathway, open up multiple possible avenues for aP vaccine improvement.

In conclusion, the duration of protection of current aP vaccines, once introduced as superior alternatives for reactogenic whole-cell vaccines, is highly disappointing[4, 5, 42]. Development of novel vaccines is a long-term investment. Our findings support the idea that adding a water soluble TLR4 ligand might be a feasible short-term solution to improve the current aP vaccine. Pathogen-specific single cell-based analysis, such as applied here, is important to establish the type, quantity, and memory potential of the relevant $CD4^+$ T cell populations, and should be part of any further adjuvant evaluation.

Acknowledgments

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Supplementary data

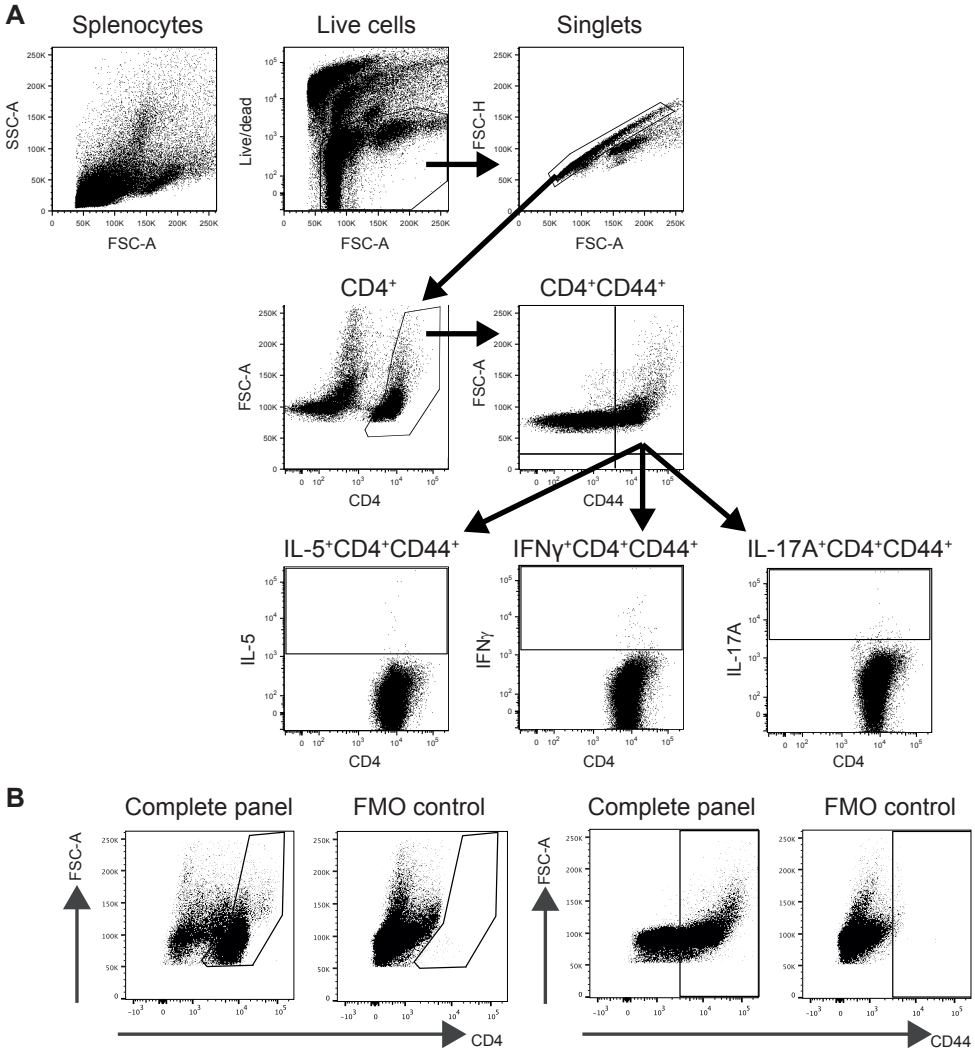

Figure S1 - Gating strategy ICS data. (A) To identify the CD4⁺CD44⁺ T cells for Figure 2, the live cells were first gated based on low Aque (live/dead staining) expression after which the doublets were excluded (FSC-A/FSC-H). Then these cells with CD4⁺ expression were gated after which the (activated) CD44⁺CD4⁺ cells were selected for further analysis. The aP vaccine antigen-specific cytokine producing CD4⁺ T cells were identified in live CD4⁺CD44⁺ cells in Figure 2. Representative dotplots for *in vitro* Ptx restimulated splenocytes from a naive mouse are shown. (B) Splenocytes of a naive mouse were cultured for 8 days, after which ICS was performed on the cells as described in Material and Methods. Gating bounds of the CD4⁺ cells and CD44⁺ cells as applied in panel A on cells that were stained with the complete mAb panel (left) and on cells that were stained with the fluorescence-minus-one (FMO) control panels of both CD4 and CD44 (right graphs) are shown.

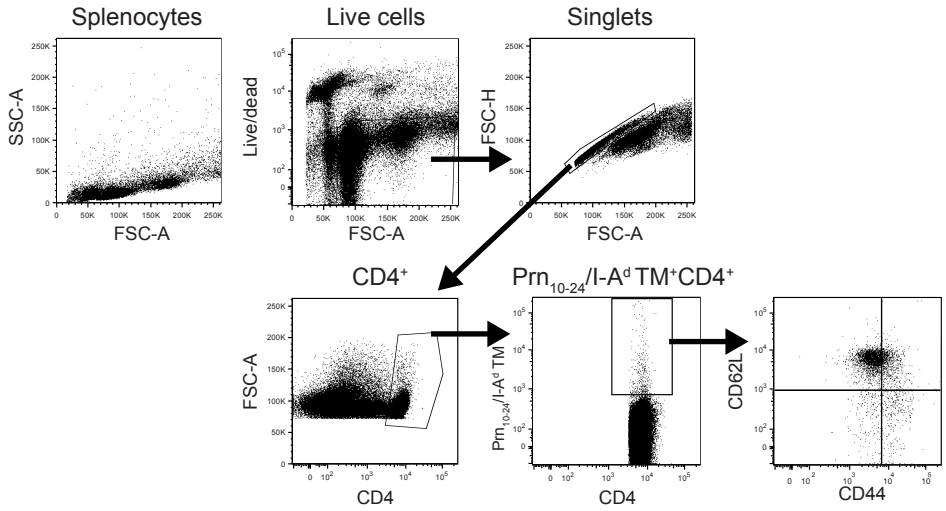

Figure S2 - Gating strategy tetramer data. To identify the Prn_{10-24}/I-A^d -specific $CD4^+$ T cells for Figure 4, the live cells were first gated based on low Aqua (live/dead staining) expression after which the doublets were excluded (FSC-A/FSC-H). Then these cells with $CD4^+$ expression were gated to select the Prn_{10-24} /I-A^d TM binding population, after which the CD44 and CD62L expression was investigated. Representative dotplots are shown

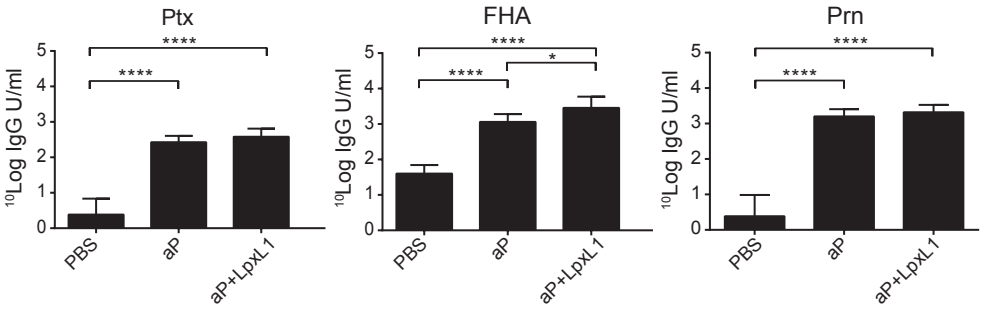

Figure S3 - Vaccine antigen-specific IgG titers in vaccinated C57BL/6 mice. C57BL/6 mice were vaccinated with PBS, aP, or aP+LpxL1 vaccine on day 0 and day 28. The Ptx-, FHA-, and Prn-specific IgG levels in sera, collected on day 52, were analyzed using a multiplex immunoassay. Data are expressed as the average ^{10}LOG U/ml ± SD of 6 mice per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

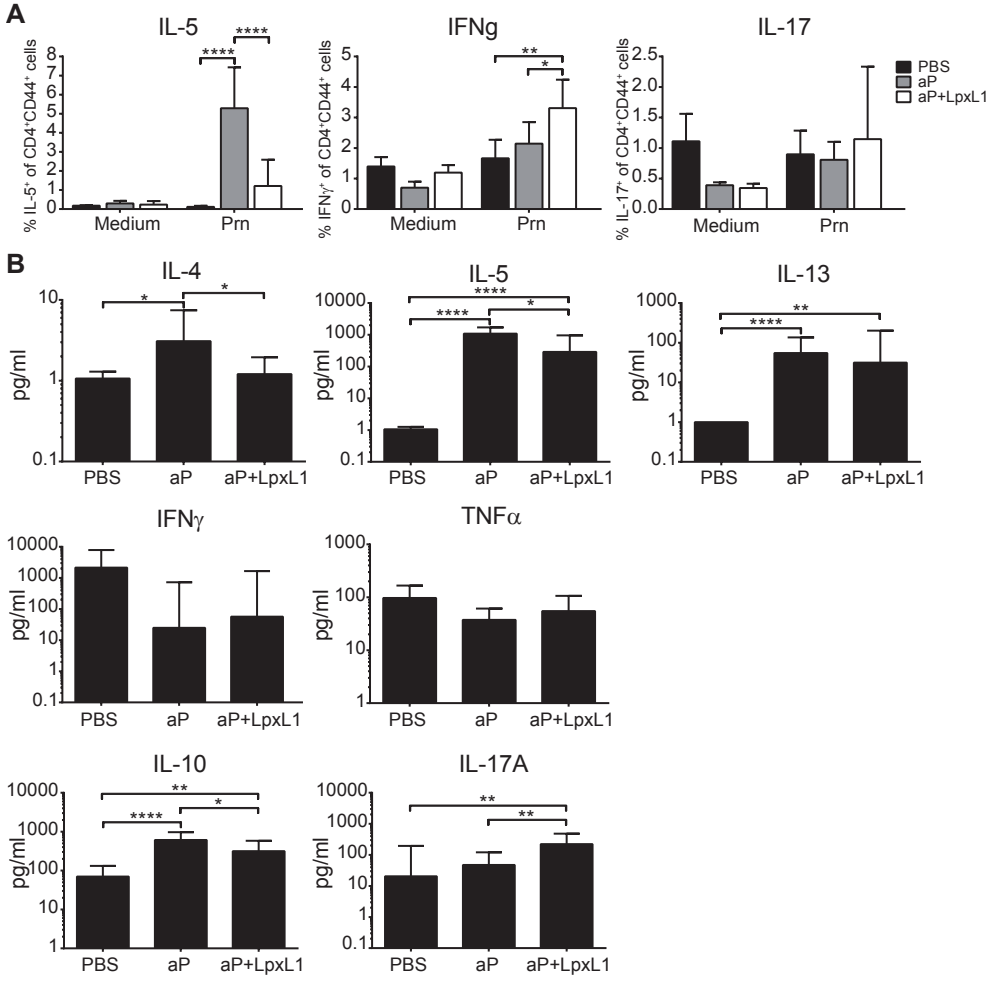

Figure S4. Th flavor of CD4⁺ T cells in C57BL/6 mice after aP or aP+LpxL1 vaccination. (A-B) C57BL/6 mice were vaccinated twice (d0 and d28) with PBS, aP, or aP+LpxL1. Splenocytes, obtained at day 52, were either unstimulated (IMDM complete medium) or stimulated with IMDM complete medium containing 1 µg/ml Prn for 8 days. (A) Percentage IL-5-, IFNγ-, and IL-17-producing CD4⁺CD44⁺ T cells were determined using an ICS assay. (B) Cytokine levels in supernatant after Prn stimulation were determined using a Luminex assay. Results are corrected for the background level (IMDM complete medium control) and are averages of 6 mice per group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Table S1 - Levels of vaccine antigen-specific IgG1 and IgG2c in sera of vaccinated C57BL/6 mice on day 52

Vaccine	IgG2c[a]			IgG1[a]			IgG2c/IgG1 ratio[a]		
	Ptx	FHA	P.69 Prn	Ptx	FHA	P.69 Prn	Ptx	FHA	P.69 Prn
PBS	0.04 ±0.10	n.d.[c]	n.d.[c]	n.d.[c]	0.83 ±0.13	n.d.[c]	n.a.[d]	n.a.[d]	n.a.[d]
aP	0.46 ±0.45	1.01 ±1.04	0.91 ±0.72	3.44 ±0.12	3.65 ±0.19	3.87 ±0.17	0.13 ±0.14	0.28 ±0.28	0.23 ±0.18
aP+LpxL1	0.99 ±1.07	1.56 ±1.00	1.01 ±0.51	3.40 ±0.26	3.87 ±0.20	3.90 ±0.15	0.29 ±0.31	0.40 ±0.25	0.26 ±0.13

[a]Represented as average ^{10}LOG MFI ± SD of 6 mice per group; [b]Represented as average ^{10}LOG MFI IgG2c/^{10}LOG MFI IgG1 ratio ± SD of individual mice.; [c]n.d., not detectable; [d]n.a., not applicable;

Chapter 6

Transcriptome signature for dampened Th2 dominance in acellular pertussis vaccine-induced CD4$^+$ T cell responses through TLR4 ligation

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Abstract

Current acellular pertussis (aP) vaccines promote a T helper 2 (Th2)-dominated response, while Th1/Th17 cells are protective. As our previous study showed, after adding a non-toxic TLR4 ligand, LpxL1, to the aP vaccine in mice, the *Bordetella pertussis*-specific Th2 response is decreased and Th1/Th17 responses are increased as measured at the cytokine protein level. However, how this shift in Th response by LpxL1 addition is regulated at the gene expression level remains unclear. Transcriptomics analysis was performed on purified $CD4^+$ T cells of control and vaccinated mice after *in vitro* restimulation with aP vaccine antigens. Multiple key factors in Th differentiation, including transcription factors, cytokines, and receptors, were identified within the differentially expressed genes. Upregulation of Th2- and downregulation of follicular helper T cell-associated genes were found in the $CD4^+$ T cells of both aP- and aP+LpxL1-vaccinated mice. Genes exclusively upregulated in $CD4^+$ T cells of aP+LpxL1-vaccinated mice included Th1 and Th17 signature cytokine genes *Ifng* and *Il17a* respectively. Overall, our study indicates that after addition of LpxL1 to the aP vaccine the Th2 component is not downregulated at the gene expression level. Rather an increase in expression of Th1- and Th17-associated genes caused the shift in Th subset outcome.

Introduction

Pertussis or whooping cough, caused by the Gram-negative bacterium *Bordetella pertussis*, remains endemic even in highly vaccinated populations[1-3]. This resurgence has been ascribed to multiple causes, including suboptimal programming of the adaptive immune response by second generation acellular pertussis (aP) vaccines. This has been supported by several studies in different models, namely mice, baboons, and humans, which have revealed that a mixed T helper 1 (Th1) and Th17 type of CD4+ T cell response is induced by *B. pertussis* infection[4-8]. Moreover, these Th subsets have been shown, by both the mice and baboon models, to be crucial in the protection against *B. pertussis*[5, 8]. In contrast, the CD4+ T cell response induced by current aP vaccines is rather Th2-dominated[5, 9-11].

Th subsets are mainly identified by the production of Th subset signature cytokines, such as IFNγ (Th1), IL-4, IL-5, and IL-13 (Th2), IL-17A (Th17), IL-10 and TGFβ (regulatory T cells (Treg)), and IL-21 (follicular helper T cells (Tfh)). CD4+ T cell differentiation has several underlying processes. After activation through their T cell receptors, the functional programming of CD4+ T cells is initiated by differentiation cytokines produced in the priming microenvironment, such as IL-12, interacting with their cognate receptors. This results in the activation of signal transducer and activator of transcription (Stat) proteins[12], which induce the expression of master transcription factors. Each Th subset can be defined by the expression of Stat proteins and master transcription factors, namely Stat4/Stat1/Tbet (Th1), Stat5/Stat6/Gata3 (Th2), Stat3/Rorγt (Th17), Stat5/FoxP3 (Treg), and Stat3/Bcl6 (Tfh)[12-14]. These master transcription factors subsequently induce expression of many Th subset-associated genes and silence genes expressed in other Th subsets. These genes include chemokine and cytokine receptors, which also can be used to discriminate between Th subsets. Th1 cells are characterized by CCR1/CCR5/CXCR3 expression, Th2 cells by CCR3/CCR4/CCR8 expression, Th17 cells by CCR4/CCR6 expression, Treg cells by CD25 expression, and Tfh cells by CXCR5 expression[15].

Recently, the programming of aP vaccine-induced CD4+ memory T cells was investigated using genome-wide gene expression profiling of human CD4+ T cells[16]. This approach revealed co-expression of both Th2- and Th1-associated gene modules in reactivated CD4+ memory T cells generated after aP vaccination in children. This raised the question of how these in principle antagonistic gene modules can establish a predominantly functional Th2 type of CD4+ T cell outcome. These gene modules, it was suggested, may exist in a dynamic equilibrium, and depending on ongoing response, the intensity of module components may tip the balance in Th subset outcome towards a Th1 or Th2 response. As several preclinical studies have demonstrated, steering the aP vaccine-induced Th2-dominated response towards a more favorable Th1 and Th17 type of response at the cytokine protein level through the use of adjuvants is feasible, for example through replacement of the currently used adjuvant alum in the aP vaccine with TLR2 or TLR9 ligands[5, 17]. We recently showed that also adding the TLR4 ligand LpxL1, a non-toxic *Neisseria meningitidis* LPS derivative, to an alum-containing aP vaccine skewed the vaccine-induced CD4+ T cell response towards

a Th1/Th17 type of $CD4^+$ T cell response at the cytokine level[11]. Yet, how the Th subset outcome in the aP vaccine-induced *B. pertussis*-specific $CD4^+$ T cell response by LpxL1 as adjuvant is regulated at the level of gene expression remains unclear. This insight is necessary to understand shortcomings and improvement of current aP vaccination.

Therefore, in the present study we compared, in mice, gene expression profiles of *B. pertussis*-specific $CD4^+$ T cells induced by aP or LpxL1-adjuvanted aP vaccination. Short stimulation of splenocytes of vaccinated mice with *B. pertussis* antigens activated the *B. pertussis*-specific $CD4^+$ T cells, after which microarray analysis was performed on RNA from isolated $CD4^+$ T cells. Distinct profiles in $CD4^+$ T cells were found that are potentially useful in the evaluation of new vaccine candidates and adjuvants.

Materials and Methods

Ethics statement

This study was approved by the Committee on Animal Experimentation of the Netherlands Vaccine Institute (Bilthoven, The Netherlands) under permit number 201200115. Animal handling in this study was carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation.

Vaccines and antigens

Pertactin P.69 (Prn) was expressed in *Escherichia coli*, purified as described previously[18] and was tested for *E. coli* LPS impurities using a Limulus Amebocyte Lysate (LAL) test. The endotoxin level was <0.015 EU/ml. Purified filamentous hemagglutinin (FHA) and pertussis toxin (Ptx) were obtained from Kaketsuken (Japan) and Ptx was heat-inactivated at 95°C for 15 minutes before use. The registered combined pentavalent diphtheria, tetanus, and acellular pertussis vaccine (Infanrix; aP) was purchased from GlaxoSmithKline and one human dose (HD) contains a minimum of 30 I.E. diphtheria toxoid, a minimum of 40 I.E. tetanus toxoid, 25 µg FHA, 25 µg Ptx, and 8 µg Prn, all absorbed to aluminumhydroxide. LpxL1, a meningococcal LPS derivative, was engineered and obtained as described elsewhere[19].

Mice and immunization

Adult (6-8 weeks old) Balb/c mice (Harlan, The Netherlands) were vaccinated s.c. on day 0 (right flank) and day 28 (left flank) with 0.3 ml of 1/4 HD aP vaccine, 1/4 HD aP vaccine supplemented with 1 µg non-adsorbed LpxL1 (aP+LpxL1), or as a control with PBS, with 5 mice per group. Mice were sacrificed on day 38, after which spleens were harvested from each mouse.

Isolation and *in vitro* restimulation of splenocytes

From each mouse, homogenized splenocytes were treated with erythrocyte lysis buffer (8.3 g/L NH_4CL, 1 g/L $NaHCO_3$, 5000 IE/L Heparin in dH_20; pH 7.4) and transferred to 24-well plates ($6x10^6$ cells/well). The cells were cultured in IMDM medium (Gibco) supplemented with 8% FCS, 100 units penicillin, 100 units streptomycin, 2.92 mg/ml L-glutamine, and 20 µM β-mercaptoethanol (Sigma) at 37°C in a humidified atmosphere of 5% CO_2. The cells were either left unstimulated or stimulated for 24 hours with a combination of Prn, Ptx, and FHA (1 µg/ml each) (2 replicate wells per condition), after which the cells were harvested and pooled per culture condition per mouse.

CD4+ T cell isolation and purity check

From each cultured splenocyte sample CD4+ T cells were isolated by positive selection using CD4 magnetic microbeads and a magnetic cell separator (Miltenyi Biotech) according to the manufacturer's instructions. The purity of the CD4+ T cells was determined using flowcytometry. Briefly, the isolated cells were stained with Pacific blue-conjugated anti-CD4 (Biolegend) in FACS buffer (PBS (pH 7.2) supplemented with 0.5% BSA (Sigma Aldrich) and

0.5 mM EDTA (ICN Biomedicals). After washing, data were acquired on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star). The purity of the isolated $CD4^+$ T cells was >95%.

RNA extraction

From each $CD4^+$ T cell preparation, cells were lysed in Qiazol (Qiagen) and RNA isolation was performed using a miRNeasy Mini Kit with DNAse treatment (Qiagen) according to the manufacturer's protocol. RNA concentrations and quality were determined using respectively UV spectroscopy (Tech3 module, Synergy Mx, BioTek) and electrophoresis (RNA nano 6000 kit, 2100 Bioanalyzer, Agilent Technologies).

Microarray analysis

Amplification, labeling and hybridization of RNA samples to microarray chips (GeneChip HT MG-430 PM Array Plate; Affymetrix) were carried out at the Microarray Department of the University of Amsterdam (The Netherlands) according to Affymetrix protocols. Array plates were scanned with a Genechip HT array plate scanner and analyzed with the Affymetrix HT software suite. Microarray analysis was performed on 3 unstimulated and 5 antigen-stimulated samples per group.

Data analysis of gene expression

Quality control and normalization of Affymetrix CEL files were performed using the ArrayAnalysis website (www.arrayanalysis.org)[20], using the Robust Multichip Average (RMA) method[21] and the MBNI custom CDF version 15[22]. Normalized data consisted of Log2 transformed signal values for 17306 genes. All slides passed quality control. Further analysis of normalized data was performed in R (www.r-project.org) and Microsoft Excel. Genes differentially expressed between the different groups of immunized mice were identified by using ANOVA. Fold ratio induction or repression of individual genes was calculated by comparing mean gene expression levels of the different immunization groups. Probes were considered differentially expressed if they met the following two criteria: (i) a p-value ≤ 0.001 (ANOVA), which corresponds to a Benjamini-Hochberg False discovery rate (FDR) of 5%; and (ii) an absolute fold ratio ≥1.5. Heatmaps visualizing differently expressed genes were made using GeneMaths XT software (Applied Maths). Hierarchical clustering of the differentially expressed genes was performed in GeneMaths XT software using Euclidean distance (with variances) as a distance metric and UPGMA linkage. Additional data visualization was done by principal component analysis in R. Functional enrichment with an over-representation analysis (ORA) was performed using DAVID[23] based on Gene Ontology biological processes (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

Gene network analysis

To construct a gene-function network, genes associated with Th subsets and metabolism were determined using text mining in murine and human studies. Interactions between genes associated with Th subsets and metabolism were determined using the STRING database (http://string.embl.de/) with high confidence (0.700) and using co-occurrence,

co-expression, experiments, databases, and text mining as types of evidence. The network visualization was performed using Cytoscape (version 2.8.3).

Results

B. pertussis-specific CD4⁺ T cell transcriptome of aP- or aP+LpxL1-vaccinated versus control mice

To determine how addition of LpxL1 to the aP vaccine regulates the Th subset outcome of the vaccine-induced *B. pertussis*-specific CD4⁺ T cells on the molecular level, gene expression profiles of these responding CD4⁺ T cells were investigated. Splenocytes from control, aP-, and aP+LpxL1-vaccinated mice were shortly stimulated with *B. pertussis* antigen Ptx, FHA, and Prn, after which microarray analysis was performed on RNA from isolated CD4⁺ T cells. The gene expression profiles of unstimulated CD4⁺ T cells of all groups were taken as a baseline, to establish whether there is an intrinsic difference between the groups. No significant differentially expressed genes could be identified between these unstimulated

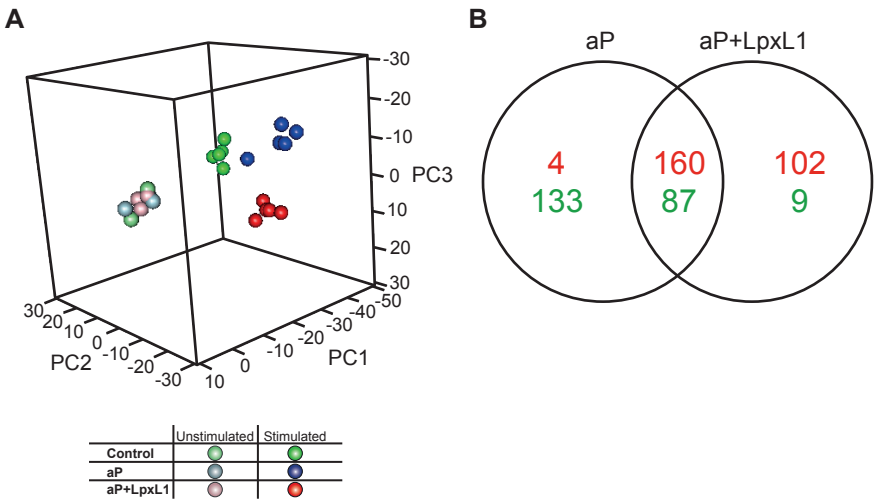
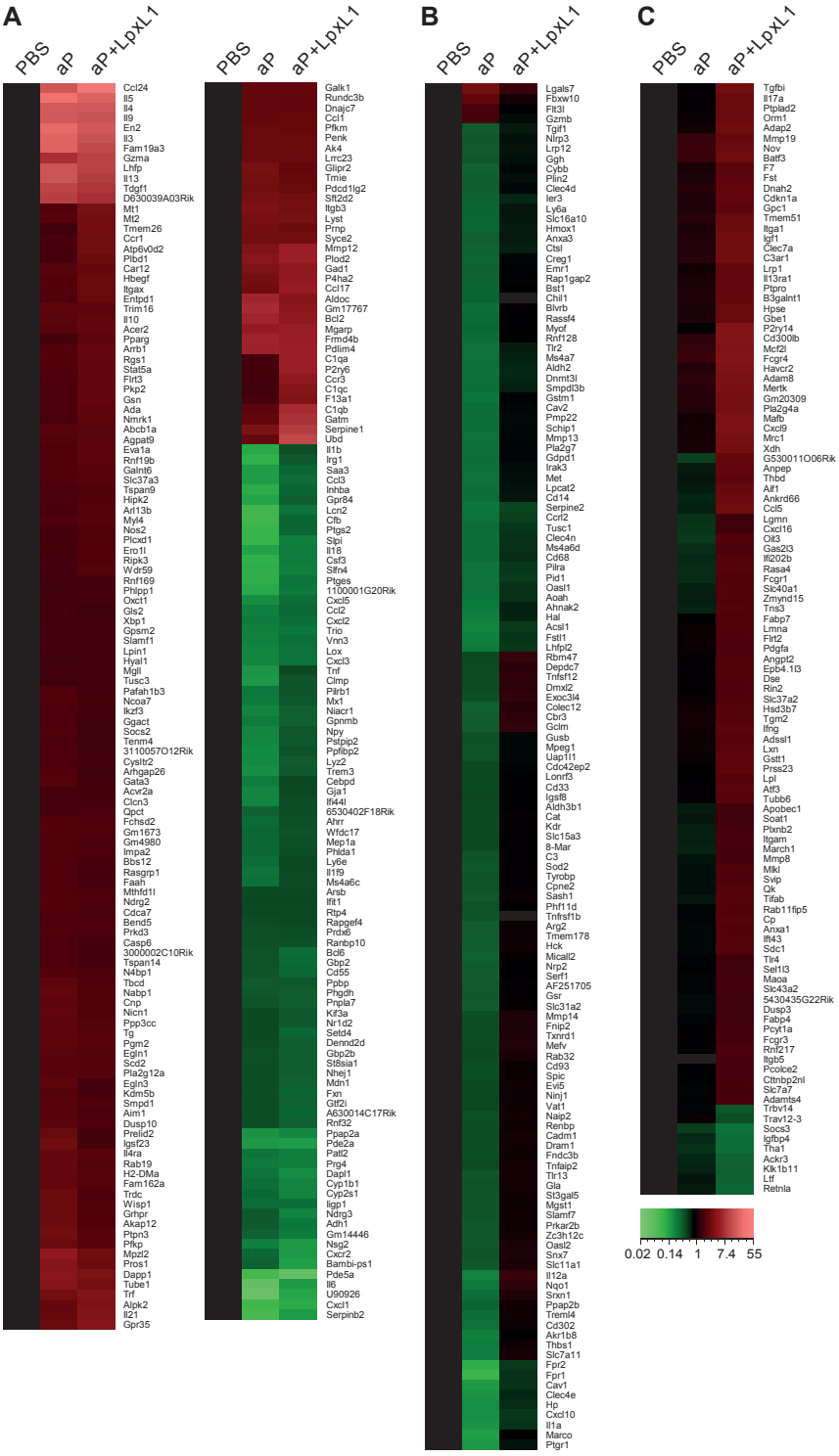


Figure 1 - Visualization of differences in gene expression in CD4⁺ T cells of control, aP-, and aP+LpxL1-vaccinated mice by principle component analysis. (A) Principal component analysis, based on the differentially expressed genes, showing (dis)similarities in gene expression in samples stimulated with the Ptx, FHA, and Prn combination (dark colors, n=5 per group) and medium controls (light colors, n=3 per group) in all vaccination groups (PBS (blue), aP (red), aP+LpxL1 (green)) are shown. (B) Venn diagram showing the amount of overlap between up- (red) and downregulated (green) genes in 24 hour *B. pertussis* antigen-stimulated CD4⁺ T cells of aP- and aP+LpxL1-vaccinated mice, as compared to control mice, based on averaged normalized gene expression levels of groups.

Figure 2 - Gene expression profiles of *B. pertussis*-specific CD4⁺ T cells of aP- and aP+LpxL1- vaccinated mice. The heatmaps depict differential up- (red) or downregulation (green) of genes observed in 24 hour *B. pertussis* antigen-stimulated CD4⁺ T cells of vaccinated compared to control mice (FR≥1.5). (A) 247 genes were differentially expressed in CD4⁺ T cells of both aP- and aP+LpxL1-vaccinated mice. (B) 137 genes were differentially expressed in CD4⁺ T cells of exclusively aP-vaccinated mice. (C) 111 genes were differentially expressed in CD4⁺ T cells of exclusively aP+LpxL1-vaccinated mice. Expression data shown are averages from the samples of 5 mice per group.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aP
aP+LpxL1
Ccl24
Il5
Il4
Il9
En2
Il3
Fam19a3
Gzma
Lhfp
Il13
Tdgf1
D630039A03Rik
Mt1
Mt2
Tmem26
Ccr1
Atp6v0d2
Plbd1
Car12
Hbegf
Itgax
Entpd1
Trim16
Il10
Acer2
Pparg
Arrb1
Rgs1
Stat5a
Flrt3
Pkp2
Gsn
Ada
Nmrk1
Abcb1a
Agpat9
Eva1a
Rnf19b
Galnt6
Slc37a3
Tspan9
Hipk2
Arl13b
Myl4
Nos2
Ptcxd1
Ero1l
Ripk3
Wdr59
Rnf169
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Oxct1
Gls2
Xbp1
Gpsm2
Slamf1
Lpin1
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Gm4980
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Faah
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Tbcd
Nabp1
Cnp
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Aim1
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Akap12
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Pfkp
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Pros1
Dapp1
Tube1
Trf
Alpk2
Il21
Gpr35
Galk1
Rundc3b
Dnajc7
Ccl1
Pfkm
Penk
Ak4
Lrrc23
Glipr2
Tmie
Pdcd1lg2
Slt2d2
Itgb3
Lyst
Prnp
Syce2
Mmp12
Plod2
Gad1
P4ha2
Ccl17
Aldoc
Gm17767
Bcl2
Mgarp
Frmd4b
Pdlim4
C1qa
P2ry6
Ccr3
C1qc
F13a1
C1qb
Gatm
Serpine1
Ubd
Il1b
Irg1
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Ppbp
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Pnpla7
Kif3a
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Dennd2d
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Prg4
Dapl1
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Cyp2s1
Iigp1
Ndrg3
Adh1
Gm14446
Nsg2
Cxcr2
Bambi-ps1
Pde5a
Il6
U90926
Cxcl1
Serpinb2
B
PBS
aP
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Lgals7
Fbxw10
Flt3l
Gzmb
Tgif1
Nlrp3
Lrp12
Ggh
Cybb
Pllp2
Clec4d
Ier3
Ly6a
Slc16a10
Hmox1
Anxa3
Ctsl
Creg1
Emr1
Rap1gap2
Bst1
Chil1
Blvrb
Rassf4
Myof
Rnf128
Tlr2
Ms4a7
Aldh2
Dnmt3l
Smpdl3b
Gstm1
Cav2
Pmp22
Schip1
Mmp13
Pla2g7
Gdpd1
Irak3
Met
Lpcat2
Cd14
Serpine2
Ccrl2
Tusc1
Clec4n
Ms4a6d
Cd68
Plira
Pid1
Oasl1
Aoah
Ahnak2
Hal
Acsl1
Fsll1
Lhfpl2
Rbm47
Depdc7
Tnfsf12
Dmxl2
Exoc3l4
Colec12
Cbr3
Gclm
Gusb
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Uap1l1
Cdo42ep2
Lonrf3
Cd33
Igsf8
Aldh3b1
Cat
Kdr
Slc15a3
8-Mar
C3
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Tyrobp
Cpne2
Sash1
Phf11d
Tnfsf1b
Arg2
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Hck
Micall2
Nrp2
Serf1
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Mefv
Rab32
Cd93
Spic
Evi5
Ninj1
Vat1
Naip2
Renbp
Cadm1
Dram1
Fndc3b
Tnfaip2
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Fpr1
Cav1
Clec4e
Hp
Cxcl10
Il1a
Marco
Ptgr1
C
PBS
aP
aP+LpxL1
Tgfbi
Il17a
Ptplad2
Orm1
Adap2
Mmp19
Nov
Batf3
F7
Fst
Dnah2
Cdkn1a
Gpc1
Tmem51
Itga1
Igf1
Clec7a
C3ar1
Lrp1
Il13ra1
Ptpro
B3galnt1
Hpse
Gbe1
P2ry14
Cd300lb
Mcf2l
Fcgr4
Havcr2
Adam8
Mertk
Gm20309
Pla2g4a
Mafb
Cxcl9
Mrc1
Xdh
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Anpep
Thbd
Aif1
Ankrd66
Ccl5
Lgmn
Cxcl16
Oit3
Gas2l3
Ifi202b
Rasa4
Fcgr1
Slc40a1
Zmynd15
Tns3
Fabp7
Lmna
Flrt2
Pdgfa
Angpt2
Epb4.1l3
Dse
Rin2
Slc37a2
Hsd3b7
Tgm2
Ifng
Adssl1
Lxn
Gstt1
Prss23
Lpl
Atf3
Tubb6
Apobec1
Soat1
Plxnb2
Itgam
March1
Mmp8
Mlkl
Svip
Qk
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Sel1l3
Maoa
Slc43a2
5430435G22Rik
Dusp3
Fabp4
Pcyt1a
Fcgr3
Rnf217
Itgb5
Pcolce2
Cttnbp2nl
Slc7a7
Adamts4
Trbv14
Trav12-3
Socs3
Igfbp4
Tha1
Ackr3
Klk1b11
Ltf
Retnla
0.02 0.14 1 7.4 55

samples (criteria: p-value ≤0.001, fold ratio (FR) ≥1.5). Nevertheless, to exclude small intrinsic non-significant differences, the expression intensities of the antigen-stimulated samples were corrected for the average expression intensities of unstimulated samples of their corresponding group. In total, 1876 differentially expressed genes (*p*-value≤0.001, FR ≥1.5) were identified between averaged unstimulated samples and antigen-stimulated samples of the control, aP-, or aP+LpxL1-vaccinated groups. A principal component analysis on these genes showed differences in gene expression profiles between unstimulated and stimulated samples of all groups, including control mice, suggesting an effect of the stimulation on naive $CD4^+$ T cells (Figure 1A). However, distinct gene expression profiles between stimulated samples of all groups were still observed, revealing functionally differently programmed *B. pertussis*-specific $CD4^+$ T cells (Figure 1B). After comparing the *B. pertussis* antigen-stimulated samples of vaccinated mice with those of control mice, differential expression (FR ≥1.5) of 384 and 358 genes was identified in the $CD4^+$ T cells of, respectively, aP- and aP+LpxL1-vaccinated mice. Overlap comparison showed that 247 genes were differentially expressed in $CD4^+$ T cells of both aP- and aP+LpxL1-vaccinated mice, 137 genes were exclusively differentially expressed in $CD4^+$ T cells of aP-vaccinated mice, and 111 genes were exclusively differentially expressed in $CD4^+$ T cells of aP+LpxL1-vaccinated mice (Figures 1 and 2).

Over-representation of immune and metabolism related terms after aP- and aP+LpxL1-vaccination

To provide more insight in the differentially expressed genes, functional annotation and over-representation analysis (Benjamini-corrected p-value ≤0.05) in GO-BP and KEGG databases were performed using DAVID[23]. Analysis of the overlapping 247 differentially expressed genes in $CD4^+$ T cells of both aP- and aP+LpxL1-vaccinated mice showed that 74 GO-BP terms and 8 KEGG pathways were enriched. Based on exclusion of overlapping terms/pathways and their relevance, a selection of these terms/pathways is shown in Figure 3A. The enriched terms/pathways are mainly involved in the regulation of the adaptive immune response, as indicated by terms as regulation of lymphocyte activation (GO:0051249), proliferation (GO:0050670), and differentiation (GO:0045597), and cytokine signaling, including chemotaxis (GO:0006935) and Jak-STAT signaling pathway (mmu4630). Moreover, the enrichment of the asthma pathway (mmu05310) indicates the presence of Th2-associated genes. Further, terms involved in metabolic processes are enriched, including positive regulation of macromolecule metabolic process (GO:0010604) and positive regulation of protein metabolic process (GO:0051247).

Functional annotation and over-representation analysis (Benjamini-corrected p-value ≤0.05) of the 137 genes differentially expressed in $CD4^+$ T cells of exclusively aP-vaccinated mice revealed enrichment of 9 GO-BP terms. Five relevant terms are depicted in Figure 3B, which includes immune response-related terms, such as immune response (GO:0006955) and regulation of cytokine production (GO:0001817), and metabolism-related terms such as oxidation-reduction process (GO:0055114) and regulation of nitric oxide biosynthetic process (GO:0045428). Functional annotation and over-representation analysis of the

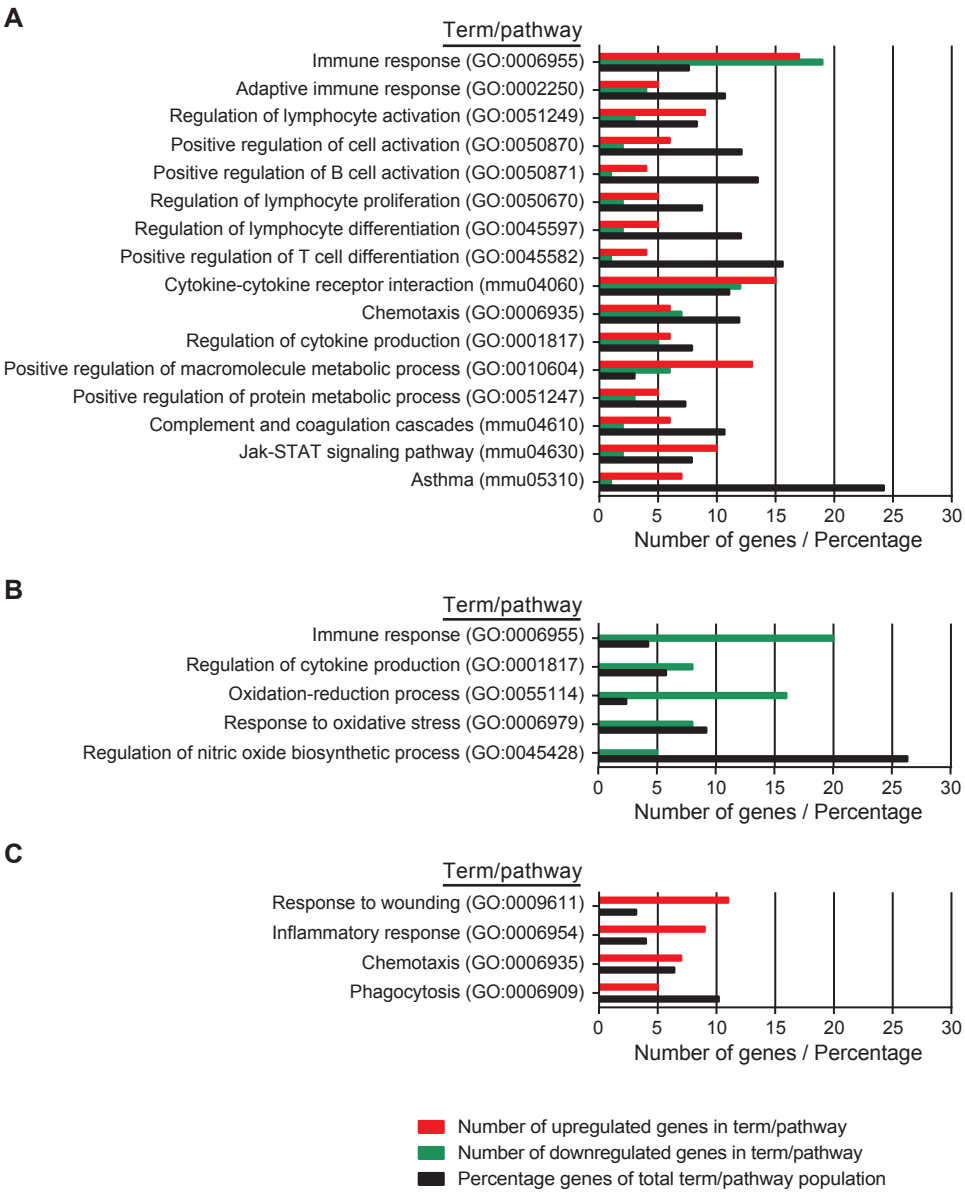

Figure 3 - Functional annotation and pathway enrichment of differentially expressed genes in *B. pertussis*-specific CD4+ T cells of aP- and aP+LpxL1-vaccinated mice. Over-representation analysis (Benjamini-corrected *p*-value ≤0.05) in GO-BP and KEGG databases was performed using genes differentially expressed in *B. pertussis* antigen-stimulated CD4+ T cells of vaccinated compared to control mice. Functional annotation and pathway enrichment are depicted from genes differentially expressed in CD4+ T cells of both aP- and aP+LpxL1 vaccinated mice (A), in CD4+ T cells of exclusively aP-vaccinated mice (B), and in CD4+ T cells of exclusively aP+LpxL1-vaccinated mice (C). The amount of up- or downregulated genes per term/pathway and the percentage of the genes in the total term/pathway population are shown.

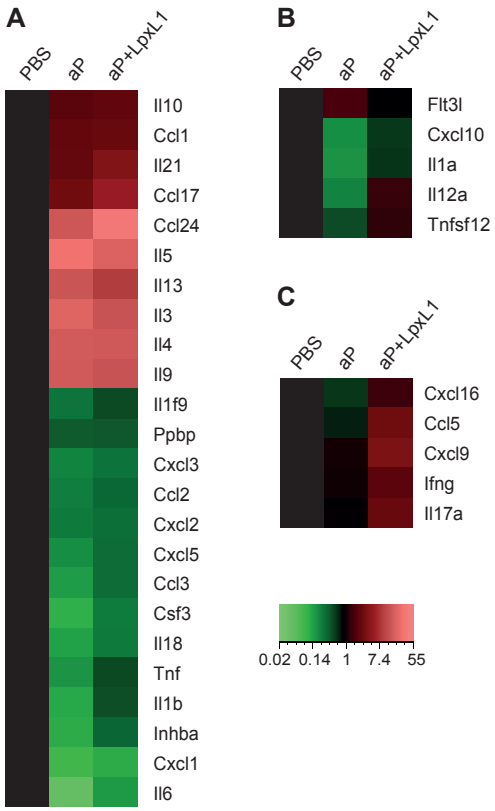

Figure 4 - Gene expression profile of cytokine-encoding genes in *B. pertussis*-specific CD4⁺ T cells of aP- and aP+LpxL1-vaccinated mice. Genes encoding cytokines differentially expressed in B. pertussis antigen-stimulated CD4⁺ T cells of both aP- and aP+LpxL1-vaccinated mice (A), in CD4⁺ T cells of exclusively aP-vaccinated mice only (B), and in CD4⁺ T cells of exclusively aP+LpxL1-vaccinated mice only (C). Expression data shown are averages from the samples of 5 mice per group.

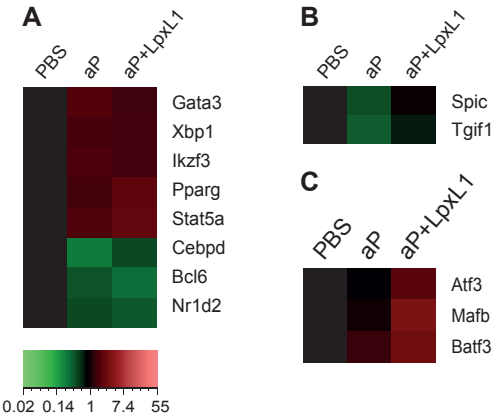

Figure 5 - Gene expression profile of transcription factor-encoding genes in *B. pertussis*-specific CD4⁺ T cells of aP- and aP+LpxL1-vaccinated mice. Genes encoding transcription factors differentially expressed in *B. pertussis* antigen-stimulated CD4⁺ T cells of both aP- and aP+LpxL1-vaccinated mice (A), in CD4⁺ T cells of exclusively aP-vaccinated mice only (B), and in CD4⁺ T cells of exclusively aP+LpxL1-vaccinated mice only (C). Expression data shown are averages from the samples of 5 mice per group.

111 genes solely altered in CD4⁺ T cells of aP+LpxL1-vaccinated mice showed enrichment of 9 GO-terms, including inflammatory response (GO:0006954), chemotaxis (GO:0006935), and phagocytosis (GO:0006909) (Figure 3C).

Differential expression of cytokine-encoding genes in vaccine-induced CD4+ T cells

Our previous study investigated the type of CD4+ T cell response at the protein level by determining the percentage of *B. pertussis* antigen-specific IL-5-, IFNγ-, and IL-17A-positive CD4+ T cells using flow cytometry and by supernatant analysis. It showed that addition of LpxL1 to the aP vaccine skews the CD4+ T cell response of a Th2-dominated to a mixed response, dominated by Th1/Th17[11]. Therefore, we investigated in more detail the expression of cytokine-encoding genes. Some Th subset signature cytokine-encoding genes could be identified which were upregulated in the CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice, such as *Il4*, *Il5*, *Il13, Il21*, and *Il10* (Figure 4A). No signature cytokine-encoding genes were found to be differentially expressed in the CD4+ T cells of solely aP-vaccinated mice, while both *Ifng* and *Il17a* were found to be upregulated exclusively in those of aP+LpxL1-vaccinated mice (Figure 4C). In addition to the Th subset signature cytokines-encoding genes, other cytokine genes were differentially expressed of which 19 were found in CD4+ T cells of aP- as well as aP+LpxL1-vaccinated mice. Genes *Il3*, *Il9*, *Ccl1*, *Ccl17*, and *Ccl24* were upregulated, whereas downregulation was found for genes encoded for chemokines, *Cxcl1*, *Ccl2*, *Cxcl2*, *Cxcl5*, *Cxcl3*, *Ccl3*, and *Csf3*, and pro-inflammatory cytokines, *Il1b*, *Il6*, *Tnf*, and *Il18* (Figure 4A). Five genes encoding other cytokines were detected in the CD4+ T cells of exclusively aP-vaccinated mice, which included downregulation of *Cxcl10*, *IL12a*, *Il1a*, and *Tnfsf12* and upregulation of *Flt3l* (Figure 4B). Three upregulated genes were found only in those of aP+LpxL1-vaccinated mice, namely *Cxcl9*, *Ccl5*, and *Cxcl16* (Figure 4C). Together, these results indicate substantial overlap in the expression of cytokine-encoding genes, including Th2 signature cytokines, after both aP- and aP+LpxL1 vaccination, while expression of genes encoding Th1 and Th17 signature cytokines is only induced by aP+LpxL1 vaccination.

Differential expression of transcription factor-encoding genes in vaccine-induced CD4+ T cells

Important in the differentiation of CD4+ T cells to different Th subsets are the master transcription factors, T-bet, Gata3, Rorγt, Bcl6, and FoxP3[12-14]. Within the CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice, *Gata3*, the gene encoding the Th2 master transcription factor was found upregulated whereas *Bcl6,* the gene encoding the Tfh master transcription factor was found downregulated (Figure 5A). Genes encoding other known master transcription factors were not found differentially expressed. The expression of master transcription factors is regulated by different Stat proteins[12]. Upregulation of only one Stat gene, namely *Stat5a*, which is involved in the differentiation of Th2 and Treg cells, was detected within CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice (Figure 5A).

In addition, five genes encoding other transcription factors were identified as differentially expressed in the CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice, including upregulation of *Pparg*, *Xbp1*, and *Ikzf3* and downregulation of *Nrld2* and *Cebpd* (Figure 5A). Transcription factors *Spic* and *Tgif1* were found downregulated only in the CD4+ T cells of the aP-vaccinated mice (Figure 5B), while transcription factors *Atf3*, *Mafb* and *Batf3* were found upregulated only in the CD4+ T cells of aP+LpxL1-

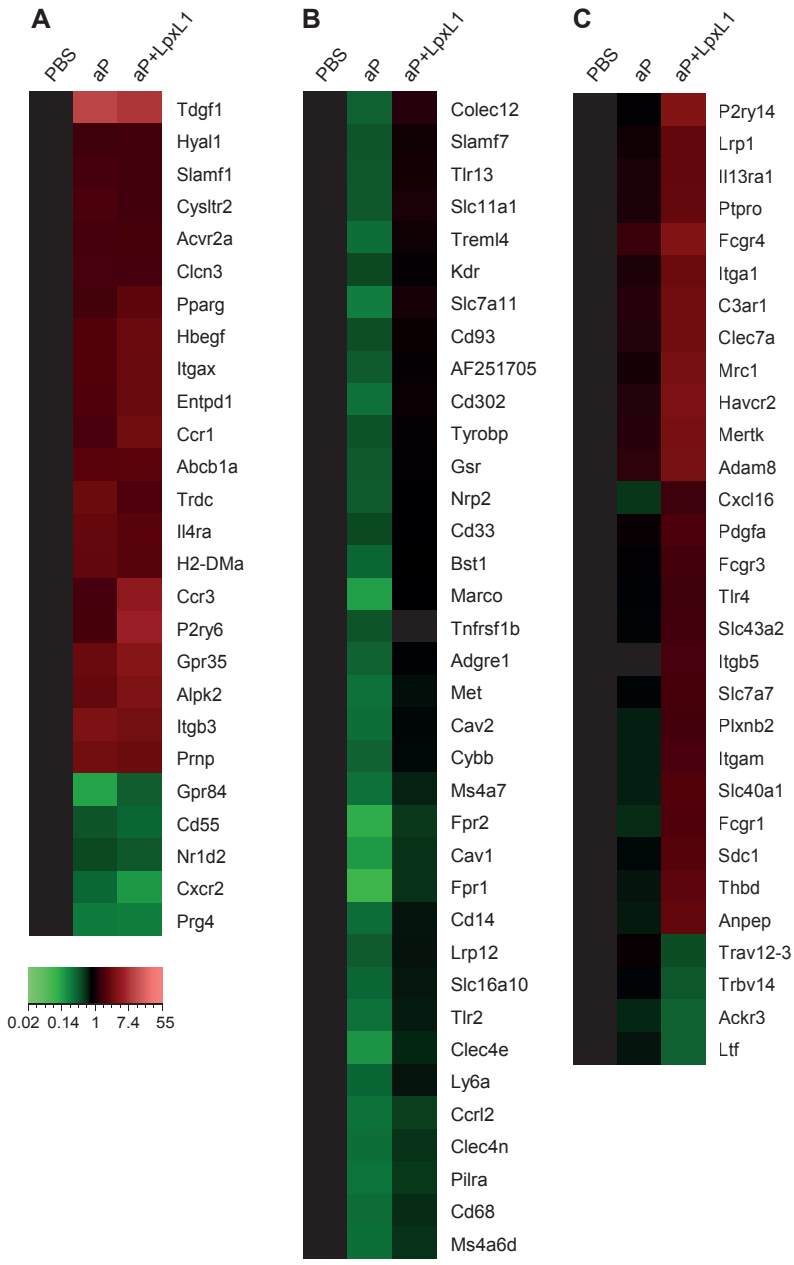

Figure 6 - Gene expression profile of genes encoding receptors and cell surface markers in *B. pertussis*-specific CD4⁺ T cells of aP- and aP+LpxL1-vaccinated mice. Genes encoding receptors and cell surface markers differentially expressed in *B. pertussis* antigen-stimulated CD4⁺ T cells of both aP- and aP+LpxL1-vaccinated mice (A), in CD4⁺ T cells of exclusively aP-vaccinated mice only (B), and in CD4⁺ T cells of exclusively aP+LpxL1-vaccinated mice only (C). Expression data shown are averages from the samples of 5 mice per group.

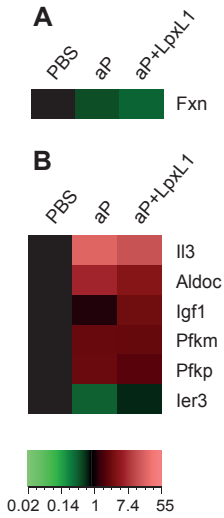

Figure 7 - Gene expression profile of genes encoding proteins involved in metabolism in *B. pertussis*-specific CD4+ T cells of aP- and aP+LpxL1-vaccinated mice. Heatmaps depict genes involved in oxidative phosphorylation (A) and glycolytic process (B) that are differentially expressed in *B. pertussis* antigen-stimulated CD4+ T cells of aP- and aP+LpxL1-vaccinated mice compared to control mice. Expression data shown are averages from the samples of 5 mice per group.

vaccinated mice (Figure 5C). Based on expression of Th differentiating transcription factors, both aP and aP+LpxL1 vaccination induce Th2 and inhibit Tfh differentiation.

Differential expression of receptor- and cell surface molecule-encoding genes in vaccine-induced CD4+ T cells

Another way to characterize CD4+ T cell subsets is by the expression of certain receptors and cell surface markers. Upregulation of markers *Ccr1* and *Ccr3* was detected in the CD4+ T cells of aP- and aP+LpxL1-vaccinated mice (Figure 6A). Remarkably, higher expression of the Th2-associated *Ccr3* was seen in aP+LpxL1 samples than in aP samples (Figure 6A). In addition to the markers used to characterize Th subsets, differential expression was found of genes encoding other receptors and cell surface molecules. Of these genes, 24 were found in CD4+ T cells of aP- and aP+LpxL1-vaccinated mice, and of these, 19 genes were upregulated, including as *Il4ra,* and 5 genes were downregulated, including *Cxcr2* (Figure 6A). Within the CD4+ T cells of aP-vaccinated mice, 36 receptor- and cell surface marker-encoding genes were downregulated, including *Ly6a*, and multiple genes encoding for proteins involved in pattern recognition, like *Tlr2*, *Tlr13*, *Clec4a*, *Clec4n*, and *Cd14* (Figure 6B). The 26 upregulated receptor- and cell surface marker-encoding genes in the CD4+ T cells of aP+LpxL1-vaccinated mice included *Havcr2, Itga1,* and genes encoding proteins involved in the innate immune response, such as *Tlr4*, *Clec7a*, *C3ar1*, *Fcgr1*, *Fcgr3*, and *Fcgr4* (Figure 6C). The 4 downregulated receptor- and cell surface marker-encoding genes in samples of aP+LpxL1-vaccinated mice were *Ackr3, Ltf, Trbv14*, and *Trav12-3*. Together, these results suggest that aP+LpxL1 vaccination induces expression of genes encoding receptors and cell surface markers associated with Th2 (*Ccr3* and *Il4ra*), Th1 (*Havcr2*), and Th17 (*Il13ra1*) subsets, while aP vaccination only induced genes associated with the Th2 (*Ccr3* and *Il4ra*) subset.

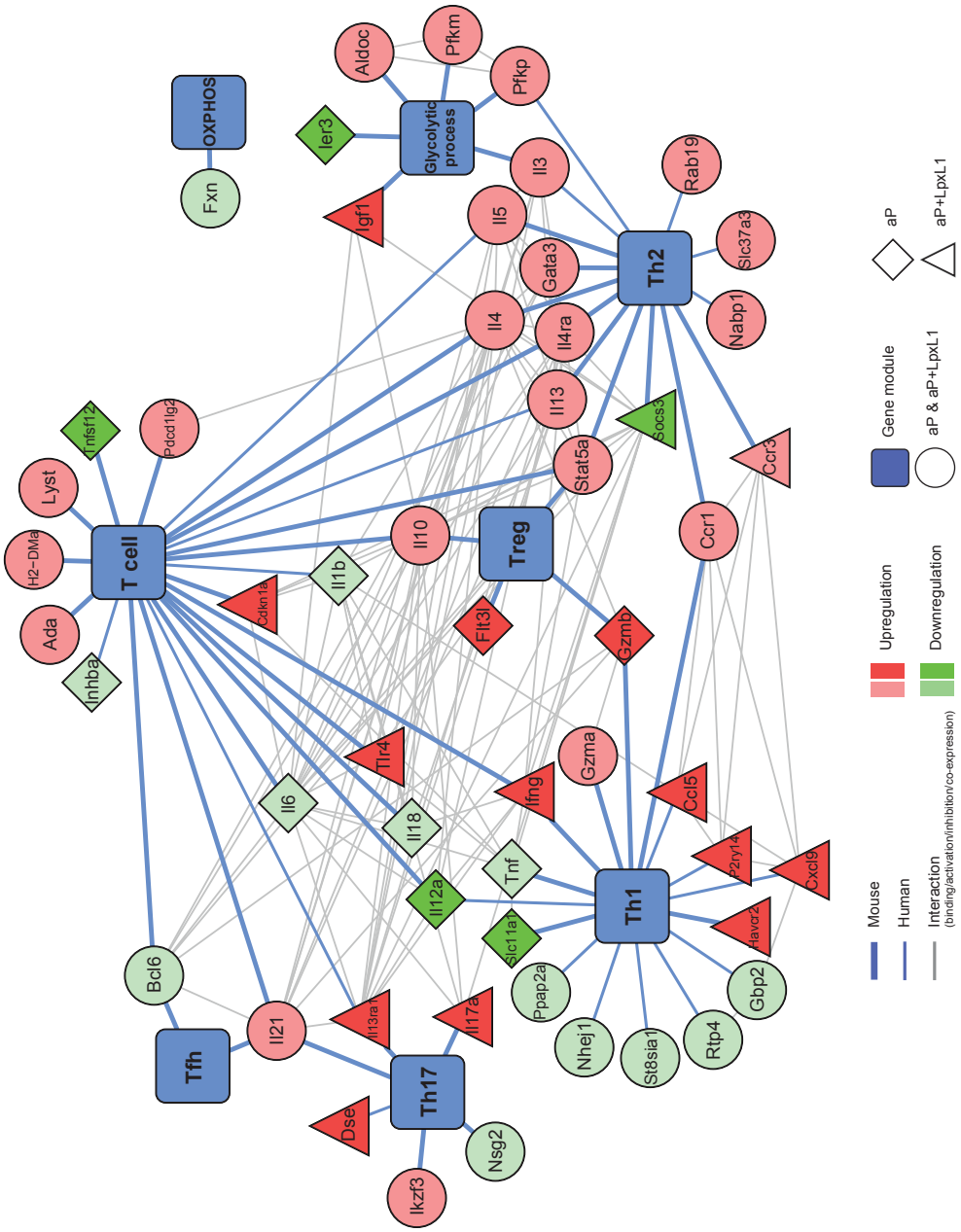
OXPHOS
Fxn
Glycolytic process
Aldoc
Pfkm
Pfkp
Ier3
Igf1
Il3
Il5
Gata3
Il4
Il4ra
Il13
Th2
Rab19
Slc37a3
Nabp1
Socs3
Stat5a
Ccr3
Ccr1
T cell
Tnfsf12
Pdcd1lg2
Lyst
H2-DMa
Ada
Inhba
Cdkn1a
Il1b
Il10
Treg
Flt3l
Gzmb
Tlr4
Il6
Il18
Il12a
Ifng
Tnf
Gzma
Ccl5
P2ry14
Cxcl9
Slc11a1
Th1
Havcr2
Ppap2a
Nhej1
St8sia1
Rtp4
Gbp2
Bcl6
Tfh
Il21
Il13ra1
Il17a
Th17
Dse
Nsg2
Ikzf3
Gene module
aP
aP+LpxL1
aP & aP+LpxL1
Upregulation
Downregulation
Mouse
Human
Interaction
(binding/activation/inhibition/co-expression)

Figure 8 - Network analysis of Th subset-associated genes differentially expressed in *B. pertussis*-specific CD4+ T cells of aP- and aP+LpxL1-vaccinated mice. A gene-function network analysis showing the Th subset-associated genes differentially expressed in *B. pertussis* antigen-stimulated CD4+ T cells of aP- and aP+LpxL1-vaccinated was performed using Cytoscape to visualize the patterns of Th subset-associated genes induced by the different vaccines. Association of genes with the gene modules (blue rectangles) was based on literature from mouse (bold blue lines) and human (thin blue lines) studies. The interactions between genes (grey lines) were determined using the STRING database. The shape of the gene nodes indicate whether genes were differentially expressed in CD4+ T cells of both vaccination groups (circles), had the highest fold-change in either the CD4+ T cells of aP-vaccinated mice (diamonds) or in those of aP-LpxL1-vaccinated mice (triangles). The color intensity of the gene nodes indicate whether genes were differentially expressed in CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice (light green and red) or in CD4+ T cells of exclusively aP-vaccinated mice or in those of exclusively aP+LpxL1-vaccinated mice (dark green and red).

Differential expression of genes encoding proteins involved in metabolism in vaccine-induced CD4+ T cells

Recent studies have revealed that a shift in metabolism from oxidative phosphorylation toward aerobic glycolysis is important in the activation of T cells[24]. Moreover, the production of IFNy in effector T cells requires aerobic glycolysis[25]. For this reason we also analyzed the expression of genes involved in these metabolic pathways. Only one gene encoding a protein involved in the oxidative phosphorylation was found differentially expressed, namely *Fxn*. The *Fxn* gene was downregulated in CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice (Figure 7A). Additionally, six genes encoding for proteins with a function in the glycolytic process could be identified in the CD4+ T cells (Figure 7B). Four genes were found upregulated in both vaccinated groups, namely *Aldoc*, *Il3*, *Pfkm*, and *Pfkp*. The *Ier3* gene was downregulated in CD4+ T cells of aP-vaccinated mice, while *Igf1* was upregulated in those of aP+LpxL1-vaccinated mice. In addition, a recent study has shown that regulation of glucose uptake induced by Notch signaling is important in the survival of memory CD4+ T cells[26]. However, no genes involved in this pathway were found to be differentially expressed in the CD4+ T cells of aP- and aP+LpxL1-vaccinated mice. Overall, these data suggest that there is no difference in the expression of genes involved in metabolic pathways in CD4+ T cells of aP- and aP+LpxL1-vaccinated mice.

Distinct Th subset-associated gene modules expressed after aP and aP+LpxL1 vaccination

Based on literature from human and murine studies, a network analysis was performed to visualize the expression patterns of genes associated with different Th subsets that were observed in the CD4+ T cells of aP- and aP+LpxL1-vaccinated mice (Figure 8). In addition to genes encoding the previously mentioned master transcription factors, signature cytokines, and surface markers, other differentially expressed genes associated with the main Th subsets were found. Mainly Th2-associated genes, such as the Th2 subset signature cytokines (*Il4*, *Il5*, and *Il13*), *Gata3*, *Il3*, *Nabp1*, and *Slc37a3*, were found upregulated in the CD4+ T cells of both aP -and aP+LpxL1-vaccinated mice. Interestingly, another Th2-associated gene, *Socs3*, was downregulated in the CD4+ T cells of exclusively aP+LpxL1-vaccinated mice. Th1-associated genes were upregulated in CD4+ T cells of aP+LpxL1-vaccinated mice, including *Havcr2* and chemokines *Cxcl9* and *Ccl5*, while downregulation of Th1-associated genes *Scl11a1* and

Il12a is observed in those of aP-vaccinated mice. Further, upregulation of Th17-associated genes *Dse*, *Il13ra1*, and *Il17a* was only observed in the $CD4^+$ T cells of aP+LpxL1-vaccinated mice. Differential expression of Treg-associated genes was found in the $CD4^+$ T cells of aP- as well as aP+LpxL1-vaccinated mice, namely *Il10* and *Stat5a*. However, other Treg-associated genes, *Flt3l* and *Gzmb*, were only upregulated in $CD4^+$ T cells of aP-vaccinated mice. Only 2 Tfh-associated genes were found in our study, *Bcl6* and *Il21*, which were respectively down- and upregulated in $CD4^+$ T cells of both the aP- and aP+LpxL1-vaccinated mice. Moreover, genes involved in glycolysis were found in $CD4^+$ T cells of both vaccination groups of which 2 genes are associated with the Th2 subset, namely *Pgkp* and *Il3*. Based on this gene expression network, our results suggest that aP vaccination induces mainly Th2 and Treg gene modules, while addition of LpxL1 to the aP vaccine induces a shift towards Th1 and Th17 gene modules.

Discussion

Addition of the TLR4 ligand LpxL1 to an aP vaccine was found to dampen the Th2 dominance of the antigen-specific CD4+ T cell response of vaccinated mice and to increase a Th1/Th17 response, based on cytokine analysis[11]. In the present study, this skewing was investigated in more detail at the gene expression level. Analysis of the expression of Th subset signature cytokine-encoding genes revealed an increased expression of *Ifng* and *Il17a* in CD4+ T cells of exclusively aP+LpxL1-vaccinated mice, which is consistent with our previous findings. Most importantly, the Th2 subset signature cytokine genes *Il4*, *Il5*, and *Il13* showed increased expression in the CD4+ T cells of aP- as well as aP+LpxL1-vaccinated mice, suggesting that the Th2 component is not downregulated at the gene expression level of Th subset signature cytokines after addition of LpxL1 to the aP vaccine.

Other Th1-, Th17-, and Th2-associated genes showed the same trend as the genes encoding Th signature cytokines. Genes associated with the Th1 subsets had increased expression in the CD4+ T cells of exclusively aP+LpxL1-vaccinated mice, including genes encoding chemokines (*Ccl5* and *Cxcl9*) and cell surface marker *Havcr2* (*Tim3*). Both Ccl5 and Cxcl9 are chemoattractants for Th1 cells and are described to be produced by human CD4+ T cells[27, 28]. Havcr2 is a cell surface marker preferentially expressed on Th1 cells and its expression is induced by Th1 master transcription factor T-bet[29]. Th17-associated genes that showed increased expression solely in the CD4+ T cells of aP+LpxL1-vaccinated mice were *Il13ra1* and *Dse*. IL-13Rα1 is a functional receptor found on both murine and human Th17 cells while it is not expressed on Th0, Th1, Th2, and Treg cells[30, 31]. Binding of IL-13 to this receptor attenuates the production of IL-17A[30, 31]. Further, Dse is an intracellular enzyme involved in epitope processing and is preferentially expressed in human Th17 cells[32].

Remarkably, several other Th2-associated genes also showed increased expression in CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice, of which most genes showed the same trend as the expression of the Th2 subset signature cytokine-encoding genes. These other Th2-associated genes include Th2 master transcription factor *Gata3*, Stat protein *Stat5a*, chemokine-receptors *Ccr1* and *Ccr3*, and cytokine receptor *Il4ra*, and other genes, namely *Rab19*, *Nabp1*, *Scl37a3*, and *Pfkp*[16]. Interestingly, downregulation of Th2-associated *Socs3* is observed in the CD4+ T cells of exclusively aP+LpxL1-vaccinated mice. Socs3, suppressor of cytokine signaling-3, is preferentially expressed in Th2 cells[33] and inhibits Th1 and Th17 differentiation by suppressing STAT4 and STAT3 activation, respectively[34, 35]. Downregulation of *Socs3* in CD4+ T cells of aP+LpxL1-vaccinated mice suggests reduced active suppression of Th1 and Th17 differentiation when LpxL1 is present in the aP vaccine and thereby favors Th1 and Th17 differentiation.

In addition to the involvement of the Th1, Th2, and Th17 subsets, this study in aP- and aP+LpxL1-vaccinated mice revealed gene expression modules pointing at the induction or inhibition of other Th subsets, namely Treg and Tfh. Treg cells were induced by both aP and aP+LpxL1 vaccination, since increased expression of a Treg subset signature cytokine gene,

Il10, as well as the Treg-associated Stat gene, *Stat5a*, was detected in CD4+ T cells of both groups. However, expression of *Gzmb*, encoding Granzyme B, which has cytolytic functions and is expressed in different cells including Tregs[36-38], showed increased expression in the CD4+ T cells of exclusively aP-vaccinated mice. Together with the increased expression of *Flt3l*, which is involved in the expansion of Treg cells[39], in only the samples of aP-vaccinated mice, this suggests that increased numbers of Treg cells were induced after vaccination with the aP vaccine alone. Tfh master transcription factor *Bcl6* showed decreased expression in the CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice, indicating that differentiation towards the Tfh subset was suppressed. This seems contradictory given the increased expression of the Tfh subset signature cytokine gene *Il21*. However, this cytokine can also be produced by Th17 cells[40]. Some induction of Th17 cells by aP vaccination might explain the increased expression of *Il21*. A study of Ross *et al.* indeed showed that Th17 cells could be detected in mice after aP vaccination[5]. These results are consistent with the increased expression of *Ikzf3* in CD4+ T cells of both aP- and aP+LpxL1-vaccinated mice, since this gene is specifically expressed in Th17 cells[41].

In addition to Th subset-associated genes, genes encoding proteins that are involved in metabolism were investigated, since a shift in metabolism from oxidative phosphorylation toward aerobic glycolysis is important in the activation of T cells[24]. Only a small number of genes involved in oxidative phosphorylation and glycolysis were differentially expressed in the CD4+ T cells of aP- and aP+LpxL1-vaccinated mice. The genes encoding proteins involved in glycolysis, namely *Il3, Pfkp*, *Aldoc,* and *Pfkm* showed increased expression in the samples of both aP- and aP+LpxL1-vaccinated mice. Interestingly, *Il3* and *Pfkp* are also associated with Th2 cells[16]. Overall, these results suggest little or no difference in the activation of CD4+ T cells based on metabolism by the different vaccines.

Within the set of genes differentially expressed in CD4+ T cells of aP- and aP+LpxL1-vaccinated mice, genes were found encoding proteins with a known function in the innate immune system, including cytokines (*Il6, Il1b, Tnf*, and *Il12a*), complement components (*C1qa*, *C1qb*, *C1qc*, *Cd55*, *Cfb, C3*, *Cd93*, *C3ar1, Itgam*), Toll-like receptors *(Tlr2*, *Tlr13*, and *Tlr4)*, C-type lectin receptors (*Clec4a*, *Clec4d*, *Clec4n*, *Clec7a*, and *Cd302*), and Fc-receptors (*Fcgr1*, *Fcgr3*, and *Fcgr4*). It is unlikely that these innate gene signatures can be fully explained by contamination of innate immune cells within the CD4+ T cell fraction, since the purity of the samples was >95%. Interestingly, several of these innate immunity genes are known to be expressed in CD4+ T cells, including complement components such as *Itgam, C3ar1*, and *CD55*[42-44]. Signaling through C3a receptor 1, upregulated gene in aP+LpxL1 samples, by binding a derivative of C3, downregulated gene in aP samples, has been associated with a Th2[45] and a Th1 response[46], and with inhibition of Treg function[47]. Moreover, some TLRs, such as TLR2 and TLR4, are also expressed on CD4+ T cells. Signaling via TLR2, which is downregulated in aP samples, has been found to induce IFNγ production by Th1 cells[48] and might even inhibit IL-4 production[49]. In addition, TLR2 signaling promotes the differentiation of Tregs into Th17 cells in human[50]. Signaling through TLR4 which gene expression is upregulated in aP+LpxL1 samples is reported to provide a signal for proliferation and cell

survival and seems to regulate persistence of Th lineages[51]. Furthermore, the Fc-gamma receptor *Fcgr3* gene, which was upregulated in aP+LpxL1 samples, was shown to be expressed on a small proportion of CD4+ T cells with an effector memory phenotype[52] and activated CD4+ T cells expressing IFNγ and T-bet[53]. Together these data indicate that the differential expression of innate genes could have a function in CD4+ T cells.

Within the CD4+ T cells of aP+LpxL1-vaccinated mice, increased expression of Th1- and Th17-associated genes, including the signature cytokine genes *Ifng* and *Il17a,* was observed. However, no increased expression of the master transcription factors of Th1 and Th17 cells, *Tbx21* and *Rorc* respectively, was found. An *in vitro* effect of 24-hour stimulation with *B. pertussis* antigens might underlie this effect, since in our previous study IFNγ and IL-17A production by naive CD4+ T cells was detected after stimulation with the *B. pertussis* antigens[11]. Indeed, in the current study, differently expressed genes found between unstimulated versus antigen-stimulated CD4+ T cells of control mice were detected, including the Th1 master transcription factor gene *Tbx21* and Th1 Stat gene *Stat1*. Therefore, we interpret the lack of differential expression of Th1 and Th17 master regulators in the samples of the vaccinated mice compared to those of control mice to be a result of an increased background expression in naive CD4+ T cells induced by the *in vitro B. pertussis* antigen stimulation. This *in vitro* activation of naive CD4+ T cells could also explain why only a few genes were found corresponding to proteins involved in metabolism, since the metabolism is altered by activation of CD4+ T cells[24].

Although addition of LpxL1 to the aP vaccine led to a decreased percentage of Th2 cells and reduced *in vitro* Th2 cytokine levels in *B. pertussis* antigen-stimulated CD4+ T cell cultures of vaccinated mice in our previous study[11], no or only a limited decrease in expression of Th2-associated genes was observed in the current study, except for *Socs3*. This might be explained by the duration of *in vitro* stimulation of the CD4+ T cells, since in the gene expression analysis the duration was shorter (24 hours) than in the functional read-out study (8 days). In addition, there might be reduced translation of the Th2 cytokine mRNA due to Th1- and Th17-associated miRNA translational repression. Such mechanism was shown for Th1-specific miR-135b[54] repressing Th2-associated genes *Stat6* and *Gata3* mRNA translation to protein[55]. Therefore, we propose that the shift towards a mixed Th1 and Th17 response is likely due to increased expression of Th1- and Th17-associated gene modules rather than downregulation of the Th2-associated gene module. Interestingly, White *et al.* also found a decisive role for the Th1 gene network module in the outcome of Th responses. In their study, extreme Th2 dominance in atopic allergy was associated with the complete absence of the Th1 gene network module[16]. A limitation of our study is that the differences on gene expression are measured on the total splenic CD4+ T cell population. Therefore, the question remains whether the shifts in gene modules observed at the population level also occur within the same cell. In future research, investigating the gene expression on single cell level can overcome this limitation, as was described by Chattopadhyay *et al.*[56].

In summary, this study provides a gene expression network model that may explain why

aP vaccination induces Th2 and Treg differentiation of $CD4^+$ T cells, and why addition of LpxL1 to the aP vaccine leads to the induction of Th1 and Th17 cells. Together with our previous data, showing a shift from a Th2-dominated response to a mixed Th1/Th17 response at the cytokine protein level, this study indicates that only a small change in the balance between the expression of Th1/Th17- and Th2-associated genes results in a shift in Th type. Moreover, this model can be used in the evaluation of the effects of new adjuvants on vaccination-induced T cell responses, in particular in the context of improving acellular pertussis vaccines.

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Chapter 7

Systems vaccinology reveals superior protection after pulmonary compared to subcutaneous administration of an outer membrane vesicle pertussis vaccine associated with local and systemic immune signatures in mice

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Manuscript in preparation

Abstract

Local immune responses in the lungs contribute to protection against *B. pertussis* infection and might improve the vaccine-elicited immunity. Therefore, the effect of the vaccine administration route on the degree of protection and the local and systemic immune response was investigated. Immunization of mice via the pulmonary route with a novel outer membrane vesicle pertussis vaccine (omvPV) led to faster clearance of *B. pertussis* upon intranasal challenge compared to immunization via the subcutaneous route. The local and systemic immune responses underlying this difference in protection were analyzed using a systems biology approach. Exclusively pulmonary immunization led to the presence of *B. pertussis*-specific IgA antibodies, IgA-producing plasma cells and Th17 cells in the lungs. Moreover, this route elicited increased levels of systemic specific IgG antibodies, IgG-producing plasma cells, memory B cells, and Th17 cells. In addition, only pulmonary immunization elicited a rapid induction of pro-inflammatory cytokines and chemoattractants, e.g. IL-6 and CXCL10, observed on transcriptomic and proteomic levels, in the lungs. Distinct cytokine profiles were measured in sera, which were overall higher after subcutaneous immunization, e.g. G-CSF and IL-5. Transcriptome analysis of lungs and draining lymph nodes revealed differences in innate and adaptive responses between both administration routes e.g. the expression of *Igha* and *Rorc*, supporting the superior IgA, IgG, and Th17 responses detected in pulmonary-immunized mice. These results show that by administering the vaccine via the pulmonary as opposed to the subcutaneous route, an omvPV can elicit superior local and systemic immunity against *B. pertussis*, resembling immunity after primary infection. The study indicates that pulmonary immunization may be key to improve pertussis vaccination strategies.

Introduction

Currently, pertussis remains an endemic disease, even in highly vaccinated populations. Approaches to increase protection include the improvement of pertussis vaccines and vaccination strategies[1, 2]. The whole-cell and acellular pertussis vaccines induce a systemic immune response characterized by the formation of IgG antibodies and a T helper (Th) response that is Th1/Th17 or Th2 dominated, respectively[3-6]. In contrast, a *B. pertussis* infection evokes a Th1/Th17 response both systemically and locally in the lungs[5, 7, 8]. Outer-membrane vesicle pertussis vaccines (omvPV) might be an improved alternative for the currently available vaccines. Subcutaneous immunization of omvPV elicited a systemic immune response comparable to that induced by *B. pertussis* infection, including high serum IgG levels against a broad antigen range[9] and a mixed Th1, Th17, and Th2 response [Raeven *et al.*, manuscript in preparation]. Unfortunately, subcutaneous omvPV immunization does not induce local immune responses in the lungs that are thought to contribute to a better protection against *B. pertussis* [Raeven *et al.*, manuscript in preparation].

Direct vaccine administration in the respiratory tract can lead to better protection compared to parenteral administration due to the induction of local immune responses as was shown for other respiratory pathogens, such as *M. tuberculosis* and influenza[10-12]. The feasibility of mucosal administration of different pertussis vaccines was proven as intranasal immunization provides protection against *B. pertussis* challenge [13-16]. Nonetheless, direct comparison of the local and systemic immune responses induced by parental and mucosal immunization of pertussis vaccines is not yet performed.

In the present study, we investigated in detail whether the route of immunization affects protection and the quality of the immune response in mice. A systems biology approach was used to compare immune responses following pulmonary and subcutaneous immunization with omvPV (Figure 1A). Such an approach was previously applied in vaccine research to predict vaccine responsiveness[17-20] and to unravel of molecular signatures of mucosal adjuvants[21] and respiratory pathogen infections[7, 22]. In our study, the novel omvPV-P93 with abolished Prn autocleavage was used, since it enables more specific readouts and provides better protection in a murine challenge model compared to the WT omvPV, even at a lower dose [Sloots *et al.* manuscript in preparation]. Besides *B. pertussis* clearance from the respiratory tract after intranasal challenge, gene expression profiles in draining lymph nodes and lungs, and cytokine profiles and antibody responses in serum and lungs of immunized mice were determined. Finally, specific B cell and T cell responses were investigated both locally and systemically. Our results demonstrate hallmarks of superior protective immunity to *B. pertussis* conferred by pulmonary vaccination with omvPV.

Materials and Methods

Vaccine and antigens

The outer membrane vesicle pertussis vaccine (omvPV) was prepared using a genetically modified *B. pertussis* B1917 strain lacking the autocleavage site in pertactin (Prn), as described previously [Sloots *et al.* manuscript in preparation]. 1 µg total protein omvPV was diluted in 50 µl and 300 µl PBS (Gibco) for pulmonary and subcutaneous immunization respectively. *B. pertussis* antigens Ptx and FHA were obtained from Kaketsuken (Japan), Prn and Fim2/3 were kindly provided by Betsy Kuipers (National Institute for Public Health and the Environment, Bilthoven, the Netherlands).

B. pertussis challenge culture

The *B. pertussis* challenge culture was prepared as described in the literature[7], except that bacteria were grown in THIJS medium[23].

Ethics statement

An independent ethical committee for animal experimentations of the Institute for Translational Vaccinology (Intravacc) approved the animal experiments with identifiers 201400125 and 201400182. Animal handling in this study was carried out in accordance to the guidelines provided by the Dutch Act on Animal Experimentation.

Immunization and challenge of mice

Female BALB/c mice (Harlan, The Netherlands), 8-week-old, were immunized with 1 µg total protein omvPV either pulmonary (P.M.; 50 µl) or subcutaneously (S.C.; 300 µl) on day 0 and 28. Non-immunized (N.I.) mice were used as a control. Pulmonary administration was performed as described by Bivas-Benita *et al.*[24] using a MicroSprayer aerosolizer (IA-1C; Penn-Century, Philadelphia, PA, USA) supplied with a high-pressure syringe (FMJ-250; Penn-Century). Mice were intranasally challenged under anesthesia (isoflurane/oxygen), with 2×10^5 colony forming units (cfu) of *B. pertussis* B1917 in 20 µL of THIJS medium on day 56. For gene expression in the lungs and draining lymph nodes (dLN), cytokine responses, and antibody responses, mice (n=4 per group) were sacrificed 4 hours and 2, 7, 14, and 28 days after primary immunization. In addition, to investigate gene expression in the dLN, cytokine responses, and antibody responses after booster vaccination, mice (n=4 per group) were sacrificed on day 35 and 56. Finally, mice (n=4 per group) were sacrificed 4 hours after challenge and on day 58, 63, 70, and 84 to measure bacterial load in the respiratory tract, cytokine responses and antibody responses. For evaluation of B cell responses, mice (n=6 per group) were sacrificed on day 35 and 63 (plasma cells) and on day 56 and 84 (memory B cells). For investigation of T cell responses, mice (n=6 per group) were sacrificed on day 28, 56, and 84. An additional control group (n=6) of completely naive mice were included for B and T cell investigation on each time point. Mice were bled under anesthesia (isoflurane/oxygen) by orbital bleeding and sacrificed by cervical dislocation for further sample collection. An overview of the study design of treatment and sample collection is schematically depicted in Figure 1A.

Sample collection

From the lungs, the left lobe was placed in 1 ml RNAlater (Qiagen), incubated overnight at 4°C, and stored at -80°C for subsequent microarray analysis. The right lobe was collected in 900 µl THIJS medium and was homogenized using a Bio-Gen PRO200 Homogenizer (Pro Scientific Inc., Oxford, CT, USA) for the lung colonization assay after the challenge and after filtration (Millex GV Filter unit 0.22 µm, Millipore), lysates of all time points were used for pulmonary cytokine and antibody analysis. Complete lungs of mice used for T and B cell assays were collected in 5 ml RPMI-1640 medium (Gibco) supplemented with 10% FCS (Hyclone), 100 units penicillin, 100 units streptomycin, and 2.92 mg/ml L-glutamine (Invitrogen), hereafter named RPMI complete medium and kept on ice until use. The draining lymph nodes (dLN), bronchial LN for P.M. immunization and inguinal LN for S.C. immunization, were isolated and placed in 5 ml RPMI complete medium and kept on ice until use, for subsequent microarray analysis and T cell analysis by tetramer staining. Whole blood for tetramer staining and analysis of B cell responses was collected in heparin tubes (MiniCollect 1 ml LH Lithium Heparin, Greiner Bio-One, Austria). Serum for cytokine and antibody responses was obtained by collecting whole blood in a serum collection tube (MiniCollect 0.8 ml Z Serum Sep GOLD, Greiner Bio-One, Austria). After coagulation (10 min. at room temperature), sera were taken after centrifugation (10 min., 3000 g) and stored at -80°C. Spleens were placed in 5 ml RPMI complete medium and kept on ice for subsequent B and T cell assays. Trachea were collected in 900 µl THIJS medium and were homogenized using the Bio-Gen PRO200 Homogenizer for the trachea colonization assay. Nose lavage was obtained by flushing the nose with 1 ml THIJS medium after the trachea was removed for nose colonization assay. After filtration (Millex GV Filter unit 0.22 µm, Millipore), nose lavages were used to determine nasal antibody responses.

Colonization assays

The tissue lysates from lungs and trachea, and lavages of the nose were serially diluted (undiluted, 1:10, 1:100, and 1:1000) in THIJS medium. The diluted samples were plated on Bordet-Gengou agar plates and incubated for 5 days at 35°C. The number of cfu per ml was determined by using a colony counter (ProtoCOL, Synbiosis, Cambridge, United Kingdom).

B cell ELISpot

Wells of filter plates (Multiscreen-HA 96 wells plates, Millipore) were coated (overnight, 4°C) with 5 µg/ml Prn (Sanofi) or 10 µg/ml wildtype B1917 OMV. As a positive control, wells were coated with a mixture of 7 µg/ml purified goat-anti-mouse kappa and 7 µg/ml purified goat-anti-mouse lambda (Southern Biotech). As a negative control, wells were left uncoated (PBS). After washing 3 times with PBS, the plates were blocked (1h, room temperature) with RPMI 1640 + 2% Protifar (Nutricia) and washed again.

Spleens and lungs were homogenized using a 70 µm cell strainer (BD Falcon, BD Biosciences) and cells were collected in RPMI complete medium. From whole blood, erythrocytes were lysed by using RBC lysis buffer (Pharm Lyse, BD Pharmingen). For detection of memory B cells, $5x10^5$ splenocytes per well of a 24 well-plate were stimulated with CpG ODN 1826

(10 µg/mL, Invivogen), PWM (10 µg/mL), Staphylococcus aureus protein A of Cowan Strain (1:5000) and β-mercaptoethanol (1:25000) (all Sigma) in RPMI complete medium for 5 days at 37°C to induce antibody secretion. Cells from blood ($0.75x10^5$ cells/well), lungs ($0.75x10^5$ cells/well), spleen ($5x10^5$ cells/well) or stimulated splenocytes ($5x10^5$ cells/well) were added to the coated plates and incubated overnight at 37°C. Plates were washed 7 times with PBS and 3 times with PBS-T (0.05% Tween-20). Then, the plates were incubated (1h, 37°C) with alkaline phosphatase-conjugated goat-anti-mouse IgA or IgG (Southern Biotech; 1:1000). Plates were washed 7 times with PBS, 3 times with PBS-T, and 5 times with tap water. Subsequently, filtered (0.45 µm) BCIP-NBT liquid substrate (Sigma) was added. Spot development was stopped by removing the substrate and extensively rinsing with distilled water. Plates were dried and stored at room temperature in the dark. Spots were counted with an AID iSpot reader (Autoimmun Diagnostika GmbH). The number of *B. pertussis* OMV-specific IgG- and IgA-secreting cells were indicated as antibody secreting cells (ASC) per $5x10^5$ cells.

Antibody measurements

OMV-, Prn-, FHA-, Ptx-, and Fim2/3-specific antibodies were measured using an in-house developed mouse multiplex immunoassay, as described previously[25]. Serum samples were diluted 1:5000 for IgG (subclass) and 1:100 for IgM and IgA measurements. Lung lysate samples were diluted 1:100 and nose lavage samples were not diluted for measuring IgA levels. Reporter antibodies were R-PE-conjugated goat-anti-mouse IgA, IgG, IgG1, IgG2a, IgG2b, IgG3 or IgM (Southern Biotech). Data were acquired with a Bio-Plex 200, analyzed using Bio-Plex Manager software (version 5.0, Bio-Rad Laboratories), and presented as fluorescence intensities (FI).

Detection of Prn_{10-24} -specific $CD4^+$ T cells

dLN were homogenized using a 70 µm cell strainer and cells were collected in RPMI complete medium. Blood was treated with erythrocyte-lysis buffer (10 g/L NH_4CL, 1.25 g/L $NaHCO_3$, 0.125 mM EDTA in dH_20; pH 7.4) for 10 minutes on ice, and then resuspended in RPMI complete medium. Cells were stained with APC-conjugated I-A^d tetramers specific for the Prn_{10-24} T cell epitope[26] (NIH Tetramer Facility, Atlanta, Georgia, USA) in RPMI complete medium for 1 hour at 37°C. Next, cells were stained with Pacific blue-conjugated anti-CD4 (Biolegend), FITC-conjugated anti-CD44 (BD Biosciences), and LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit for 30 minutes at 4°C in FACS buffer (PBS (pH 7.2) supplemented with 0.5% BSA (Sigma Aldrich) and 0.5 mM EDTA (ICN Biomedicals). Data were acquired on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Intracellular cytokine staining (ICS)

Lungs and spleens were homogenized using a 70 µm cell strainer and cells, collected in RPMI complete medium, were treated with erythrocyte lysis buffer. The cells were cultured in 24-well plates ($6x10^6$ cells/well) for 3 days at 37°C in the presence of IMDM complete medium (IMDM medium (Gibco) supplemented with 8% FCS, 100 units penicillin, 100 units streptomycin, 2.92 mg/ml L-glutamine, and 20 µM β-mercaptoethanol (Sigma)).

For restimulation of cells, 1 µg/ml Prn or 1.5 µg/ml wildtype B1917 OMVs was added. On day 3, supernatant was collected for cytokine analysis, and the cells were transferred to U-bottom 96-well plates ($5x10^5$ cells/well) and restimulated overnight using the same antigen conditions.

ICS was performed on restimulated splenocytes and lung cells by using the BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), according to the manufacturer's protocol. Briefly, cells were incubated with 10 µg/ml Golgiplug (BD Biosciences), 1 µg/ml αCD28 (BD Pharmingen), and 1 µg/ml αCD49d (BD Pharmingen) during the last 5 hours of restimulation. Cells were then stained in FACS buffer with Pacific blue-conjugated anti-CD4 (Biolegend), FITC-conjugated anti-CD44 (BD Biosciences), PE-Cy7-conjugated anti-CD103 (Biolegend; only the lung cells), and with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen). Thereafter, cells were fixed, permeabilized, and stained with PE-conjugated anti-IFNγ (BD Biosciences), APC-conjugated anti-IL-5 (Biolegend), and PerCP-Cy5.5-conjugated anti-IL-17a (eBioscience). Data were acquired on a FACS Canto II and analyzed by using FlowJo software.

Multiplex cytokine analysis (MIA)

Concentrations (pg/ml) of 32 cytokines (Eotaxin, G-CSF, GM-CSF, IFNγ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IL- 1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IP-10, KC, LIF, LIX, M-CSF, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, and VEGF) present in serum and lung lysates were determined by using a MIA (Milliplex MAP Mouse Cytokine/ Chemokine - Premixed 32 Plex; Merck KGaA). The concentration of various Th subset cytokines (IL-4, IL-5, IL-10, IL-13, IL-17A, TNFα, and IFNγ) was determined in splenic culture supernatant using a Milliplex mouse cytokine 7-plex luminex kit (Millipore), according to the manufacturer's protocol. Measurements and data analysis were performed with a Bio-Plex 200 and using Bio-PlexManager software (version 5.0, Bio-Rad Laboratories). Results of the Th subset cytokines were corrected for the background (IMDM complete medium control) per mouse per stimulation per cytokine and calculated in pg/ml.

RNA isolation and microarray analysis

Isolation of RNA from lung tissue with additional determination of RNA concentrations and integrity was performed as described previously[7]. For isolation of RNA from cells in the dLN, the dLN were homogenized using a 70 µm cell strainer (BD Falcon, BD Biosciences) and cells were collected in RPMI complete medium and then washed with PBS. By using the MagNA Pure LC RNA Isolation High Performance kit (Roche) according to the manufactures protocol, the cells were lysed, in 1 ml lysis buffer, and RNA was isolated with the MagNA Pure System (Roche). For lung tissue, samples of naive mice and P.M. vaccinated mice were analyzed as individual samples (n=3), whereas the RNA concentrates of S.C. vaccinated mice were pooled (n=3) for the following time points: Naive, 4 hours, 2 days, 7 days, 14 days, and 28 days post primary vaccination. RNA concentrates from the lymph node suspensions, bronchial for P.M. vaccination and inguinal for S.C. vaccination, of individual mice (n=3) were analyzed for eight time points (naive, 4 hours, 2 days, 7 days, 14 days, 28 days, 35 days

and 56 days post primary vaccination). From the naive mice, both bronchial and inguinal lymph nodes were analyzed. Amplification, labeling and hybridization of RNA samples for either lung tissue or lymph nodes for microarray (HT MG-430 PM Array Plates, Affymetrix, Santa Clara, Calif, USA) was carried out at the Microarray Department of the University of Amsterdam, The Netherlands.

Transcriptomic data analysis

Quality control and normalization of raw Affymetrix CEL files were performed using the ArrayAnalysis website (www.arrayanalysis.org)[27], using the Robust Multichip Average (RMA) method[28] and the MBNI custom CDF version 19[29]. Normalized data consisted of Log2 transformed signal values for 17856 genes. Subsequent analysis of normalized data was performed in R (www.r-project.org) and Microsoft Excel. To identify differentially expressed genes between experimental groups (naive and various time points post vaccination) an ANOVA was applied. The induction or repression of individual genes was expressed as fold ratio by comparing mean gene expression levels of experimental groups to the naive mice. For pulmonary transcriptome analysis, average normalized gene expression levels contain individual data of three mice per group (P.M. and naive mice) and pooled data of 3 mice for the S.C. group. For dLN transcriptome analysis, average normalized gene expression levels contain individual data of three mice per group. The criteria for differential expression for the pulmonary transcriptome analysis were p-value <0.01 (ANOVA) and an absolute fold ratio >2.0 (experimental groups compared to naive mice). For dLN transcriptome analysis, the criteria were set at p-value < 0.001 and fold ratio > 1.5. GeneMaths XT (Applied Maths, St-Martens-Latem, Belgium) was used to visualize differences in gene expression in heatmaps. Genes were arranged according to similar expression patterns in time at which genes exceeded the fold ratio cut-off. To facilitate visual interpretation of heatmaps, upregulation (red) and downregulation (green) of gene expression levels are only visualized above the fold ratio cutoffs and presenting fold ratios below the cutoffs as an unchanged value (black). Additional data visualization was done by principal component analysis based on expression profiles of all differentially expressed genes in R. Functional enrichment was determined with an over-representation analysis (ORA) based on Gene Ontology Biological Processes (GO-BP) and Kyoto Encyclopedia of Genes and Genomes (KEGG) by using DAVID[30]. Involvement of type I and II interferon-signaling pathway was performed by using the Interferome database (http://www.interferome.org/interferome/home.jspx)[31].

Gene network analysis

To construct a gene-function network, genes associated with five modules, namely acute phase, cytokine response, humoral response, pathogen recognition receptor (PRR) signaling, and T cell responses, were determined using the ORA results and additional text mining. Interactions between genes were determined using the STRING database (http://string.embl.de/) with high confidence (0.700) and using co-occurrence, co-expression, experiments, databases, and text mining as types of evidence. Gene-function associations and gene-gene interactions were combined into one network file. The network visualization was performed using Cytoscape (version 2.8.3).

Immunoproteomic profiling

One-dimensional (1D) and two-dimensional (2D) electrophoresis in combination with Western blotting (1DEWB, 2DEWB) and LC-MS analysis were performed for the identification of antigen specificity of the antibody responses as described in the literature[9]. Acrylamide gels were loaded with 10-15 µg protein of a *B. pertussis* (B1917) lysate for 1D and with 25 µg protein of the lysate for 2D electrophoresis and blotted. Blots were treated with diluted sera (1:100), diluted lung lysates (1:10-1:50) and diluted nose lavages (1:10) prior to immunostaining with two different IR-800-labeled goat-anti-mouse secondary antibodies (anti-IgG or -IgA). Blots were scanned using an Odyssey infrared imager (Westburg) and analyzed with Delta2D software (Version 4.5) (Decodon, Germany).

Statistics

To determine significance of differences in the outcome of B cell ELIspot, T cell ICS, and T cell tetramer analysis between groups, a Mann-Whitney t-test was used. Data of the cytokine, antibody, and colonization assays were log-transformed after which a t-test was performed. p values ≤0.05 were considered to indicate significance of differences.

Results

Superior protection against *B. pertussis* infection by P.M. compared to S.C. omvPV immunization

The colonization of the respiratory tract after intranasal *B. pertussis* challenge of pulmonary immunized mice (P.M. mice), subcutaneously immunized mice (S.C. mice), and non-immunized control mice (N.I. mice) differed substantially. Lungs, trachea, and noses of N.I. mice were heavily colonized by *B. pertussis* after a challenge. The highest numbers of colony forming units were found 7 days post challenge (p.c.) (Figures 1B-D). In contrast, *B. pertussis* was mostly cleared from lungs of P.M. mice already 2 days p.c., whereas bacteria from lungs of S.C. mice were cleared 5 days later (Figure 1B). In the trachea of both S.C. and P.M. mice, bacteria were mostly cleared 7 days p.c. (Figure 1C). In the nose, no complete clearance of *B. pertussis* was observed in the P.M., S.C., and N.I. mice within 28 days p.c. However, the number of bacteria in P.M. mice was significantly lower than in N.I. mice on day 70 (Figure 1D). Together, these data show that P.M. immunization with omvPV induced enhanced protection against *B. pertussis* infection compared to S.C. immunization.

IgG antibody responses

B.pertussis-specific antibodies are important contributors to pertussis immunity[32] and currently IgG serology still is the gold standard in pertussis vaccine research. The IgG (subclass) levels were determined after P.M. and S.C. immunization. Both immunization routes induced high and comparable levels of anti-OMV IgG antibodies in serum, but with significantly higher levels after P.M. immunization on day 56 (Figure 2A). S.C. immunization elicited already anti-Prn IgG antibodies after primary immunization, while these were observed only after a booster immunization in P.M. mice (Figure S1A).

The IgG subclasses distribution induced by P.M. and S.C. immunization were comparable for OMV-specific responses (Figure 2B). The primary immunization stimulated production of IgG1 antibodies, while the booster vaccination promoted the formation of IgG3 antibodies. For Prn-specific responses, booster P.M. immunization led to more IgG2a/b than S.C. immunization, while IgG1 still was the predominant subclass for both immunization routes (Figure S1B).

The immunogenic proteins to which the anti-OMV antibodies are directed were identified (Figure 2E and Figure S3A-B). On 2DEWB, twelve and seventeen immunogenic pertussis proteins were detected after P.M. and S.C. immunization, respectively (Figure 2E and Table S1). Spots showing high staining intensities in both groups corresponded with BP3755, Vag8, BrkA, and U2. Antibodies against ahcY, also visible at 55 kDa on 1DEWB (Figure S3B), and ompA(2) were solely induced by P.M. immunization, whereas antibody formation against ompA(1), U5, bfrG, GroEI, purH, U3, and U4 were only found after S.C. immunization.

Additionally, anti-LPS antibodies (10 kDa) were observed after booster vaccination in both immunization groups (Figure S3B). Notably, following omvPV immunization via either

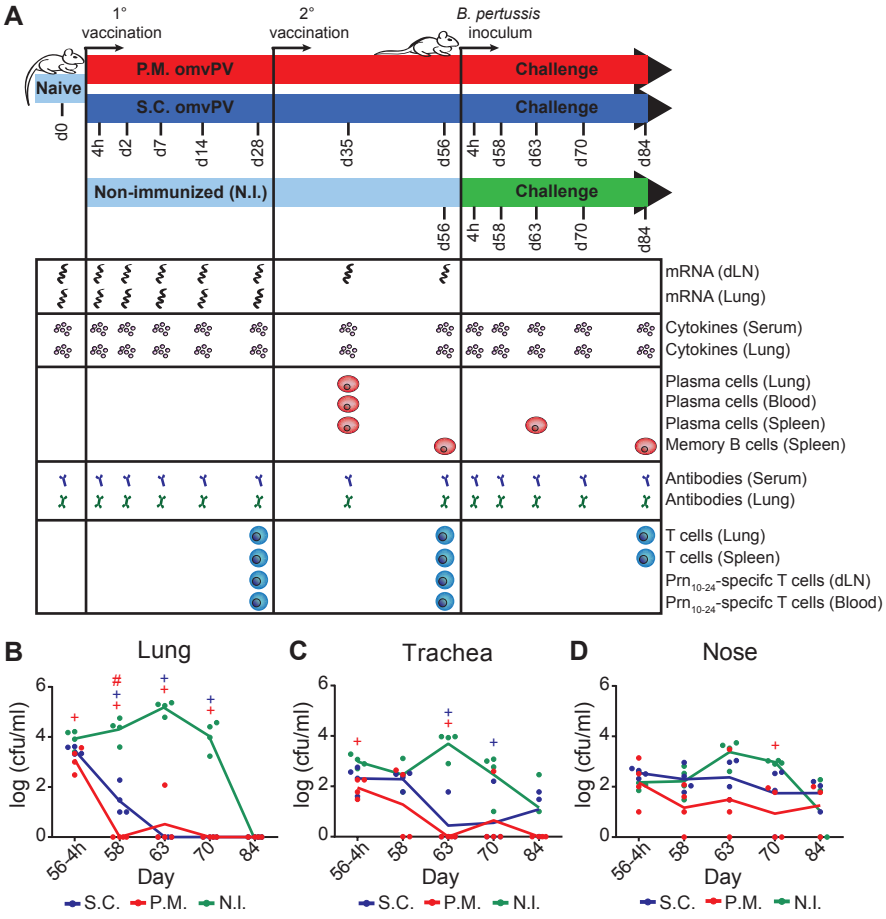


Figure 1 - Study design and *B. pertussis* colonization measured in the respiratory tract. (A) BALB/c mice were immunized with 1 µg omvPV pulmonary (P.M.; red) or subcutaneously (S.C.; blue) twice on day 0 and day 28. Subsequently, for both routes the vaccination-induced responses were characterized over a period of 56 days at 7 different time points. Additionally, a *B. pertussis* challenge (2x10^5 CFU) was performed on day 56 in both vaccinated groups and non-immunized (N.I.) mice (green). Vaccination responses and *in vivo* recall responses were characterized at the transcriptomic, proteomic and cellular level on given time points, as depicted. (B-D) The number of colony-forming units (cfu) were determined after *B. pertussis* challenge (B) in lungs, (C) trachea and (D) nose lavages of S.C. and P.M. immunized and N.I. mice. #p< 0.05 versus S.C. mice; +p<0.05 versus N.I. mice.

route, no IgG antibodies could be detected that were directed against Ptx, FHA, and Fim2/3 (data not shown), the antigens present in acellular pertussis vaccines in addition to Prn. Altogether, the systemic anti-OMV IgG response and subclass distribution after both P.M. and S.C. immunization were comparable and could not explain the increased protection of P.M. mice following challenge.

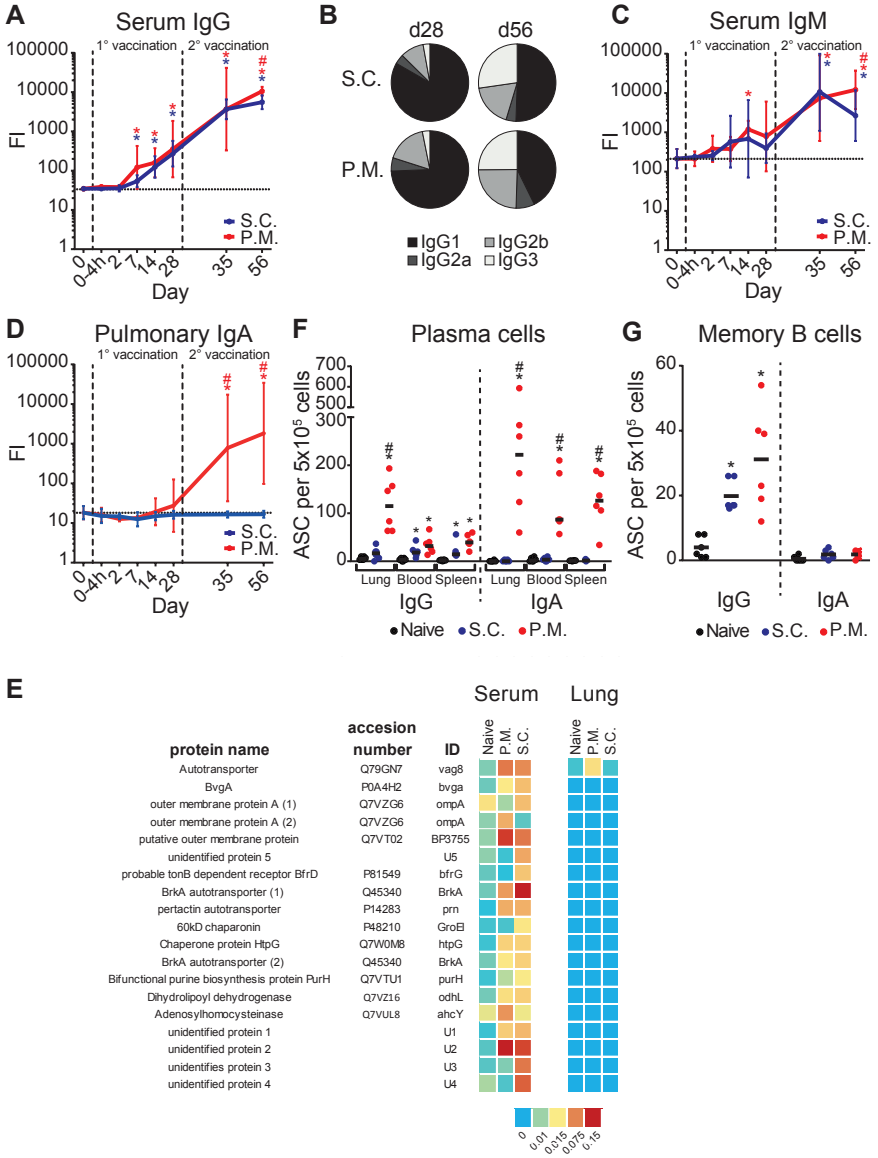


Figure 2 - *B. pertussis* OMV-specific B cell responses induced by P.M. and S.C. immunization with omvPV. (A-D) By using MIA, anti-OMV IgG antibody levels (A), IgG subclass distribution (B), IgM antibody levels (C) were determined in sera and anti-OMV IgA antibody levels (D) in lung lysates. Results are expressed as fluorescence intensities (FI) of 4 mice per group per time point. (E) Specificity of serum IgG and pulmonary IgA elicited by P.M. and S.C. immunization as determined by 2DEWB. Analysis was performed using pooled serum and lung lysates of 4 mice per group. Fluorescence intensities for each spot were obtained from 1 blot for IgA and the average of 3 blots for IgG. *p<0.05 versus naive mice (day 0); #p<0.05 versus S.C. mice. (F-G) Numbers of OMV-specific IgG- and IgA-secreting plasma cells in lungs, blood, and spleens (F) and numbers of IgG- and IgA-producing memory cells in spleens (G) were determined by B cell ELISpot of 6 mice per group at day 35 and day 56, respectively. Results are indicated as antibody secreting cells (ASC) per $5x10^5$ cells. *p<0.05 versus naive mice; #p<0.05 versus S.C. mice.

IgM antibody responses

Next, IgM antibodies were determined in sera from P.M. and S.C mice. IgM antibodies were only found directed against OMV and were induced significant on day 14 in P.M. mice. For both immunization routes, booster vaccination resulted in enhanced IgM antibody levels (Figure 2C). Similar to the findings for IgG, the level of anti-OMV IgM was significantly increased in P.M. versus S.C. immunized mice on day 56 (Figure 2C).

IgA antibody responses

IgA antibodies were found exclusively in P.M. mice, both in serum (Figures S2A-B) and lungs (Figures 2D and S1D). Serum IgA antibodies were directed against OMV and Prn, but not against other antigens present in acellular vaccines (Figures S2A-E). The specificity of pulmonary IgA was determined by immunoblotting and subsequent mass spectrometric identification of immunogenic proteins. The anti-OMV antibodies in the lungs were directed against Vag8 and LPS (Figures 2E, S3A, and S3C).

Pulmonary and systemic B cell responses

The effect of the immunization route on the numbers of *B. pertussis*-specific plasma cells was investigated in cell suspensions from lungs, peripheral blood, and spleen. At the peak of the plasma cell response on day 35, anti-OMV and anti-Prn IgG-secreting cells were detected in the lungs of P.M. mice only (Figures 2F and S1D). However, in blood and spleen of S.C. and P.M. mice similar and modest numbers of IgG-secreting cells were found (Figures 2F and S1D). In addition, anti-OMV and anti-Prn IgA-secreting cells were detected in the lungs, spleen and blood, exclusively after P.M. immunization (Figures 2F and S1D).

Anti-OMV IgG-producing memory B cells were measured in the spleen of P.M. and S.C. mice on day 56, with a trend towards higher numbers after P.M. immunization (Figure 2G). In contrast, anti-OMV IgA-producing memory B cells could not be detected at all (Figure 2G). The Prn-specific memory B cell responses on day 56 showed a similar trend as was found for the OMV-specific responses (Figure S1G). Thus, these data show that both S.C. and P.M. immunization elicited IgG-producing plasma and memory B cells, while P.M. immunization also evoked IgA-producing plasma cells.

Pulmonary T cell responses

Pulmonary *B. pertussis*-specific T cell responses were investigated by analyzing cytokine levels in the culture supernatants of lung cells that were restimulated with OMVs *in vitro*. Moderate IL-5 and vigorous IL-17A responses were detected after P.M. immunization only (Figure 3A). To investigate whether these cytokines were produced by $CD4^+$ T cells, single cell analysis by ICS was performed on these cultured cells. Only P.M. immunization induced OMV-specific IL-17A-producing $CD4^+CD44^+$ T cells in the lungs (Figure 3B). No OMV-specific IFNγ-producing and IL-5-producing $CD4^+CD44^+$ T cells could be detected by ICS in the lungs of any of the immunized mice (data not shown).

Recently, it has been demonstrated that tissue-resident memory T cells are important in the

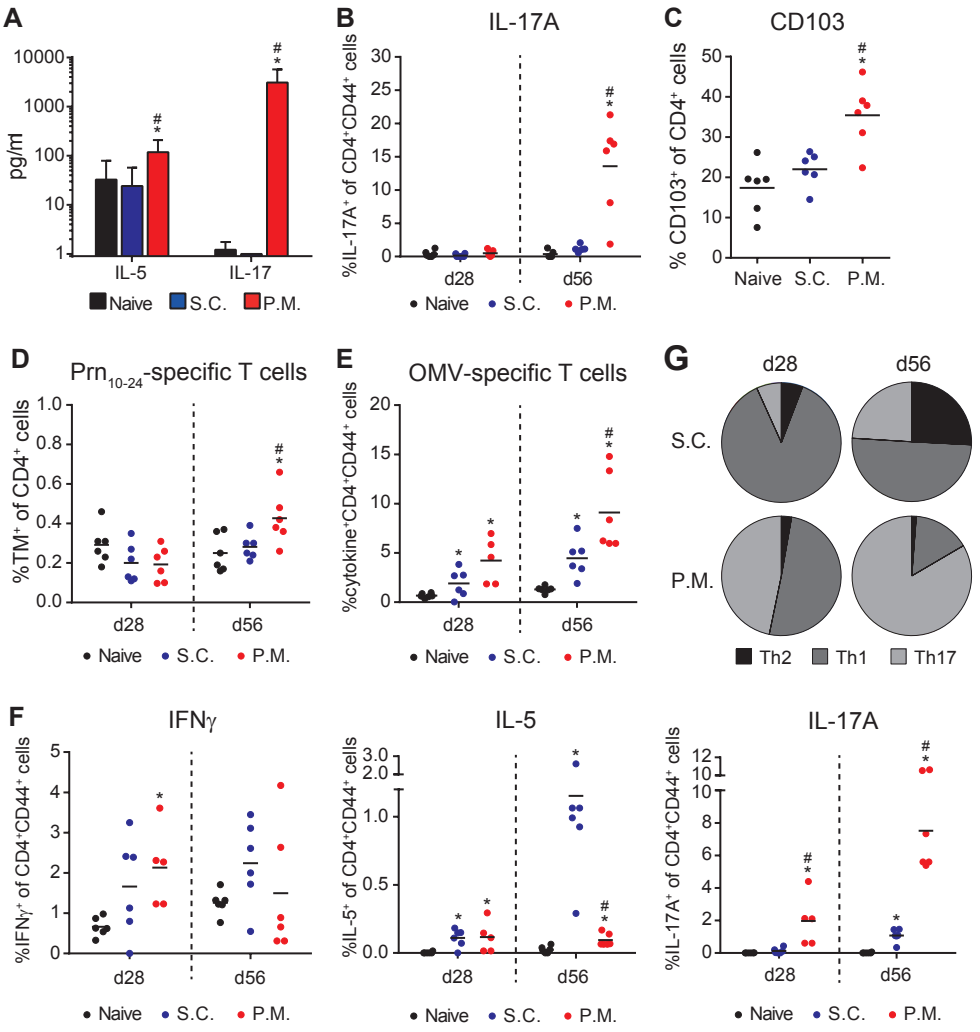


Figure 3 - Pulmonary and systemic *B. pertussis* OMV-specific T cell responses induced by P.M. and S.C. omvPV immunization. (A) IL-5 and IL-17A levels in 3 day culture supernatant of lung cells, isolated on day 56, after *in vitro* stimulation with OMVs as determined by MIA. Results in pg/ml are corrected for the background level (IMDM complete medium control) and are given as mean ± SD of 6 mice per group. (B) Percentage of IL-17-producing $CD4^+CD44^+$ T cells in the lungs, harvested on day 28 and day 56, as measured by ICS and flow cytometry after *in vitro* stimulation for 4 days with OMVs. (C) Flow cytometry for expression of CD103 on gated $CD4^+CD44^+$ T cells in the lungs on day 56. (D) Frequency of Prn_{10-24}-specific $CD4^+$ T cells in blood determined directly *ex vivo* on day 28 and day 56 using a tetramer staining and flow cytometry. (E) Magnitude of the systemic OMV-specific $CD4^+$ T cell response after *in vitro* stimulation with OMV for 4 days was determined using ICS on splenocytes, calculated as the total percentage cytokine (IL-5, IFNγ, and IL-17A)-producing $CD4^+CD44^+$ T cells. (F) Percentage IL-5-, IFNγ-, and IL-17-producing cells of $CD4^+CD44^+$ T cells of spleens harvested on day 38 and day 56 and stimulated *in vitro* for 4 days with OMVs. Results of each analysis are given of 6 mice per group. (G) Distribution of Th subsets based on IL-5 (Th2), IFNγ (Th1), and IL-17A (Th17) production, as determined by ICS and flow cytometry. *p<0.05 versus naive mice; #p< 0.05 versus S.C. mice.

protection against respiratory pathogens[33]. Expression of CD103, a marker for tissue-resident memory T cells, was determined on the OMV-specific IL-17A-producing $CD4^+CD44^+$ T cells, which were solely detected in P.M. mice. Of these cells, 57 ± 24 percent expressed CD103 (data not shown). Moreover, an increased percentage of pulmonary $CD103^+CD4^+$ T cells was detected in P.M. mice on day 56 compared to both S.C. and naive mice (Figure 3C). In conclusion, only P.M. immunization elicited pulmonary tissue-resident Th17 $CD4^+$ T cells.

Magnitude of the systemic $CD4^+$ T cell response

The magnitude of the $Prn_{10\text{-}24}$-specific $CD4^+$ T cell response was determined *ex vivo* using tetramers specific for this immunodominant I-A^d restricted T cell epitope of Prn in BALB/c mice. No $Prn_{10\text{-}24}$-specific $CD4^+$ T cells were detected in the dLN, bronchial and inguinal for P.M. and S.C. mice, respectively, on day 28 and day 56 (data not shown). However, $Prn_{10\text{-}24}$-specific $CD4^+$ T cells were observed in blood of exclusively P.M. mice on day 56 (Figure 3D).

Furthermore, the magnitude of the $CD4^+$ T cell response was investigated by determining the total percentage of *B. pertussis*-specific cytokine-producing (IFNγ, IL-5, or IL-17A) $CD4^+$ T cells. Both P.M. and S.C. immunization induced a significant increase of OMV- and Prn-specific cytokine-producing $CD4^+CD44^+$ T cells already on day 28 (Figures 3E and S4B). On day 56, a significantly higher percentage of these cells was detected after P.M. compared to S.C. immunization.

The effect of the immunization route on the Th-subset differentiation was determined. Analysis of cytokine levels in the culture supernatants of OMV-stimulated splenocytes revealed increased production of Th2 cytokine IL-4 and IL-5 after S.C. immunization, while the level of IL-13 was comparable after S.C. and P.M. immunization on day 28 and day 56 (Figures S5A-B). Stimulation of splenocytes with OMVs led to a high background production of Th1 cytokines IFNγ and TNFα of naive mice, possibly due to the presence of LPS in the OMVs (Figures S5A-C). Increased production of IFNγ by splenocytes from S.C. mice on day 28 and from P.M. on day 56 was still observed (Figures S5A-B). In addition, increased production of IL-17A and IL-10 was found by splenocytes of P.M. mice compared to S.C. mice on day 56. ICS analysis showed a significantly increased percentage of OMV-specific IFNγ-producing $CD4^+CD44^+$ T cells after P.M. immunization on day 28 (Figure 3F, left panel). OMV-specific IL-5-producing $CD4^+CD44^+$ T cells were detectable in P.M. and S.C. mice on day 28 and day 56. Notably, the percentage was significantly higher after S.C. immunization on day 56 (Figure 3F, middle panel). Significant OMV-specific IL-17A-producing $CD4^+CD44^+$ T cells were induced after S.C. immunization on day 56. Notably, significantly higher percentages of these cells were detected after P.M. immunization at both day 28 and day 56 (Figure 3F, right panel). A similar trend for Prn-specific $CD4^+CD44^+$ T cells were observed as the OMV-specific $CD4^+CD44^+$ T cells (Figure S4C).

The distribution of OMV- and Prn-specific $CD4^+CD44^+$ T cells, based on the production of IFNγ, IL-5, or IL-17A, indicates that S.C. immunization induced systemically a Th1-dominated

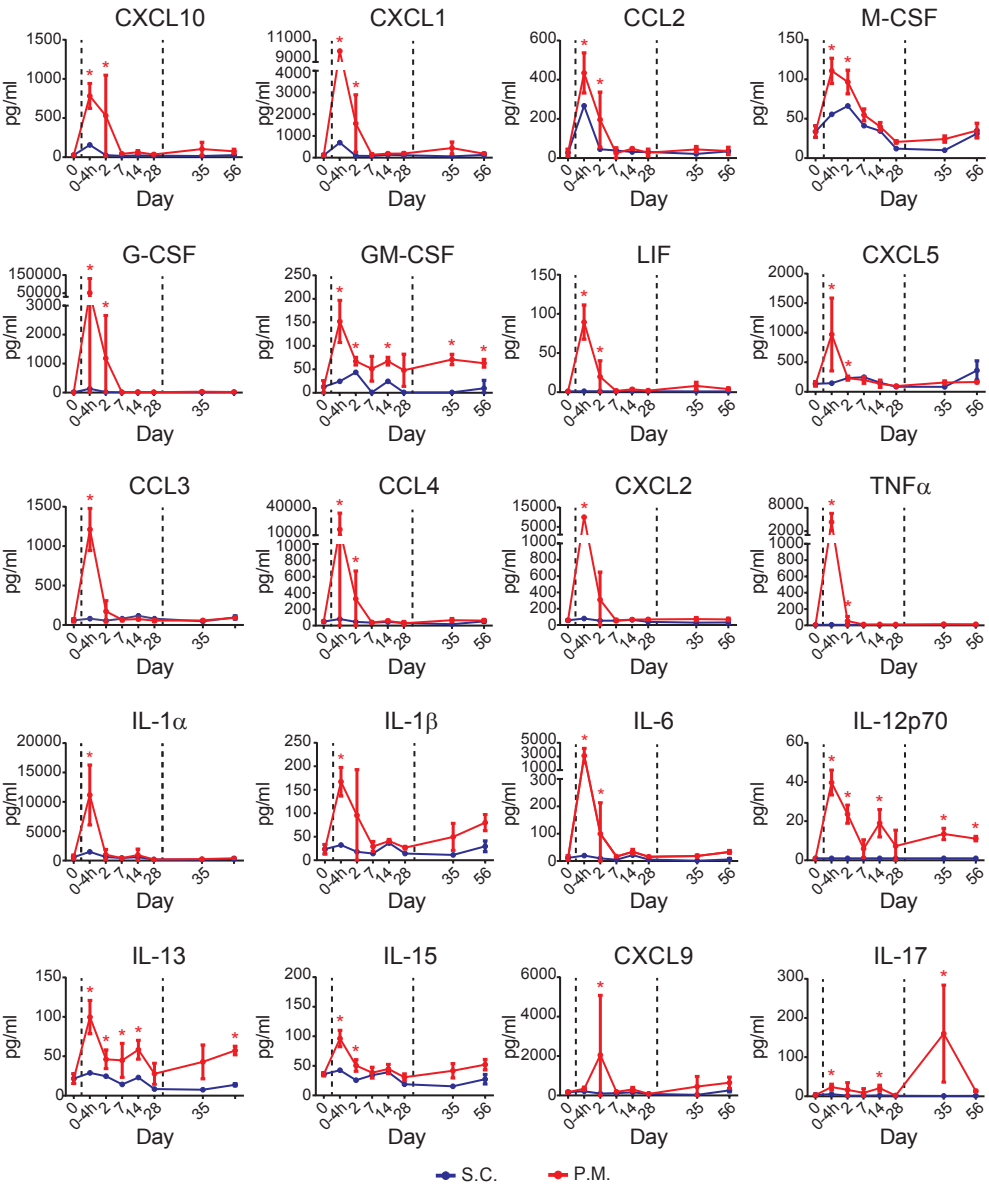


Figure 4 - Pulmonary cytokine responses after P.M. and S.C. omvPV immunization. The concentrations of cytokines in lung lysates of immunized mice were analyzed over time by using MIA. The cytokine concentrations were measured in the individual lung lysates of 4 mice per time point for P.M. mice and in a pool of the lung lysates of 4 mice per time point for S.C. mice.*p<0.05 versus naive mice (day 0).

response on day 28 and a mixed Th1/Th17/Th2 response on day 56 (Figures 3F and S4D). In contrast, P.M. immunization induced a mixed Th1/Th17 response on day 28, which shifted towards a Th17-dominated response on day 56. In summary, more Th1/Th17-skewed $CD4^+$ T cells were elicited by P.M. compared to S.C. immunization.

Cytokine profiles

The immunization-induced cytokine profile was determined in the lung lysates as well as serum. The largest differences were observed 4 hours post primary immunization (Figures 4 and 5). Concentrations of IL-6, IL-12p70, G-CSF, GM-CSF, IL-1α, IL-1β, IL-13, IL-15, LIF, CXCL5, CCL3, CCL4, CXCL2, CXCL10, CXCL1, CCL2, M-CSF, and TNFα were increased 4 hours post primary immunization in the lungs of P.M. mice. A trend towards increased levels of CXCL10, CXCL1, CCL2, and M-CSF was observed in the lungs of S.C. mice (Figure 4). At later time points, CXCL9 and IL-17A were found in P.M. mice on day 2 and day 35, respectively. In serum, increased levels of CXCL1, CCL4, TNFα, and IL-10 levels were found in both S.C. and P.M. mice (Figure 5A). In addition, higher levels of G-CSF, CXCL10, CCL2, CCL5, and IL-5 were detected after S.C. compared to P.M. immunization (Figure 5B), whereas the levels of IL-6 were higher in P.M mice (Figure 5C). Notably, CXCL1, CCL4, TNFα, G-CSF, CXCL10, CCL2, and IL-6 were found in both the lungs and sera of P.M. mice. Altogether, while S.C. induced only a systemic cytokine response, P.M. immunization induced a distinct systemic and pulmonary cytokine response.

Transcriptomic signatures in draining lymph nodes

Microarray analysis on bronchial and inguinal dLN from P.M. and S.C. mice, respectively, revealed 1921 genes that were differentially expressed (p-value ≤ 0.001, FR ≥ 1.5) over time compared to naive mice (Figure 6A). 951 genes were upregulated and 466 downregulated solely after P.M. immunization, 211 were upregulated and 145 were downregulated in both groups, and 109 were upregulated and 39 were downregulated exclusively after S.C. immunization. ORA using DAVID indicated enrichment of 141 GO-BP terms and KEGG pathways. A selection of these terms was sorted by the most significant enrichment and included *Cell cycle, apoptosis, immune response, T cell activation,* and *B cell mediated immunity* (Figure 6B). Cell cycle genes were mainly upregulated in P.M. mice. Innate signatures in the S.C. mice mainly involved genes downstream of IFN signaling (Figure 6C).

Genes related to T and B cells, based on gene ontology and text mining, are shown in Figures 6D-E. In total, 50 genes were related to T cells of which many were associated with distinct Th subsets (Figure 6D). Upregulation of most genes occurred 4 hours after immunization and from day 7 onwards. More genes were upregulated in P.M. mice than in S.C. mice. The upregulated genes in P.M. mice included Th17-associated genes, such as the master regulator for Th17 differentiation (*Rorc*), *Hif1a*, and *Havcr1*. The few T cell related genes exclusively expressed in S.C. mice comprised Th1- and Th17-associated genes, such as *Irf5*, *Ccr6*, *Syt11*.

Expression of B cell-related genes in S.C. mice mainly occurred on day 7 and day 35 (Figure 6E). In contrast, in P.M. mice elevated expression of B cell-related genes post primary immunization was detected earlier (4 hours) and persisted until day 28. Moreover, a smaller number of genes was induced on day 35 by booster immunization in P.M. compared to S.C. mice. Expression of *Mzb1*, *Ighm, Igkc*, *Ighg2b, Jchain, Ighg1,* and *Aicda* suggested antibody production and the presence of B cells in dLN, which was more pronounced in P.M. mice.

In addition, upregulation of specific B cell membrane, activation, and homing markers were observed. Both immunization routes induced *Cxcr5, Cd22*, *Cd40,* and *Cd83* expression, while exclusively the S.C. route induced *Cd19*, *Cd72*, *Ccr6*, and *Siglecg* and exclusively the P.M. route induced *Cd38*. In conclusion, P.M. and S.C. immunization with omvPV evoke distinct innate and adaptive responses in the dLN as detected on transcriptome level.

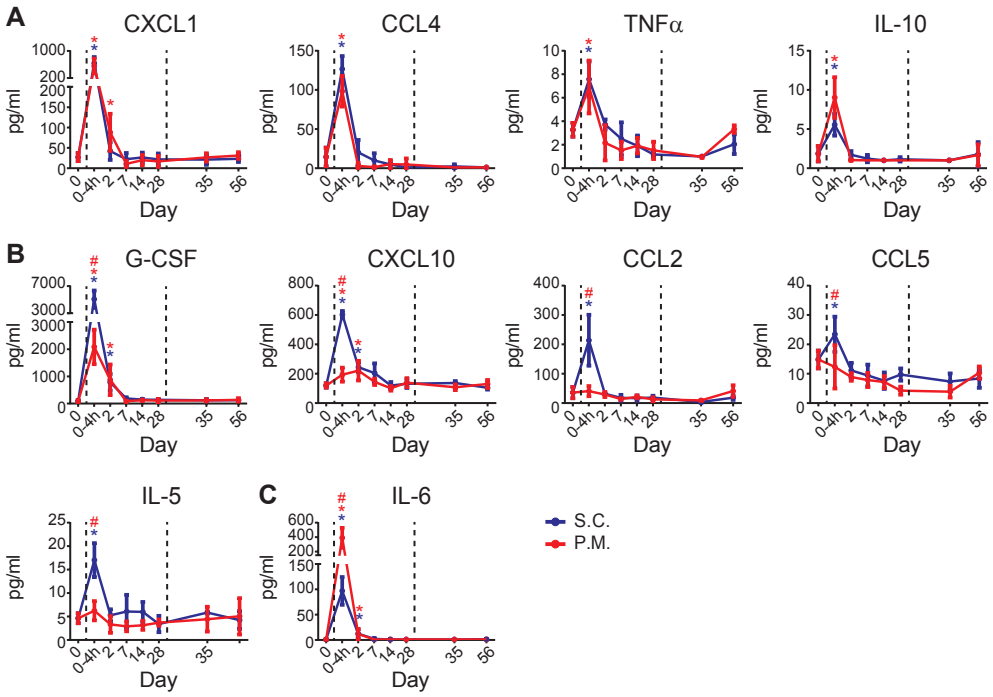
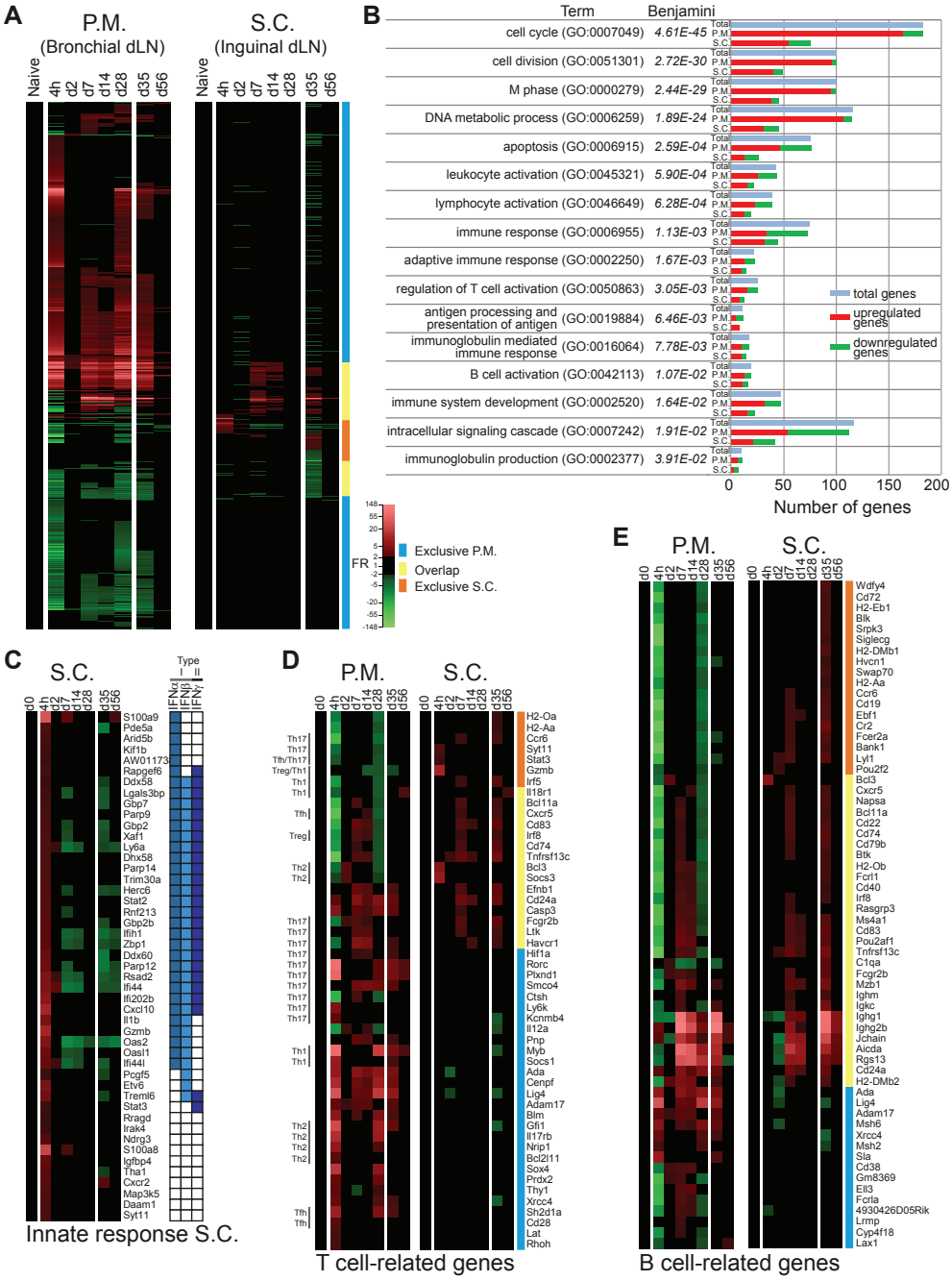


Figure 5 - Systemic cytokine responses after P.M. and S.C. omvPV immunization. The concentrations of cytokines in serum of immunized mice were analyzed over time by using MIA. (A) Cytokines with comparable levels in P.M. and S.C. mice. (B) Cytokines with elevated levels in S.C. compared to P.M. mice. (C) Cytokine with elevated level in P.M. compared to S.C. mice. Data is given as mean concentrations for 4 mice per group per time point. *p<0.05 versus naive mice (day 0); #p<0.05 versus S.C. mice.

Figure 6 - Transcriptomic profiles in the draining lymph nodes following P.M. and S.C. omvPV immunization. (A-E) Gene expression in P.M. and S.C. mice was compared to naive mice (day 0) (FR≥1.5, p-value≤0.001). (A) 1921 genes upregulated (red) or downregulated (green) are visualized in heatmaps (mean of n=3). Genes not surpassing a fold change of 1.5 are shown as basal level (black). The overlap (yellow) and the exclusive presence of genes in either the P.M. (blue) or S.C. (orange) immunization groups is depicted next to the heatmap. (B) Over-representation analysis on all 1921 genes revealed the involvement of specific GO-BP terms with corresponding total amount of genes (blue), upregulated (red) and downregulated (green) genes. (C) The 47 genes exclusively found upregulated during the innate response of the S.C. mice were shown in a heatmap (left panel) and the genes were compared to the Interferome database to determine involvement of the Type I IFN (IFNα and IFNβ) and/or Type II IFN (IFNγ) signaling pathway (right panel). (D) T cell-related genes, including association with distinct Th subsets, are depicted for P.M. and S.C. mice. (E) B cell-related genes are depicted for P.M. and S.C. mice.


A
P.M.
(Bronchial dLN)
S.C.
(Inguinal dLN)
Naive
4h
d2
d7
d14
d28
d35
d56
FR
Exclusive P.M.
Overlap
Exclusive S.C.
B
Term
Benjamini
cell cycle (GO:0007049) 4.61E-45
cell division (GO:0051301) 2.72E-30
M phase (GO:0000279) 2.44E-29
DNA metabolic process (GO:0006259) 1.89E-24
apoptosis (GO:0006915) 2.59E-04
leukocyte activation (GO:0045321) 5.90E-04
lymphocyte activation (GO:0046649) 6.28E-04
immune response (GO:0006955) 1.13E-03
adaptive immune response (GO:0002250) 1.67E-03
regulation of T cell activation (GO:0050863) 3.05E-03
antigen processing and presentation of antigen (GO:0019884) 6.46E-03
immunoglobulin mediated immune response (GO:0016064) 7.78E-03
B cell activation (GO:0042113) 1.07E-02
immune system development (GO:0002520) 1.64E-02
intracellular signaling cascade (GO:0007242) 1.91E-02
immunoglobulin production (GO:0002377) 3.91E-02
Total
P.M.
S.C.
total genes
upregulated genes
downregulated genes
Number of genes
C
S.C.
Type
I
II
IFNα
IFNβ
IFNγ
Innate response S.C.
D
P.M.
S.C.
T cell-related genes
E
P.M.
S.C.
B cell-related genes

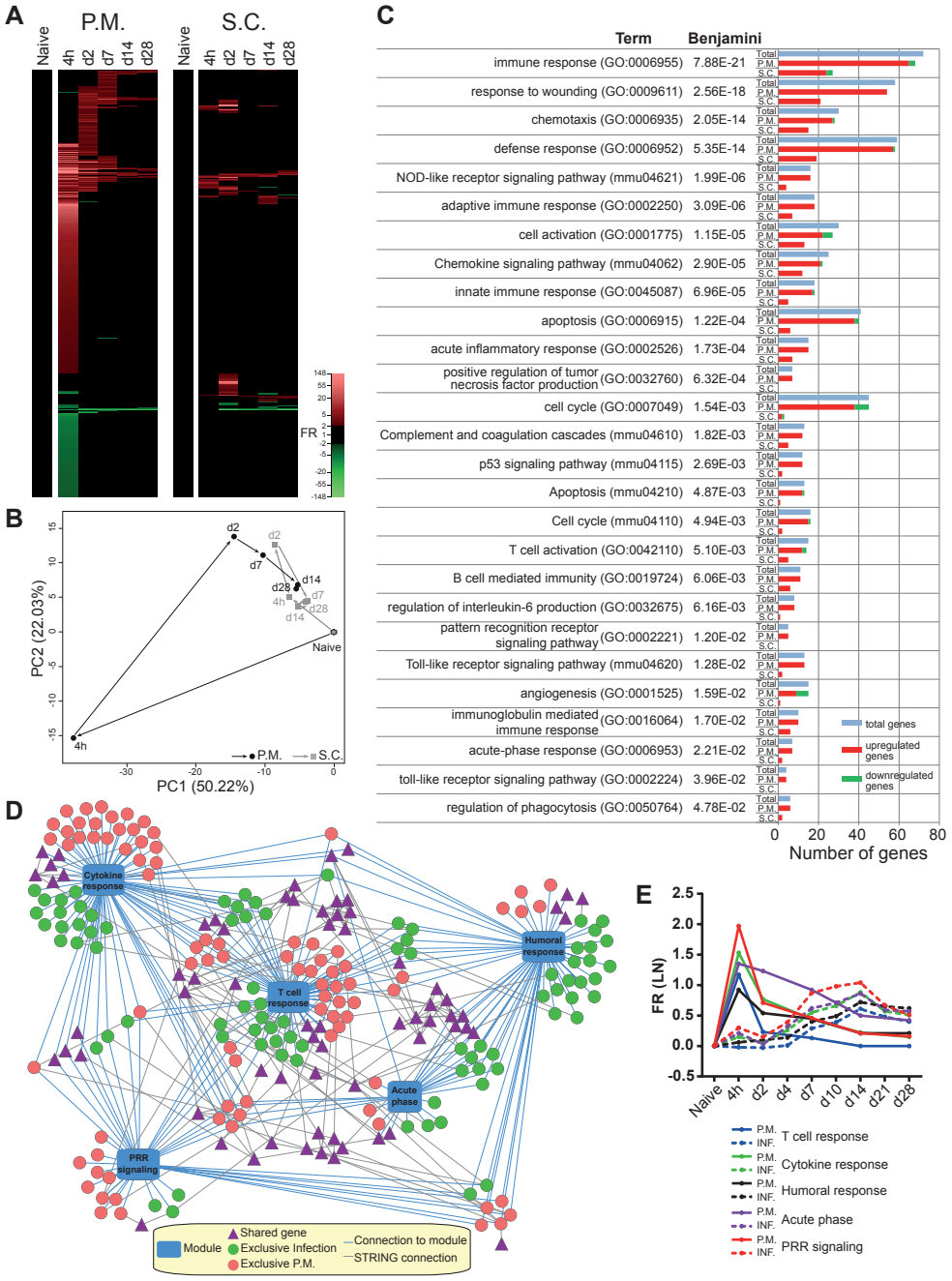

A
P.M.
S.C.
Naive
4h
d2
d7
d14
d28
FR
B
PC2 (22.03%)
PC1 (50.22%)
P.M.
S.C.
C
Term
Benjamini
immune response (GO:0006955) 7.88E-21
response to wounding (GO:0009611) 2.56E-18
chemotaxis (GO:0006935) 2.05E-14
defense response (GO:0006952) 5.35E-14
NOD-like receptor signaling pathway (mmu04621) 1.99E-06
adaptive immune response (GO:0002250) 3.09E-06
cell activation (GO:0001775) 1.15E-05
Chemokine signaling pathway (mmu04062) 2.90E-05
innate immune response (GO:0045087) 6.96E-05
apoptosis (GO:0006915) 1.22E-04
acute inflammatory response (GO:0002526) 1.73E-04
positive regulation of tumor necrosis factor production (GO:0032760) 6.32E-04
cell cycle (GO:0007049) 1.54E-03
Complement and coagulation cascades (mmu04610) 1.82E-03
p53 signaling pathway (mmu04115) 2.69E-03
Apoptosis (mmu04210) 4.87E-03
Cell cycle (mmu04110) 4.94E-03
T cell activation (GO:0042110) 5.10E-03
B cell mediated immunity (GO:0019724) 6.06E-03
regulation of interleukin-6 production (GO:0032675) 6.16E-03
pattern recognition receptor signaling pathway (GO:0002221) 1.20E-02
Toll-like receptor signaling pathway (mmu04620) 1.28E-02
angiogenesis (GO:0001525) 1.59E-02
immunoglobulin mediated immune response (GO:0016064) 1.70E-02
acute-phase response (GO:0006953) 2.21E-02
toll-like receptor signaling pathway (GO:0002224) 3.96E-02
regulation of phagocytosis (GO:0050764) 4.78E-02
Total
P.M.
S.C.
total genes
upregulated genes
downregulated genes
Number of genes
D
Cytokine response
T cell response
Humoral response
Acute phase
PRR signaling
Module
Shared gene
Exclusive Infection
Exclusive P.M.
Connection to module
STRING connection
E
FR (LN)
Naive
4h
d2
d4
d7
d10
d14
d21
d28
P.M.
INF.
T cell response
Cytokine response
Humoral response
Acute phase
PRR signaling

Figure 7 - Pulmonary transcriptomic profiles following P.M. and S.C. omvPV immunization. (A-C) Gene expression was compared to naive mice (day 0) (FR≥2.0, p-value≤0.01). (A) 691 genes upregulated (red) or downregulated (green) are visualized in heatmaps (mean of n=3 for P.M. mice and n=1 (pool of 3 mice) for S.C. mice). Genes not surpassing a fold change of 2.0 are shown as basal level (black). (B) Principal component analysis (PC1 and PC2) shows the (dis)similarity of the five time points for both P.M. and S.C. mice. (C) ORA on 691 genes uncovered the involvement of specific GO-BP terms and KEGG pathways with corresponding total amount of genes (blue), upregulated (red) and downregulated (green) genes. (D-E) Pulmonary transcriptomes were compared after P.M. immunization and a *B. pertussis* infection[7]. (D) A gene-function network analysis showing the connection of genes, expressed within 28 days after P.M. immunization or infection, to a specific module (blue line) based on involvement in acute phase, cytokine response, humoral response, pathogen-recognition receptor (PRR) signaling, and T cell responses. Genes that were expressed in both datasets (shared, triangle, purple), exclusively expressed in infected mice (circle, green), or exclusively expressed in P.M. mice (circle, red) are depicted. Additional, indirect linkage of shared genes with other genes in the network (grey line) by using the STRING database are shown. (E) For infected mice (9 time points) and P.M. mice (6 time points) an average fold ratio (FR) (expressed as LN-transformed numbers) was calculated to determine the expression intensity and kinetics for each module corresponding to those included in the network analysis.

Pulmonary transcriptomic signatures

Microarray analysis was performed on lung tissue collected during the primary immunization and comprised 691 genes that were differentially expressed (p-value ≤ 0.01, FR ≥ 2.0) over time compared to naive mice (Figure 7A). The largest dissimilarity was observed 4 hours after P.M. immunization as revealed by a principal component analysis (PCA) (Figure 7B). Many of these genes were no longer expressed on day 2 and later, while upregulation of other genes was observed (Figure 7A), as was also observed in the direction of variance in the PCA (Figure 7B). The pulmonary gene expression on day 28 did not return completely to basal levels. Notably, also differential gene expression was observed mainly on day 2 in the lungs of S.C. mice (Figure 7A-B). ORA (Benjamini<0.05) on the 691 genes revealed enrichment of 127 terms (11 KEGG Pathways, 116 GO-BP terms). A selection is shown in Figure 7C. Genes involved in pathogen recognition reflected by expression of PRR genes and members of corresponding downstream signaling pathways (inflammasome, TNF, NFκB, and MAPK) and effector molecules (e.g. pro-inflammatory responses) (Figures 7C and S6A). Moreover, genes involved in innate lymphoid cells (ILCs), CSF signaling, T cell activation, humoral response, and genes encoding complement, cytokines, cytokine receptors, and membrane markers were upregulated (Figures 7C and S6B).

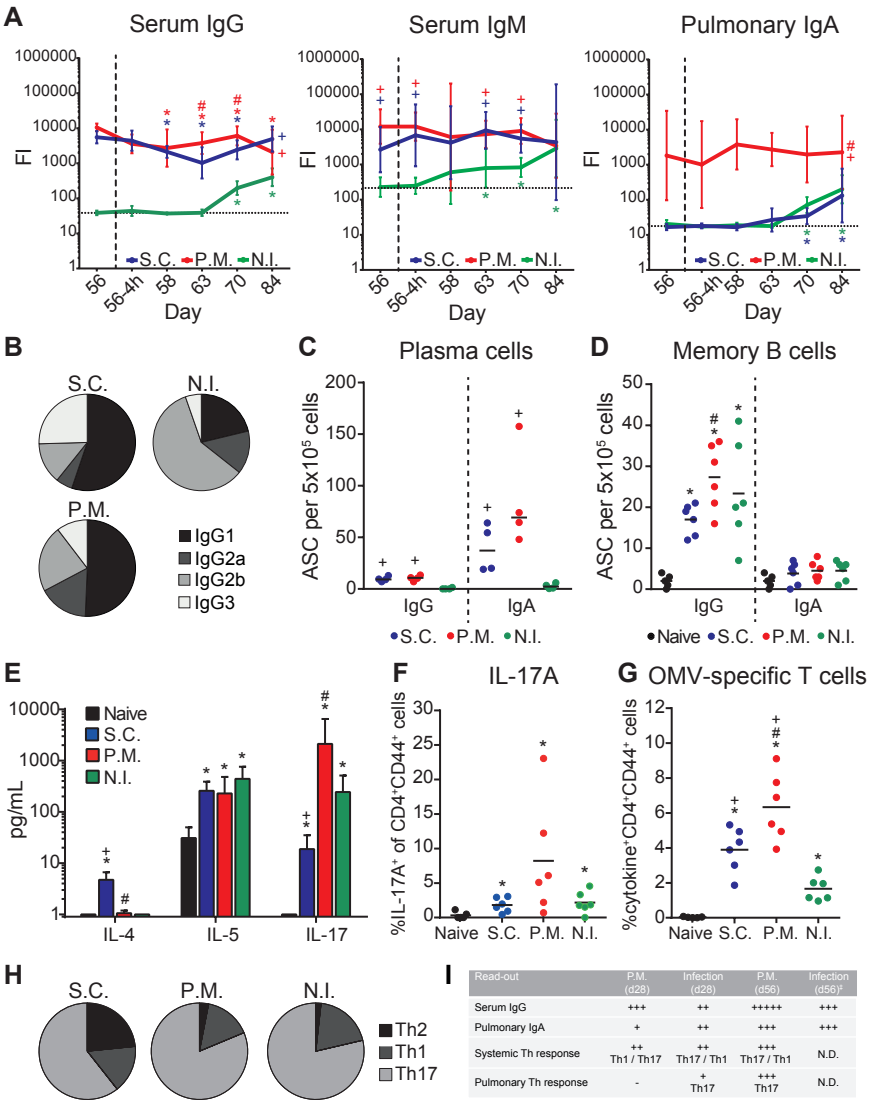
The transcriptomic signatures obtained after P.M. immunization in the lungs (691 genes) were compared to the dataset of gene expression profiles (558 genes) discovered in the lungs of mice after experimental *B. pertussis* infection in a previous study[7]. In total, 98 genes were found in both datasets. A gene-function network analysis investigating the involvement of genes in modules PRR signaling, acute phase, cytokine response, humoral response, and T cell responses, showed differences between both datasets (Figure 7D). Notably, the infection dataset comprised more genes related to the modules acute phase and humoral response, whereas the P.M. immunization dataset included more genes related to cytokine response and PRR signaling. Only a few shared genes (27 genes) were directly connected to the investigated modules. However, by using the STRING database many shared genes (42 genes) could be indirectly linked to the modules by their interaction with other genes in

the network. A comparison of intensity and kinetics of the average gene expression of each module revealed that P.M. immunization induced faster and more intense expression of all modules than infection (Figure 7E). In conclusion, both P.M. immunization and *B. pertussis* infection induced genes involved in PRR signaling, acute phase, cytokine response, humoral response, and T cell responses, yet with different intensity and kinetics. Moreover, the genes included in these modules are distinct for P.M. immunization and infection.

In vivo recall response after *B. pertussis* challenge

After unraveling the immune status of both of P.M. and S.C. mice on day 56, we determined the recall responses after an intranasal *B. pertussis* challenge. The serum anti-OMV IgG levels decreased after the challenge in P.M. and S.C. mice (Figure 8A). The challenge of non-immunized (N.I.) mice developed anti-OMV IgG and IgM, but the IgG levels remained lower compared to immunized mice (Figure 8A). For anti-Prn IgG levels a similar trend was observed after challenge (Figure S1A). However, no anti-Prn IgM was detected in any of the mice (data not shown). Further, no detectable levels of IgG and IgM to Ptx, FHA, or Fim2/3 were observed (data not shown). The challenge did not substantially alter the IgG subclass distribution of the anti-OMV and anti-Prn serum IgG response in P.M. and S.C. mice, present on day 56 (Figures 2B and S1B), since the IgG1 subclass remained dominant in both groups on day 84 (Figures 8B and S1B). However, a distinct distribution was found in challenged N.I. mice, since the IgG2b subclass response was more prominent in this group (Figures 8B and S1B). Enhanced serum IgA antibodies specific for OMV, Prn, and Fim2/3 were present in all challenged mice, whereas serum anti-Ptx and anti-FHA IgA were absent (Figures S2A-E). The pulmonary anti-OMV IgA antibody levels in P.M. mice remained high after the challenge, while in S.C. and N.I. mice these antibodies could only be detected on days 70 and day 84 (Figure 8A). The same trend was observed for pulmonary anti-Prn IgA levels (Figure S1C). In addition, no pulmonary anti-Ptx and anti-FHA IgA antibodies were detected after challenge (data not shown), while pulmonary anti-Fim2/3 IgA antibodies were found in S.C. and N.I. mice on day 84 (Figure S2F). Anti-OMV IgA antibodies were only detected in nose lavages of P.M. mice 4 hours after challenge, which were most likely induced by the earlier immunization (Figure S7A). These nasal IgA antibodies were directed against Vag8 (Figure S3D) and Prn (Figure S7B). The challenge led to enhanced anti-OMV IgA antibodies in all groups on day 84 (Figure S7A). In addition to higher anti-Vag8 IgA levels, the challenge induced anti-LPS antibodies in the P.M. and S.C. mice, but not in the N.I. control mice (Figure S3D). Furthermore, nasal anti-Prn, -Ptx, and -FHA IgA levels were significantly increased in S.C. mice following challenge (Figures S7B-D). Significantly higher anti-Fim2/3 IgA antibody concentrations were detected in N.I. mice on day 84 as compared to 4 hours after challenge, with the same trend in both immunized groups, but not significant (Figure S7E).

The numbers of anti-OMV IgG- and IgA-secreting plasma cells in the spleen of S.C. and P.M. mice were comparable and significantly higher than the numbers in N.I. mice on day 63 (Figure 8C). Furthermore, high numbers of anti-OMV IgG-producing memory B cells were present in the spleen of P.M., S.C., and N.I. mice compared to the numbers in naive mice (Figure 8D). Moreover, higher numbers were induced by P.M. than S.C. immunization. In contrast, even



Read-out	P.M. (d28)	Infection (d28)	P.M. (d56)	Infection (d56)[7]
Serum IgG	+++	++	+++++	+++
Pulmonary IgA	+	++	+++	+++
Systemic Th response	++ Th1 / Th17	++ Th17 / Th1	+++ Th17 / Th1	N.D.
Pulmonary Th response	-	+ Th17	+++ Th17	N.D.

Figure 8 - *In vivo* recall response after *B. pertussis* challenge of P.M., S.C. and N.I. mice. (A) Serum anti-OMV IgG antibodies (left panel), IgM antibodies (middle panel), and pulmonary anti-OMV IgA antibodies (right panel) determined in time after *B. pertussis* challenge on day 56 using MIA. Results are expressed as fluorescence intensities (FI) of 4 mice per group per time point. *p<0.05 versus levels on day 56; #p<0.05 versus S.C. mice; +p<0.05 versus N.I. mice. (B) Distribution of the anti-OMV IgG subclasses on day 84. (C-D) Numbers of OMV-specific IgG- and IgA-secreting plasma cells (C) and memory cells (D) in spleens on day 84, as determined by B cell ELISpot of 6 mice per group. (E) Cytokine levels in 3 day culture supernatant of lung cells, isolated on day 84, after *in vitro* stimulation with OMVs, as determined by MIA. Results in pg/ml are corrected for the background level (IMDM complete medium control) and are given as mean ± SD of 6 mice per group. (F) Percentage of IL-17-producing $CD4^+CD44^+$ T cells in the lungs, harvested on day 84, as measured by ICS and flow cytometry after *in vitro* stimulation for 4 days with OMVs. (G) Magnitude of the systemic OMV-specific $CD4^+$ T cell response on day 84 after *in vitro* stimulation with OMV for 4 days, as determined by ICS on splenocytes and calculated as the total percentage cytokine (IL-5, IFNγ, and IL-17A)-producing $CD4^+CD44^+$ T cells. (H) Distribution of Th subsets based on IL-5 (Th2),

IFNγ (Th1), and IL-17A (Th17) production on day 84, as determined by using ICS and flow cytometry. *p<0.05 versus naive mice; #p<0.05 versus S.C. mice; +p<0.05 versus N.I. mice. (I) The characteristics of the adaptive immune response obtained by primary (day 28) and booster (day 56) P.M. omvPV immunization in comparison to primary *B. pertussis* infection on day 28 and 56 are summarized. ‡ Obtained from a previous study [Raeven *et al.*, manuscript in preparation]. N.D. = not determined.

after challenge, anti-OMV IgA-producing memory B cells were not detected in any of the groups (Figure 8D). The Prn-specific antibody-secreting cells and memory B cell responses showed a similar trend as found for the OMV-specific responses, except that no Prn-specific IgG-producing memory B cells could be detected in challenged N.I. mice (Figures S1F-G).

Investigation of the culture supernatants of OMV-stimulated cells from the lungs showed IL-4 production solely by cells from S.C. mice (Figure 8E). In contrast, lung cells from all three challenged groups produced IL-5 and IL-17A, although IL-17A secretion by P.M. mice was significantly increased as compared to S.C. and N.I. mice (Figure 8E). However, a trend towards a higher percentage of OMV-specific IL-17A-producing $CD4^+CD44^+$ T cells was observed in the lungs after challenge in P.M. compared to S.C. or N.I. mice (Figure 8F). Similar results were found after Prn stimulation, except that IL-17A-producing $CD4^+CD44^+$ T cells were not detectable in N.I. mice (Figure S4C). OMV- and Prn-specific IFNγ- and IL-5-producing $CD4^+CD44^+$ T cells were absent in the lungs of all challenged groups (data not shown). Systemic T cell responses, as detected by ICS, showed a higher magnitude, as calculated by the total percentage of *B. pertussis*-specific cytokine-producing (IFNγ, IL-5, or IL-17A) splenic $CD4^+$ T cells, in P.M. compared to S.C. and N.I. mice (Figure 8G). Moreover, distinct Th subset distributions were observed in S.C. and P.M. mice after challenge (Figure 8H). In S.C. mice, the pre-challenge Th response (Figure 3F) underwent a shift towards a Th17 response at the expense of the Th1 response. Notably, the Th2 component remained responsible for a quarter of the Th response. In P.M. mice, the Th response was not altered after challenge with little or no Th2 component (Figure 8H).

The systemic and local adaptive immune responses after a primary (day 28) and booster (day 56) P.M. immunization were schematically summarized and compared with those induced by a primary *B. pertussis* infection on day 28, obtained in this study, and day 56, obtained in our previous study [Raeven et al., manuscript in preparation]. The comparison demonstrated that the adaptive immune responses were comparable after P.M. omvPV immunization and a *B. pertussis* infection (Figure 8I). Notably, there were however quantitative differences as the systemic IgG levels were higher after P.M. immunization compared to infection while pulmonary IgA levels were enhanced upon infection on day 28. Collectively, the data of the *in vivo* recall responses indicate that the systemic and local memory B and T cell responses after P.M. versus S.C. omvPV-immunized mice were different in function and magnitude. The systemic ratio of the Th subsets and the IgA response induced by P.M. immunization was reminiscent of the response after primary *B. pertussis* infection.

Discussion

Pulmonary immunization induces local immunity important for protection as was demonstrated for several respiratory pathogens[10-12]. In this study, the immune responses evoked by pulmonary and subcutaneous immunization of an omvPV were compared in detail. Pulmonary immunization provided superior protection against *B. pertussis* challenge over the subcutaneous route. A systems biology approach revealed innate and adaptive immune signatures associated with this superior protection. The pulmonary gene expression and cytokine profiles exclusively evoked by P.M. immunization revealed omvPV recognition by PRRs, induction of pro-inflammatory and IL-17 effector cytokines, and IgA responses. Furthermore, in dLNs of S.C. mice, genes downstream of IFN signaling were expressed, while in dLNs of both P.M. and S.C. mice genes involved in the production of IgG were expressed. Moreover, based on gene expression, Th17 cells were detected in the dLNs of P.M. mice. The systemic cytokines, however, most were higher after S.C. immunization. Although both P.M. and S.C. immunization induced strong systemic adaptive immune response, P.M. immunization evoked higher IgM levels, IgG levels, and $CD4^+$ T cell responses. Moreover, the induced Th subsets differed, since P.M. immunization led to a mixed Th1/Th17, yet Th17 dominated, response, whereas S.C. immunization led to a mixed Th1/Th2/Th17 response. More importantly, P.M. immunization elicited a robust mucosal adaptive immune response as indicated by the presence of IgA antibodies, IgA- and IgG-producing plasma cells, and Th17 differentiated $CD4^+$ T cells in the lungs of mice.

An overview of the main findings is presented in Figure 9 and discussed in detail hereafter. During the innate phase, expression of genes associated with pathogen recognition and the PRR downstream-signaling cascade were detected in the lungs of P.M. mice, which included TLRs, MyD88, NFκB, and pro-inflammatory cytokines (Figure 9A). This demonstrates that the omvPV is sensed in the lung by TLR4, based on the expression of *Lbp* and *Cd14*, and by TLR2 on innate cells, which is similar to the recognition of wPV and whole bacteria[34-36]. Interestingly, also enhanced expression of *Tlr7* and *Nlrp3* was observed indicating activation of intracellular PRRs by omvPV. The expression of pro-inflammatory cytokines, such as *Il1b*, *Il6*, and *Cxcl10*, was also detected in the lungs on protein level. Multiple known IL-17 effector cytokines[8, 37] were measured in the lungs of P.M. mice, including CXCL1, CXCL2, CCL2, G-CSF, GM-CSF, and TNFα. These IL-17 effector cytokines facilitate, together with IL-1β and IL-6, a Th17-differentiating environment. This was reflected by the presence of Th17 cells in the dLN, based on expression of *Rorc*, *Hif1a*, and *Havcr1.* Moreover, Th17 cells were detected in the spleen and more importantly in the lungs on single cell level, suggesting induction of both systemic and tissue-resident cells. In addition, the presence of CCL3, CCL4, CXCL10, and IL-12p70 in the lungs promotes differentiation towards Th1 cells[38, 39]. Indeed Th1 differentiation was promoted, since Th1 cells were detected systemically on single cell level. A Th1/Th17 response is favorable for providing protection against *B. pertussis* infection[5, 7, 8] and additionally, the presence of tissue-resident T cells in the lungs might even enhance protection[33]. The humoral response after P.M. immunization was characterized by pulmonary expression of IgA-related genes and the IgA transporter (*Pigr)* and the

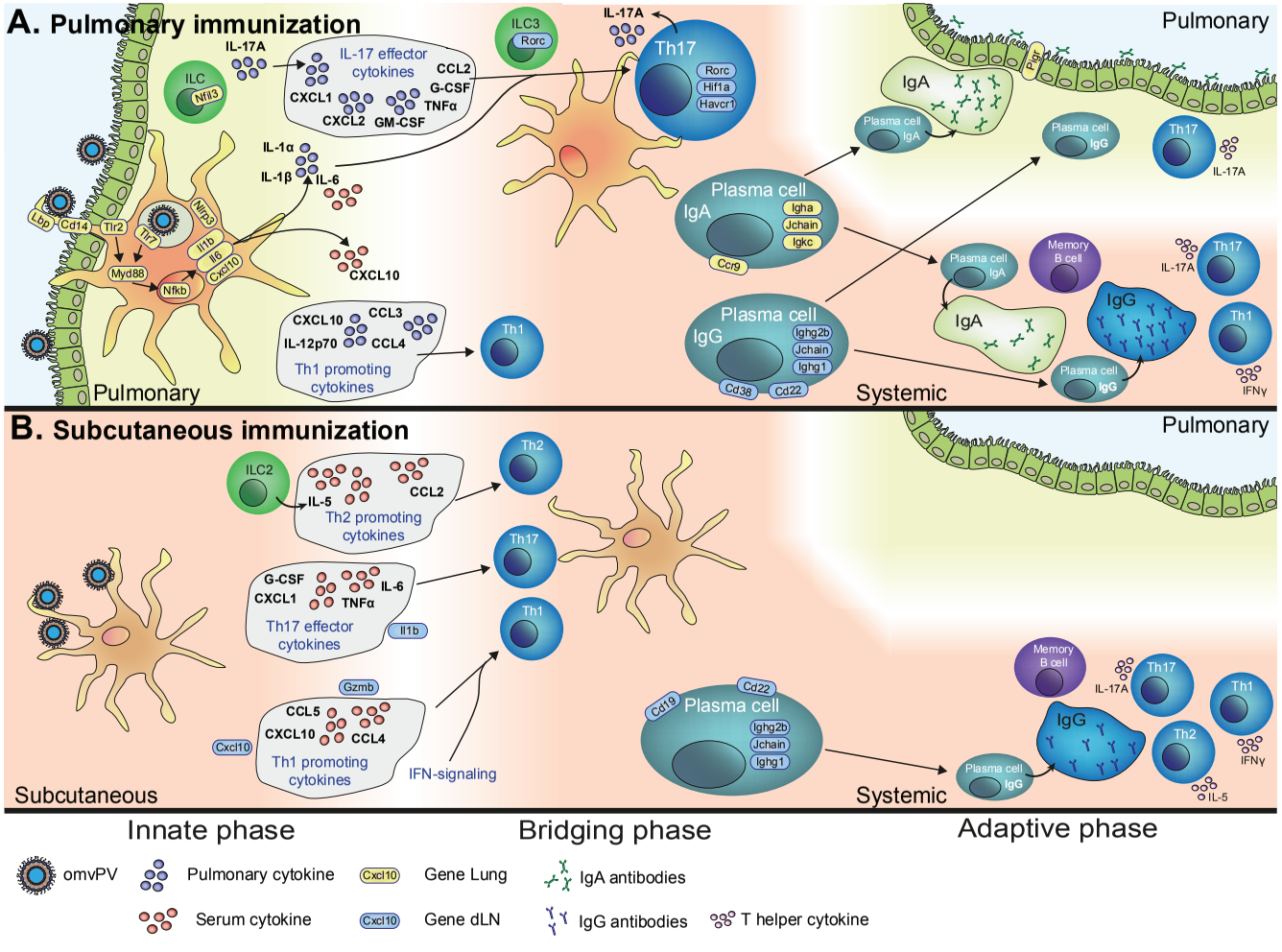

A. Pulmonary immunization
IL-17A
ILC
Nfil3
IL-17 effector cytokines
CCL2
G-CSF
CXCL1
TNFα
CXCL2
GM-CSF
ILC3
Rorc
IL-17A
Th17
Rorc
Hif1a
Havcr1
IL-1α
IL-1β
IL-6
Lbp
Cd14
Tlr2
Tlr7
Nlrp3
Il1b
Il6
Cxcl10
Myd88
Nfkb
CXCL10
CXCL10
CCL3
IL-12p70
CCL4
Th1 promoting cytokines
Th1
Pulmonary
Pulmonary
Plasma cell
IgA
Igha
Jchain
Igkc
Ccr9
Plasma cell
IgA
Plasma cell
IgG
Th17
IL-17A
Plasma cell
IgA
Memory B cell
Th17
IL-17A
IgA
IgG
Th1
IFNγ
Plasma cell
IgG
Plasma cell
IgG
Ighg2b
Jchain
Ighg1
Cd38
Cd22
Systemic
B. Subcutaneous immunization
Pulmonary
ILC2
IL-5
CCL2
Th2 promoting cytokines
Th2
Th17
Th1
G-CSF
CXCL1
TNFα
IL-6
Il1b
Th17 effector cytokines
Gzmb
CCL5
CXCL10
CCL4
Cxcl10
Th1 promoting cytokines
IFN-signaling
Subcutaneous
Cd22
Cd19
Plasma cell
Ighg2b
Jchain
Ighg1
Memory B cell
IL-17A
Th17
Th1
IgG
Th2
IFNγ
Plasma cell
IgG
IL-5
Systemic
Innate phase
Bridging phase
Adaptive phase
omvPV
Pulmonary cytokine
Serum cytokine
Cxcl10
Gene Lung
Cxcl10
Gene dLN
IgA antibodies
IgG antibodies
T helper cytokine

Figure 9 - Overview of the immune responses after P.M. and S.C. omvPV immunization. Based on the results obtained in this study, the main findings of the innate and adaptive immune responses were connected for (A) P.M. immunization and (B) S.C. immunization.

subsequent IgA presence in lungs and serum. In line with these findings, IgA-producing plasma cells were detected in spleen, blood, and lungs. Furthermore, high serum IgG levels in P.M. mice corresponded with specific gene expression (*Ighg1*, *Igh2b*, and *Jchain*) of IgG-producing plasma cells in the dLN, and with detected IgG-producing plasma cells on single cell level in spleen, blood, and lungs. In addition, IgG-producing memory B cells were detected in spleens of P.M. mice. Interestingly, pulmonary expression of *Nfil3* and early expression of *Rorc* in the dLN suggest involvement of ILCs in the immune response upon P.M. immunization. *Nfil3* is an ILC marker and *Rorc* is expressed by the ILC3 subset[40].

After S.C. immunization, omvPV is most likely sensed in a similar manner as after P.M. immunization, mainly by TLR2 and TLR4, although this was not investigated at the injection site in this study. The strong systemic cytokine response demonstrated a Th1- (CCL4, CCL5, and CXCL10), Th2- (IL-5 and CCL2), and Th17-differentiating (CXCL1, G-CSF, IL-6, and TNFα) environment, which was later reflected by presence of splenic Th1, Th2, and Th17 cells on single cell level (Figure 9B). Moreover, the induction of Th1 cells was linked to the early expression of genes downstream of IFN signaling found in the dLN[41]. The source of the early IL-5 production might be ILC2s[40], suggesting the involvement of these cells after S.M. omvPV immunization. Furthermore, the expression of B cell markers (*Cd19* and *Cd22)* in addition to immunoglobulin-related genes (*Igh2b, Jchain*, and *Ighg1*) revealed the presence of IgG-producing plasma cells in the dLNs. Subsequently, IgG-producing plasma cells were detected on single cell level in spleen and blood. Additionally, S.C. omvPV immunization led to IgG-producing memory B cells in the spleen and high serum IgG levels.

Based on the IgA responses, three interesting observations were revealed. First, full clearance of *B. pertussis* from the nose was not achieved despite the presence of anti-Vag8 IgA antibodies in the nose lavage in P.M. mice. This suggests that under the conditions used in our study with respect to vaccine and challenge dose, anti-Vag8 IgA induction in the nose alone may not be sufficient for prevention of nasal *B. pertussis* colonization. Second, 28 days after *B. pertussis* challenge, nasal anti-LPS IgA antibodies were detected in P.M. and S.C. mice, but not in non-immunized (N.I.) mice. Also, a lack of OMV-specific IgA-secreting plasma cells in N.I. mice after challenge was observed. Because no IgA response was detectable before challenge in S.C. mice, it was expected that challenge of S.C. and N.I. mice would evoke a similar IgA response. This faster induction of IgA responses in S.C. compared to N.I. mice may indicate the presence of low numbers of LPS-specific IgA-producing memory B cells in S.C. mice stimulated upon a challenge. Third, large numbers of IgA-secreting plasma cells were present in the spleen 7 days post P.M. booster vaccination (day 35). However, IgA-producing memory B cells were non-detectable on day 56. Nonetheless, the recall of memory B cells in response to infection led to a rapid increase in numbers of IgA-secreting plasma cells in the spleen (day 63). This suggests that IgA-producing memory B cells may not reside in the

spleen but elsewhere, presumably as tissue-resident IgA-producing memory B cells in the mucosa[42]. Since IgA responses may contribute to protection against *B. pertussis* infection, these different aspects of the IgA responses require further investigation.

The immunity induced by P.M. immunization with an omvPV substantially resembles the infection-induced immunity, still considered as benchmark for optimal immunity. Interestingly, P.M. immunization with omvPV confers sterilizing immunity to a similar extent as is observed for infection-induced immunity in mice and baboons[5, 7, 8]. After both P.M. immunization and *B. pertussis* infection, a strong IgG response is induced. Furthermore, circulating and pulmonary IgA-producing B cells were detected after P.M. immunization, leading to the production of mucosal IgA. This IgA was mainly directed against Vag8 as was observed after a challenge of mice with *B. pertussis* [Raeven *et al.* manuscript in preparation]. Moreover, both after P.M. immunization and *B. pertussis* infection, the $CD4^+$ T cell responses, pulmonary and systemically, are Th17 dominated[5, 7, 8] [Raeven *et al.* manuscript in preparation].

Remarkably, there were also differences between P.M. immunization and infection-induced immunity. Serum IgG responses were already higher after primary P.M. immunization compared to *B. pertussis* infection on day 28. Moreover, increased IgG levels were found after booster immunization compared to the levels observed 56 days after infection [Raeven *et al.* manuscript in preparation]. Moreover, the omvPV vaccination in general evoked a distinct IgG subclass response compared to infection, which was partly caused by high anti-LPS IgG3 induction after the booster vaccination. IgG subclass distribution is thought to be indirect evidence for Th responses[43-45]. In our study the Th responses are not mirrored in the IgG subclass responses, since the Th responses are comparable after P.M. immunization and infection, while the IgG subclass distribution is distinct. Given that both S.C. and P.M. administration of omvPV result in an equal IgG subclass response, suggests that the differences compared to infection are probably caused by the vaccine composition and vaccination scheme rather than administration route. Furthermore, this study and our previous study[9] demonstrate that the antigen specificity of the serum IgG induced by P.M. immunization differs from infection, most likely due to distinct antigen composition. Whereas omvPV contains only outer-membrane and periplasmic proteins, live bacteria are comprised of additional cytosolic proteins and are capable of *de novo* production and secretion of antigens, such as Ptx[9]. Pulmonary IgA levels on day 28 induced by infection were higher compared to primary P.M. immunization. Booster P.M. immunization equalized the IgA levels with those after infection on day 56 [Raeven *et al.* manuscript in preparation].

Furthermore, with respect to cytokine profiles, P.M. immunization leads to faster and more intense pulmonary and serum cytokine production as compared to infection. However, higher cytokine levels were expected during *B. pertussis* infection because of a prolonged antigen exposure[7] and a higher antigen dose compared to P.M. immunization with non-replicating omvPV. Weaker cytokine responses upon an infection might be explained by the immune evasion strategies of *B. pertussis*[46], such as dampening of host

cytokine responses[47]. Extensive comparison of early pulmonary transcriptomic profiles induced by P.M. vaccination obtained from this study and those induced by *B. pertussis* infection[7] demonstrated that although both responses included genes involved in acute phase, PRR-signaling, cytokine responses, humoral response and T cell responses, these responses comprised distinct genes. Moreover, P.M. immunization leads to faster and more intense pulmonary gene expression, which is in line with the cytokine profiles. Together, the comparison of omvPV-induced immunity through P.M. administration and infection-induced immunity revealed overall comparability, notwithstanding some distinct innate responses.

Another advantage of pulmonary immunization, besides the induction of mucosal immune responses resembling that induced by *B. pertussis* infection, is that P.M. vaccine administration is needle-free. Currently, no pulmonary vaccines are licensed and only a few mucosal vaccines have been approved for human use. These include nasal vaccine against influenza virus and oral vaccines against poliovirus, rotavirus, *Salmonella typhi*, and *Vibrio cholerae*[48]. Pulmonary vaccine administration in humans is in early clinical development. Both powder inhalers[49] and fluid, nebulized vaccines are clinically tested, the latter in infants of 9 months of age[50]. Nasal vaccine administration might also be an option and may be suitable for infants. Intranasal immunization with an omvPV is also feasible since it provides protection against *B. pertussis* infection[13]. In addition, nasal immunization has been shown to result in IgA antibodies not only in the nasal cavity, but also lower in the respiratory tract [48, 51]. However, in depth analysis of the immune response induced by nasal immunization route in the context of pertussis is required.

In conclusion, this study showed that an omvPV can elicit superior local and systemic immunity against *B. pertussis* infection, with a similar type of adaptive immune response as induced by infection, by administering the vaccine pulmonary instead of subcutaneously. Therefore, pulmonary immunization may be key to the development of novel candidate pertussis vaccines. Further, in addition to serology, the gold standard in pertussis research, a systems biology approach is invaluable in order to unravel and understand the immunological signatures underlying the vaccine-induced immunity. In turn, this facilitates a rational choice for a vaccine candidate and route of administration, mitigating the risk of failure in the late-stage of development.

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Supplementary data

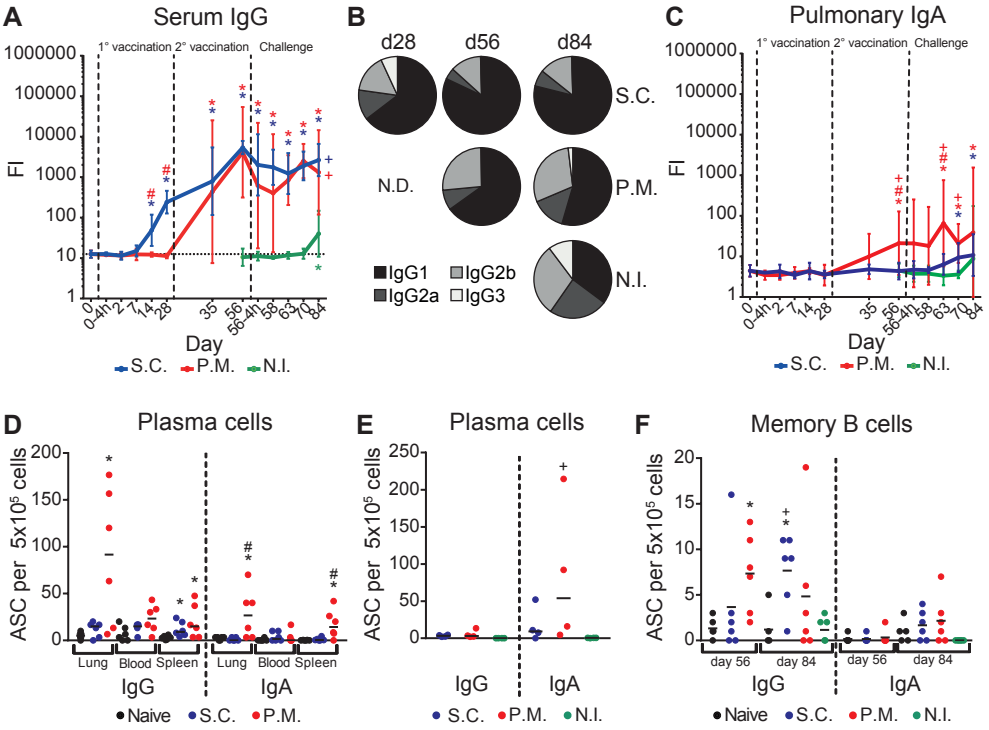

Figure S1 - Prn-specific B cell responses induced by P.M. and S.C. omvPV immunization. (A-C) Serum anti-Prn IgG antibodies (A), IgG subclass distribution (B), and pulmonary anti-Prn IgA antibodies (C) were determined in immunized mice using MIA. Results are expressed as fluorescence intensities (FI) of 4 mice per group per time point. *p<0.05 versus naive mice (day 0); #p<0.05 versus S.C. mice; +p<0.05 versus N.I. mice. (D-E) Numbers of Prn-specific IgG- and IgA-secreting plasma cells (n=6) in (D) lungs, blood, and spleens on day 35 and in (E) spleens (n=4) on day 63. (F) Numbers of Prn-specific IgG- and IgA-producing memory cells were determined in spleens (n=6) on day 84. *p<0.05 versus naive mice; #p<0.05 versus S.C. mice; +p<0.05 versus N.I. mice. (N.D = not detected).

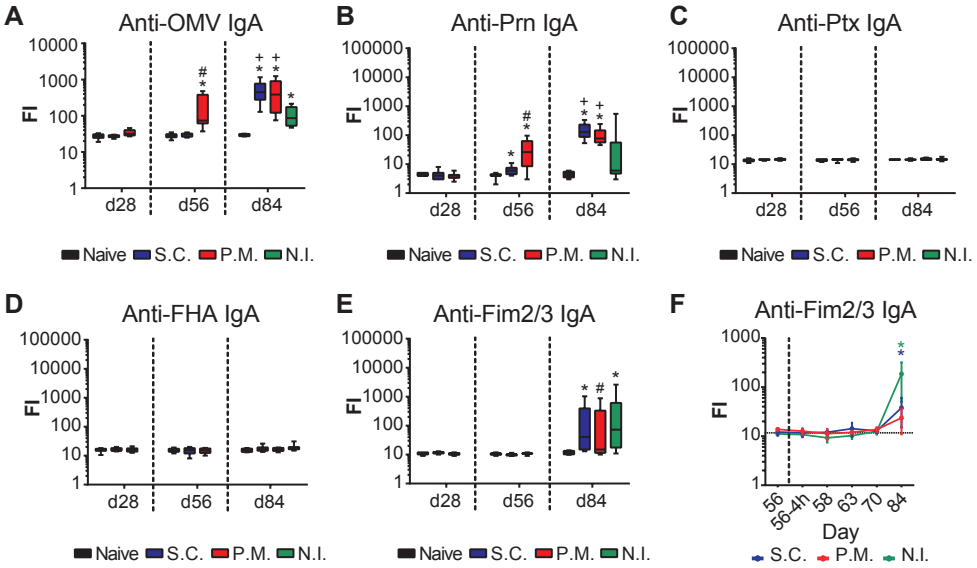

Figure S2. Serum IgA levels against OMV, Prn, Ptx, FHA and Fim2/3 and pulmonary anti-Fim2/3 IgA induced by P.M. and S.C. omvPV immunization. (A-E) The levels of IgA antibodies directed against OMV (A), Prn (B), Ptx (C), FHA (D), and Fim2/3 (E) were determined in serum on day 28 and day 56 (vaccine-induced) and on day 84 (infection-induced) in naive, S.C., P.M., and N.I. mice. *p<0.05 versus naive mice; #p<0.05 versus S.C. mice; +p<0.05 versus N.I. mice. (F) The levels of pulmonary anti-Fim2/3 IgA following challenge were determined in S.C., P.M. and N.I. mice. Results are expressed as fluorescence intensities (FI) of 4 mice per group per time point. *p<0.05 versus levels day 56.

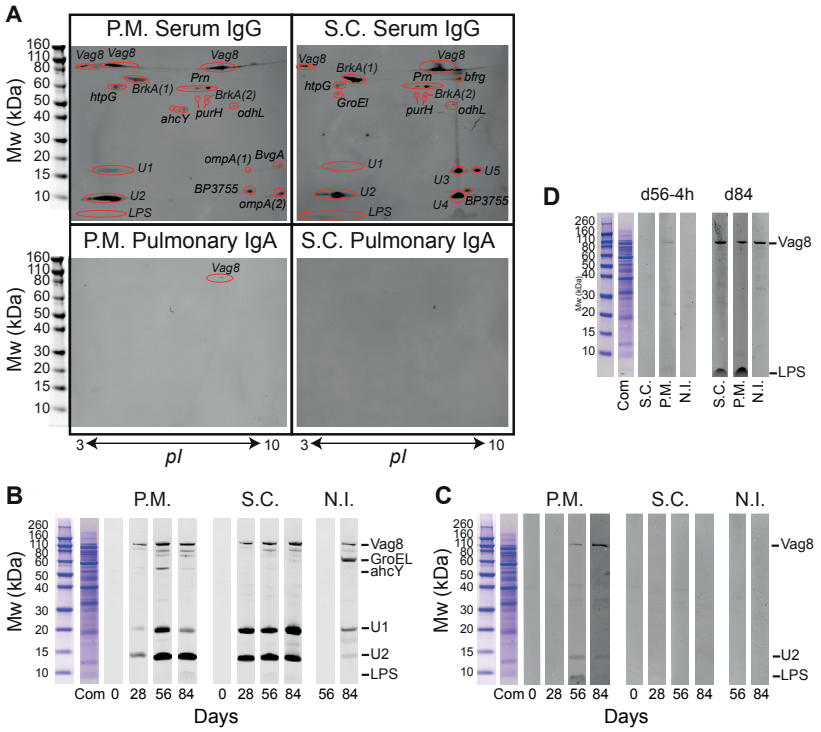

Figure S3 - Specificity of antibodies induced by P.M. and S.C. omvPV immunization. (A) Specificity of IgG and IgA in respectively serum and lung lysates of S.C. and P.M. mice on day 56 was determined using 2DEWB. Figure srepresents one blot for each condition. Individual spots with corresponding protein ID, as determined by LC-MS, are depicted. (B-D) Antibody specificity as determined by 1DEWB for serum IgG (B) and pulmonary IgA (C) on day 28, 56, and 84 and nasal IgA (D) 4 hours and 28 days (day 84) after challenge. Identification of bands representing GroEL, LPS, Vag8, ahcY, and two unidentified antigens (U1-2) are shown. As a control a Coomassie staining (Com) was performed on the 1DE gel to stain the *B. pertussis* proteins.

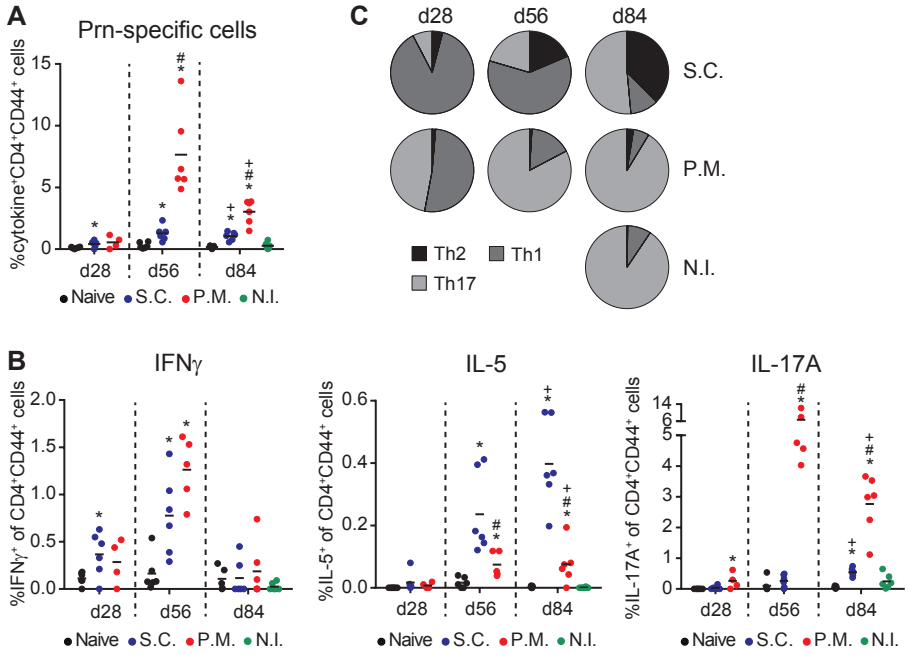

Figure S4 - Pulmonary and systemic Prn-specific T cell responses induced by P.M. and S.C. omvPV immunization. (A) Magnitude of the Prn-specific CD4+ T cell response on day 28, 56, and 84 after *in vitro* stimulation with Prn for 4 days was determined using ICS and calculated as the total percentage cytokine (IL-5, IFNγ, and IL-17A)-producing CD4+CD44+ T cells. (B) Percentage IL-5-, IFNγ-, and IL-17-producing cells of CD4+CD44+ T cells of spleens harvested on day 38 and day 56 and stimulated *in vitro* for 4 days with Prn. Results of each analysis are given of 6 mice per group. (C) Distribution of Th subsets based on IL-5 (Th2), IFNγ (Th1), and IL-17A (Th17) production as determined using ICS and flow cytometry. *p<0.05 versus naive mice; #p<0.05 versus S.C. mice; +p<0.05 versus S.C. mice.

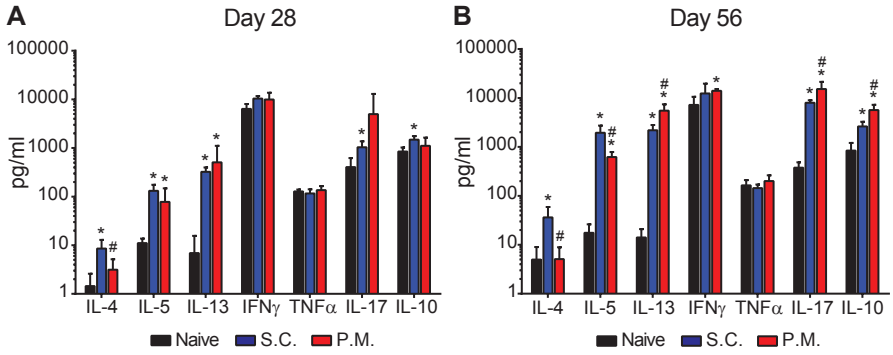

Figure S5 - Cytokine levels in supernatants of OMV stimulated splenocytes. (A-B) The presence of cytokines in day 3 culture supernatants of splenocytes collected on day 28 (A) and day 56 (B) was tested using a multiplex assay. Results in pg/ml are corrected for the background level (IMDM complete medium control) and are given as mean ± SD of 6 mice per group. *p<0.05 versus naive mice; #p<0.05 versus S.C. mice.

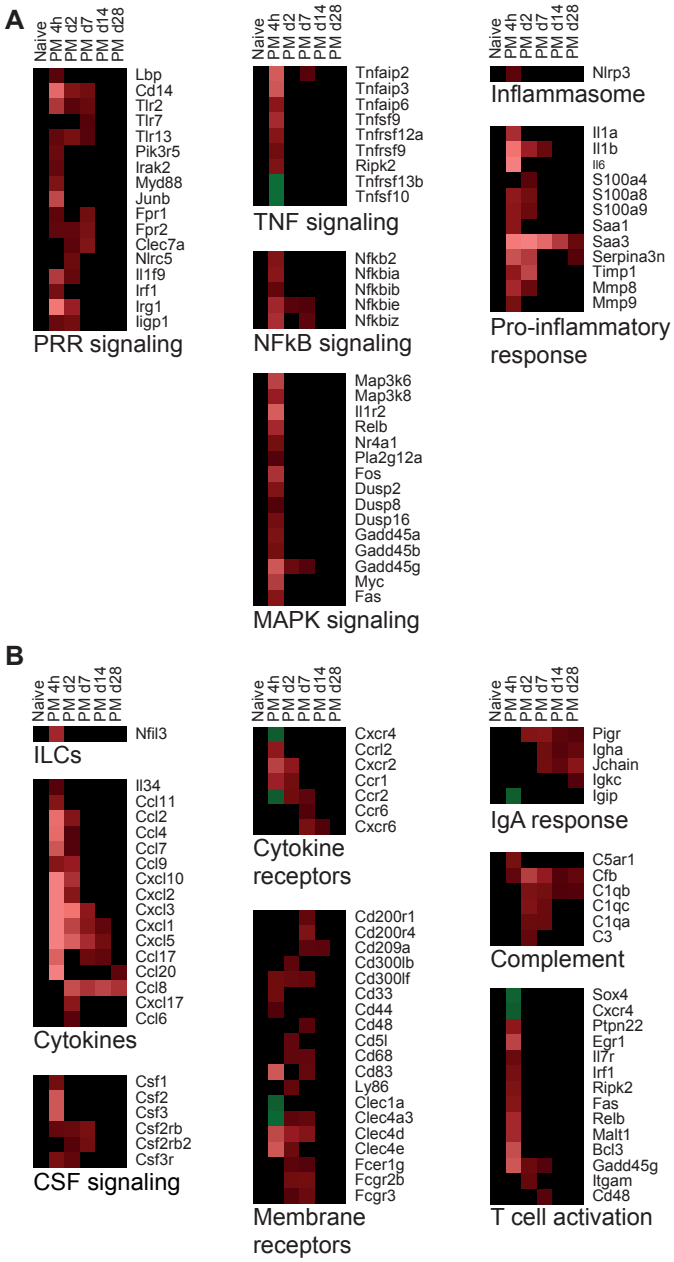

Figure S6 - Pulmonary transcriptome following P.M. immunization. (A-B) Differential upregulated (red) or downregulated (green) gene expression of P.M. mice (mean of n=3) compared to naive mice (day 0) (FR≥2.0, p-value≤0.01). Genes not surpassing a fold change of 2.0 are shown as basal level (black). (A) Genes are depicted involved in pathogen recognition, such as the inflammasome and the pathogen-recognition receptor (PRR), TNF, NFκB, and MAPK signaling pathways and pro-inflammatory responses. (B) Genes are depicted involved in ILCs, CSF signaling, T cell activation, humoral response, and complement in addition to genes encoding cytokines, cytokine receptors, and membrane markers.

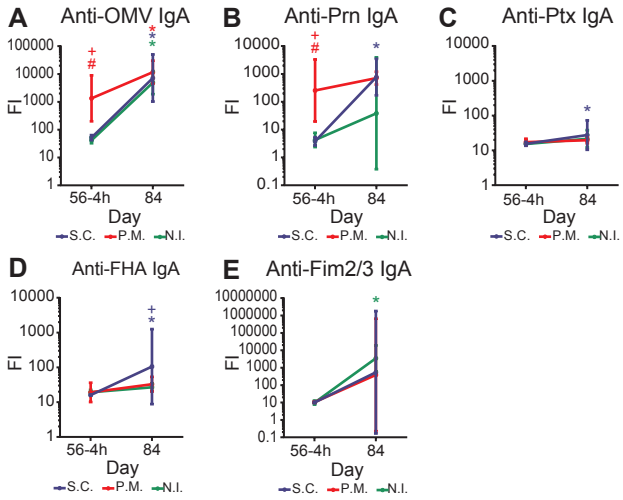

Figure S7 - IgA responses in the nose lavages of P.M., S.C., and N.I. mice. IgA antibodies specific for OMV (A), Prn (B), Ptx (C), FHA (D), and Fim2/3 (E) were determined in the nose lavage of P.M., S.C., and I.N. mice 4 hours after *B. pertussis* challenge (d56-4h) and at day 84. Results are expressed as fluorescence intensities (FI) of 4 mice per group per time point. *p<0.05 versus levels on d56-4h; #p<0.05 versus S.C. mice; +p<0.05 versus N.I. mice.

Table S1 - Immunogenic proteins determined by LC/MS of excised spots found by immunoblotting of 2DE gels

Protein description[a]	Accession number[b]	ID[b]	Mw (kDA)[c]	pI[c]	Coverage (%)[d]	Number of peptides assigned with high confidence[d]
60kD chaparonine	Q7W0M8	*GroEl*	57.4	5.2	69	33
adenosylhomocysteinase	Q7VUL8	*ahcY*	51.6	6.1	53	20
autotransporter	Q79GN7	*vag8*	94.8	6.8	41	27
bifunctional purine biosynthesis protein	Q7VTU1	*purH*	55.8	6.3	36	16
BrkA autotransporter (1)	Q45340	*BrkA*	103.3	7.1	30	21
BrkA autotransporter (2)	Q45340	*BrkA*	103.3	7.1	18	14
chaperonine protein HtpG	Q7W0M8	*htpG*	71.1	5.2	65	39
dihidrolipoyl dehydrogenase	Q7VZ16	*odhL*	50.1	6.8	61	25
outer membrane protein A (1)	Q7VZG6	*ompA*	21.0	8.7	17	3
outer membrane protein A (2)	Q7VZG6	*ompA*	21.0	8.7	35	5
pertactin autotransporter	P14283	*prn*	93.4	9.2	28	16
probable tonB dependent receptor	P81549	*BfrD*	79.3	8.2	58	37
putative outer membrane protein	Q7VT02	*BP3755*	23.0	9.2	26	5
Virulence factors putative positive transcription regulator	P0A4H2	*bvga*	22.9	8.4	78	14

[a]Proteins in excised gel spots identified by LC–MS analysis. [b]Accession numbers of proteins and corresponding ID provided by uniprot (http://www.uniprot.org). [c]Molecular weight (Mw) and isoelectric point (pI) are calculated based on protein sequence. [d]Percentage of a protein sequence covered by identified peptides from the corresponding protein and the number of peptides assigned with high confidence.

Chapter 8

Summarizing Discussion

The introduction of whole-cell pertussis (wP) vaccines in the 1950s led to a massive decrease in pertussis incidence and mortality. However, since a few decades, resurgence of pertussis is observed in highly vaccinated populations[1-3]. Moreover, there is a shift in incidence towards older age groups, including adolescents and adults[4]. These older age groups form a reservoir for transmission of *Bordetella pertussis*, the causative agent of pertussis, to vulnerable non- or partially-immunized infants. Several explanations have been proposed to underlie the resurgence of pertussis, including the suboptimal efficacy of the currently used acellular pertussis (aP) vaccines. In this thesis, several alternative approaches for current pertussis vaccination were applied and compared using innovative and systems based technologies, with the main focus to understand the molecular basis for the different outcomes in the $CD4^+$ T cell response.

B. pertussis infection-induced immune responses

Infection with *B. pertussis* provides the most durable protection against subsequent infection[5]. **Chapter 2** comprises an overview of the literature describing the immune responses found after *B. pertussis* infection in humans and animal models. In **Chapter 3**, a study is described in which we elucidated in detail the molecular and cellular events upon *B. pertussis* infection in the different phases of the immune response in mice using a systems biology approach. For this purpose, responses were analyzed at the cellular, proteome, and transcriptome level in systemic and local tissues and fluids, e.g. spleen, lung (lysates), and serum. The insight gained in the *B. pertussis* infection-induced immune responses may serve as a benchmark for pertussis vaccination research. The study in **Chapter 3** revealed expression of genes involved in different innate responses, such as antimicrobial peptides, acute phase, and chemokines, in the lung shortly after infection and were maintained, somewhat unexpectedly, until 28 days after infection, when the bacteria were already cleared from the lungs. The expression of chemokine encoding genes revealed the recruitment of neutrophils, macrophages, and DCs, which is consistent with the findings of other studies[6, 7].

After the rapid responses, antigen presentation was observed, as indicated by the expression of genes involved in MHC class I and class II presentation (**Chapter 3**). This stresses the involvement of respectively $CD8^+$ and $CD4^+$ T cells in the immune response upon *B. pertussis* infection. The involvement of $CD8^+$ T cells in the immune response against *B. pertussis* was disregarded for a long time, since formalin-fixed *B. pertussis* immunized $CD8^+$ T cell-deficient mice did not show decreased protection against *B. pertussis* challenge compared to their immunized WT counterparts[8]. In contrast, influx of $CD8^+$ T cells, although to a lesser extent than observed for $CD4^+$ T cells, was detected based on flowcytometry in the lungs of *B. pertussis*-infected mice[9]. This is consistent with our findings based on gene expression profiles in the lung upon infection. Interrogation of the $CD4^+$ T cell response induced by *B. pertussis* infection revealed the presence of Th1 and Th17 cells in the lung at day 14 based on expression of genes encoding transcription factors, cytokines and chemokine

receptors, namely *Stat1*, *Ifng*, and *Cxcr3* for Th1 cells and *Il17a*, *Il17f*, and *Ccr6* for Th17 cells (**Chapter 3**). The induction of Th1 and Th17 cells was further confirmed systemically in the memory phase at the single cell level by using intracellular cytokine staining of splenocytes. The induction of Th1 and Th17 cells by *B. pertussis* was also found in other studies in mice, baboon, and human[10-13], and was shown essential in providing protection against infection[10, 11].

The induction of a humoral response was reflected by the expression of genes involved in B cell receptor signaling and genes encoding immunoglobulins, observed in the lung 7 days after *B. pertussis* infection (**Chapter 3**). In contrast, IgA antibodies in the lung and IgG antibodies in serum were only detected at day 28. The detection of IgG antibodies only after the bacteria are almost cleared is also found by others[14], and might suggest a redundant role of B cells during primary infection.

Sensing of *B. pertussis* by innate immune cells and immune evasion

The innate immune response is the first line of defense and is important in the activation and steering of the adaptive immune response. Involvement of different immune cells, namely neutrophils, macrophages, and dendritic cells, was found in **Chapter 3**. Innate immune cells are able to sense pathogens via pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs) and C-type lectin receptors. These receptors recognize pathogen-associated molecular patterns (PAMPs) expressed by pathogens. The most well known PAMPS expressed by *B. pertussis* are lipprotein BP1516, ligand for TLR2[15], and lipooligosaccharide (LOS), ligand for TLR4[16].

Adaptation of *B. pertussis*, possibly accelerated due to vaccination pressure, has been observed that could interfere with the recognition of the bacterium by innate cells[17-19]. In **Chapter 4,** we interrogated the capacity of different strains, representing distinct families of *B. pertussis* identified over time, to activate TLR2 and TLR4 signaling. All nineteen tested strains induced TLR2 signaling, whereas three of them failed to activate TLR4. Mass spectrometric analysis revealed that these strains had altered structures in their lipid A moiety, which is the part of the LOS that is recognized by TLR4. These alterations were a shorter C3' acyl chain (10 or 12 carbons instead of 14 carbons long) and the lack of glucosamine modification. One of the strains, B1121, was already described in literature, as strain 18-323, to have these alterations in its lipid A moiety [20]. Shah *et al*. identified that the difference in length of the C3' acyl chain in this strain was due to a mutation in the *LpxlA* gene[20]. In our study, this mutation was also identified in the other two strains with altered lipid A moieties. In addition, Shah *et al*. found that the lack of glucosamine modification of the lipid A moiety of the 18-323 strain was due to a mutation in the lipid A GlcN modification B (*lgmB*) gene and the absence of *lgmC* and *lgmD* genes. Interestingly, in our study this mutation and the absence of the *lgmC* and *lgmD* genes was not observed in the other two strains with altered LOS moieties. The underlying cause of the lack of

glucosamine modification in these strains requires further investigation. As a consequence of the absence of TLR4 signaling, the strains with the altered lipid A moieties also failed to fully mature monocyte-derived dendritic cells. Remarkably, these strains induced a decreased production of cytokines known to induce Th1 and Th17 differentiation, respectively IL-12p70[21] and IL-6 and IL-23[22, 23], compared to the other TLR4 signaling strains. Circulating strains that possess low TLR4 signaling capacities might have a survival advantage over the strains that do activate the host immune system properly. Both **Chapter 3** and **Chapter 4** indicate that PAMPs, and more specific TLR4 ligands, are important in the activation of the innate immune response and subsequent steering of the $CD4^+$ T cell response and should be taken into account when improving vaccine-induced immunity.

Shortcomings of aP vaccines and considerations for improving pertussis vaccines

The current aP vaccines have some shortcomings that might lead to the decreased long-term effectiveness compared to wP vaccines[24-27] and the lack of protection against *B. pertussis* colonization and transmission, as found in the baboon model[28]. These shortcomings and potential immunology-guided solutions were reviewed in detail in **Chapter 2**. One shortcoming is the induction of a distinct functional programming of $CD4^+$ T cell response by aP vaccines compared to infection. The aP vaccines induce a Th2-dominated response[10, 29, 30], while the protection against *B. pertussis* infection require Th1 and Th17 responses[10, 11]. These Th subsets are induced by *B. pertussis* infection (**Chapter 3**)[10-12, 31] and wP vaccines[11, 30, 31]. The presence of Th1 and Th17 skewing PAMPs, like the TLR4 ligand LOS, in the bacterium and wP vaccines could explain the differentiation of *B. pertussis*-specific $CD4^+$ T cells towards these Th subsets. aP vaccines do not contain PAMPs, although it has been described that Ptx could induce TLR4 signaling[32, 33]. This signaling, however, is not sufficient to skew towards a Th1 response, most likely due to the aldehyde treatment of the Ptx preparations for the aP vaccines. Including PAMPs in the pertussis vaccines could improve the functional programming of the vaccine-induced $CD4^+$ T cell response.

Second, the aP vaccines induce a narrow immune response since these vaccines consist of only one to five *B. pertussis* antigens in a high dose. Variations or even deletion of the aP vaccine antigens in circulating strains have been observed[17-19, 34-37], most likely due to vaccination pressure, and these strains could in this way partially escape the aP vaccine-induced immune responses. A broad antigen composition of new vaccines could reduce vaccination pressure and possibly prevent evasion of the vaccine-induce immune response by the bacterium.

Third, the aP vaccines induce end-stage differentiation of the $CD4^+$ T cells[38]. We hypothesize that this is due to the high antigen dose in the aP vaccines (**Chapter 2**). Several studies show that high antigen and adjuvant dose may induce vigorous short-term immunity, yet can lead to exhaustion and impede long-term immunity[39-42]. This was shown in a *Leishmania*

model, where high dose vaccination of mice elicited high numbers of total IFNγ-producing CD4+ T cells yet resulted in poor protective immunity[39]. In contrast, although low dose vaccination led to lower numbers of IFNγ-producing CD4+ T cells, it did induce protective immunity. To prevent end-stage differentiation of the CD4+ T cell response and rather induce long-term immunity, new vaccines should carefully consider the doses of the antigens.

Fourth, the current intramuscular route of immunization with the aP vaccines most likely does not result in local immune responses in the respiratory tract. Emerging evidence from other respiratory diseases indicate the role of lung tissue-resident memory T cells in long-term protection[43, 44], yet their role in the immunity against *B. pertussis* remains unclear. Interestingly, in the study described in **Chapter 3**, we found that even 28 days after infection, when all bacteria are cleared from the lung, the gene expression in the lungs was not back to basal level. A follow-up study showed that even 56 days after *B. pertussis* infection 320 genes were still differentially expressed [Raeven *et al.* manuscript in preparation]. These genes included innate and adaptive response-associated genes. Due to the absence of viable bacteria at this time point, this differential expression is most likely not due to continuous stimulation. We hypothesize that this prolonged differential expression reflects the presence of *B. pertussis*-specific tissue-resident B and T cells in the lungs. The differential expression of innate response-associated genes could suggest long-term functional programming of innate immune cells, which status is defined as trained immunity by Netea *et al.*[45].

In view of these insights, new vaccine approaches should tackle known shortcomings, trying to mimic the response found after *B. pertussis* infection as much as possible, including the induction of local immunity at the site of entry of the bacterium. In this thesis, we evaluated some approaches that aim to improve the outcome of pertussis vaccination by addressing one or more of the known shortcomings. The effect of addition of a TLR4 ligand, namely a LPS derivative, on the aP vaccine-induced CD4+ T cell response was assessed in **Chapter 5** and **Chapter 6**. We described in **Chapter 7** a study investigating a more complex vaccine candidate, namely a *B. pertussis*-derived outer-membrane vesicles (OMV) vaccine. This vaccine contains a broad antigen composition in a 'natural' low dose and contains multiple PAMPs that are known to promote Th1 and Th17 responses. In addition, we investigated whether pulmonary administration of this vaccine will lead to the induction of a pulmonary immune response.

Improvement of the functional programming of aP vaccine-induced CD4+ T cells by TLR4 ligation

In **Chapter 5** and **Chapter 6**, we investigated whether the functional programming of the CD4+ T cells induced by the aP vaccine could be improved by addition of a TLR4 ligand. For this purpose, a well-defined detoxified derivative from *Neisseria meningitidis* LPS, named LpxL1, was used. This penta-acylated LPS lacks a 2' secondary C_{12} acyl chain, making it less

reactogenic, yet it retains its Th1 promoting capacity in mice [46]. In **Chapter 5**, we determined which Th subsets were induced by the aP vaccine in the presence or absence of TLR4 ligation by investigating aP vaccine antigen-specific cytokine production using intracellular cytokine staining and supernatant analysis of splenic cell cultures. A decrease in the Th2 component and an increase in the Th1 and Th17 components were observed in the presence of the TLR4 agonist.

The molecular signatures underlying this shift in Th response were further determined in the study described in **Chapter 6**. For this purpose, splenocytes of aP- or aP+LpxL1-vaccinated mice were stimulated for a short period with the aP vaccine antigens, Ptx, FHA, and Prn, after which a microarray analysis on the RNA of isolated $CD4^+$ T cells was performed. This method was previously applied in a study of White *et al*. describing gene expression profiles of aP vaccination-induced $CD4^+$ T cells in children[47]. In our study, we found that the expression of Th2-associated genes in $CD4^+$ T cells is not downregulated after addition of LpxL1 to the aP vaccine, despite a reduced Th2 responsiveness seen at the cytokine level (**Chapter 6**). Rather an increase in expression of Th1- and Th17-associated genes caused not only the increase in Th1 and Th17 cytokine components, but also the observed and desired decrease in Th2 cytokine components. In literature, Th subset-associated proteins are described to inhibit expression of genes associated with other Th subsets[48], which seems contradictory with our findings. Single cell transcriptome analysis, as described by Chattopadhyay *et al.* might provide additional insight in whether the in principle antagonistic gene modules are expressed in the same cell[49]. Another mechanism underlying the observed shift in Th subsets on the cytokine protein level, even though there is co-expression of Th2- with Th1- and Th17-associated genes, could be reduced translation of Th2 cytokine mRNA due to Th1- and Th17-associated miRNA translational repression. Such mechanism was shown for Th1-specific miR-135b[50] repressing Th2-associated genes *Stat6* and *Gata3* mRNA translation to protein[51]. However, the exact role of miRNA in the programming of the $CD4^+$ T cell response after aP vaccination in the presence or absence of a TLR4 agonist needs to be investigated.

The studies described in **Chapter 5** and **Chapter 6** show that addition of a TLR4 ligand can improve the functional programming of aP-vaccine induced $CD4^+$ T cells (Figure 1). The same effect has been described for TLR2 and TLR9 agonist, since replacement of alum with these agonists led to increased numbers of Th1 and Th17 cells[10, 52].

Effect of TLR4 ligation on magnitude and phenotype of aP vaccine-induced $CD4^+$ T cell response

The effect of the presence or absence of TLR4 ligation on the magnitude and memory potential of the aP vaccine antigen-specific $CD4^+$ T cell response was also investigated in **Chapter 5**. A risk of addition of a pro-inflammatory adjuvant is that only a strong short-term effector response will be induced instead of a long-term memory response. Memory T cells

can be divided into different subsets with their own memory potential, in mice based on the expression of CD44 and CD62L. Central memory T cells (T_{CM}, $CD44^+CD62L^+$) have a higher memory potential than effector memory T cells ($T_{EM,}$ $CD44^+CD62L^-$) and are preferred for long-term immunity[53-56]. The memory potential of the $CD4^+$ T cells is influenced by the amount of inflammatory signals (Figure 2 of **Chapter 2**)[40, 41]. Using a tetramer specific for the immunodominant Prn epitope, Prn_{10-24}[57], we found indeed increased numbers of specific $CD4^+$ T cells when the TLR4 ligand was added to the aP vaccine (**Chapter 5)**. However, when investigating the memory phenotype of these cells, no altered ratio between T_{CM} and T_{EM} cells was observed. Yet, due to the increase in the total number of specific cells, there was a net increase in numbers of T_{CM}. This indicates that addition of a TLR4 ligand to the aP vaccine did not have a negative effect on the longevity of the aP vaccination-induced $CD4^+$ T cells. Investigation of the memory phenotype of the specific $CD4^+$ T cell response provides insight in the induction of long-term protection against *B. pertussis* infection.

In literature, the effect of vaccination on protection is usually assessed by a challenge. In the study described in **Chapter 5**, we also performed an intranasal *B. pertussis* challenge in aP-vaccinated mice, however, the bacteria were already cleared from the lungs of all mice, including in those that were vaccinated with 1/100 human dose of the aP vaccine alone, at the time the colonization assay was performed, i.e. 7 days after challenge. Investigation of the lung colonization at multiple time points after challenge, as was used in **Chapter 3** and **Chapter 7**, might have provided more information about the level of protection induced by aP vaccination with or without TLR4 ligation. However, Geurtsen *et al.* showed that addition of TLR4 ligand monophosphory lipid A derived from *Salmonella minnesota* to an aP vaccine indeed led to increased protection compared to the aP vaccine alone against *B. pertussis* challenge in mice[58]. Together with our findings, this indicates that addition of a TLR4 ligand to the aP vaccine improves the functional programming of aP-vaccine induced $CD4^+$ T cells and protective immunity.

Infection-resembling immunity induced by *B. pertussis* OMVs, a third generation pertussis vaccine

Addition of an adjuvant to or replacing the current adjuvant in the current aP vaccines helps to overcome the induction of suboptimal Th programming by the aP vaccine, without negatively influencing the memory potential of these cells. However, the other shortcomings of the aP vaccines, the narrow response and the lack of induction of mucosal immune responses, are not addressed by this approach. Development of third generation pertussis vaccines might overcome these other shortcomings. In **Chapter 7**, using a comprehensive systems biology approach, we evaluated in depth a third generation pertussis vaccine, namely *B. pertussis*-derived OMVs, in its capacity to induce a protective immune response against *B. pertussis*. Moreover, we compared the induced immune responses and level of protection after pulmonary and subcutaneous administration of the vaccine. Protection against intranasal *B. pertussis* challenge was found after immunization of mice with the

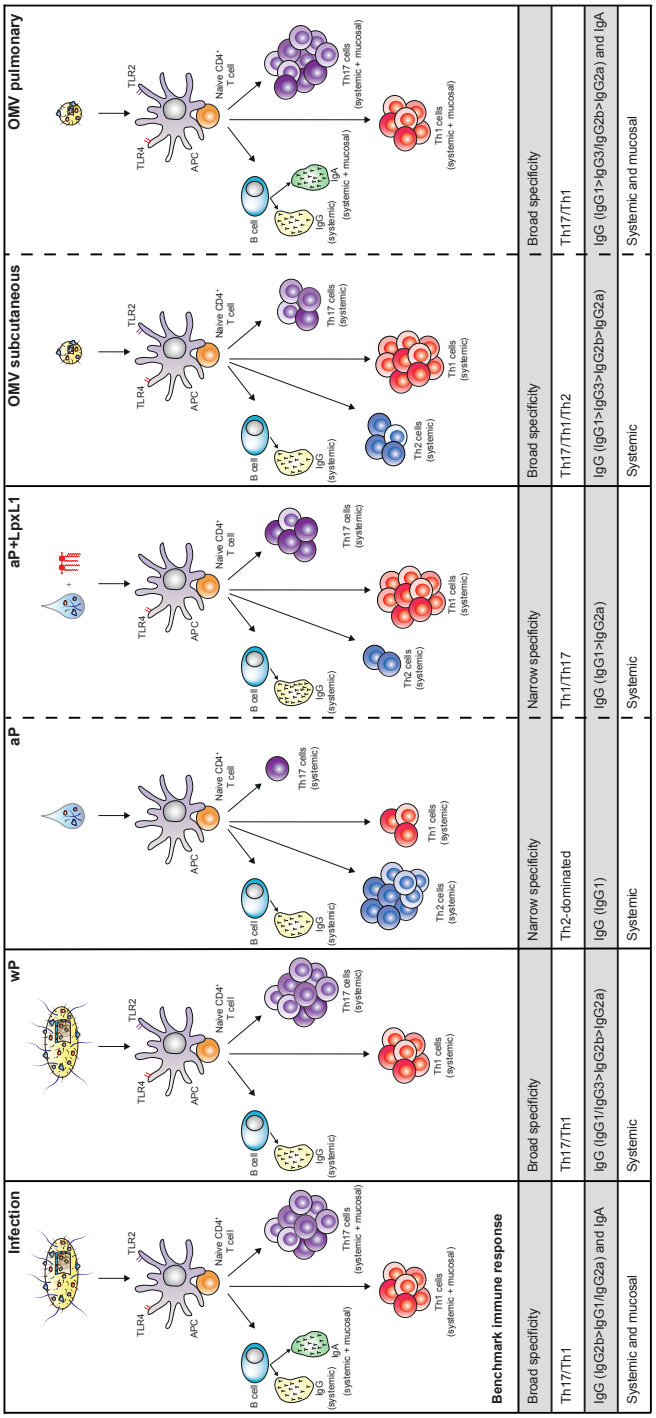
Infection
wP
aP
aP+LpxL1
OMV subcutaneous
OMV pulmonary
TLR2
TLR4
APC
Naive CD4+ T cell
B cell
IgG (systemic)
IgA (systemic + mucosal)
Th1 cells (systemic + mucosal)
Th17 cells (systemic + mucosal)
Th1 cells (systemic)
Th2 cells (systemic)
Th17 cells (systemic)
Benchmark immune response
Broad specificity
Th17/Th1
IgG (IgG2b>IgG1/IgG2a) and IgA
Systemic and mucosal
Narrow specificity
Th2-dominated
IgG (IgG1)
Systemic
Narrow specificity
Th1/Th17
IgG (IgG1>IgG2a)
Systemic
Broad specificity
Th17/Th1/Th2
IgG (IgG1>IgG3/IgG2b>IgG2a)
Systemic
Broad specificity
Th17/Th1
IgG (IgG1>IgG3/IgG2b>IgG2a) and IgA
Systemic and mucosal

Figure 1 - Adaptive immune response induced by *B. pertussis* infection or vaccination with wP, aP, aP+LpxL1, OMV (subcutaneous), or OMV (pulmonary) vaccines. The adaptive immune response was determined after *B. pertussis* infection in **Chapter 3** and that induced by wP vaccination was described in another study [Reaven *et al.* manuscript in preparation] and in literature [10, 28, 29]. The aP- and aP+LpxL1-induced adaptive responses were elucidated in **Chapter 5**. The adaptive responses by pulmonary and subcutaneous immunization with a OMV vaccine was investigated in **Chapter 7**. The charateristics (breadth of the specificity, type of $CD4^+$ T cell response, type of Ig response, location of the response) of the responses were summarized in the table. A dotted line between responses indicates that the responses can be compared directly, since they were investigated in the same studies.

OMVs via both routes. This is in line with the findings in the study of Roberts *et al.* showing that intraperitoneal and intranasal immunization with *B. pertussis*-derived OMVs induced protection against *B. pertussis* challenge. A direct comparison between the routes, however, was not possible in their study, since different formulations of the vaccine per route were used[59]. Remarkably, our study revealed that pulmonary compared to subcutaneous immunization led to faster clearance of *B. pertussis* from the lungs upon challenge (**Chapter 7**). Interestingly, only pulmonary immunization of mice with the OMV vaccine led to local immune responses in the lungs, characterized by the presence of IgA, IgA-producing plasma cells, and tissue-resident Th17 cells. In addition, the study in **Chapter 7** revealed that the administration of the OMV vaccine via different routes led to distinct systemic responses. The $CD4^+$ T cell response after subcutaneous immunization had a mixed Th1/Th17/Th2 profile, whereas pulmonary immunization induced a mixed Th1/Th17 response resembling the response induced by *B. pertussis* infection. Induction of a distinct systemic T cell response by immunization via different routes has also been shown for influenza, *Salmonella typhimurium*, and *Mycobacterium tuberculosis*, mainly based on IgG subclass levels and cytokines in splenic culture supernatants[60-62]. In **Chapter 7**, we showed that OMVs as a third generation vaccine, can tackle multiple shortcomings of the current aP vaccines. Moreover, administration of this vaccine in the respiratory tract induces, in addition to a systemic response, a mucosal response in the lungs, mimicking the *B. pertussis* infection-induced responses (Figure 1).

Future perspectives

The resurgence of pertussis and the fact that the incidence of pertussis is higher than of any other vaccine-preventable infectious disease shows the need for improvement of pertussis vaccination. Therefore, short- and long-term solutions need to be evaluated. In this thesis the *B. pertussis* infection-induced immune responses were unraveled to provide a benchmark for future pertussis vaccine research. In addition, we found that there are naturally occurring *B. pertussis* isolates that have altered LOS molecules, leading to the lack of TLR4 signaling and failure of fully maturating dendritic cells. This indicates that especially TLR4 signaling is important in the induction of the correct differentiation environment for $CD4^+$ T cell differentiation. Further, we evaluated the immune responses induced by new vaccine concepts (Figure 1).

Since no new pertussis vaccine is yet available, alternative short-term solutions for the pertussis problem include maternal immunization, additional booster vaccination of adolescents and adults, and cocooning strategies. Maternal immunization will protect infants, too young to be vaccinated, against infection by passive transfer of anti-*B. pertussis* antibodies. This approach was found to be very effective in several countries, such as UK and Belgium[63, 64]. However, there is evidence of some interference of the maternal antibodies with infant vaccine responses[64, 65], yet the effect of this interference needs to be investigated. The additional booster vaccination of adolescents and adults with current aP vaccines will tackle the problem of waning immunity and reduce spread of *B. pertussis*. However, additional boosters might only postpone the pertussis problem, since the other shortcomings of aP vaccines are not tackled. Moreover, repeated exposure to high doses of the antigens might even lead to end-differentiation of the immune response, which could lead to faster waning immunity. Cocooning strategies, including vaccination of individuals that have close contacts with infants, such as parents, will minimize transmission of the bacteria to these infants. Data on effectiveness of this approach is limited and data in baboons suggest that aP vaccines do not protect against transmission of the bacterium[28].

A rationally designed third generation pertussis vaccines would appear to be an ideal long-term solution to improve pertussis vaccination. In this thesis we examined the $CD4^+$ T cell response after an alternative formulated aP vaccine, which included a TLR4 ligand. An advantage of this approach would be that such a vaccine would be more quickly ready for market application than a new pertussis vaccine. However, this adjusted aP vaccine still contains a high dose of a small amount of antigens. This might still promote end-differentiation of the $CD4^+$ T cells and put pressure on the bacterium to evade the vaccine-induced immune responses.

Increasing the number of antigens in the current aP vaccines would broaden the vaccine-induced immune response and might reduce vaccination pressure and possible strain adaptation. Several *B. pertussis* antigens were investigated for this purpose, such as adenylate cyclase toxin (ACT)[66], Bordetella resistance killing antigen (BrkA)[67], and iron

regulated proteins, IRP1-3 and AfuA[68]. These antigens are immunogenic and addition to an aP vaccine enhanced the vaccination-induced protection.

A more complex pertussis vaccine would be required in the long-term. Such vaccine should contain a broad set of antigens and include PAMPs that could steer the *B. pertussis*-specific CD4$^+$ T cells towards a Th1 and Th17 response. One complex vaccine concept is the live-attenuated BPZE1 vaccine, which is designed for intranasal delivery. The BPZE1 strain is attenuated by genetic alterations of Ptx, tracheal cytotoxin, and dermonecrotic toxin, leading to absence or detoxification of the toxins, yet the strain retained immunogenic properties[69]. Immunization of mice with BPZE1 indeed induces Th1 and Th17 CD4$^+$ T cells, high systemic IgG levels, dominated by the IgG2a subclass, and IgG-secreting cells[70, 71]. Moreover, BPZE1 has been shown in a phase I clinical trial to induce B cell responses[72]. These studies demonstrate that intranasal BPZE1 immunization evokes an adaptive immune response comparable to that induced by *B. pertussis* infection. A disadvantage of this vaccine concept is that it contains live bacteria, which could make the acceptance by the public difficult.

Another complex vaccine concept is a vaccine based on *B. pertussis*-derived OMVs, which was evaluated in this thesis (**Chapter 7**). Administration of this vaccine pulmonary in mice, the CD4$^+$ T cell response resembled that induced by *B. pertussis* infection, both systemically as locally in the lungs. This suggests that this type of vaccine in combination with mucosal delivery could induce long-lasting immunity. Another advantage of OMVs is that the bacteria from which they are derived can be genetically engineered to overexpress [Sloots *et al.* manuscript in preparation] or lack certain antigens. Moreover, the LOS of these strains can be detoxified to reduce reactogenicity[73].

In conclusion, for designing new pertussis vaccines, it is important to include Th1 and Th17 skewing PAMPs or adjuvants and a broad antigen composition with an optimized dose. However, careful evaluation of the choice of antigen, adjuvant, and their dose is required to ensure that the new vaccine programs induce the appropriate functional programming and memory potential of the specific CD4$^+$ T cells for providing sustained protective immunity against infection. Moreover, this thesis showed that administration of a vaccine in the respiratory tract can improve the vaccine-induced CD4$^+$ T cell programming and enhance protection against *B. pertussis* infection, yet there is a long road ahead in the development of these types of vaccines.

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Appendices

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Nederlandse Samenvatting

Kinkhoest

Kinkhoest is een ernstige en zeer besmettelijke infectieziekte van de bovenste luchtwegen. De ziekte wordt veroorzaakt door de bacterie *Bordetella pertussis*. Het ziekteverloop van kinkhoest wordt gekenmerkt door drie fases. Tijdens de eerste fase vertoont de patiënt milde aspecifieke symptomen zoals hoesten, niezen en een loopneus. De tweede fase wordt gekarakteriseerd door hevige en langdurige hoestbuien, waarna de patiënt met gierende ademhaling lucht schept en een “whoop” geluid te horen is. Tijdens fase drie nemen de symptomen geleidelijk in frequentie en hevigheid af. De ziekte verloopt het meest ernstig bij jonge kinderen die (nog) niet of onvolledig zijn gevaccineerd. Naast kinderen kunnen ook (jong) volwassenen geïnfecteerd worden, bij deze leeftijdsgroepen verloopt de ziekte vaak milder of zelfs zonder symptomen.

Kinkhoestvaccinatie

In de jaren ’50 werd kinkhoestvaccinatie grootschalig ingevoerd wat leidde tot de reductie van de kinkhoestincidentie. De eerste kinkhoestvaccins waren de zogenaamde cellulaire vaccins, die bestonden uit hele gedode kinkhoestbacteriën. Ondanks de effectieve werking van deze cellulaire vaccins, gaven deze relatief veel bijwerkingen. Dit kan veelal worden toegeschreven aan de zogenaamd reactogene eigenschappen van het aanwezige lipooligosaccharide (LOS) molecuul. Daarom werden tweede generatie vaccins, de acellulaire vaccins, ontwikkeld. Deze bestaan uit enkele gezuiverde eiwitten van de kinkhoestbacterie. Vanaf 2005 wordt er in Nederland gevaccineerd met deze acellulaire vaccins.

Kinkhoest epidemiologie

Vaccinatie tegen kinkhoest heeft het aantal kinkhoestgevallen drastisch verlaagd. Desondanks is er sinds 1996 weer een stijging te zien van het aantal kinkhoestgevallen, met elke 2 tot 3 jaar een epidemie. Kinkhoest blijft de meest gemelde bacteriële infectieziekte in Nederland met de laatste jaren 3.000 tot 14.000 gerapporteerde ziektegevallen per jaar. Daarnaast is er ook een verschuiving waargenomen in de leeftijd van de personen die kinkhoest krijgen. Vóór het kinkhoestvaccinatie tijdperk kregen vooral 4-5 jarige kinderen kinkhoest. Dit is verschoven naar oudere personen, inclusief (jong) volwassenen. Deze oudere personen zijn nu een besmettingsbron voor (nog) niet of onvolledig gevaccineerde kinderen. De toename in kinkhoestgevallen is niet uniek voor Nederland, maar wordt ook gezien in andere Europese landen, de Verenigde Staten, Canada en Japan. Verschillende oorzaken voor de aanhoudende toename van het aantal kinkhoestgevallen zijn beschreven in de wetenschappelijke literatuur. Onder andere veranderingen van de kinkhoestbacterie waardoor deze niet meer of minder snel herkend wordt door de immuunrespons (afweerreactie van het immuunsysteem) die is opgewekt door de vaccinatie. Ook de duur van de bescherming na vaccinatie speelt hoogstwaarschijnlijk een rol, deze is namelijk niet levenslang. Dit kan komen door het opwekken van een suboptimale immuunrespons door de vaccins.

Immuunsysteem

Het immuunsysteem van zoogdieren beschermt tegen pathogenen (ziekteverwekkers) en is grofweg in te delen in twee onderdelen, de niet-specifieke aangeboren (innate) afweer en de specifieke aangeleerde (adaptieve) afweer. De cellen van het innate immuunsysteem zijn in staat om een pathogeen snel te herkennen en te helpen verwijderen. Indien een innate immuunrespons niet afdoende is om de pathogeen snel te verwijderen, zal het adaptieve immuunsysteem in werking treden. De cellen van het adaptieve immuunsysteem hebben het vermogen om pathogenen of lichaamscellen waarin deze pathogenen zich schuilhouden heel specifiek te herkennen en onschadelijk te maken. Hiervoor heeft het adaptieve immuunsysteem tijd nodig, omdat de cellen die specifiek zijn voor het pathogeen zich eerst klonaal moeten vermeerderen tot celpopulaties van enige omvang. Een andere belangrijke eigenschap van het adaptieve immuunsysteem is het opbouwen van een zogenaamd immunologisch geheugen tegen pathogenen die al eens zijn herkend. Hierdoor zal een herhaalde infectie met hetzelfde pathogeen leiden tot een snellere specifieke immuunrespons. Deze eigenschap van het immuunsysteem wordt gebruikt bij vaccinatie. Door middel van vaccinatie worden stukjes van een pathogeen aangeboden aan het immuunsysteem, waardoor een infectie wordt nagebootst en het adaptieve immuunsysteem een immunologisch geheugen opbouwt tegen het pathogeen.

Cellen van het adaptieve immuunsysteem worden geactiveerd door cellen van het innate immuunsysteem, de zogenaamde antigeen-presenterende cellen (APCs). De APCs herkennen de pathogenen met verschillende receptoren op hun celoppervlak. Deze receptoren herkennen bepaalde patronen van pathogenen, de zogenaamde pathogen-associated molecular patterns (PAMPs), waardoor de APCs onderscheid kunnen maken tussen verschillende pathogenen. De APCs nemen de pathogenen op en presenteren kleine stukjes van de pathogeeneiwitten op hun celoppervlak. Deze kleine stukjes eiwitten worden specifiek herkend door T cellen van het adaptieve immuunsysteem. Het type T cel dat voornamelijk belangrijk is voor de bescherming tegen kinkhoest is de CD4 T cel. Deze CD4 T cellen kunnen onder andere cellen van het innate immuunsysteem rekruteren om pathogenen op te ruimen en B cellen, een ander celtype van het adaptieve immuunsysteem, stimuleren om antistoffen te produceren. Een CD4 T cel herkent door zijn unieke klonale T cel receptor heel specifiek één bepaald klein stukje eiwit van het pathogeen dat door APCs wordt gepresenteerd. Deze eigenschap geldt ook voor alle dochtercellen van zo'n specifeke CD4 T cel. Omdat er veel verschillende stukjes eiwitten gepresenteerd worden door de APCs kunnen er meerdere klonale CD4 T cel responsen naast elkaar worden opgewekt. Het aantal verschillende reagerende CD4 T cel klonen, allemaal met een andere specificiteit, vormen een basis voor het succes van de afweer, hoe meer CD4 T cellen mee doen, hoe breder en effectiever de respons. Door de signalen die de APCs afgeven worden deze CD4 T cellen geïnstrueerd om bepaalde functies uit te oefenen, waardoor er verschillende CD4 T cel types ontstaan, zoals T helper 1 (Th1), T helper 2 (Th2) en T helper 17 (Th17) cellen. Th1 en Th17 cellen zijn belangrijk in de bescherming tegen kinkhoest. Deze laatste typen cellen worden opgewekt door cellulaire vaccins en ook door natuurlijke infectie met de bacterie, maar de acellulaire vaccins wekken meer Th2 cellen op. Het induceren van een

ander, minder effectief type CD4 T cel immuunrespons door acellulaire vaccins zou verder kunnen bijdragen aan de toename in kinkhoestmeldingen.

In dit proefschrift

Het voornaamste doel van het onderzoek beschreven in dit proefschrift was om meer inzicht te krijgen in de eigenschappen die de *B. pertussis*-specifieke immuunrespons moet hebben om langdurige en effectieve bescherming te bieden. Hierbij ligt de focus op de kwaliteit en de hoeveelheid van de specifieke CD4 T cellen, aangezien deze belangrijk zijn voor de langdurige bescherming tegen kinkhoestinfectie.

Het literatuuronderzoek dat beschreven is in **Hoofdstuk 2** geeft een overzicht over wat er bekend is over de immuunresponsen, die opgewekt worden door kinkhoestinfectie en vaccinatie met cellulaire en acellulaire vaccins. Er wordt in detail ingegaan op onderzoek in proefdiermodellen en in mensen waaruit blijkt dat de immuunrespons, en met name de CD4 T cel respons, na acellulaire vaccinatie inderdaad nogal afwijkt van de respons die wordt opgewekt door infectie. Daarnaast wordt beschreven op grond van welke eigenschappen de respons die wordt opgewekt door de cellulaire vaccins het meest vergelijkbaar is met een infectie-geïnduceerde respons.

De immuunrespons opgewekt door kinkhoestinfectie geeft tot nu toe de langst durende bescherming vergeleken met vaccinatie-geïnduceerde immuunresponsen. De immuunrespons na natuurlijke infectie zou daarom gebruikt kunnen worden als blauwdruk voor vaccin ontwikkeling. Om deze reden hebben we in het onderzoek dat wordt beschreven in **Hoofdstuk 3** de immuunrespons na *B. pertussis* infectie gedetailleerd in kaart gebracht. Hiervoor is gebruik gemaakt van een muismodel waardoor je de immuunrespons na kinkhoestinfectie op verschillende plaatsen en tijdstippen kunt bestuderen. Dit is in de mens niet mogelijk. Het onderzoek is gedaan met een vernieuwende aanpak, namelijk systems biology. Met deze aanpak wordt op verschillende niveaus, bijvoorbeeld op die van genexpressie, eiwitexpressie en cel populaties, gekeken naar de werking van de immuunrespons na bijvoorbeeld vaccinatie of infectie. Zo kon in dit hoofdstuk de immuunrespons na infectie in detail worden gekarakteriseerd, zowel lokaal in de longen als systemisch in het bloed of het lymfoïde orgaanstelsel. De geïnduceerde CD4 T cel respons werd gekenmerkt door de aanwezigheid van Th1 en Th17 cellen.

Om te bepalen waardoor uiteindelijk een Th1 en Th17 type CD4 T cel respons wordt opgewekt door infectie met *B. pertussis*, is het belangrijk om te weten hoe de eerste herkenning van de kinkhoestbacterie door humane APCs plaatsvindt. In **Hoofdstuk 4** wordt daarom de herkenning van negentien circulerende kinkhoestbacterie stammen, die zijn geïsoleerd uit verschillende kinkhoest patiënten, door APCs bestudeerd. Belangrijke receptoren op het celoppervlak van APCs in het herkennen van kinkhoestbacteriën bleken Toll-like receptor 2 (TLR2) en Toll-like receptor 4 (TLR4) te zijn. Opvallend was dat drie van de negentien stammen TLR4 niet activeerden. Deze drie bacteriestammen bleken structurele veranderingen te hebben in het molecuul dat bij de andere stammen zorgt voor

TLR4 activatie, namelijk het lipooligosaccharide (LOS). De verminderde activatie van de TLR4 leidde tot een verminderde respons van de APCs, die mogelijk geassocieerd is met een niet optimale sturing en activatie van de CD4 T cel respons. Uit deze studie blijkt dat TLR4 activatie een belangrijke rol speelt in aansturing van de adaptieve immuunrespons tegen kinkhoestbacteriën.

De acellulaire kinkhoestvaccins zijn niet optimaal in het opwekken van de benodigde Th1 en Th17 type CD4 T cel respons, maar induceren vooral een niet gewenste Th2 respons. Dit zou kunnen komen doordat deze vaccins geen PAMPs zoals LOS bevatten die APCs instrueren om een Th1 en Th17 respons te induceren. Daarom is in **Hoofdstuk 5**, op eiwit niveau, en in **Hoofdstuk 6**, op genexpressie niveau, de CD4 T cel respons na het vaccineren van muizen met een acellulair vaccin vergeleken met de respons na het geven van een acellulair vaccin waar een TLR4 activerende maar niet-toxische variant van LOS aan is toegevoegd. De studie in **Hoofdstuk 5** liet op eiwit niveau zien dat wanneer TLR4 geactiveerd wordt door een LOS-gecomplementeerd acellulair vaccin, er minder Th2 cellen en meer Th1 en Th17 cellen worden opgewekt. Op genexpressie niveau was er echter geen verschil in de expressie van de Th2-gerelateerde genen, en was er alleen verhoogde expressie van Th1- en Th17-gerelateerde genen (**Hoofdstuk 6**). Dit geeft aan dat hier de verschuiving van een Th2 naar een Th1 en Th17 respons vooral verklaard wordt door de verhoogde expressie van de Th1- en Th17-gerelateerde genen en niet door een verlaagde expressie van Th2-gerelateerde genen. Het toevoegen van een extra PAMP aan een vaccin zou ervoor kunnen zorgen dat er alleen maar kort levende CD4 T cellen ontstaan. Echter uit het onderzoek beschreven in **Hoofdstuk 5** is gebleken dat het toevoegen van een niet-toxisch LOS aan het acellulaire vaccin niet leidt tot meer kort levende CD4 T cellen. De ratio tussen kort- en lang-levende CD4 T cellen in deze situatie was namelijk vergelijkbaar met de ratio na het vaccineren van muizen met alleen het acellulaire vaccin. Deze studies hebben laten zien dat het complementeren van het huidige acelluaire vaccin met een 'veilig' LOS leidt tot een verandering in het type CD4 T cel respons, die meer gaat lijken op de respons die wordt geïnduceerd door infectie.

Naast het evalueren van een aangepaste vorm van het huidige acellulair vaccin, is er ook een nieuw vaccin concept geëvalueerd. In het onderzoek in **Hoofdstuk 7** is eveneens de systems biology aanpak toegepast om een gedetailleerd overzicht te geven van de beschermende immuunrespons die kan worden opgewekt door een zogenaamd outer-membrane vesicle (OMV) vaccin in muizen. Dit OMV vaccin bevat een groot aantal kinkhoesteiwitten, waardoor er een immuunrespons wordt opgewekt die een brede specificiteit heeft. Het vaccin bevat ook LOS, waardoor TLR4 op APCs wordt geactiveerd en deze cellen de juiste adaptieve immuunrespons kunnen induceren. Het gebruikte muismodel werd eerst geoptimaliseerd om de beschermende werking van dit type vaccin tegen een intranasale challenge met levende kinkhoestbacteriën aan te kunnen tonen. Daarnaast werd het OMV vaccin via twee verschillende routes toegediend om te bepalen wat het effect van de route op de gedetailleerde immuunrespons is. Uit dit onderzoek bleek dat toediening van dit vaccin direct in de longen, in tegenstelling tot toediening via de subcutane (onderhuidse)

route, een immuunrespons opwekt die in een aantal facetten veel lijkt op die na *B. pertussis* infectie. Het induceerde namelijk een lokale en systemische immuunrespons en de CD4 T cel respons werd gekenmerkt door Th1 en Th17 cellen. Dit systems biology onderzoek geeft voor het eerst een diepgaande immunologische blauwdruk van een vernieuwend kinkhoestvaccin dat via twee routes is aangeboden.

Samenvattend, het onderzoek in dit proefschrift heeft geleid tot de ontwikkeling en optimalisatie van verschillende muismodellen en onderzoekstechnieken waarmee verschillen tussen effectieve en minder effectieve *B. pertussis*-specifieke immuunresponsen gedetailleerd in kaart kunnen worden gebracht. Het onderzoek kan bijdragen aan het vinden van een blauwdruk voor de meest optimale immuunrespons voor de bescherming tegen kinkhoest. De fundamentele kennis die verkregen is in dit onderzoek kan daarom bijdragen aan de ontwikkeling van nieuwe kinkhoestvaccins en vaccinatiestrategieën. Daarnaast is deze kennis belangrijk ter ondersteuning van de overheid in haar beleid rondom de preventie van kinkhoest door middel van vaccinatie.

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Curriculum Vitae

Jolanda Brummelman was born in Delft on the 12th of October in 1987. After graduating from the RSG Lingecollege (Atheneum) in Tiel in 2006, she started her study Biomedical Sciences at Utrecht University. After obtaining her Bachelor's degree in 2009, she continued her study Biomedical Sciences with the Master's program Biology of Disease. During her first internship at the department of Nephrology and Hypertension of the University Medical Center Utrecht, she investigated the role of endothelial cell-derived and endothelial progenitor cell-derived exosomes in vascular damage repair. She performed her second internship at the department of Vaccine Research of the Netherlands Vaccine Institute, which later became part of the National Institute for Public Health and the Environment (RIVM). During this internship she investigated the immunomodulatory effects of pertactin derived from *Bordetella pertussis* on dendritic cell function and T cell polarization in vitro. In 2011, she obtained her Master's degree. In the same year, she started her PhD project at the National Institute for Public Health and the Environment (RIVM) under supervision of dr. Cécile van Els, dr. Wanda Han, and prof. dr. Willem van Eden (department of Infectious Diseases and Immunology, Utrecht University), which resulted in this thesis. Temporarily, she will continue working at the same department on T cell responses after pertussis infection and vaccination as a post-doc.

List of Publications

Brummelman J, Raeven RHM, Helm K, Pennings JLA, Metz B, van Eden W, van Els CACM, Han WGH. *Transcriptome signature for dampened Th2 dominance in acellular pertussis vaccine-induced CD4$^+$ T cell responses through TLR4 ligation*.[Submitted for publication]

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Brummelman J, Wilk MM, Han WG, van Els CACM, Mills KM. *Roads to the development of improved pertussis vaccines paved by immunology*. Pathogens and Disease 2015, 73(8):ftv067.

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