Pertussis specific T-cell immunity in Dutch children:

Differences after whole-cell versus acellular vaccination

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Pertussis specific T-cell immunity in Dutch children:
Differences after whole-cell versus acellular vaccination

Kinkhoest specifieke T-cel immuniteit in Nederlandse kinderen: verschillen tussen cellulaire en acellulaire vaccinatie

Proefschrift

(met een samenvatting in het Nederlands)

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 25 februari 2014 te 12.45

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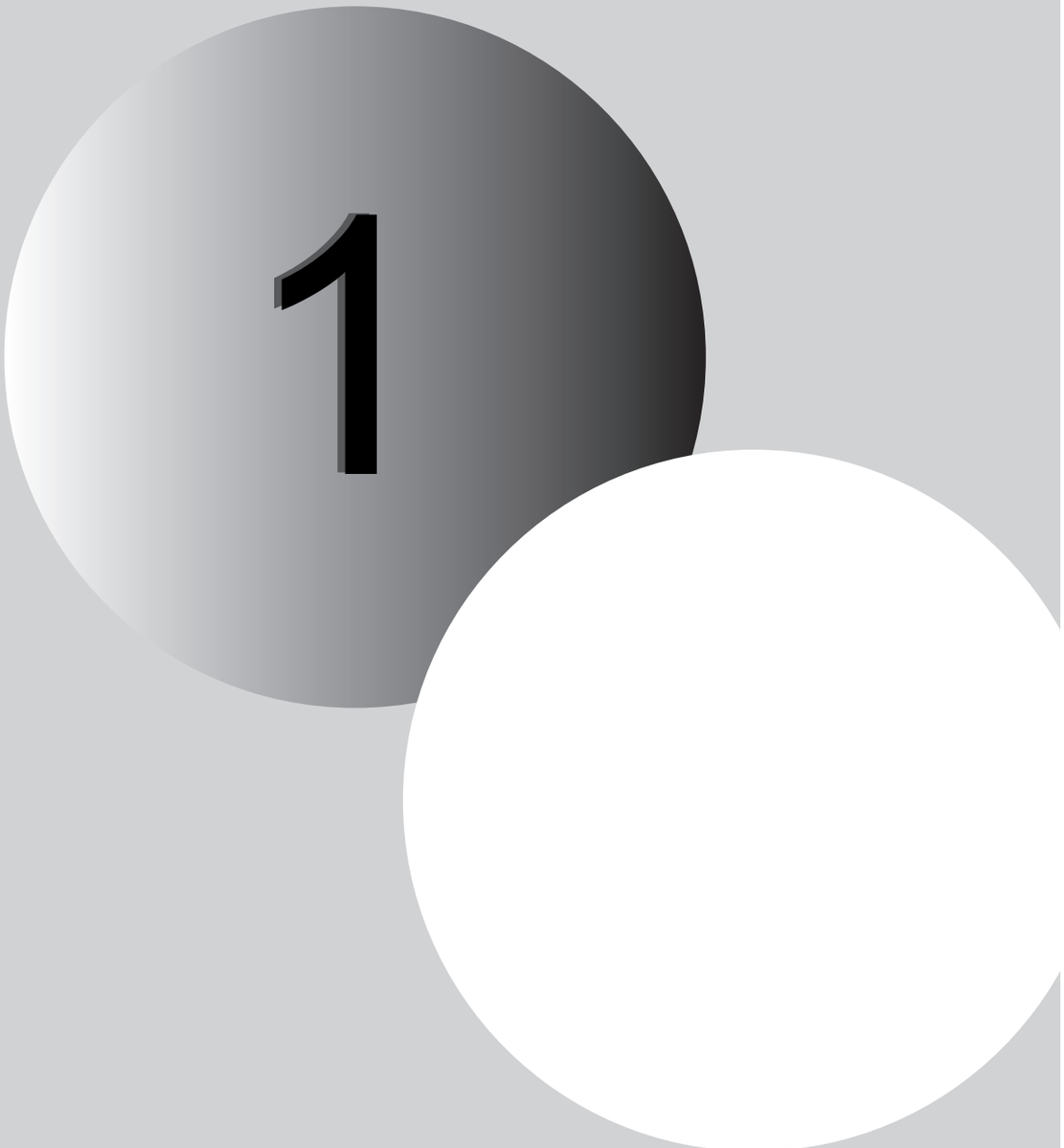

Whooping cough

Bordetella pertussis, the causative agent of whooping cough is a coccus-shaped Gram-negative bacterium. It is a strictly human pathogen [1]. Whooping cough is a highly contagious infection, which is characterized by three stages of disease of which the most important hallmark is paroxysmal coughing with whooping and post-tussive vomiting. Typical symptomatic disease starts after 7-20 days of incubation time. The first catarrhal stage of infection starts with mild respiratory symptoms, like a runny nose, mild coughing and sneezing. This will last for 1-2 weeks after which it progresses to the paroxysmal stage. This stage has the typical whooping cough as hallmark, in which severe spells of coughing end in a distinct 'whooping' high-pitched sound, when a person gasps to breathe again. It can be accompanied by post-tussive vomiting, apnoea and gagging [2]. The paroxysmal stage can last for several months to transit into the convalescent stage. In these last weeks of the disease, the coughing gradually decreases in frequency and severity and the vomiting subsides [1].

Complications frequently associated with classical pertussis include pneumonia, otitis media, seizures, encephalopathy, (brain) haemorrhages and fainting [2-4]. However, it should be noted that *B. pertussis* infections, particularly in hosts with partial immunity to the bacterium, will usually follow a subclinical or milder course [5]. Particularly, infants under 6 months who have not been fully vaccinated are at risk for serious complications [2, 6, 7]. Most of these infants get infected via transmission by siblings (41%), their mother (38%) or their father (17%) [6, 8].

Pertussis epidemiology and vaccination

Worldwide, pertussis currently affects about 48.5 million children yearly, resulting in about 300.000 deaths [9]. More than 90 % of these deaths occur among infants under 6 months of age [2]. Case fatality rates usually are about 4 percent. In the first half of the 20th century, before vaccination was introduced in the Netherlands, annually 150.000 got ill and several hundreds of people died due to pertussis [10]. From 1950 onwards the pertussis vaccine became available in the Netherlands. Initially, pertussis vaccination was successful in protecting young children against whooping cough [11], and between 1946 and 1954 the incidence decreased from 9 to 0,2 per 100.000 persons while the number of deaths dropped from 846 to 25 [10]. A mass vaccination campaign in 1957 against polio with the DTP-IPV vaccine was the start of the National Immunization Programme (NIP) and vaccination was provided against diphtheria, tetanus, whooping cough and polio. In the 1960s and 1970s, the number of deaths due to pertussis decreased even further to less than 5 per year [12].

Since 1996 the incidence of pertussis started to increase again in the Netherlands. Since then every 2-3 years epidemic cycles are seen in the Netherlands (Figure 1). This resurgence of pertussis, however, is worldwide including most European countries as well as the USA, Canada, Japan and Australia [13-17]. Annually, the pertussis incidence peaks in the third quarter of the year in the Netherlands [15], as in most European countries and the USA [17-20]. This resurgence of pertussis might be due to increased awareness and reporting, improved diagnosis, suboptimal vaccines, antigenic divergence and waning immunity [7]. Waning immunity and strain variation are considered the most important causes of this resurgence.

From the 1950s onwards the whole cell pertussis vaccine (wP) was used for vaccination at 3, 4, 5 and 11 months of age in the Netherlands and vaccination coverage for infants has been more than 96% for decades [7]. After the pertussis resurgence in 1996, changes in the national vaccination schedule have been implemented. First, the potency of the wP vaccine has been elevated from 4 to 7 IU/HD in 1997. In 1999 the primary schedule for newborns at 3, 4, 5 months of age was accelerated to 2, 3 and 4 months of age. From 2001 onwards an additional acellular booster vaccination was implemented at 4 years of age. In 2005 the whole cell vaccine was replaced by acellular vaccines in the first year of life. The main reason for this change was that aP vaccines showed less adverse effects [22]. The current Dutch pertussis vaccination schedule consists of four primary doses of the combined DTaP-IPV/Hib/HepB vaccine administered at 2, 3, 4 and 11 months of age and an additional preschool booster dose of acellular vaccine (aP) at the age of 4 years.

After implementation of an aP preschool booster vaccination in November 2001, the peak incidence of whooping cough in children shifted from 4-5 to 9-10 years of age in 2008 [23] and to 11-12 years of age in 2011. In the winter-spring of 2011-2012 a new pertussis outbreak occurred with a particularly high incidence, that also affected children from 8-9 years of age up, who all had been vaccinated with wP vaccine in the primary schedule and with an aP booster at 4 years [24]. In the same 2011-2012 epidemic in the USA, a whole cell vaccine as priming dose may prove to be more protective for 12-14 year old children compared with acellular priming [25].

Moreover, over time the pertussis incidence also has increased in adolescents and adults, who had been vaccinated only in their first year of life with whole cell pertussis vaccine [26]. In recent years 3,000-10,000 pertussis cases were notified in the Netherlands annually, from which about 200 were hospitalized, concerning mainly infants less than 6 months old. On average, 1 infant died due to pertussis every 10 years [8]. In the outbreak of 2011-2012 notifications in the Netherlands drastically increased to over 1,000 per month – about 4 times more than recorded in the same months of the preceding year with an increase in infants and hospitalizations for pertussis [21]. This pertussis rise in the Netherlands is not unique.

Late in 2009, an upsurge of pertussis was observed in California, resulting in 10 infant deaths in 2010 [27, 28]. Notably, the pertussis incidence during this outbreak was highest among 8-11 year old children, who had received the aP-vaccine already five times during childhood suggesting that waning efficacy of the aP vaccine played an important role in both allowing and sustaining the pertussis outbreak [29]. To illustrate the severity of the last epidemic, nearly 2,300 cases of pertussis were reported in the USA in the first half of 2012 -13 times more than recorded during the same time in 2011 [30]. In the UK, a 10-fold rise in notifications was observed in 2012 compared to 2008 [31, 32] and 10 infant deaths are reported so far [33], while in the Netherlands 3 infants died in this last 2011-2012 epidemic [34]. Recently, the emergence of *B. pertussis* isolates not expressing a vaccine antigen like Prn or PT, has been noticed in a number of countries, where the acellular vaccines have been implemented [35-38]. However, up till now the biological relevance of these vaccine-antigen-deficient mutants on pertussis epidemiology and disease is not clarified.

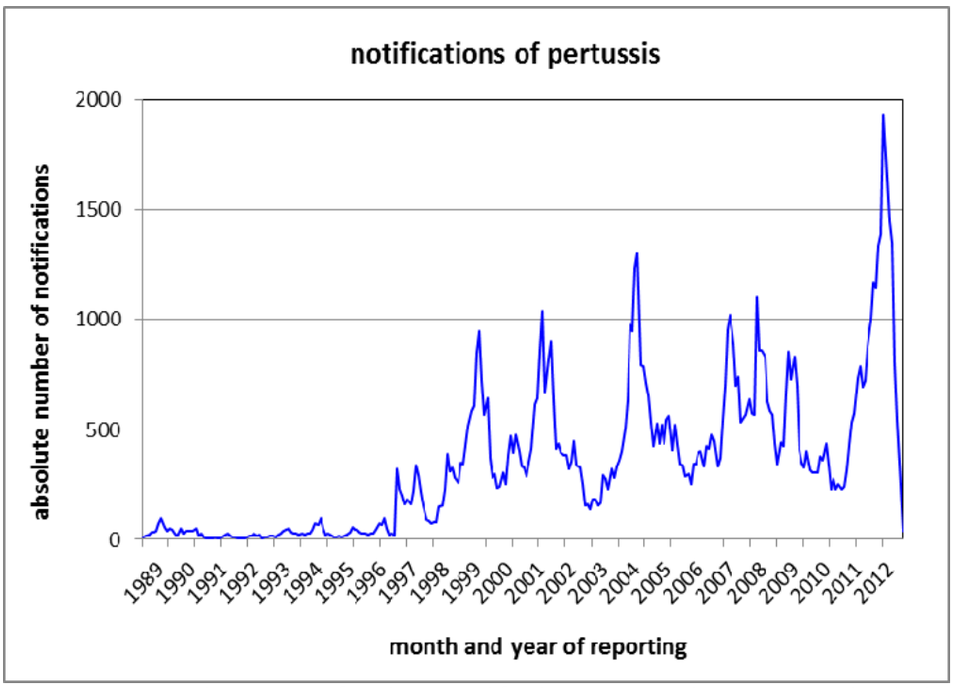

Fig. 1. The absolute number of pertussis notifications per month and year in the Netherlands between 1989 and 2012. Since 1996 every 2-3 years epidemic cycles are present [21].

Vaccines

The Dutch whole cell vaccine of 1997 consisted of 7 IU/HD of killed bacteria in contrast to the different acellular vaccines, which consist of relatively high amounts of 1 to 5 highly purified pertussis proteins. These *B. pertussis* proteins have immunomodulating functions. Filamentous heamagluttinin (FHA), pertactin (Prn) and fimbriae (Fim2/3) all are adherence factors, which facilitate the survival of the bacterium in the respiratory tract by entering the

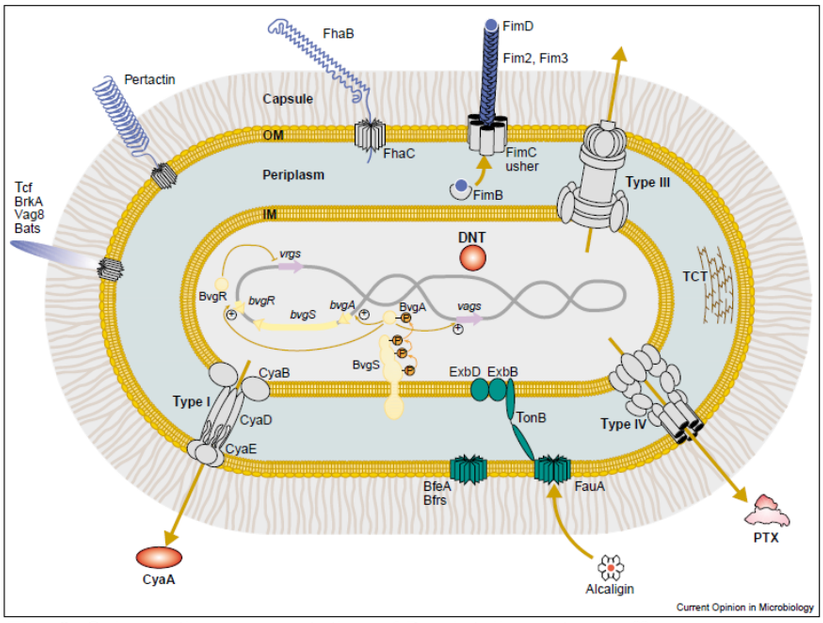

Fig. 2. Schematic representation of Bordetella pertussis [39].

epithelial cells (shown in Figure 2). After adherence the bacterium can produce several toxins. The most important toxin, pertussis toxin (PT), is excreted by the bacterium during illness and causes most of the clinical symptoms.

The available acellular vaccines consist of 1 to 5 purified pertussis antigens: PT, FHA, Prn and Fim2 and Fim3. In the Netherlands only acellular vaccines that contain 3 or more pertussis antigens are being used. Several switches between 3- and 5-component aP vaccines for infants as well as preschool booster vaccination have been made in the Netherlands since their implementation (shown in Table 1).

Differences in composition and antigen concentration of these aP vaccines may have consequences for the degree of acquired immunity and protection post vaccination. Little attention has been paid to these consequences so far. The vaccines, which have been used in the Netherlands, are shown in Table 1.

Table 1 Pertussis antigen compositions of the pertussis vaccines in the NIP.

Vaccine	Pertussis antigen concentration				Used in Dutch NIP
(manufacturer)	**PT**	**FHA**	**Prn**	**Fim 2/3**	
wP-IPV (RIVM)	0.16 µg	2.6 µg	n.d.	n.d.	at 2, 3, 4 and 11 months until 2005
Triaxis™ (GSK)	2.5 µg	5 µg	3 µg	5 µg	at 4 years of age: 2006-2008
Infanrix™ (GSK)	25 µg	25 µg	8 µg	-	at 2, 3, 4 and 11months and at 4 years
Pediacel™(SP)	20 µg	20 µg	3 µg	5 µg	at 2, 3, 4 and 11 months in 2006-2008

wP = whole cell pertussis, IPV = inactivated polio virus, GSK = GlaxoSmithKline, SP = Sanofi Pasteur, µg = microgram, n.d. = not determined.

Natural and vaccine induced immunity

B. pertussis can survive in the host both intra- and extracellularly. This dual location of pertussis is consistent with a role for both cellular and humoral immunity [40, 41]. The immune response to *B. pertussis* is rather complex and the bacterium has gained ways to intervene with certain aspects of host immune mechanisms [5].

The first line of defence in the battle against pertussis infection is regulated by the innate immunity, subsequently followed by several mechanisms of adaptive immunity [40]. The respiratory epithelium, together with resident antigen presenting cells including dendritic cells, sense and initiate local immune responses against the pertussis bacterium. However, the bacterium has evolved strategies to prevent detection by B- and T-cells of the adaptive immune system [5]. Later, when the disease progresses the adaptive immune system becomes involved.

B. pertussis induced humoral immunity

Optimal vaccines induce long-term immunity. Some live viral vaccinations induce antibody responses that persist over time with half-lives of 50 years or more [42]. The smallpox vaccine for example also showed a strong virus-specific T-cell immunity over time with no measurable decrease. In contrast, vaccine induced antibodies to non-replicating bacterial protein vaccine antigens, like tetanus and diphtheria toxoid, decrease at a relatively rapid pace in adults, with half-lives estimated to be 11 to 19 years, respectively [43]. The pertussis component of DTaP-IPV/Hib/HepB vaccine, however, seems to induce only limited long-term memory and protection is thought to last relatively short [44]. During the 2010 outbreak in California, aP-primed children attributed to the pertussis burden even at 8-11 years of age, only 4-6 years after the aP preschool booster vaccination. Clinically, cases of pertussis in this age group were only mild to moderate in severity [29, 45]. However, the rate of disease markedly increased from ages 8-12 years, proportionate to the interval since the last scheduled vaccination [46].

The humoral part of adaptive immunity was initially considered the main effector of protection against *B. pertussis*, though there is no evidence for a direct correlation between serum antibody levels and protection [47]. Indeed, several vaccine studies tried to correlate antibody levels and protection, but no genuine correlate of protection could be established [48-50]. Nevertheless, serum IgG levels specific for PT, FHA, Prn, and Fim 2/3 are supposed to play an important role in protection against pertussis [49, 51], though the role for FHA antibodies remains disputable [48, 49]. Antibodies to pertussis may operate in three ways: (1) by neutralizing bacterial toxins, (2) by inhibiting extracellular bacteria from binding to cells in the mucosal tract or (3) by enabling bacterial uptake and destruction by macrophages and neutrophils [40].

Since PT is specific for *B. pertussis*, IgG-PT antibodies are being used as a serologic marker for whooping cough [52]. Prn is also found in other *Bordetellae* species [53] and FHA in other bacteria like *Haemophilus influenzae*, *Mycoplasma pneumonia* and *Chlamydia pneumonia* [1]. Because PT is specific for *B. pertussis*, an IgG-PT antibody level above a certain cut-off value is assumed indicative for recent pertussis infection [26]. The used cut-off value differs per country mainly due to laboratory assay differences [54]. In the Netherlands we defined a level above 62.5 EU/ml to be indicative for a recent infection in the past year, while above 125 EU/ml is thought to be indicative for a current infection [26]. Additionally, an elevated IgA-PT level can supplement the IgG-PT criterion to ascertain active or recent infection [55]. A value above 20 EU/ml for IgG-PT has been used occasionally as a minimal protective level after vaccination [56].

The longevity of the diverse antibody responses upon the different vaccines and schedules has been studied in several countries. Overall, protective IgG levels after either wP or aP vaccinations seem to last only for a few years [5, 44, 57]. Infection acquired PT-IgG-levels reach a higher level and seem to remain longer compared to vaccine-induced PT-IgG-levels [58]. However, upon subclinical infection, PT-IgG-levels remain at lower levels and drop more rapidly [59, 60]. In general, higher IgG responses were observed in aP-primed children than in wP-primed children at 4 years of age [58, 61]. When an additional booster vaccination was given to wP-primed children at 9 years of age, both IgG-levels and their affinity increased [62]. However, regardless of vaccinations or infections, antibody levels wane rather rapidly over the years and cellular immunity is also required for long-lasting immunity to pertussis.

B. pertussis specific cellular immune responses

Protection against pertussis requires complementary roles for both the humoral and cellular part of the adaptive immune system [40]. Memory B-cell responses in children 4 years of age vary depending on infant priming with cellular or acellular vaccine and the administered acellular booster vaccination with different antigen concentrations. Before and after booster vaccination, higher memory B-cell responses were present in aP-primed children compared to wP-primed children, though the number of memory B-cells increased post booster vaccination in both wP- and aP-primed children. Interestingly, in aP-primed children a 5th aP vaccination with a high dose of antigens even tended to induce lower memory B-cell responses than a low-dose aP booster [63].

The number of pertussis protein-specific memory B-cells present in wP-primed children of 3, 4, 6 and 9 years of age and aP-primed children 4 and 6 years of age increased with age, despite waning circulating antibody levels. Memory B-cells build up over time maybe due to natural exposition that boosts this long-term memory pool, and this may contribute to protection against pertussis [63]. In wP-primed children 9 years of age, an extra booster vaccination further increased the memory B-cell levels. These responses had declined slightly 1 year post booster vaccination, but still substantially exceeded pre-booster levels and may further enhance immune protection after the pre-adolescent booster [62].

However, besides the role of B-cells, T-cell responses are also essential in immunity to pertussis, e.g. for the induction of high-affinity antibodies and memory [40, 64]. Only a limited number of studies have investigated the effect of pertussis on T-cell immunity, because most pertussis vaccine studies focussed on humoral immunity. With respect to T-cell immunity, protective immunity generated by wP vaccination appears to be mediated largely by Th1 cells, whereas aP vaccines induce stronger antibody responses and stronger Th2 and Th17 responses [65]. Infant priming with either wP or aP vaccination potentially modulates infant immune responses [66], skewing responses respectively more towards Th1- and Th2-mediated responses. This may explain partly why we found a more pronounced Th2-response at 4 years of age after acellular infant priming. Remarkably, high cellular responses upon the 5th acellular booster dose also are suggested to be associated with large local reactions such as extensive swelling of the whole limb in a number of children [67]. In general, about a fifth of the children aP vaccinated with a high doses of antigens at 4 years develops local adverse effects, but luckily severe local adverse effects are only present in a minor portion of these children [68]. Mice studies demonstrated that wP vaccinations confer immunity by more similar mechanisms as generated by natural infection, and suggest that aP and wP vaccinations confer protection by distinct combinations of immune effector mechanisms [40]. These findings were confirmed in humans, where they found that the wP vaccine induced a Th1-mediated response more similar as to infected persons and the aP vaccine resulted in a more Th2-mediated response [66] or mixed Th1/Th2 response [69].

Phenotypical and functional characterization of T-cells

There are several types of T-cells characterized by phenotypical and functional differences. The phenotype of induced T-cells is dependent upon the antigen load, costimulation and cytokine environment [69]. Both CD4+ and CD8+ cells probably play an important role in immunity against pertussis infection as well as responses upon vaccination [70, 71]. CCR7 and CD45RA are used to determine whether CD4+ and CD8+ cells are differentiated in an effector type (CCR7-CD45RA-), central memory type (CCR7+CD45RA-), naïve (CCR7+CD45RA+) or terminally differentiated state (CCR7-CD45RA+) [72]. The effector type can act rapidly by cytokine production (CD4+) or by cytotoxic (CD8+) cells, which can destroy infected or damaged cells. The memory subtypes can be activated upon secondary challenge e.g. by infection, and expand than rapidly [72]. After the invading pathogen has been cleared, typically more than 90% of the effector T lymphocytes die via apoptosis whereas a few effector cells express survival factors and become long-lived T effector-memory or T central-memory cells [73]. A memory immune response compared to a primary immune response has the ability to generate more rapidly and to generate larger numbers of antigen-specific T effector-memory CD4+ and CD8+ cells, while from the pool of specific T central-memory cells new effector cells can be derived to replenish the effector cells [73].

Apart from the phenotype, CD4+ effector cells or T-helper cells excrete cytokines to orchestrate and direct an immune response [73]. There are multiple CD4+ effector cells T-cell lineages e.g. the Th1, Th2, Tregulatory (Treg) and Th17 cells, which are functionally distinguished by the subset of cytokines they secrete (Figure 3). Th1 cells secrete IL-2, IL-12, IFN-γ and TNF-α. Th2 cells secrete IL-5 and IL-13 and Treg cells secrete IL-10, which can down-regulate Th1 cells. Th17 cells produce IL-17.

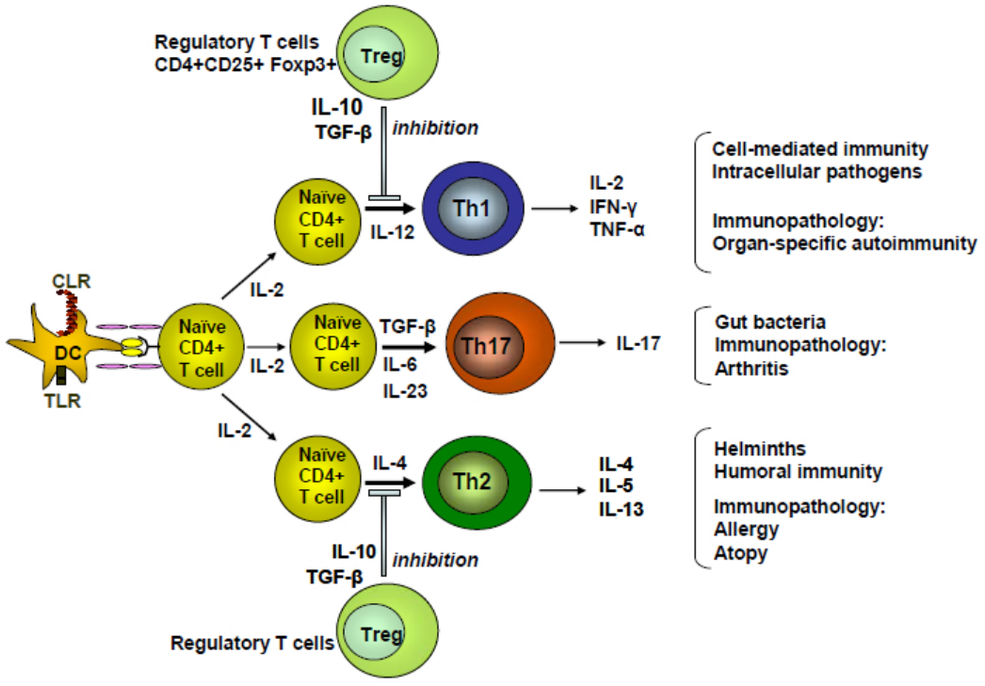

Fig. 3. Functional development and activity of the T helper cell subpopulations [74].

Th1 cells act by mobilizing the cellular arm of the immune system to combat intracellular pathogens, but are also essential in B-cell class switching. Th2 cytokines are essential for the generation of appropriate classes of antibodies and for the elimination of extracellular pathogens [75]. The Th17 lineage is centrally involved in mediating the host response to extracellular pathogens. The Th17 cytokines also play a central role both in primary infection and in recall responses seen in vaccine studies [76]. Tregs produce IL-10 and suppress the functions of other T-cells to regulate cytokine production. Th1/Th2 responses need to be in balance. An imbalance, for instance by an excess of Th1 and Th17 cells, is thought to be related to autoimmunity, while high Th2 responses might give rise to allergic diseases [77]. Notably, even a single cell can simultaneously excrete multiple cytokines and there is consensus that these multi- or polyfunctional responses, particularly of Th1 cytokines IL-2, IFN-γ, TNF-α play an important role upon antigen challenge in controlling infection [78] or providing vaccine-induced protection [79].

Study design

Since the upsurge of pertussis a number of changes have been implemented in the Dutch vaccination programme concerning the pertussis component of the DTaP-IPV vaccine. Whole cell pertussis vaccines have been effective in the past, but have been replaced in most developed countries, including the Netherlands, by acellular pertussis vaccines that are less reactogenic. Moreover, in the Netherlands switches between acellular vaccines with different antigen doses have also been implemented. This thesis describes the first attempt to monitor pertussis-specific T-cell responses in Dutch children. The children under study have been vaccinated with either wP- or aP-vaccines at 2, 3, 4 and 11 months of age and have been administered an aP booster vaccine at 4 of age and in one trial an extra low-dose aP vaccine at 9 years of age

The principal aim of the studies presented in this thesis was to evaluate pertussis specific immune responses shortly after a preschool booster vaccination but also on the long-term. Importantly, the influence of wP and aP priming was compared in these studies. The cellular immune responses were measured prebooster and at 10 and 28 days post-booster in wP- and aP-primed children 4 years of age, 2 years post-booster in wP- and aP-primed children 6 years of age and 5 years post-booster in wP- primed children 9 years of age. Qualitative and quantitative differences in T-cell immunity induced by wP- and aP-priming and by aP booster vaccination with either a high or a low dose were studied. The goal was to gain a better understanding of the immune mechanisms involved in protection against pertussis that is needed to advise on changes in the pertussis vaccination schedule in order to improve protection of Dutch children against pertussis.

For this thesis blood samples from 3 different cohort studies have been used. In a first cross-sectional observational study started in 2006, the Memory study, children of 3, 4, 6 and 9 years of age were included, who all had received 3+1 wP vaccines in the first year of life. Children of 4 years of age, who all had received 3+1 aP vaccines during infancy were included

at the end of 2008, after the introduction of aP vaccines for all children in 2005. Blood samples from children 4 years of age either primed with wP or aP vaccine were collected before and at 10 days and at 28 days after a preschool aP booster vaccination. Later, blood was collected from aP-primed children of 6 years of age who also had received an aP preschool booster vaccine in November 2010. All groups in the memory study consisted of about 60 children per group.

In a second cohort study, the Booster study, the effects of an extra (low-dose) booster vaccine in wP-primed children 9 years of age was evaluated longitudinally. This study started in March 2009 and blood samples were collected from 83 children prevaccination and at 1 month and 1 year post booster vaccination.

Lastly, in a European collaboration study called Child Innovac, it was attempted to assess the effects of the current different pertussis vaccination schedules on pertussis specific T-cell immunity in several European countries. The aim was to assess functional and phenotypical T-cell responses to pertussis antigens in Dutch vaccinated children of 4, 6 and 9 years of age and to compare these responses to those in children from other participating European countries.

Outline

In **chapter 2,** we described the pertussis-specific memory T-cell responses over time in the peripheral blood of Dutch wP-primed children aged 4 to 9 years. In **chapter 3**, we studied the effects of wP- and aP-priming in the first year of life and in combination with either a low-dose or a high-dose aP preschool booster vaccine on the T-cell responses in children of 4 years of age. In **chapter 4**, we further characterized T-cell responses functionally and phenotypically before and after the aP booster vaccination in wP- and aP-primed children 4 years of age. In **chapter 5** we evaluated the differences in IgG-subclass responses in wP- and aP-primed children and the Th1 and Th2 immune response after the booster vaccination in aP-primed children. In **chapter 6** we investigated both B-cell and T-cell immunity 2 years after the preschool aP booster vaccination related to wP- and aP-priming at infant age. Finally, in **chapter 7** we summarized and discussed our main findings and propose recommendations to optimize the pertussis vaccination programme in the future.

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Pertussis Circulation Has Increased T-Cell Immunity during Childhood More than a Second Acellular Booster Vaccination in Dutch Children 9 Years of Age

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Abstract

Here we report the first evaluation of T-cell responses upon a second acellular pertussis booster vaccination in Dutch children at 9 years of age, 5 years after a preschool booster vaccination. Blood samples of children 9 years of age were studied longitudinally till 1 year after the second aP booster and compared with those after the first aP booster in children 4 and 6 years of age from a cross-sectional study. After stimulation with pertussis-vaccine antigens, Th1, Th2 and Th17 cytokine responses were measured and effector memory cells (CCR7-CD45RA-) were characterized by 8-colour FACS analysis. The second aP booster vaccination at pre-adolescent age in wP-primed individuals did increase pertussis-specific Th-1 and Th-2 cytokine responses. Noticeably, almost all T-cell responses had increased with age and were already high before the booster vaccination at 9 years of age. The enhancement of T-cell immunity during the 5 year following the booster at 4 years of age is probably caused by natural boosting due to the a high circulation of pertussis. However, the incidence of pertussis is high in adolescents and adults who have only received the Dutch wP vaccine during infancy and no booster at 4 years of age. Therefore, an aP booster vaccination at adolescence or later in these populations might improve long-term immunity against pertussis and reduce the transmission to the vulnerable newborns. ISRCTN register, ISRCTN64117538, http://www.controlled-trials.com/ISRCTN64117538/

Introduction

Since the introduction of pertussis vaccination in the developed world, disease incidence, morbidity and mortality have decreased [1]. Over the last two decades, however, pertussis has reemerged [2]. In the Netherlands rises in pertussis incidence are seen every 2-3 years from 1996 onwards [3]. For this reason, several changes in the Dutch pertussis immunization program have been implemented. In 1999, primary vaccinations with the whole cell (wP) vaccine were advanced to 2, 3 and 4 months of age, followed by a booster at 11 months of age. In 2001, a high-dose acellular pertussis (aP) vaccination was introduced at 4 years of age as a preschool booster. Additionally, in 2005 the wP-component was replaced by an aP component in the DTP-IPV-Hib primary schedule for infants in the first year of life. As a result, the previous pertussis peak-incidence in the age-cohort of children of 4-5 years in 2001 has shifted towards the age-cohort of 12-13 years nowadays in the Netherlands [4] (F.R. Mooi, personal communication). In general, the burden of whooping cough has shifted from young children to pre-adolescents and adults [5].

The primary sources of infection of the unvaccinated or not fully vaccinated neonates who have the highest risk for both severe symptoms of disease and pertussis-related deaths [6] appear to be household contacts, like mothers and siblings [7]. A number of countries have therefore implemented an acellular pertussis booster vaccination in adolescents and young adults, also hoping to protect infants via herd effects [8].

The mechanism of immunity to pertussis involves a range of both humoral and cellular immune responses, directed at several pertussis antigens included in the vaccines [9]. Although antibody levels wane relatively fast after both natural infection and vaccination [10], a certain level of protection via cell-mediated immunity seems to persist [11]. We recently demonstrated that an extra aP booster vaccination at 9 years of age induced elevated antibody responses that persisted even after one year due to enhanced memory B-cell levels one month post booster [12]. However, data on pertussis-specific T-cell immunity in response to a pre-adolescent booster vaccination are lacking, though T-cells are suggested to be relevant for clinical protection [13].

The aim of this study was to investigate longitudinal T-cell immunity before and after an pre-adolescent aP booster vaccination at 9 years of age. Pertussis-specific T-cell immunity was measured before, 1 month and 1 year after booster vaccination. For comparison, T-cell immunity was evaluated in 4- and 6-year-old children. All children had previously been vaccinated at infant age with four wP vaccinations and had received an acellular pertussis booster vaccine at 4 years of age.

Material and methods

Subjects and study design

In this study (ISRCTN64117538), blood samples were collected to evaluate pertussis booster vaccination in 20 children aged 9 years as described in the Consort 2010 Flow Diagram (Fig. 1). The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist and Protocol S1. Blood samples (15 ml) were collected

before, at 1 month and at 1 year post booster aP vaccination. Data were compared with those obtained from children aged 4 and 6 years (n=15) who had participated in a cross-sectional observational study in the Netherlands (ISRCTN65428640), in 2007-2008 [10,14,15]. From children of 4 years of age, PBMCs prebooster (n=15) as well as those at 28 days after booster (n=12) were available.

These studies were conducted according to the Declaration of Helsinki, Good Clinical Practice Guidelines with the approval of the relevant ethics review committee (Medisch Ethische Toestingscommissie (METC) in Almere, the Netherlands and the Central Committee on Research Involving Human Subjects (CCMO), the Hague, the Netherlands). Written informed consent was obtained from both parents or legal representatives. In both studies, sexes were equally distributed.

Vaccines

The 9 years old children received Boostrix-IPV™ (GlaxoSmithKline Biologicals S.A., Rixensart, Belgium) containing 8 µg pertussis toxin (PT), 8 µg filamentous heamagglutinin (FHA) and 2.5 µg pertactin (Prn) for the pertussis antigens. All children of 4, 6 and 9 years of age received DTwP-IPV + Hib (NVI, Bilthoven, the Netherlands) at 2, 3, 4 and 11 months of infant age. At 4 years of age a high-dose booster ACV-SB™ containing 25 µg PT, 25 µg FHA and 8 µg Prn was administered according to the Dutch NIP.

Cell stimulation and IFN-γ ELISPOT

PBMCs were isolated for measurement of IFN-γ spots per 100,000 PBMCs directed against the 3 *B. pertussis* vaccine antigens included in the booster vaccine. Cells were stimulated with 2 µg/ml PT (inactivated for 10 minutes at 80˚ C) or FHA (Kaketsuken, Kumamoto, Japan) and 4 µg/ml of recombinant Prn [16] as previously tested to be the optimal antigen concentrations to stimulate PBMCs to produce cytokines. As positive controls 5 µg/ml pokeweed mitogen (PWM) (Sigma Chemicals, St. Louis, Mo.) and 150 Lf/ml tetanus toxoid (Td) (NVI, Bilthoven, the Netherlands) were used. Non stimulated (NS) cells served as negative controls. B-cells were removed by anti-CD19 coupled magnetic beads and used for B-cell stimulations [14]. PBMCs depleted of CD19+ cells were counted and 300,000 cells were cultured for 5 days incubated at 37 º C and 5 % CO_2. Preliminary experiments indicated similar results in cytokine production at day 5 from PBMCs and PBMCs depleted from B-cells, both stimulated with pertussis and tetanus antigens. At day 5, all cells were harvested and incubated on 96-well anti-IFN-γ (Mabtech, Nacka Strand, Sweden) coated plates (Millipore MSIP4510, Danvers, Ma.). The next day, plates were developed by anti-IFN-γ-biotin (Mabtech), extravidin (Sigma) and BCIP/NBT. After each development step the plates were washed. Dried plates were analysed and spot numbers assessed by automatic computer-assisted ImmunoScanPro reader (CTL Europe, Bonn, Germany).

Multiplex assay

Cell culture supernatants were collected after 24 hours for IL-2 and after 5 days for IL-10, IL-13, IFN-γ and IL-17 and frozen at -80 ºC until further use. Cytokine concentrations were

measured with the Bioplex pro human cytokine plex (Bio-rad, Hercules, CA, USA) according to manufacturer's protocol, utilizing standard curve concentrations and no sample dilution. Bioplex validation kits were used to calibrate Bioplex systems (Bio-rad).

8-colour FACS analysis

To measure proliferation of cells, one million PBMCs, not depleted from B-cells, were stained with 5 µM CFSE for 10 minutes in the dark at 4 ºC and stimulated for 5 days with 5 µg/ml PT or 10 µg/ml FHA (Novartis Siena Italy, kindly donated by Dr. Clara Ausiello) or 4 µg/ml Prn [16]; not-stimulated cells (NS) served as controls. In preliminary experiments within an international collaboration, the optimal antigen concentrations for FACS analysis have been tested elsewhere [17]. Moreover, the PT and FHA antigens provided by Novartis and purchased from Kaketsuken as described for the IFN-γ ELIspot-assay have been compared and showed similar results. At day 5 golgiplug (BD Biosciences, San José, USA) was added to block intracellular transport processes before further intracellular cytokines staining. Cells were collected, washed and stained for life-dead-staining by Aqua (Invitrogen, Paisley, Scotland, UK) and the cell surface markers APC-7-labelled CD4 (BD), PE-Cy7-labelled CD45RA (BD), and PE-labelled CCR7 (R&D systems, Minneapolis, USA). Subsequently, cells were resuspended in Cytofix/Cytoperm Plus kit (BD) and stained for V450-labelled CD3 (BD), PerCP-Cy5.5 labelled TNFα (Biolegend, San Diego, CA, USA) and APC labelled IFN-γ (BD). After washing, the cells were analysed using the FACS canto cytometer (BD) in combination with Diva software (version 5.2 BD) and FlowJo software (Mac-version 9.0.2, Treestar US, Ashland, OR). Proliferated (CFSE-) viable T-helper-cell populations (CD3+CD4+) were divided in central memory (CCR7+CD45RA-) and effector memory T-cell (CCR7-CD45RA-) populations and analysed for Th1 cytokine production as described recently (17). CD3+CD4- cells were considered to be CD8+ cells.

Flow Cytometric Data Analysis

Flow Cytometry Standard format 3.0 files were exported and data were evaluated using FlowJo software. Dead cells were excluded if stained with Aqua amine-reactive dye. Lymphocytes were gated based on SSC/FSC characteristics. Using FSC-A and FSC-H, singlets were gated on, based on CD3 and CD4 staining, CD3+CD4+ cells were gated within the viable lymphocyte singlet gate.

Statistical methods

Cytokine concentrations and IFN-γ spots per 100,000 PBMCs were measured against the different pertussis vaccine antigens and geometric mean concentrations (GMC's) and 95% confidence intervals (CIs) of PT-, FHA-, Prn-specific T-cell cytokines were calculated. Cytokine concentrations and numbers of IFN-γ producing cells in the different groups were compared with the Mann-Whitney-Wilcoxon signed-rank test. For the longitudinal samples of the 9-year old children, the Wilcoxon-matched-pairs signed rank test was performed. Moreover, we analysed the number of IFN-γ producing cells with a random effects regression model to evaluate the effect of the booster vaccination (9 years of age, longitudinally) and the effect of age (children

4, 6 and 9 years of age, cross-sectionally) separately. We used parametric repeated measurements in this model (R package version 3.1-103 R Foundation for Statistical Computing, Vienna, Austria). Significant differences between groups were found when $p < 0.05$.

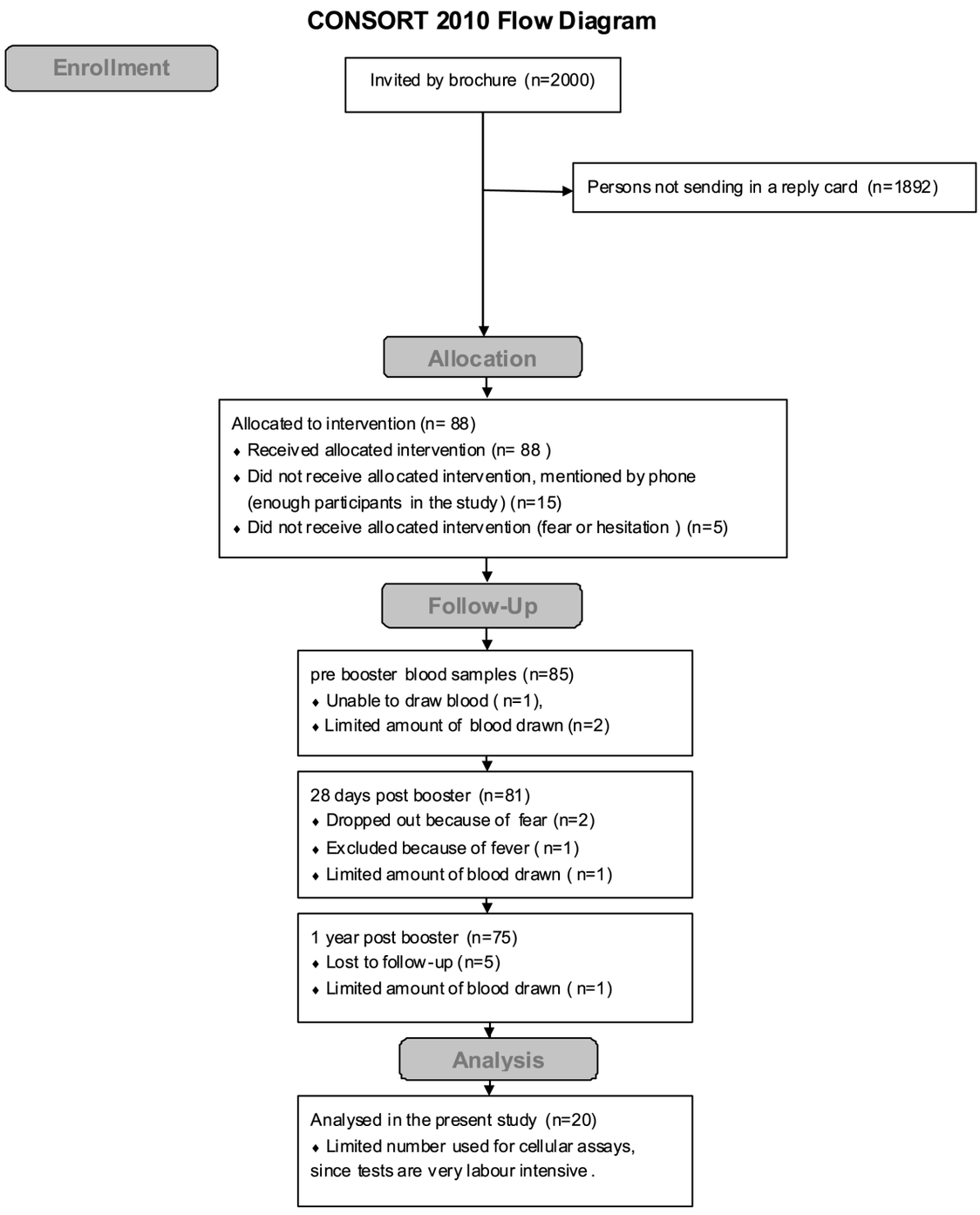


Fig. 1. Consort 2010 Flow Diagram
Study participants, during the recruitment of children 9 years of age who received Boostrix-IPV™ as a second aP booster vaccination.

Results

The number of pertussis-specific IFN-γ producing cells in vaccinated children

The number of the IFN-γ producing cells specific for PT, FHA and Prn in children 4, 6 and 9 years of age are represented in figure 2. The second aP booster vaccination at 9 years of age did not increase the numbers of pertussis specific IFN-γ producing cells. However, in general, the number of IFN-γ producing cells in these children increased with age and children aged 9 years showed significantly higher numbers of PT-, FHA- and Prn-specific IFN-γ producing cells than children at 4 years of age. Analysis with the regression model confirmed that this age-effect resulted in significantly increased numbers of IFN-γ producing cells specific for all three pertussis proteins (PT p=0.001, FHA p=0.0001, Prn p=0.0072). Also, children 6 years of age showed significantly higher numbers of PT- and FHA-specific IFN-γ producing cells as compared to those of 4 years of age. Furthermore, 1 month after the second booster vaccination at 9 years of age, the geomean values of IFN-γ producing cells were higher than those at 1 month after the first booster vaccination at 4 years.

The numbers of IFN-γ producing cells had increased only slightly during a month after the second aP booster at 9 years and a significant increase was only found for Prn-specific cells. Nevertheless, only a minimal decrease after one year was observed, indicating persistently high levels of T-cell immunity both before and after the booster at 9 years of age. However, with the regression model an significantly increased booster response irrespective of age was found not only for the numbers of Prn-specific IFN-γ producing cells but also for PT at 1 month postbooster (p=0.037 and p=0.026, respectively).

Table 1. Th-1 and Th-2 cytokine responses in children of 9 years of age.

		PT			FHA			Prn		
Cytokine	Blood sampling	n	GMC	95% CI	n	GMC	95% CI	n	GMC	95% CI
IFN-γ	Pre booster	17	429	24-7538	17	23555	7003-79231	17	790.1	70-8965
IFN-γ	+1 month	17	4883*	1620-14713	17	25725	8426-78540	17	5267 *	1932-14359
IFN-γ	+1 year	17	601	33-10911	17	16402	4964-54190	17	673	37-12387
IL-13	Pre booster	17	146	30-717	17	685	307-1532	17	64	10-410
IL-13	+1 month	17	573*	282-1165	17	1055	536-2075	17	451*	186-1096
IL-13	+1 year	17	439	192-1003	17	730	419-1269	17	61	7-558

* = Significantly higher post booster compared to pre-booster p>0.05

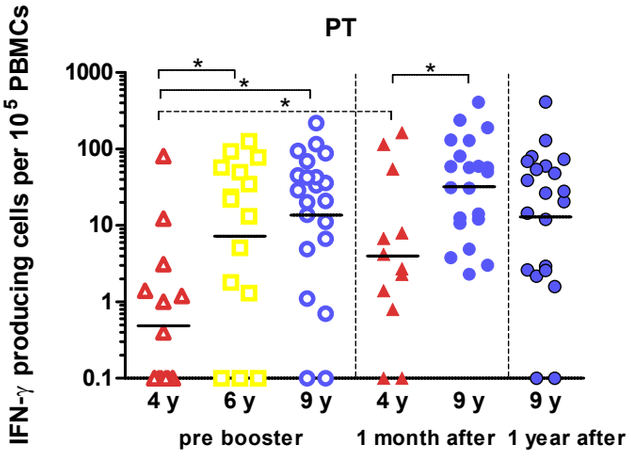
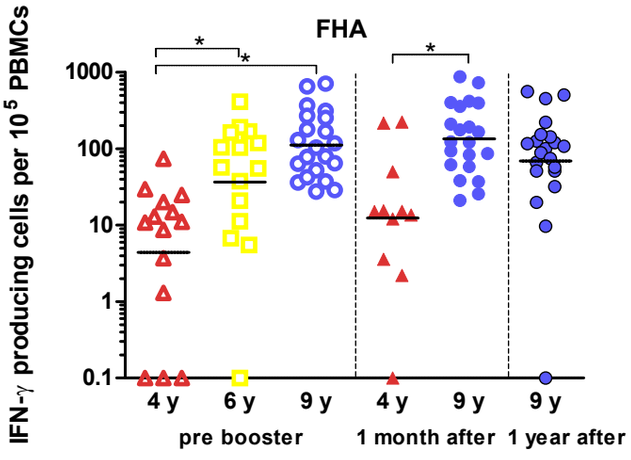
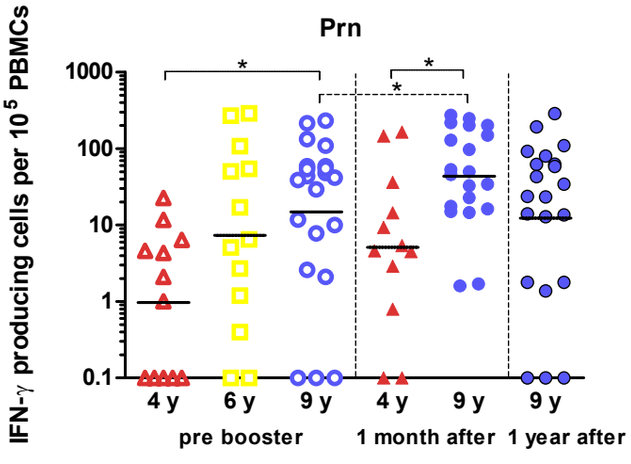


Fig. 2. Numbers of IFN-γ producing cells. PBMCs of children 4 years of age (red open triangles) (n=14), 6 years of age (yellow open squares) (n=15) and 9 years of age (blue open circles) (n=20) pre-booster have been stimulated with PT, FHA or Prn for 5 days and subsequently numbers of IFN-γ producing cells have been determined. Children 9 years of age have been studied longitudinally at 1 month (blue closed circles) and 1 year (blue filled circles) after a second aP booster vaccine (n=20) and children 4 years of age have been studied cross-sectionally at 1 month after a first aP booster vaccine (red filled triangles) (n=11). Horizontal lines represent geometric means of IFN-γ producing cells per 100.000 stimulated PBMCs.
* = significant difference between groups.

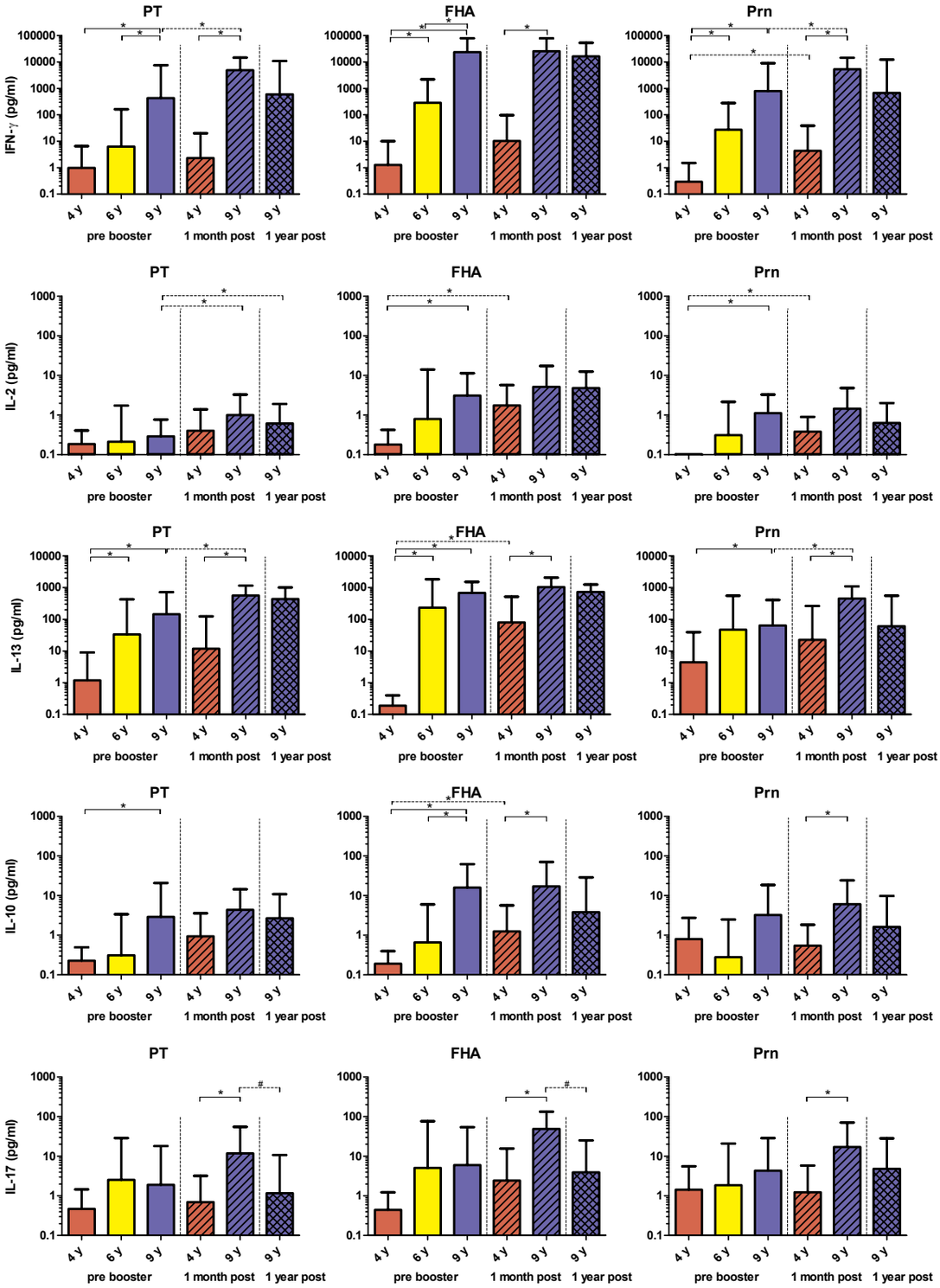




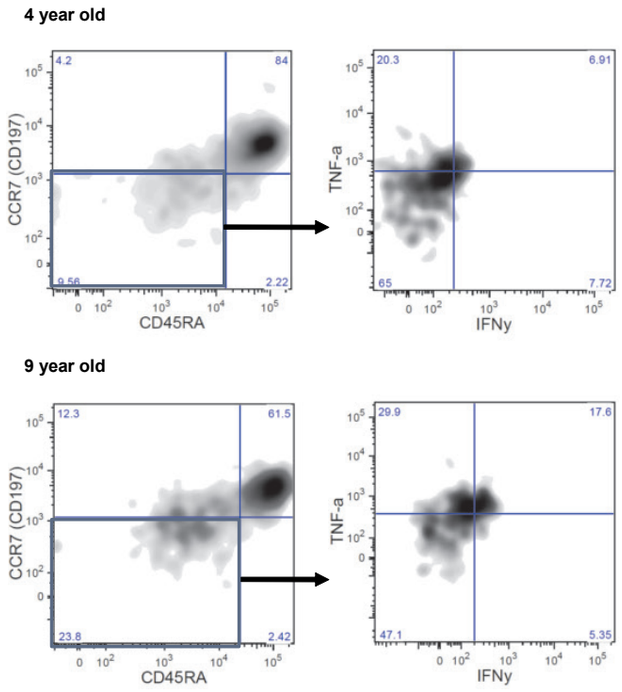
Cytokine profiles in 4, 6 and 9 years old pertussis vaccine recipients

In the same groups of children, Th1 (IFN-γ and IL-2), Th2 (IL-13), Th17 (IL-17) and T regulatory (IL-10) responses specific for PT, FHA and Prn (Fig. 3) have been measured. In general, the *B. pertussis*-specific T-cell cytokine concentrations increased significantly with age already before the booster vaccinations and independently of the stimulatory antigen; all pertussis-specific Th1 and Th2 and IL-10 responses were higher at 9 years compared with children of 4 of age, except for PT-specific IL-2 and Prn-specific IL-10. Furthermore, FHA- and Prn-specific IFN-γ as well as PT- and FHA-specific IL-13 concentrations were significantly higher in children aged 6 as compared to 4 years of age. Additionally, at 9 years of age PT- and FHA-specific IFN-γ and FHA-specific IL-10 GMCs were higher than at 6 years of age.

Fig. 3. Pertussis protein-specific cytokine responses.
Th1, Th2 and Th17 and IL-10 responses in supernatants of PT, FHA and Prn stimulated PBMCs of children of 4 years of age (red bars), 6 years of age (yellow bars) and 9 years of age (blue bars) are presented as GMCs with 95% confidence intervals. Additionally, cytokine responses of children 4 years of age at 1 month after a first aP booster vaccine (blue hatched bars) and children 9 years of age at 1 month (blue hatched bars) and 1 year (cross-hatched bars) after a second aP booster vaccine are shown.
* = significant increase between groups
# = significant decrease between groups



A. PT-specific effector memory T-cells



B. Prn-specific effector memory T -cells

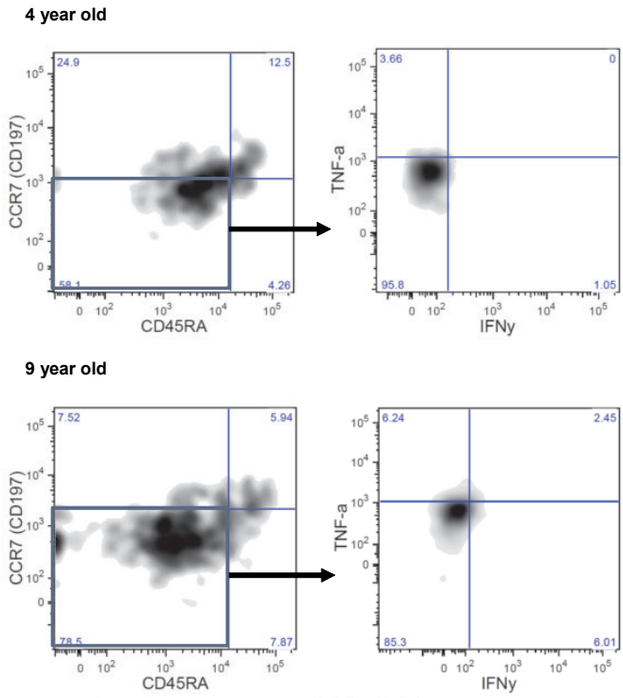


Fig. 4. Flow-cytometry analysis of pertussis-specific CD3+CD4+ T-cells.
PBMCs of children 4 and 9 years of age pre-booster were stimulated with PT and Prn for 5 days and analysed by 8-colour FACS analysis. T-cells which have proliferated upon stimulation (CFSE-) were characterized phenotypically by CD45RA and CCR7 and the effector memory cells (CD45RA- and CCR7-) were further analysed functionally (IFNγ+ and TNFα+). The results of a representative child of 4 years and 9 years of age specific for PT (A) and Prn (B) are presented.

Effect booster vaccination in 9 years old children

In general, at 9 years of age, prebooster IFN-γ, IL-13, IL-10 and IL-17 responses were already elevated, and the effect of the booster vaccination was mostly minimal. Nonetheless, the PT- and Prn-specific IFN-γ, as well as IL-13, and PT-specific IL-2 significantly increased 1 month after booster compared to prebooster values also at 9 years (Table 1, Fig. 3). Most cytokine responses tended to decrease again one year after the booster, but this was significant only for FHA-specific IL-17 (Fig. 3).

Cytokine profile after booster compared in 4 years old and 9 years old children

We also compared the cytokine profiles in children of 9 years of age one month after the second aP booster vaccination with the values one month after the first aP booster in children of 4 years of age (Fig. 3). In general, the effect of the first booster vaccination at 4 years was higher than the effect upon the second booster at 9 years of age, as illustrated by significantly increased production of FHA-specific IL-2, IL-13 and IL-10 and Prn-specific Th1 responses after the first booster in children 4 years of age. Nevertheless, because of the high pre-booster levels at 9 years of age, all cytokine concentrations 1 month after the booster were significantly higher than the levels in the children at 4 years except for IL-2 and PT-specific IL-10.

Characterization of CD3+CD4+ T-cells comparing 4 and 9 years old children

We found differences in the phenotypes of T-cells (CD3+CD4+) when children of 4 and of 9 years of age were compared upon stimulation with PT and Prn, as illustrated for one child per age-group. The T-cells were characterized by their proliferation capacity (CFSE-), expression of CCR7 and CD45RA and the production of the Th-1 cytokines IFN-γ and TNF-α (Fig. 4). Several children 9 years of age showed higher percentages of pertussis-specific effector memory T-cells (CCR7-CD45RA-), and showed more Th1- cytokine production in this T-cell subpopulation (Fig 4). Also, significantly higher percentages of PT-specific effector memory T-cells (CCR7-CD45RA-) were present in 9 years old children in the total CD3+CD4+ T-cells and also the proliferated T-cells (CFSE-, CD3+CD4+) (Geomean (GM) 20% and 43%, respectively) as compared to those in 4 years old children (GM 5% and 11%, respectively) (Fig. 5). In contrast, these two age groups exhibited similar percentages of FHA- and Prn-specific effector memory T-cells (Fig. 5A). They differed in that the percentages of effector memory T-cells that proliferated (CD3+CD4+CFSE-) upon stimulation with FHA and Prn were higher only in some of the children at 9 years of age and not among the 4 year olds (Fig. 5B).

The percentages of effector memory T-cells in children 9 years of age and those 4 years of age producing Th1 cytokines, appeared comparable by both CD3+CD4+ T-cells as well as proliferated CD3+CD4+CFSE- T-cells, (GM about 1.5 to 4 % and GM 0.3 to 16%, respectively) (data not shown). Notably, in both groups of children effector memory cells producing both IFN-γ and TNF-α were found (GM 0.01% to 2.6 %), although the percentages were low (Fig. 4)

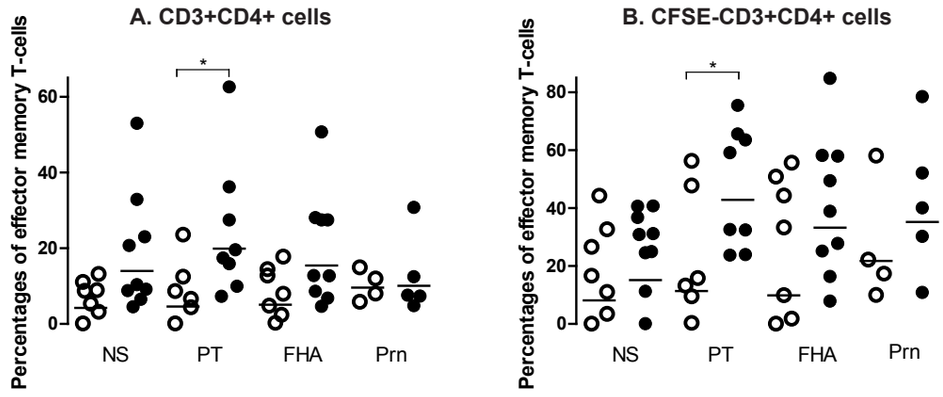


Fig. 5. Effector memory T-cell responses.
Percentages of effector memory T-cells (CD45RA-,CCR7-) in children of 4 years (open circles) and 9 years of age (black circles) found in CD3+CD4+ T-cells (A) and proliferated (CFSE-) CD3+CD4+ T-cells (B) upon stimulation with the pertussis antigens and in non-stimulated cells (NS). Horizontal lines represent geomean values. * = significant difference between children of 4 and 9 years of age

Discussion

The present study represents the first reported evaluation of T-cell responses upon a second acellular pertussis booster vaccination at the pre-adolescent age, 9 years of age, 5 years after a preschool booster vaccination in children 4 years of age. The second aP booster vaccination at pre-adolescent age in wP-primed individuals did increase pertussis-specific Th-1 and Th-2 cytokine responses. However, almost all T-cell responses were already high before the booster vaccination at 9 years of age and had enhanced significantly during the 5 year period between the two booster vaccinations. Higher T-cell booster responses were also booster responses were also observed 1 month after the extra booster vaccination at 9 years of age than 1 month after the preschool booster vaccination at 4 years of age. The fact that T-cell responses at 9 years of age were higher than those directly after the preschool booster at 4 years and at 6 years of age implies that natural boosting has stimulated these T-cell responses, which can be explained by the high circulation of pertussis in the Netherlands [5]. This seems in line with the presence of higher numbers of PT-specific (proliferated) effector memory CD4+ T-cells in children 9 years compared to those at 4 years of age.

In the Netherlands, an increased, high circulation of pertussis was found in the last decade as shown by carefully monitoring the incidence of pertussis as well as the evaluation of pertussis IgG levels in persons from 0 till 80 years of age in two large cross-sectional serosurveillance studies performed in 1995/6 and 2006/7 [3]. Also, the incidence of pertussis has shifted from young to older children since the implementation of the preschool aP booster vaccination at 4 years of age [4]. Very recent data have shown that the positive effect of the preschool booster lasts till the age of 13. Therefore, nowadays the increased incidence of whooping cough is mainly found in adolescents and adults [5].

It is difficult to compare our study with other studies on cell-mediated immune responses against pertussis and the effects of booster vaccinations, because each study used different age groups of children, vaccination schedules or measured different T-cell parameters [11,18,19,20,21,22].

In contrast to other studies [18,23], we have shown just a small increase of pertussis-specific T-cell immunity induced by a second booster at pre-adolescent age, because T-cell immunity was already elevated in these 9 years old children. The limited increase and the discrepancy with other publications describing a longer persistence of cell-mediated immune responses after booster vaccination [11,20,24] can be explained by the effect of a preschool booster vaccination 5 years earlier, at 4 years of age, in combination with boosting by natural infection in our study cohort.

Our results additionally showed higher numbers of pertussis-specific CD3+CD4+ effector memory T-cells in 9 years old children compared with 4 years old children. Although the percentages of effector memory T-cells producing Th1 cytokines between 4 and 9 years old children were similar, the higher number of these cells in older children resulted into higher amounts of Th1 cytokines. Notably, in both groups of children, CD3+CD4+ effector memory cells producing simultaneously IFN-γ and TNF-α upon stimulation with pertussis antigens were found, indicating that one cell is able to produce more than 1 cytokine [25].

The concentrations of T-cell cytokines in 9 years old children both before and at 1 month after booster vaccination predominantly showed Th1 IFN-γ responses and fewer IL-13 responses, associated with Th-2 cytokine lineage, or IL-17 responses. Therefore, particularly in children, Th1 responses might play an important role in protection against clinical pertussis infection, while in general IL-17 was suggested to play a more prominent role in older individuals [26].

Phenotypical characterization of T-cells in adolescents by Rieber et al. after an aP booster following wP or aP priming at infancy confirmed a predominant Th1 response especially by activation of CD8 T-cells [13]. However, in contrast to these results, we found a higher percentage of CD4 effector memory T-cells (CCR7+CD45RA-) than CD8 effector memory cells in the children in our study. Next to pertussis-specific CD8+ memory T cells that may contribute to protection against clinical pertussis [13], we believe that also CD4+ T cells producing Th1-cytokines may play an important role in protection of children against clinical pertussis.

We recently showed increased pertussis-specific memory B-cell immune responses after the second aP booster vaccination in Dutch wP primed children 9 years of age that sustained at least for one year [12]. The correlation between the high numbers of pertussis-specific memory B-cells at one month after this second booster vaccination with the corresponding antibody responses still present after one year indicates the important role of the memory B-cell pool in the maintenance of antibody levels. A second aP booster vaccine affects B-cell memory more than the T-cell memory responses. We speculate that a certain level of T helper-cell memory immunity is needed to be able to increase the B-cell responses upon booster vaccination. Epidemiological data reveal an improved protection against pertussis after the implementation of aP booster vaccines at 4 years of age [4,5]. These studies together with the increased T-cell and B-cell responses upon the second aP-booster vaccinations at pre-adolescent age which is strengthened by the circulation of pertussis, might result into a better protection against pertussis during adolescence. For a better understanding of the duration of pertussis-specific memory immunity, this needs to be monitored over the longer term.

Although other West European countries had already switched from wP to aP infant vaccinations in the 1990s, pertussis still circulates among these aP primed populations too and notable pertussis incidences have been equally observed in these countries as in the Netherlands, that implemented the vaccine switch only in 2005. Meanwhile, concerns about the efficacy of repetitive aP booster vaccinations have risen [27]. In Canada a low vaccination coverage was observed 5 years after the implementation of adult booster vaccinations [28]. Moreover, repeated booster vaccinations in adults in general are not considered cost-effective [29], since the yet unvaccinated newborns are particularly the risk group susceptible for severe infection. As transmission studies have shown, mothers are one of the main sources of infection for the young infant [7,30]. Thus although natural boosting of the population will also provide pertussis-specific immune responses that improve protection, the vulnerable unprotected young infant is put at risk by a high incidence of pertussis in the adult population. This means that selective vaccination of those adults who are in close contact with infants, the so-called cocooning strategy, will better reduce transmission to infants and will probably be more (cost)-effective [29,30].

Given that a majority of the Dutch population born before 1997 has only received the Dutch wP vaccine during infancy, we believe that aP booster vaccination in this part of the population will improve long-term immunity against pertussis substantially. Such a vaccine strategy might enlarge also the antibody levels in woman at child-bearing age, in turn increasing the maternal transfer of antibodies to newborns who subsequently will be better protected. However, pertussis booster vaccinations in adults are expensive and the vaccination coverage in the adult population has proven to be generally low [31,32]. Therefore, a good information and communication strategy must accompany such a booster vaccination in order to make its implementation in the late adolescent or young adult population successfully.

In conclusion, we demonstrated, in Dutch wP-primed children 9 years of age, an enhanced Th-1 memory immune response upon a second aP booster vaccination. These results, together with enhanced memory B-cell responses upon booster vaccination support the introduction of an aP booster vaccination for pre-adolescents. The positive effect of the preschool aP booster vaccination at age 4 years in combination with natural boosting of the immune responses by circulation of pertussis will probably protect wP-primed children until teenage. An aP booster vaccination at adolescence or later might improve long-term immunity against pertussis and reduce the transmission to the vulnerable newborns.

Acknowledgements

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T-cell Responses before and after the Fifth Consecutive Acellular Pertussis Vaccination in 4-Year-Old Dutch Children

3

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Abstract

Immunization with acellular pertussis vaccine (aP) induces higher specific antibody levels and less adverse reactions as compared to the whole-cell vaccine (wP). However, antibody levels in infants induced by both types of pertussis vaccines wane already after one year. Therefore, long-term T-cell responses upon vaccination might play a role in protection against pertussis. In a cross-sectional study (ISRCTN65428640) we investigated T-helper (Th) cell immune responses in wP- or aP-vaccinated children before and after an aP low-dose or high-dose preschool booster at 4 years of age in the Netherlands.

T-cells were stimulated with pertussis vaccine antigens. Interferon-γ producing cells and Th1, Th2, Th17 and IL-10 cytokines were determined. In addition, pertussis-specific IgE-levels were measured in plasma.

Children being vaccinated with aP vaccinations at 2, 3, 4 and 11 months of age still showed higher pertussis-specific T-cell responses at 4 years of age in contrast to wP vaccinated children. These T-cell responses failed to show a typical increase in cytokine production after a fifth aP vaccination, but remained high after a low-dose booster and seemed to decline even after a high-dose booster. Importantly, elevated IgE-levels were induced after this booster vaccination. In contrast, wP-vaccinated children had just low prebooster T-cell responses and these children showed a clear post booster T-cell memory response even after a low-dose booster vaccine.

Four high-dose aP vaccinations at infant age induce high T-cell responses present even three years after vaccination and enhanced IgE responses after preschool booster vaccination. Therefore, studies into changes in vaccine dosage, timing of pertussis (booster) vaccinations and the possible association with local side effects are necessary.

Introduction

Recently, during a large pertussis outbreak in California 10 infants have died (19) and 9154 cases of whooping cough have been reported by the California department of Public Health (4). Already in the 1990s, many developed countries replaced the whole-cell pertussis component (wP) with the acellular pertussis component (aP) in the DTP-IPV-HIb combination vaccine in order to achieve higher antigen specific antibody levels and fewer side effects. This however did not stop the reemergence of pertussis in these countries (8, 38, 39). The high incidence of pertussis worldwide can be (partly) explained by adaptation of the circulating bacterial strains to vaccine pressure as well as waning immunity after vaccination and natural infection (2, 27).

In the Netherlands, wP vaccines have been used since the early 1950s resulting in a decline of pertussis disease. Despite a high vaccination coverage, the incidence of pertussis increased again after 1996 and for this reason an aP preschool booster vaccination (aP) has been introduced at 4 years of age in 2001. From 2005 onwards, the wP infant vaccine component administered at 2, 3, 4 and 11 months of age has also been replaced by the aP vaccine.

Pertussis-specific antibody levels are induced by vaccination and natural infection and protect against disease, however these levels decline very rapidly after vaccination (9, 13). Several studies have shown that protection against disease also relies on T-helper cells (Th), next to antibodies (7, 26). Multiple Th-cell lineages may be involved, like the Th1, Th2 and Th17 cells, and each lineage is characterized by specific cytokine repertoires (6). However, the induction of long-term T-cell memory responses and the relative contribution of each Th-cell lineage upon vaccination is largely unknown. Previous studies have shown that aP may lead to Th2 cytokine repertoires in infants and children, whereas wP rather primes for Th1 immune responses (1, 23). The aP vaccines consist of some purified pertussis proteins that may differ in the induction of Th-cell responses as compared to wP which include many other biological components. Information about T-cell immunity after pertussis vaccination and comparison between wP- and aP-primed infants is scarce. Moreover, Th2 responses might be associated with atopic reactions (31) and pertussis-specific IgE has been found after aP vaccinations in infants (28). The aim of this study is to assess the Th1, Th2, Th17 as well as IL-10 cytokine responses to pertussis vaccine antigens in children 4 years of age who received either a low-dose or a high-dose antigen aP preschool booster vaccination. We compared groups of children who have been primed either by wP or aP in infancy. Apart from T-cell kinetics, also pertussis-antigen specific IgE responses in these groups of children are studied.

Material and methods

Subjects and study design

In this study a cohort of children 4 years of age forms a subset of a cross-sectional observational study in the Netherlands (ISRCTN65428640) performed from 2007 (wP-primed children) onwards until 2008 (aP-primed children), which aimed to investigate pertussis-specific immunity in children 3 to 9 years of age. In these 4 years old children, the pertussis-

vaccine-specific IgG antibody responses have been published previously (13). Now, we evaluated T-cell immune responses in a randomly selected subset of these children (N=92). As previously described (13), we divided the children in 8 different groups, according to (1) the vaccination history (wP- or aP-priming), (2) the type of preschool booster vaccine (low-dose or high-dose aP) and (3) time of blood sampling i.e. before, 10 or 28 days after the booster (Fig 1). This study was conducted according to the Declaration of Helsinki, Good Clinical Practice Guidelines with the approval of the relevant ethics review committee. Written informed consent was obtained from either parents or legal representatives.

Vaccines

All wP-primed children had received DTwP-IPV-Hib (NVI, Bilthoven, the Netherlands and all aP-primed children had received DTaP-IPV-Hib (Infanrix-IPV-Hib™, GlaxoSmithKline Biologicals S.A., Rixensart, Belgium) at 2, 3, 4 and 11 months of age according to the Dutch National Immunisation Programme. The wP vaccine contained among other bacterial proteins about 0.16 µg pertussis toxin (PT), 2.6 µg filamentuous heamagglutinin (FHA) and an unknown amount of pertactin (Prn) while the aP vaccine contained 25 µg PT, 25 µg FHA and 8 µg Prn. During the inclusion period of this study, children received randomly either a low-dose or high-dose preschool booster vaccine due to a shortage in supply of the vaccine. Therefore, we included children who had received a low-dose booster vaccine, Triaxis™ (Sanofi Pasteur, Lille, France), containing 2.5 µg PT, 5 µg FHA, 3 µg Prn and 5 µg fimbriae type 2 and 3, or a high-dose booster vaccine, Infanrix™, in wP- and aP-primed children at 28 days post booster. At 10 days post booster, we were only able to include wP-primed children who received a low-dose booster and aP-primed children who received a high-dose booster, because the booster vaccine in the NIP had changed during the inclusion of our study.

T- cell stimulation

PBMCs were isolated from blood as described earlier and frozen (3). After thawing, 3.0 x 10^5 viable cells per well were cultured in AIMV medium (Gibco Invitrogen, Grand Island, NY, USA) containing 5% human AB serum (Harlan Laboratories, Leicestershire, UK) (AIMV+) for 5 days at 37 °C and 5% CO_2 in 96 well roundbottom culture plates (Greiner, Invitrogen, Breda, the Netherlands) and stimulated in triplo with 2 µg/ml inactivated PT, FHA (Kaketsuken, Kumamoto, Japan; endotoxin content <1,17 EU/ml), 4 µg/ml of recombinant Prn (22) and 5 µg/ml pokeweed mitogen (Sigma Chemicals, St. Louis, MO, USA) as a positive control. Non stimulated (NS) cells served as negative controls. The purified PT was heat inactivated by incubation for 10 minutes at 80°C. The batch of Prn was controlled for endotoxin contents by use of the Limulus amoebocyte lysate assay (17). One batch of each antigen was used for the whole study. In preliminary experiments, we determined the optimal pertussis antigen concentrations for cell stimulation and the culture period needed to measure optimal numbers of IFN-γ producing cells. After both 1 day and 5 days of stimulation, culture supernatants were collected and stored at -80 °C to be able to measure early and late cytokine responses. T-cell responses were measured in culture supernatants and 5-13 samples in all groups indicated in figure 1 were analyzed.

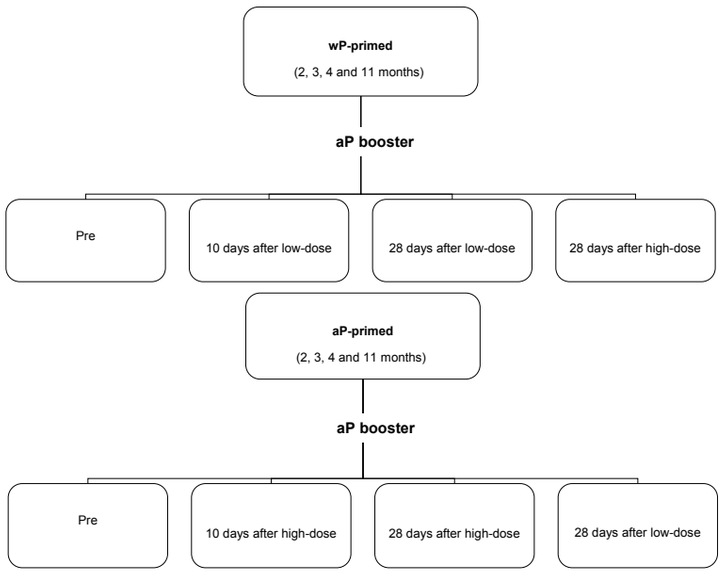

Fig. 1. Different groups of children were used in this study: wP-primed and aP-primed children at 2, 3, 4 and 11 months of age received a booster vaccine at 4 years of age with either low-dose or high-dose. Groups of children were studied before booster and at 10 and 28 days after booster. Numbers of individuals used varied in the different assays as indicated.

IFN-γ ELIspot assay

ELIspot plates (Millipore MSIP4510, Danvers, MA, USA) prewetted with 70% ethanol, were coated overnight with 5 µg/ml anti-human IFN-γ (Mabtech, Nacka Strand, Sweden) in PBS. After three washes with PBS and blocking the plates for 2 h with AIMV+, 1.4 x 10^5 stimulated cells per well were serially diluted twofold and cultured for 18 hours at 37 °C and 5% CO_2. After four washes with PBS/0.05% Tween20 (PBST) (washed) and cell lysis with water, plates were incubated with 1 µg/ml anti-human-IFN-γ (Mabtech) for 2 h, in combination with peroxidase labeled extravidin (Sigma) for 1 h. Spots were developed as described (14).

Multiplex bead-based immunoassay for cytokines

The cytokines IFN-γ, TNF-α, IL-5, IL-13, IL-17 and IL-10 were determined in supernatants after 5 days and IL-2 was measured after 1 day of stimulation by commercial multiplex bead-based immunoassay kits according to manufacturers' instructions (Bio-Rad Laboratories, Hercules, CA, USA). All samples presented in this study have been stored just for a few months before testing, and have been randomly divided over the different tests to avoid interassay variation between the samples. Measurements were performed with a Bio-Plex 200 in combination with Bio-Plex Manager software (Bio-Rad).

Bordetella pertussis-specific IgE

Plasma samples were depleted from IgG by adding Gulsorb (1:10 V/V) (Meridian Bioscience Inc., Cincinnati, OH, USA). Afterwards, the concentration of pertussis-specific IgE was measured using goat anti-human IgE-PE (Epsilon, Fisher Scientific) in a multiplex bead-based assay as described (15, 37). IgE responses were measured in 16-52 plasma samples of all groups presented in figure 1.

Definitions, data presentation and statistical analysis

All individual data or geometric means values (GM) and (95 % confidence intervals) per group were presented. Background values of negative control cells were deducted from each sample. Significant differences between groups ($p<0.05$) were determined as indicated.

Results

Induction of T-cell cytokines in wP- and aP-primed children in infancy

The concentrations of the T-cell cytokines TNF-α, IFN-γ, IL-5, IL-17 and IL-10 specific for the pertussis proteins PT, FHA and Prn in PBMCs of aP- and wP- primed children pre and at 28 days after an high-dose aP preschool booster are illustrated in figure 2. At 4 years of age, three years after four aP vaccinations in the first year of life, most pre-booster T-cell cytokine levels specific for all three pertussis-proteins (11/15) were significantly higher as compared to those of wP-primed children. The PT-specific IFN-γ, IL-17 and IL-10 as well as the Prn-specific IL-10 were also higher in aP-primed children but did not reach significance (Fig.2).

In wP-primed children the aP booster did enhance the pertussis-specific TNF-α, IFN-γ, IL-5 production, an effect which was significant for FHA- and Prn specific IL-5 as well as the Prn-specific IFN-γ. In contrast, the aP booster in aP-primed children did not enhance the T-cell cytokine concentrations. Moreover, after administration of a fifth high-dose aP vaccine, the FHA-specific IFN-γ and IL-17 response even showed a decline. In general, the pertussis-specific IL-17 and IL-10 values were low compared with the other cytokine values, especially for PT (Fig. 2).

To compare the production of the Th2 cytokine and the Th1 cytokines levels the (GMs) geomean values of the IL-5 levels per group pre- and post booster were compared to those of IFN-γ. The GM of the Th2 cytokine IL-5 specific for PT was about 6-fold higher in aP-primed children as compared to that of the Th1 IFN-γ GM before booster vaccination. At 28 days after a fifth, high-dose aP vaccine the IL-5 GM was about 10 to 20-fold higher than that of IFN-γ for all three pertussis antigens. In contrast, similar values of Th1 and Th2 cytokines were found in wP-primed children (Fig. 2).

Kinetics of the T-cell cytokine production in wP- and aP-primed children after a preschool aP booster vaccination.

To illustrate the kinetics of the T-cell cytokine production after an aP booster vaccination at 4 years of age, the concentrations of two other cytokines were presented in table 1 showing data for day 10 after an aP booster vaccination too. Also for the Th1 cytokine IL-2 and the Th2 cytokine IL-13, prebooster T-cell cytokine levels specific for all pertussis antigens were significantly higher in aP-primed children compared with those of wP-primed children as shown for the other cytokines (Table 1).

In general, the T-cell cytokine concentrations of wP-primed children specific for all pertussis antigens showed increased values already at day 10 after a low-dose booster vaccination. These values were significantly higher for FHA- and Prn-specific IL-2 as well as PT and Prn-specific IL-13. During the next 18 days no significant differences in cytokine production were observed. In contrast, no increased values or a tendency to lower values was observed at day 10 after even a high dose aP booster in aP-primed children (Table 1).

Table 1. IL-2 and IL-13 (pg/ml) produced by PBMCs of wP- and aP-primed children pre and post booster upon stimulation with PT, FHA and Prn

	wP-primed				aP- primed			
IL-2	pre booster	10 days low-dose	28 days low- dose	28 days high-dose	pre booster	10 days high-dose	28 days high-dose	28 days low-dose
	n=10	n=8-9	n=9	n=8	n=9	n=9	n=6	
PT	0.01 (-)	0.04 (7.2)	1.5 # (501)	0.05 (1.1)	7.0 * (55.2)	3.0 (25.5)	19.6 (36.6)	nd
FHA	0.01 (-)	0.6 # (74.2)	6.0 # (401)	1.6 # (44.1)	36.1 * (250)	22.5 (116)	37.9 (340)	nd
Prn	0.01 (-)	0.1 # (2.4)	0.5 # (12.3)	0.3 # (6.4)	10.6 * (42.0)	5.1 (66.8)	7.1 (20.6)	nd
IL-13	pre booster	10 days low-dose	28 days low- dose	28 days high-dose	pre booster	10 days high-dose	28 days high-dose	28 days low-dose
	n=13	n=10-11	n=9-10	n=8-9	n=13	n=11-12	n=5-6	n=5-7
PT	0.5 (28.4)	15.1 # (143)	8.4 (111)	13.8 # (443)	132 * (3877)	99.7 (948)	65.5 (255)	82.3 (693)
FHA	1.9 (79.0)	16.9 (267)	7.0 (152)	47.7 # (230)	502 * (7322)	326 (2754)	45.8 (799)	644 (1543)
Prn	1.6 (45.1)	39.3 # (174)	43.4 # (157)	39.8 # (212)	158 * (450)	43.3 (999)	38.7 (270)	135 (148)

children of 4 years of age have been vaccinated with either a low-dose (Triaxis) or a high dose (Infanrix) aP booster vaccine
data are presented in geometric mean values and (sd)
*significant higher pre booster values in aP-primed children compared to wP-primed children
significant increase post booster compared to pre booster values in wP-primed children

Due to the high prebooster values, the IL-13 cytokine levels for all three proteins in aP-primed children at day 28 after a low-dose booster were still higher than found in wP-primed children after a low-dose booster vaccination. This was also found for PT-specifc IL-2 and IL-13 at 28 days after a high-dose booster, although the differences were not significant due to the high variation in the relatively low amount of samples (Table 1).

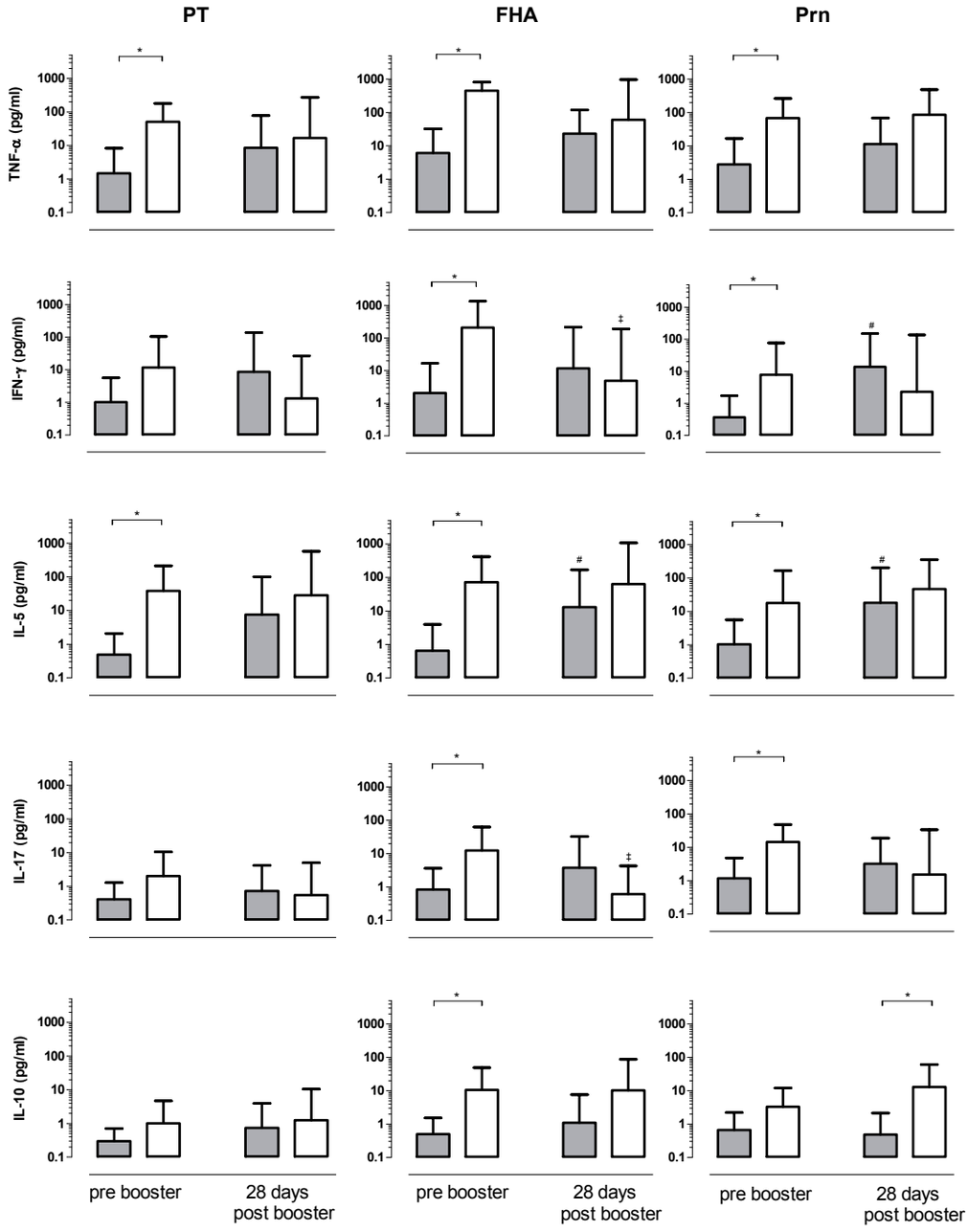

Fig. 2. Concentrations of Th1 (TNF-α and IFN-γ), Th2 (IL-5), Th17 (IL-17) and IL-10 cytokines in supernatants of PBMCs in wP- (gray) and aP-primed (white) children specific for the pertussis-proteins PT, FHA and Prn. Bars represent GMs and 95%-confidence intervals of data prebooster and at 28 days post booster vaccination with a high-dose aP vaccine. Groups consisted of 13 individuals pre booster vaccination, and 5 to 8 persons at 28 days post booster vaccination.

* = Significant increased values in aP-primed children compared to wP-primed children

= Significant increase at 28 days after booster vaccination compared to pre booster values in wP-primed children.

‡ = Significant decrease at 28 days after booster compared to pre booster values in aP-primed children.

Induction of IFN-γ producing T-cells in wP- and aP-primed children

The numbers of IFN-γ producing T-cells in wP- and aP-primed children pre and at 10 and 28 days post booster showed the same pattern as found for the cytokine values in the T-cell supernatants. At three years after four aP vaccinations in the first year of life, the number of IFN-γ producing cells specific for all three pertussis antigens were about a ten-fold higher compared with children vaccinated with wP at infancy (Fig. 3).

In wP-primed children the numbers of IFN-γ producing cells specific for PT increased significantly at both day 10 and day 28 after a low-dose booster (p= 0.02 and p= 0.04 respectively) as well as after a high-dose booster (p=0.02). Those numbers specific for FHA and Prn were significantly increased at day 10 (p=0.04) and (p= 0.03) respectively, and were just slightly increased at day 28 after either a low-dose or a high-dose booster. At day 28 a high-dose booster in wP-primed children induced comparable numbers of IFN-γ producing cells compared with a low-dose booster for all three antigens (Fig. 3).

The already high numbers of IFN-γ producing cells at 4 years of age prebooster in aP-primed children did not further increase after receiving either a low-dose or a high-dose booster. Surprisingly, at 28 days after a high-dose aP booster vaccine, these numbers even tended to decrease for all three antigens, which resulted in significantly lower numbers (p= 0.04) for Prn as compared to those after a low-dose booster (Fig. 3). Furthermore, at 28 days after a low-dose preschool booster, the numbers of pertussis-specific IFN-γ producing cells in aP-primed children were higher than those in wP-primed children which reached significance for the antigens PT and FHA (p= 0.04 and p=0.03, respectively) and just not for Prn (p= 0.06) (Fig. 3).

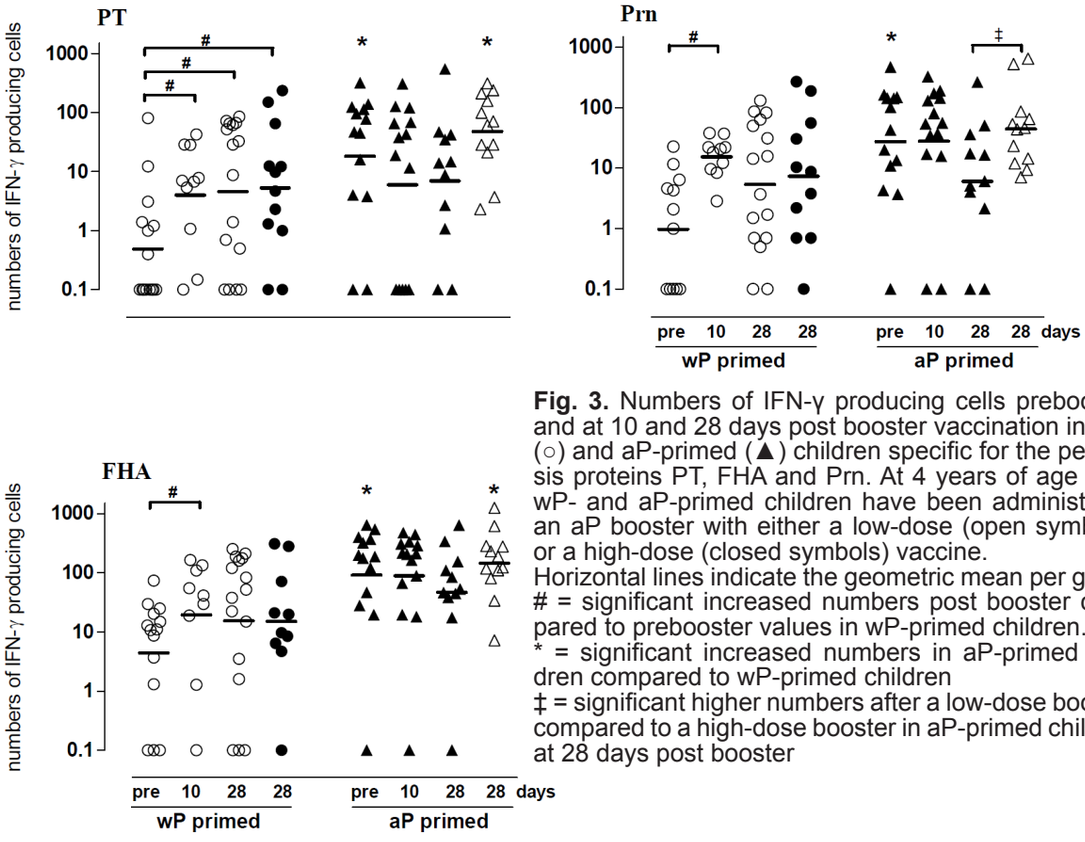

Fig. 3. Numbers of IFN-γ producing cells prebooster and at 10 and 28 days post booster vaccination in wP- (○) and aP-primed (▲) children specific for the pertussis proteins PT, FHA and Prn. At 4 years of age both wP- and aP-primed children have been administered an aP booster with either a low-dose (open symbols) or a high-dose (closed symbols) vaccine.
Horizontal lines indicate the geometric mean per group
= significant increased numbers post booster compared to prebooster values in wP-primed children.
* = significant increased numbers in aP-primed children compared to wP-primed children
‡ = significant higher numbers after a low-dose booster compared to a high-dose booster in aP-primed children at 28 days post booster

Correlations of cytokine levels

Significant correlations were found for the concentrations of the Th2 cytokines IL-5 with IL-13 (R=0.81), for the Th1 cytokines IFN-γ with TNF-α (R=0.45) and even for the Th17 (IL-17) response with that of IFN-γ (R= 0.64) using the values (>10 pg/ml) of all wP- and aP-primed children in this study (Fig. 4). Additionally, the correlation of the Th1 cytokines IL-2, measured at 24 hrs, with IFN-γ at day 5 was similar (R=0.48). The correlation between Th1 and Th2 cytokines mutually was lower (data not shown).

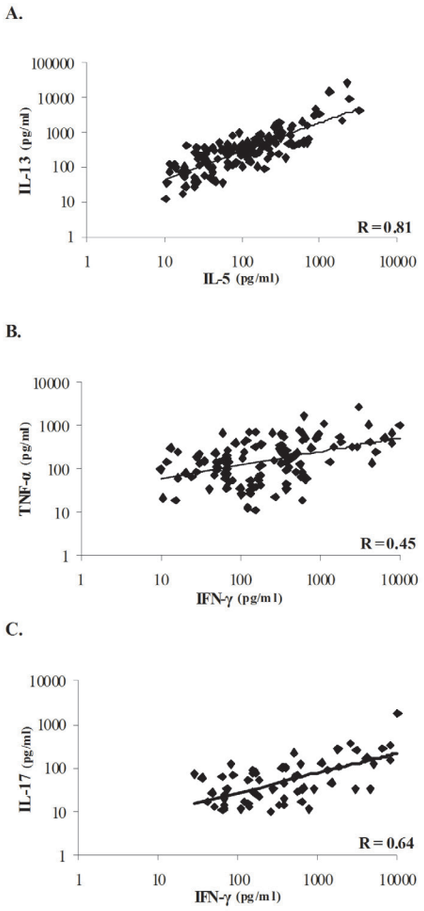

Fig. 4. Correlation between concentrations of
A) The Th2 cytokines IL-5 and IL-13
B) The Th1 cytokines IFN-γ and TNF-α
C) The Th1 cytokine IFN-γ with the Th17 cytokine IL-17 present in T-cell culture supernatants of all wP- and aP-primed children used in this study.

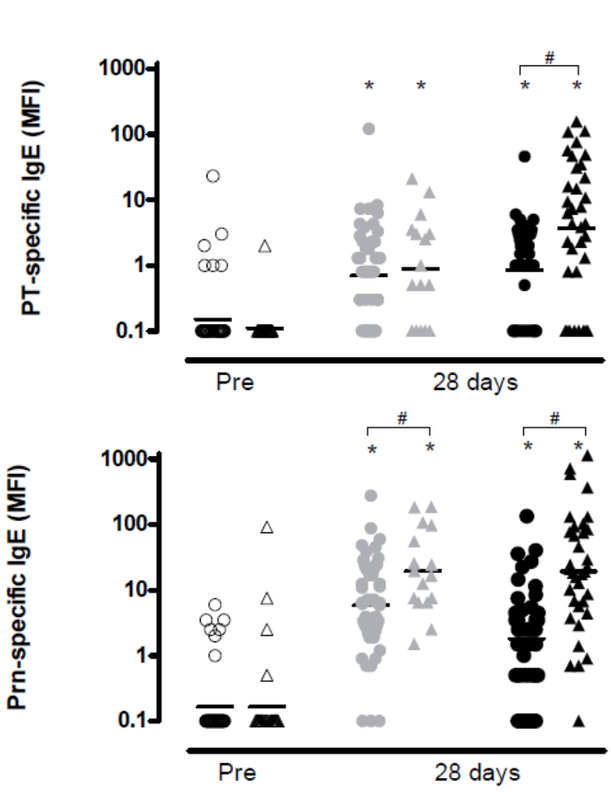

Fig. 5. A) PT- and **B)** Prn-specific plasma IgE-values in wP- (○) and aP-primed (Δ) children pre booster (open symbols) (n=46 and n=32) and at 28 days after a low-dose booster (gray symbols) (n=46 and n=16) or a high-dose booster (black symbols) (n=52 and n=35). A horizontal line indicates the geometric mean per group.
* = Significant increased values post booster compared to prebooster
# = Significant increased values in aP-primed children compared to wP-primed children.

Differences in plasma pertussis-specific IgE levels of wP- and aP-primed children

In addition to pertussis-specific IgG levels that were previously reported (6) we also evaluated pertussis-specific IgE values upon the preschool booster vaccinations of 4 years old children. Pertussis-specific IgE values were similar in wP- and aP-primed children before booster vaccination (Fig. 5). Both PT- and Prn-specific IgE values increased after either a low-dose or a high-dose booster vaccination in aP-primed and wP-primed children (Fig. 5). However, in aP-primed children IgE values specific for Prn were significantly higher after both booster doses ($p=0.01$ and < 0.0001, respectively) compared with wP-primed children and this was also the case for the IgE values specific for PT after a high-dose booster ($p = 0.001$). Total IgE values were also higher in aP-primed children than in wP-primed children after a low-dose or a high-dose booster vaccination (data not shown).

Discussion

We demonstrated that infant vaccinations with high-dose acellular pertussis vaccines resulted in high pertussis antigen-specific Th1 and Th2 T-cell responses that persisted in children at least until 4 years of age in aP-primed children, despite waning IgG levels. Importantly, a fifth aP preschool booster vaccine at 4 years of age did not show a typical memory response by increasing these T-cell responses. In contrast, an aP booster in wP-primed children did induce memory T-cell responses. Apart from inducing high IgG levels (13), the fifth aP vaccine elevated IgE-antibody values, which are associated with Th2 and atopic responses. Moreover, pertussis-specific IgE values were increased in aP-primed children after both low- and high-dose preschool booster vaccination.

Still limited information on T-cell cytokine responses in young children is available since most studies that evaluated pediatric DTaP-IPV-Hib(HebB) and adult TdaP vaccines have focused on antibody responses against the different vaccine components (13, 21, 24, 25). Pertussis-specific antibody levels found in the 4 years old children in this study (13) vaccinated at 2, 3, 4 and 11 months of age were comparable with data from French children of the same age (12) vaccinated at 2, 4 and 18 months of age. In both countries the pre-booster anti-PT antibody levels were similarly low in 4 years old children and those specific for Prn- and FHA were higher after aP vaccination than found after wP vaccination (12, 13). The comparison of the few studies on cell-mediated immune responses against pertussis and the replacement of wP- with aP-vaccines is complicated since different vaccines, varying vaccination schedules and different age groups have been used (1, 5, 12, 23, 32, 34). Similar Th1 responses have been found before and 5 weeks post booster in 4-6 years old aP-primed children, whereas increased Th2 responses were found after the 4th aP vaccination (34). However, we did not found such an increase after a 5th aP booster vaccine. This discrepancy probably results from the additional booster given after the primary series in the first year of life in our children, which might explain the already higher levels of prebooster IL-5 at 4 years of age. These IL-5 levels resemble the data of 4-6 years old French aP-vaccinated children, although their IFN-γ data were higher. This can be explained by the comparison of mean values with geomean values in combination with a high variation between samples. The higher cytokine levels in French wP-vaccinated children could have been induced by the use of a different wP vaccine in France compared to the one used in the Netherlands (12).

The immunological basis of long-term vaccine induced protection against pertussis is not clear yet. In the present study, we determined pertussis-specific Th1, Th2, Th17 and T-regulatory cytokine responses three years after infant aP or wP vaccinations but also shortly after a preschool booster with two aP vaccines containing different doses of proteins. After a fifth aP vaccination in our study, the already high levels of all T-cell cytokines remained elevated, whereas the IFN-γ levels and the numbers of IFN-γ producing cells even slightly declined. The results of the IFN-γ producing cells confirm the kinetics of the Th1 responses found in T-cell supernatants corroborating the differences in pre- and post booster T-cell responses between wP- and aP-primed children. Surprisingly, we did not found a typical T-cell memory response by an increase in cytokine production shortly after a fifth high-dose aP vaccine. This might suggest that the T-cell responses were still high enough to assist the T-cell dependent recall antibody production upon an aP booster in aP-primed children, which has resulted in significantly increased post booster antibody levels (13). Although we were not able to study the effects of a high-dose aP booster in wP-primed children, we clearly showed that even a low-dose aP booster vaccine did increase the post booster T-cell cytokine responses in these children at 10 days post booster. It can be expected that a high-dose aP booster vaccine will induce the same kinetics as a low-dose vaccine.

In aP-primed children, we did observe slightly higher Th2 responses compared to Th1 responses and low levels of IL-10 and IL-17 compared with the Th1 and Th2 cytokines. Although IL-17 plays a role in protection against intracellular pathogens (10) and in protection against *Bordetella* in mice (16), we do not know much about IL-17 induction in vaccinated children. In English infants vaccinated with BCG also low IL-17 and Il-10 levels were found, whereas in infants from Malawi higher levels of these cytokines have been shown suggesting that this is influenced by the genetic background (20). In pertussis infected adults however, we did measure higher levels of IL-17 (about 100-300 pg/ml) by using the same method as in the present study (36) (R.-M. Schure, K. Öztürk, L. de Rond, E.A.M. Sanders, G.A.M. Berbers, and A.M. Buisman, unpublished data). Anyway, the exact mechanism of T-cell responsiveness or regulation in children is rather complex and needs to be subject of further investigation.

After the fifth aP vaccination at 4 years of age the number of children reporting various side effects has doubled in comparison with that after an aP booster in wP-primed children. The local adverse effects range from non-tender redness (<5 cm) to severe swelling (>5cm) at the injection site (18). Moreover, the aP-primed children in this study showed increased pertussis-specific IgE levels after both a low-dose and a high-dose booster vaccine. This finding is in agreement with other studies (11, 34), however the clinical relevance of these increased IgE responses is not clear. Since antihistamine was not effective in treating the adverse effects, the time course of these symptoms (mostly occurring within one or two days after vaccination (30)) reflects the possibility of a delayed-type hypersensitivity reaction caused by T-cells and macrophages (29). We speculate that the four infant high-dose aP vaccinations might be responsible for the high prebooster Th1 and Th2 responses. The preschool booster vaccination additive to ongoing T-cell immunity may explain the adverse local reactions, which have been associated with Th2 responses by Rowe et al. (33) and T-cell responses by Scheifele et al. (35).

Further research is necessary to assess the direct association between these high T-cell responses and local side effects and at the moment a study that recruits samples of children who actually report the local side effects is ongoing.

Our findings are in agreement with other studies (1, 23), showing that memory T-cells persist for years while antibody responses wane rapidly after vaccination with aP or wP. This strongly suggests that memory T-cells might play an important role in pertussis-specific long-term immunity. The T-cell responses in aP-primed children can be induced by vaccination and natural boosting due to the high circulation of pertussis in the population. However, since wP-primed children showed much lower T-cell responses than aP-primed children of 4 years of age and the incidence of pertussis during the inclusion period of both groups was constant, the high T-cell responses were most likely induced by the aP vaccines administered early in life. The immune system of young infants is still under development and therefore very sensitive to stimulation. We propose that the high doses of proteins in the aP vaccines administered repeatedly within a short period of time, at 2, 3, and 4 months of age followed by a booster 7 months later, initiate high T-cell responses, which are maintained till even three years later.

The aP-priming at infant age in combination with a preschool aP-booster also induced high serum antibody levels (13) and memory B-cell responses (14). These responses together with epidemiological data indicate that protection against pertussis has improved in children after replacing the Dutch wP vaccine with aP vaccines. Since the effectiveness of vaccination is normally evaluated by antibody levels alone, we would like to stress that monitoring T-cell immunity in follow-up studies is necessary to better elucidate the efficacy and safety of infant vaccines. Moreover, the aP vaccines not only contain purified proteins but also an adjuvant probing the immune system to a Th2 response. In order to improve the immune response induced by aP vaccines in the future, the nature of the vaccine, the adjuvants and the vaccination intervals need to be reconsidered. A vaccination schedule of 2, 4 and 6 months is employed by a third of the European countries (euvac.net), but also by the USA, Canada and the South American countries. Therefore, we also recommend a more dispersed aP vaccination schedule starting at 2 months of age in the Netherlands. A better understanding of T-cell responses and their effectivity upon vaccination could result in a further improvement of the pertussis vaccination schedules for infants. The improved schedules should stimulate the immune system of infants in a better way to protect against pertussis.

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Identification of pertussis specific effector memory T-cells in preschool children

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Abstract

Whooping cough remains a worldwide problem even in countries that have implemented extensive pertussis vaccination programs. Since next to antibodies, cellular immunity plays a role in long-term protection against pertussis, we studied pertussis-specific T-cell responses in children 4 years of age. We phenotypically and functionally characterized memory T-cells in preschool children.

All children were primed during infancy three times before the age of six months and a booster at 11 months of age with either the acellular pertussis (aP) or the whole cell pertussis (wP) vaccine and received an aP booster vaccine at 4 years of age. PBMCs were isolated and stimulated with pertussis-vaccine antigens for 5 days. T-cells were characterized by cell-staining for CFSE, CD4, CD3, CD45RA, CCR7, IFN-γ, and TNF-α.

Before the aP booster vaccination at 4 years of age, both the proliferated CD4+ and CD8+ T-cell fraction ($CFSE^{dim}$) was higher in aP- compared to wP-primed children. Post-booster, more pertussis-specific CD4+ effector memory cells (CD45RA-CCR7-) were induced in aP-primed children compared to those primed with wP. Although the percentages of Th1 cytokine producing cells were alike in aP and wP-primed children before boosting, aP-primed children showed a higher Th1 cytokine production due to higher numbers of proliferated pertussis-specific effector memory cells. Infant vaccinations with four aP vaccines in the first year of life result in pertussis-specific CD4+ and CD8+ effector memory T-cell responses that persist in children until 4 years of age and are higher than in wP-primed children. The preschool booster vaccination at 4 years of age did not appear to significantly affect the T-cell phenotypes and functionality neither in aP-primed children nor in wP-primed children.

At present all children receive four high-dose aP vaccines in infancy. The need for a repeat booster already at 4 years of age is therefore questionable and may be postponed to a later age.

Introduction

Whooping cough remains a worldwide problem in high income countries despite high pertussis vaccination coverage. Already since the 1990s acellular pertussis vaccines (aP) have been implemented in the immunisation programmes to replace whole cell vaccines (wP) in many countries. In the last decade, several studies have shown that the immunity to pertussis will wane within several years after primary wP or aP vaccinations but also after the subsequent aP booster vaccinations at preschool age [1-3]. In the Netherlands, three-yearly peaks in the incidence of whooping cough are observed since 1996 [4, 5]. Since 2001 preschool children in the Netherlands have been boosted with an aP vaccine at 4 years of age. In the beginning of 2005 the Dutch wP vaccine administered at infant age was replaced by an aP vaccine. Nowadays, Dutch infants are immunized at 2, 3, 4 and 11 months and boosted at 4 years of age with a high dose aP vaccine.

Remarkably, in 2012 an enormous rise in pertussis disease was observed starting at 8 years of age and in teenagers and young adults. This unexpected rise in pertussis was not restricted to the Netherlands, but also was observed in many other countries worldwide [1, 6]. It is known that antibodies to the different pertussis vaccine components wane within 2 years both after wP and aP infant vaccinations [3, 7-11]. We have found that the priming vaccination history in infancy also influences the pertussis-specific memory response, resulting in higher memory B-cell responses in aP-primed children compared to wP-primed children [12]. This suggests a different effect of aP and wP vaccines on B-cell memory immunity.

Besides the memory B-cell response, T-cell immune responses play an important role in the maintenance of immunological memory and may be relevant for clinical protection to pertussis [13, 14]. We have demonstrated that aP immunized children still show high pertussis-specific T-cell responses at 4 years of age just before the preschool booster. Surprisingly, these responses did not increase after booster vaccination despite a further rise in antibody levels. However, in wP-primed children the booster vaccination induced a rise in T-cell memory responses [15].

We now have further characterized these memory T-cells phenotypically and functionally. Different subsets of T-cells have been identified based on expression patterns of CD45RA and the chemokine receptor CCR7 [16, 17] starting with the CD45RA+CCR7+ naïve T-cells. The CD45RA−CCR7+ were described as central memory T-cells (T_{CM}) which have the capacity to proliferate and differentiate to CD45RA−CCR7− effector memory T-cells (T_{EM}) in response to antigenic stimulation. The CD45RA+CCR7− terminally differentiated T-cells (T_{TD}) were defined as the most differentiated T-cells, still capable to produce cytokines [16-18].

The aim of the present study was to improve the insight in the immunological T-cell expression patterns of proliferated CD4+ and CD8+ T-cells generated by pertussis vaccine antigens to show potential differences between aP and wP primed children. T-cells were characterized by cell staining for CD3, CD4, proliferation (CFSE), the memory markers CD45RA, CCR7 and the intracellular cytokines IFN-γ, TNF-α. In this way, the induction of T-cell memory immunity was studied just before and 10 days after the aP preschool booster vaccination in children 4 years of age primed with either aP or wP in their first year of life.

Subjects and Methods

2.1 Study population

In this study, T-cells of children 4 years of age were analysed. The children were a subset of a cross-sectional observational study in the Netherlands (ISRCTN65428640), which aimed to investigate pertussis-specific immunity in children 3 to 9 years of age. The cohorts of 4-year-old children were enrolled in 2007 and 2008 and children were either wP or aP primed in infancy. The pertussis vaccine-specific IgG antibody and the T-cell cytokine responses in these 4-year-old children have been published previously [7, 15]. Now, we evaluated T-cell expression patterns in a randomly selected subset of these children (n=27).

As previously described [7], we divided the children in 4 different groups, according to the vaccination history in infancy (aP- or wP-priming in the first year of life) and time of blood sampling i.e. pre-booster and 10 days post-booster vaccination (Fig. 1). This study was conducted according to the Declaration of Helsinki, Good Clinical Practice Guidelines with the approval of the relevant ethics review committee. Written informed consent was obtained from both parents or legal representatives.

2.2 Vaccines

All aP-primed children had received DTaP-IPV-Hib (Infanrix-IPV-Hib™, GlaxoSmith-Kline Biologicals S.A., Rixensart, Belgium), containing 25 µg PT, 25 µg FHA and 8 µg Prn (high-dose vaccine), at 2, 3, 4 and 11 months of age according to the Dutch National Immunisation Programme. All wP primed children had received DTwP-IPV-Hib (NVI, Bilthoven, the Netherlands) at the same age. At 4 years of age, the aP-primed children received a high-dose preschool booster vaccine Infanrix™, and the wP-primed children received a low-dose preschool booster vaccine, Triaxis™ (Sanofi Pasteur, Lille, France), containing 2.5 µg PT, 5 µg FHA, 3 µg Prn and 5 µg fimbriae type 2 and 3 (Fig. 1).

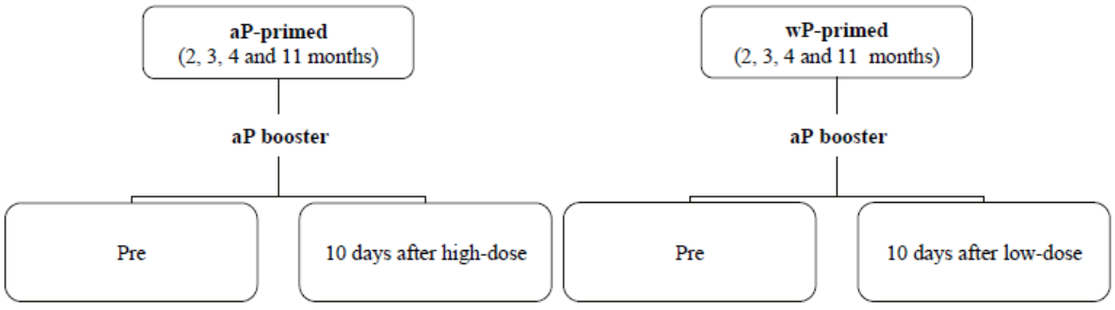


Fig. 1. Four different groups of children were used in this study. Children primed with either aP or wP vaccine at 2, 3, 4 and 11 months of age received an aP booster vaccine at 4 years age. The aP-primed group received a high-dose and wP-primed group a low-dose vaccine. The groups of children were studied pre-booster and at 10 days post-booster. Numbers of individuals used varied in the different stimulations as indicated.

2.3 8-colour FACS analysis

PBMCs were isolated from blood as described earlier and frozen [19]. After thawing, $1x10^6$ PBMCs were stained with 5 µM CFSE for 10 minutes in the dark at 4 ºC to measure proliferation of cells. After washing the PBMCs, cells were cultured in AIMV medium (Gibco Invitrogen, Grand Island, NY) containing 5% human AB serum (Harlan Laboratories,

Leicestershire, UK). The cells were stimulated with 5 µg/ml PT or 10 µg/ml FHA (Novartis, Siena, Italy) or 4 µg/ml recombinant Prn [20] at 37 °C and 5% CO_2 in 24 well culture plates (Greiner, Invitrogen, Breda, the Netherlands) for 5 days. PT and FHA were heat inactivated at 95°C for 15 min to avoid any mitogenicity [21]. Non-stimulated cells (NS) and cells stimulated with 1 µg/ml pokeweed mitogen ((PWM) Sigma Chemicals, St. Louis, MO) served as negative and positive controls, respectively. Cells were stimulated subsequently with PWM, NS, PT, FHA and PRN if enough cells were available. Within an international collaboration, the optimal antigen concentrations for FACS analysis had been tested in preliminary experiments [21, 22]. At day 5 golgiplug (BD Biosciences, San José, CA) was added to block intracellular transport processes 4 hours before further intracellular cytokines staining. Cells were collected, washed and stained for dead cell discrimination by Aqua amine-reactive dye (Invitrogen, Paisley, Scotland, UK) and for the cell surface markers APC-H7-labelled CD4 (BD), PE-Cy7-labelled CD45RA (BD), and PE-labelled CCR7 (R&D systems, Minneapolis, MN). Subsequently, cells were resuspended in a Cytofix/Cytoperm Plus kit (BD) and stained for V450-labelled CD3 (BD), PerCP-Cy5.5-labelled TNF-α (Biolegend, San Diego, CA) and APC-labelled IFN-γ (BD). After washing, the cells were analysed using the FACS canto cytometer (BD) in combination with Diva software (version 5.2 BD) and FlowJo software (Mac-version 9.3.2., Treestar US, Ashland, OR). Proliferated ($CFSE^{dim}$) viable CD3+CD4+ and CD3+CD4- T-cell populations were futher divided in naïve (CCR7+CD45RA+), T_{CM} (CCR7+CD45RA-), T_{EM} (CCR7-CD45RA-) and T_{TD} (CCR7-CD45RA+) phenotypes and analysed for intracellular Th1 cytokine production as described recently [21, 22]. The CD3+CD4+ and CD3+CD4- T-cell populations are further described as CD4+ and CD8+ T-cells.

2.4 Flow Cytometric Data Analysis

Flow Cytometry Standard format 3.0 files were exported and data were evaluated using FlowJo software. Dead cells were excluded if stained with Aqua amine-reactive dye as showed in figure 2A. Singlets were selected using FSC-A and FSC-H and lymphocytes were gated based on SSC/FSC characteristics (Fig. 2A). Both CD4+ and CD8+ cells were gated within the viable lymphocyte singlet gate (Fig. 2A). Total CD4+ (Fig. 2B) and CD8+ (Fig. 2D) gated cells as well as proliferated cells ($CFSE^{dim}$) (Fig. 2C and 2E, respectively) were further analysed for phenotype populations (CD45RA, CCR7) and intracellular Th1 cytokine production (IFN-γ, TNF-α). Using the morphological parameters, FSC/SSC we identified the blast region of the CD4+ proliferated cells (Fig. 3A). Only samples showing both more than $1x10^4$ lymphocytes and proliferation after positive control (PWM) stimulation were included in the data analysis. Because of the limited numbers of PBMC available per sample, not all antigens could be tested in each sample.

2.5 Statistical methods

Results were expressed in median (25-75% percentile) or mean ± standard deviation of the mean (SD). The Mann-Whitney U-test was used to determine differences between groups. $p<0.05$ was considered significantly different. Correlations were compared by linear regression and by calculating the correlation coefficients (R2 values).

Results

3.1 Proliferation of pertussis antigen-specific T-cells

In general, both before and after the boosting with aP vaccine 4 years of age, the frequencies of proliferated CD4+ and CD8+ T-cells after stimulation with the 3 pertussis-specific antigens separately were higher in aP-primed children than in wP-primed children, although not significant due to low numbers of tested samples (Table 1). With regard to the various antigens used for stimulation, we found that in particular after PT stimulation, the total frequencies of proliferating T-cells of aP-primed children were even more enhanced pre-booster compared to post-booster. However, the pre-booster T-cell proliferation of aP-primed children showed a high variation after PT stimulation and concerned only 7-9 children, so the difference between pre- and post-booster children cannot be considered significant. The FHA and Prn stimulation of T-cells of aP-primed children resulted in comparable frequencies of proliferated CD4+ and CD8+ T-cells pre- and post-booster vaccination although Prn-specific T-cells showed a tendency to increase post-booster. In wP-primed children, who were boosted with a low dose aP vaccine at 4 years, all pertussis stimulations resulted in similar T-cell proliferation pre- and post-booster. The frequencies of proliferated CD8+ T-cells were highly comparable to CD4+ T-cells after pertussis stimulation at all time points in both groups (Table 1).

TABLE 1

Proliferation of $CD4^+$ and $CD8^+$ T-cells determined by CFSE staining

	pre-booster				10 days post-booster			
	aP-primed (n=7-9)	n	wP-primed (n=2-6)	n	aP-primed Infranrix™ (n=6-7)	n	wP-primed Triaxis™ (n=4-5)	n
$CD4^+$								
PT	14.5 (5.2-18.5)	7	1.3 (0.4-10.2)	6	3.9 (1.5-7.9)	6	0.72 (0.06-3.5)	4
FHA	3.0 (1.0-9.2)	9	0.30 0.001-5.0)	5	4.7 (1.7-8.8)	7	1.7 (0.2-4.2)	5
Prn	8.9 (3.9-12.6)	7	0.09 (0.001-0.19)	2	5.8 (2.5-12.5)	6	1.5 (1.3-3.0)	4
$CD8^+$								
PT	15.6 (8.0-27.1)	7	1.6 (0.3-7.4)	6	5.1 (3.3-12.0)	6	1.6 (0.3-3.9)	4
FHA	2.0 (0.8-7.8)	9	0.20 (0.05-5.3)	5	2.6 (0.7-7.6)	7	0.60 (0.001-3.0)	5
Prn	8.1 (2.2-14.1)	7	0.08 (0.001-0.16)	2	3.9 (1.6-7.5)	6	1.7 (1.0-2.3)	4

Children 4 years of age were vaccinated with either Infranrix™ (high-dose) or Triaxis™ (low-dose) aP booster vaccine. PBMCs have been stimulated for 5 days with PT, FHA or Prn. Per sample, the non-stimulated T-cell proliferation data were subtracted. Data are presented as median (25-75% percentile).

3.2 Phenotypic characterisation of pertussis antigen-specific T-cells

We determined the distribution of the T-cell phenotypes of proliferated $CD4+CFSE^{dim}$ T-cells both pre-booster and at 10 days post-booster. The results of three representative subjects 4 years of age primed with either aP or wP is illustrated in Fig. 4. Overall, the non-stimulated (NS) samples in all four different groups consisted of high amounts of naïve T-cells. Upon pertussis specific stimulation T-cells of aP-primed children displayed higher proportions of T_{CM}, especially T_{EM} and T_{TD} cells compared to the control non-stimulated cells (Fig. 4A/B).

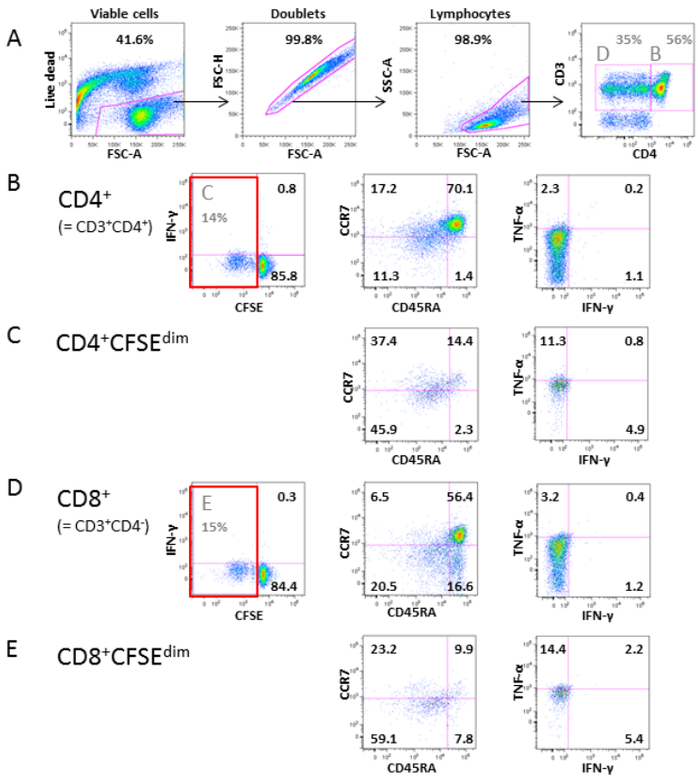
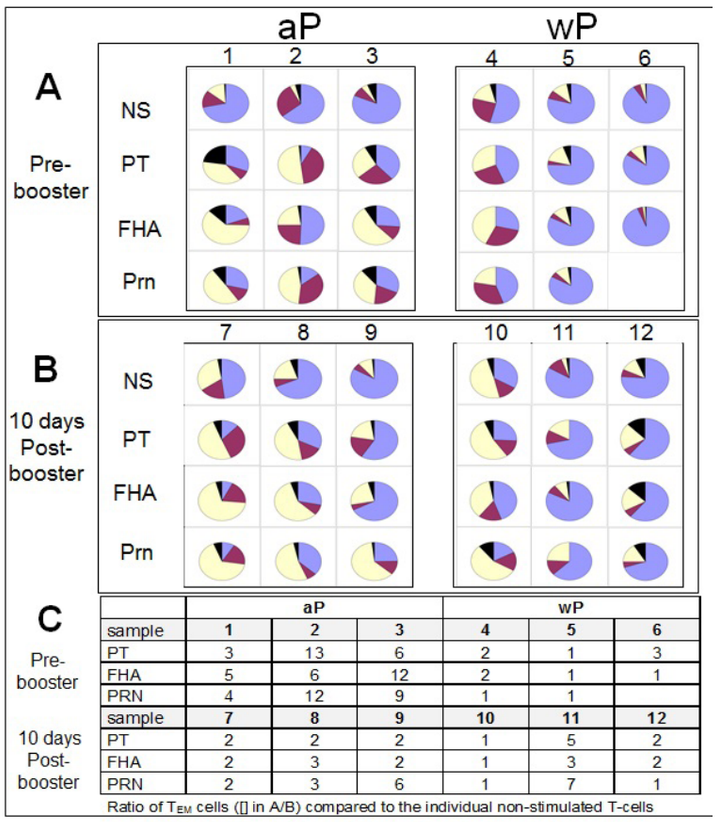


Fig. 2. Example of the 8 color FACS analysis of T-cells stimulated with pertussis antigen.
(A) Viable cells were gated, doublet cells excluded and lymphocytes were selected using forward scatter (FSC-A) and side scatter (SSC-A), subsequently CD4+ and CD8+ cells were gated. Total **(B)** CD4+ and **(D)** CD8+ gated cells as well as **(C)** proliferated ($CFSE^{dim}$) CD4+ and **(E)** proliferated ($CFSE^{dim}$) CD8+ gated cells were further characterized by CCR7 and CD45RA surface markers and analyzed for intracellular Th1 cytokine production of IFN-γ and TNF-α.



C

	aP			wP		
sample	1	2	3	4	5	6
PT	3	13	6	2	1	3
FHA	5	6	12	2	1	1
PRN	4	12	9	1	1	
sample	7	8	9	10	11	12
PT	2	2	2	1	5	2
FHA	2	3	2	1	3	2
PRN	2	3	6	1	7	1

Ratio of T_{EM} cells ([] in A/B) compared to the individual non-stimulated T-cells

Fig. 4. Phenotypes of proliferated ($CFSE^{dim}$) CD4+ T-cells of three individual aP- and wP-primed children sampled **(A)** pre- and **(B)** 10 days post-booster are presented. The proportion of naïve (CCR7+CD45RA+) ▉, central memory (T_{CM}) (CCR7+CD45RA-) ▉, effector memory (T_{EM}) (CCR7-CD45RA-) [] and terminally differentiated T-cells (T_{TD}) (CCR7-CD45RA+) ▉ specific for PT, FHA and PRN antigen is shown. **(C)** The ratio of the proportion of proliferated ($CFSE^{dim}$) T^{EM} cells ([] in A/B) related to the individual non-stimulated (NS) proliferated ($CFSE^{dim}$) T-cells is shown.

In contrast, T-cells phenotypes of wP-primed children before the booster did not really change upon the stimulation (Fig. 4A). The distribution of the T-cell phenotypes of either aP- or wP-primed children did not really change upon booster vaccination (Fig. 4B).

Within a European collaboration the T-cell proliferation have been determined by either identifying the blast region using the morphological parameters FSC/SSC or by staining for CFSE [21] (Fig. 3A/B). Pre-booster a good correlation (R2 = 0.95) between the blasts and the CFSE^{dim} CD4+ T-cells of the aP-primed children was found (Fig. 3C) and a lower correlation was found with the T-cells of the wP-primed children (R2 = 0.61) (Fig. 3D). This was caused by the lower cell proliferation of some samples. The post-booster correlation of these two parameters was similar (R2 = 0.75) for aP- and wP-primed children.

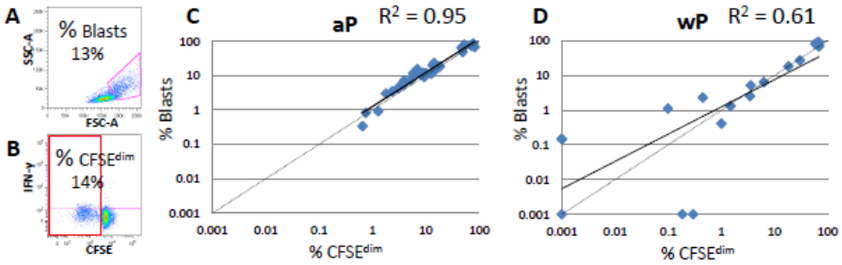


Fig. 3 A/B. Representative example of pertussis-specific T-cell proliferation determined by FACS analysis of (A) the blast region of T-cells using the morphological parameters FSC/SSC or (B) the CFSE^{dim} frequencies of T-cells.

C/D. Correlation of proliferated T-cells identified by the blasts region with proliferated T-cells determined by the CFSE^{dim} frequencies of (C) aP and (D) wP-primed children 4 years of age before booster vaccination. Per sample, data of non-stimulated (NS) proliferated cells were subtracted from those of the pertussis-specific stimulated cells.

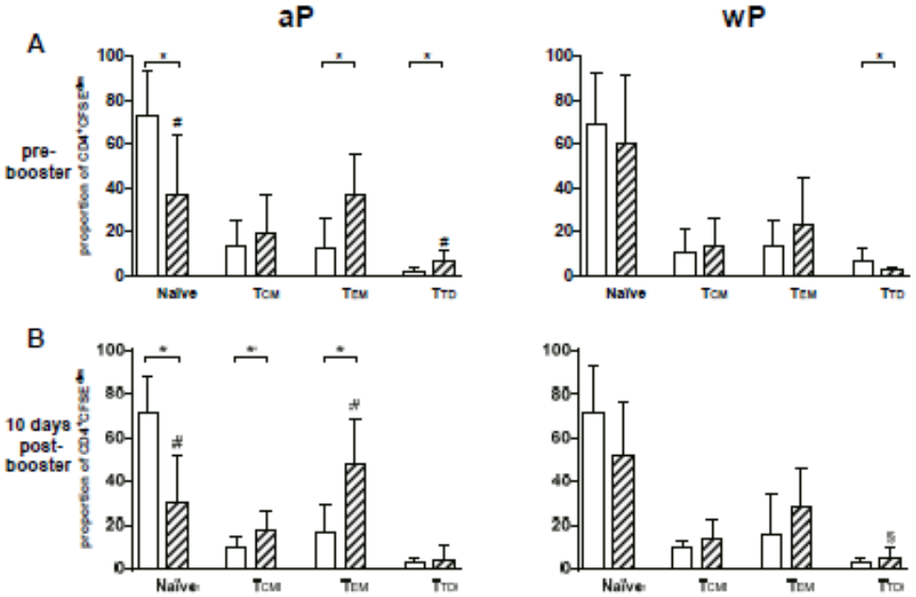


Fig. 5. Phenotype of proliferated (CFSE^{dim}) CD4+ T-cells of aP-primed and wP-primed children, (A) pre- and (B) 10 days post-booster vaccination at 4 years of age. Data are presented in proportions of naïve (CCR7+CD45RA+), central memory (T_{CM}) (CCR7+CD45RA-), effector memory (T_{EM}) (CCR7-CD45RA-) and terminally differentiated (T_{TD}) (CCR7-CD45RA+) T-cell phenotypes. Bars represent mean and sd for non-stimulated (NS) (open bars) and pertussis stimulated T-cells (hatched bars). Pertussis stimulation is the mean of the three pertussis antigen (PT, FHA and Prn) stimulation data, n=13-23 as indicated in more detail in table 1.
* = significant difference between non-stimulated and pertussis-stimulated cells.
# = significant difference between aP- and wP-primed children.
§ = significant difference between pre- and post booster T-cells of wP-primed children.

The ratio in proportion of T_{EM} cells after stimulation with pertussis antigen related to non-stimulated cells per individual is presented in figure 4C. In aP-primed children, the proportion of T_{EM} cells before the booster showed an increase up to 13-fold whereas post-booster a 2- to 6-fold increase with less variation was observed. In wP-primed children, the proportion of T_{EM} cells before the booster at 4 years increased just up to 3-fold and was lower compared to aP-primed children. Post booster the proportion of T_{EM} cells of only one wP-primed child showed an increase up to 7-fold (Fig. 4C).

In aP-primed children, several differences between non-stimulated cells and pertussis stimulation were detected. Pre-booster, the proportions of naïve T-cells of these children declined and the T_{EM} and T_{TD} cells were significantly higher upon pertussis stimulation compared to the non-stimulated T-cells (Fig. 5A). Post-booster vaccination in aP-primed children, the proportions of T_{CM} and T_{EM} cells were significantly higher compared to the non-stimulated T-cells (Fig. 5B). Within the aP-primed children there were no significant differences in phenotype proportions between the pre- and post-booster vaccination samples.

In the pre-booster samples of wP-primed children, the only significant decrease after pertussis stimulation was the decline in number of T_{TD} cells compared to non-stimulated T-cells (Fig. 5A). Post-booster no significant differences in the T-cell distribution of these children compared to non-stimulated T-cells (Fig. 5B) were found. Between wP-primed children, there was a significant rise in pertussis specific T_{TD} cells post-booster compared to pre-booster vaccination although the proportions are very low.

Comparing the T-cell phenotypes after pertussis stimulation of aP-primed children to wP-primed children pre-booster, the proportion of naïve T-cells is lower in aP-primed children, whereas that of T_{TD} cells was higher (Fig. 5A). Post-booster the proportion of the naïve T-cells is lower and the T_{EM} cells of aP-primed children are significantly higher upon pertussis stimulation in comparison to wP-primed children (Fig. 5B).

Overall, the CD8+CFSE^{dim} T-cells and the frequencies of the different phenotypes were comparable with that of the CD4+CFSE^{dim} T-cells in both aP- and wP- primed children. Notably, all CD8+CFSE^{dim} T-cells of aP- or wP-primed children had elevated T_{TD} cells (about 20%) compared to CD4+CFSE^{dim} T-cells and a lower proportion was of the T_{CM} phenotype (about 5%) upon pertussis stimulation (data not shown).

3.3 Th1 intracellular cytokine production of pertussis antigen-specific CD4+CFSEdim and CD8+-CFSEdim T-cells

The T-cells producing intracellular IFN-γ and TNF-α have been identified in aP- and wP-primed children pre- and 10 days post-booster vaccination both in the proliferated (CFSE^{dim}) CD4+ and CD8+ T-cells (Fig. 6).

In both aP- and wP-primed children the same proportion of cytokine producing CD4+CFSE^{dim} T-cells were found before the booster. Pre-booster only a few aP-primed children had a high proportion of both CD4+ and CD8+ Th1 cytokine producing cells. Post-booster vaccination a significantly higher proportion of Th1 cytokine producing CD4+CFSE^{dim} T-cells was detected in aP-primed compared to wP-primed children. Moreover, the proportion of cytokine-producing CD4+CFSE^{dim} T-cells of aP-primed children post-booster displayed less variation than that of wP-primed children.

In general, in the same individual higher proportions of Th1 cytokine producing CD4+ T-cells were found after pertussis antigen stimulation compared to those of the CD8+ T-cells.

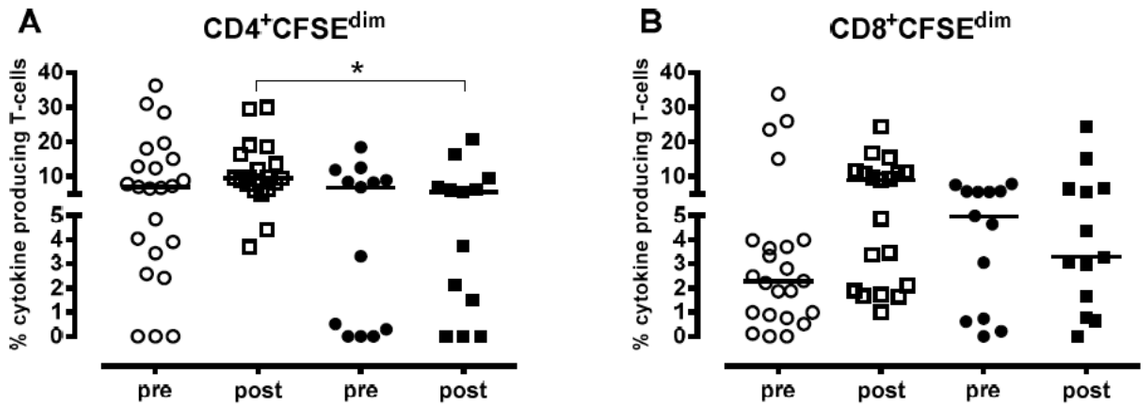


Fig. 6. Percentages of IFN-γ and/or TNF-α producing CD4+CFSE^{dim} (A) and CD8+CFSE^{dim} (B) proliferated T-cells of aP-primed (○) and wP-primed (●) children, pre- (○) and 10 days (□) post-booster vaccination at 4 years of age. Per sample, the non-stimulated (NS) data of cytokine producing T-cells were subtracted. Percentage of IFN-γ and/or TNF-α producing proliferated T-cells is the mean of the three pertussis antigen (PT, FHA and Prn) stimulation data. Horizontal lines indicate the median per group. * = $p \leq 0.05$.

Discussion

We investigated possible differences in T-cell phenotypes of proliferated CD4+ and CD8+ T-cells specific for pertussis vaccine antigens in pre- and post-booster vaccination samples of children 4 years of age who have been primed with either aP or wP in their first year of life.

We demonstrated that both the CD4+ and the CD8+ pertussis-specific T-cells of aP-primed children proliferated more than those of wP-primed children before the preschool booster. Moreover, priming with four acellular infant vaccinations in the first year of life resulted in a higher proportion of effector memory T-cells already before the preschool booster compared to wP-primed children at 4 years of age. The T-cells of aP-primed children contained more effector memory T-cells and terminally differentiated T-cells compared to that of wP-primed children. In contrast, the proliferated T-cells of wP-primed children still had a significant higher amount of naïve T-cells upon pertussis stimulation compared to aP-primed children both pre and post booster vaccination. Although the percentages of Th1 cytokine excreting cells were almost similar in both groups of children, the total amount of the accumulated Th1 cytokines is higher in aP-primed children already before the booster because of the higher total numbers of proliferated T-cells producing these cytokines. The preschool booster vaccination at 4 years of age appeared not to significantly affect the T-cell phenotypes and functionality neither in aP-primed children nor in wP-primed children.

In this study, we focused on proliferated T-cells since these cells are mainly involved in the development of T_{CM}, T_{EM} and T_{TD} cells as agreed upon within a European collaboration network. Our results showing a clear proliferation of CD4+ and CD8+ T-cells of aP-primed children upon pertussis stimulation are in agreement with studies who showed that CD4+ and CD8+ T-cells are involved in the immune response against pertussis [13, 21, 23]. Moreover, the observation that pertussis-specific T_{CM} cells were more prevalent in CD4+ compared to

CD8+ T-cells and that the relative proportions of T_{CM} and T_{EM} cells do not change after a booster immunization is in line with other studies on T-cell phenotypes [16, 24]. In general, we confirmed the presence of higher proportions of T_{TD} cells in non-stimulated CD8+ T-cells compared to CD4 + cells both in wP and aP-primed children [16, 24].

Pertussis specific T-cell phenotypes have been studied in relation to the T-cell activation status after vaccination in other age-groups [13, 14]. Sharma et al. found that pertussis-specific T-cell responses of infants who have received 3 doses of DTaP vaccine were restricted to T_{CM} CD4+ T-cells and that adults had more fully differentiated pertussis-specific CD4+ T-cells compared to infants due to multiple vaccinations [14]. We earlier have demonstrated that children 9 years of age showed higher numbers of pertussis-specific T_{EM} cells compared to children 4 years of age due to the preschool booster vaccination in combination with the high circulation of pertussis [25]. So, specific T-cells are able to differentiate further upon pertussis vaccination and infection.

Other groups have also reported higher cell proliferation responses in aP versus wP vaccinated children although they studied children of different ages and at other time-points after immunization [8, 26]. Since the optimum response of antigen-specific human CD4+ T cells following re-immunization lies between 5 and 15 days after vaccination [27], the 10-days post-booster T-cell responses in the vaccinated groups of children were analysed in this study. Both CD4+ and CD8+ T-cells of aP- or wP-primed children contain pertussis-specific T-cells producing IFN-γ and/or TNF-α before and 10 days after the preschool booster vaccination at 4 years of age. Some studies indicate that cytokine production is dependent of the kind of T-cell subset involved and that naïve T-cells can also produce cytokines IFN-γ and TNF-α but at a lower level than T_{CM} and T_{EM} cells [16-18, 28]. Because of the limited numbers of cells available, we could not determinate the single IFN-γ or TNF-α cytokine producing T-cells.

We have already shown by measuring cytokine profiles in the supernatants of T-cell cultures [15] that most of the prebooster Th1 cytokine responses specific for all three pertussis proteins were significantly higher in aP- than in wP-primed children. The high cytokine levels in aP-primed children remained elevated post booster and were enhanced in wP-primed children following the booster vaccination. This is in agreement with the higher proliferation of effector memory T-cells of aP-primed children producing Th1 cytokines upon pertussis specific stimulation already before the booster as shown in the present study. Altogether, the higher numbers of pertussis-specific T_{EM} cells producing Th1 cytokines in aP-primed children leads to a higher total production of these cytokines compared to wP-primed children.

Several limitations of this study need to be discussed. The aP- and wP-primed children were boosted with different aP (high-dose vs low-dose) vaccine. However, we did not find any difference in the induction of effector memory cells producing cytokines at 10 days after booster vaccination. Also, we previously showed that cytokine responses at 10 days were similar to 28 days post booster. We found that even the low-dose booster vaccine in wP-primed children did induce a T-cell memory response by increasing the T-cell cytokine responses whereas a high-dose booster in aP-primed children did not [15]. This indicates that a difference in booster vaccine dose at 4 years of age did not appear to influence the T-cell responses.

Differences in T-cell responses seem to have been induced already by the different priming vaccinations in infancy. Interestingly, in the recent peak in pertussis in Europe, USA and Australia, it appeared that priming with an aP vaccine in the first year of life induces a shorter immune protection later in childhood compared to priming with wP vaccine [29-32]. The priming and boosting may thus prove critical for long term protection.

Another limitation is that the method we used for identifying the phenotypes of pertussis-specific proliferating T-cells and their Th1 cytokine production is rather complex and the amount of cells was limited. The CFSE staining of T-cells resulted in a high loss of cells. Carollo et al. showed that the identification of pertussis-specific proliferation of T-cell blasts was comparable to that found with CFSE staining [21]. We also observed a good correlation between the numbers of proliferated cells identified by blasts or by CFSE staining. This indicates that the enumeration of blasts instead of $CFSE^{dim}$ cells is a better proliferation marker for pertussis-specific T-cells.

The FACS analysis provides the benefit of possible identification of a specific cytokine profile per T-cell phenotype. In this study we were able to measure pertussis-specific Th1 cytokine production by intracellular FACS analysis. However, for the measurement of Th1 cytokine responses at cellular levels in population studies the IFN-γ ELIspot method showed a higher sensitivity [33, 34] and is easier to handle. Because of the limited cell numbers, we were unable to assess detailed intracellular cytokines per T-cell phenotype or to determine Th2 intracellular cytokines. However, in the same groups of vaccinated children we have previously already shown Th1 as well as Th2 and Th17 responses by measuring cytokines in the supernatant of pertussis-stimulated T-cells [15].

To summarize, we demonstrated that both effector memory CD4+ and CD8+ T-cells are induced by acellular and whole cell priming vaccinations, but the aP priming led to higherT-cell immunity already before the boosting. The 5^{th} aP vaccination at age 4 years had no effect on the phenotype and functionality of these T-cells. In the present national immunisation program, infants are primed with four high-dose aP vaccine in the first year of life. Therefore, it is tempting to speculate that the pertussis specific T-cells in these aP-primed children are not boosted in an efficient way, although antibody levels and B-cell memory responses are enhanced after the aP booster. Possibly the fifth consecutive aP vaccination at 4 years of age is too early for the aP-primed children and could be better postponed to a later time point contributing to a longer protection. Also, the dosage may have impact, though we were not able to investigate this. Several other countries have implemented an aP booster at 6 years of age without an increase in clinical pertussis cases before that age, indicating that the postponement could be a good possibility [8, 35]. Moreover, better memory immunity might be induced when children are vaccinated at a later age when the immune system is more mature [36]. In the recent epidemic of 2012, however, it became clear that pertussis-specific immunity induced by a booster vaccination at 6 years of age is only short-lived in many children and the role or rather the lack of T-cell immunity needs to be carefully investigated in these cases. Priming, dosage, vaccine type (wP/aP) and timing of vaccinations all need to be taken into account in new studies aiming to improve the memory immune responses to pertussis.

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Different IgG-subclass distributions after whole-cell and acellular pertussis infant primary vaccinations in healthy and pertussis infected children

5

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Abstract

The distribution of IgG-subclasses provides insight in the immunological mechanisms of protection against whooping cough. We investigated the effect of Dutch whole-cell pertussis and acellular pertussis vaccines administered in infancy on the IgG-subclass distributions in healthy children aged 12 months, 4 years and 9 years as well as in children who have been infected with *Bordetella pertussis*.

A fluorescent bead-based multiplex immunoassay was used for the measurement of IgG1, IgG2, IgG3 and IgG4 responses against pertussis toxin, filamentous heamagglutinin and pertactin.

Although IgG1 was the predominant subclass for all pertussis antigens in both healthy and infected children, elevated IgG4 levels were only present in children who had received repeated number of acellular pertussis vaccinations. IgG2 and IgG3 antibodies did not contribute to the IgG response. No differences in IgG-subclasses between healthy vaccinated or infected children were found.

The pertussis vaccine used for priming seems to determine the IgG-subclass composition elicited after a secondary antibody response either induced by pertussis vaccination or infection. The pronounced anti-pertussis IgG4 response might reflect the Th2-skewing of the immune response after aP vaccination.

Introduction

Whooping cough is reemerging despite wide spread pertussis immunization programs in high-income countries. During the 1990s acellular pertussis (aP) vaccines have replaced whole-cell pertussis (wP) vaccines. In general, aP vaccines induce high antibody levels against the pertussis vaccine components. IgG is the most common antibody isotype in blood after vaccination and infection and consists of four IgG-subclasses. The IgG1 and IgG3 bind to monocytes and neutrophils and prevalently mediate the T-cell dependent response to proteins, whereas IgG2 is predominant in the T-cell independent response to polysaccharide antigens [1]. Moreover, IgG1 and IgG3 activate the complement system more easily than IgG2. In contrast, IgG4 uses the alternative route in the complement system and is associated with IgE-mediated allergy [2, 3]. In general, the distribution of the subclasses is relatively stable with IgG1 as the dominant subtype, followed by IgG2, IgG3 and IgG4 [3]. However, this distribution varies during an antibody response depending on age, nature and dose of the antigens, mucosal or parenteral immunization route and genetic factors of the host [1, 4]. In T-cell dependent responses, T-helper (Th) 1 and Th2 cell cytokine production influence IgG-subclass switching [5-8].

Since the protective immunological mechanisms against whooping cough are not clearly understood, the determination of the different IgG-subclass responses induced by cellular or acellular vaccination or by infection can provide useful information.

In the Netherlands the aP vaccine has replaced the Dutch wP vaccine at 2, 3, 4 and 11 months of age since 2005. Previously, we have shown differences in antibody and memory B-cell responses in children primed with either the Dutch wP or with aP vaccines [9, 10]. This indicates that wP and aP vaccines trigger the immune system differently. The aim of the present study was to compare the effect of both infant vaccinations as well as the impact of natural infection with *Bordetella pertussis* in children on the IgG1, IgG2, IgG3 and IgG4 subclass responses specific for the three *B. pertussis* vaccine antigens (pertussis toxin (PT), filamentous heamagglutinin (FHA) and pertactin (Prn)).

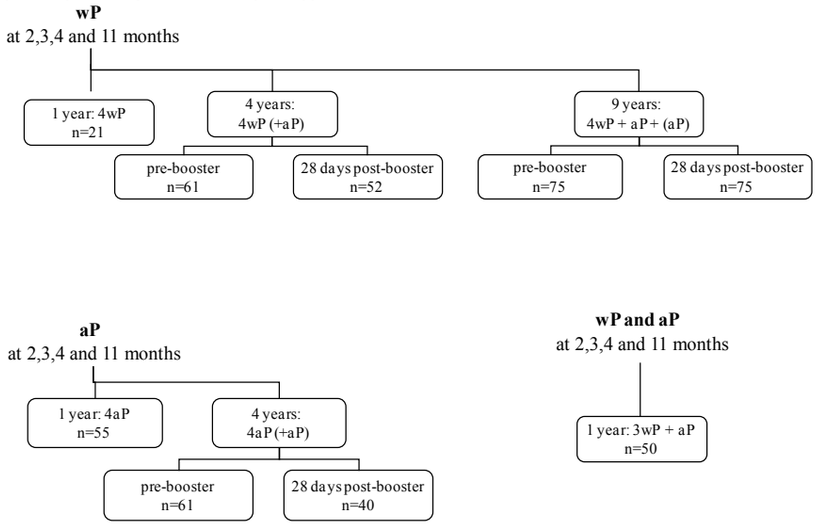


Fig. 1. The number of healthy children per age group who were primed with either Dutch wP or aP vaccines. As one subset of 1 year old children had received 3wP and 1aP vaccination.

Material and methods

Study population

Healthy children of different ages (12 months, 4 and 9 years) included in this study originated from three different cohort studies: (1) between 2004 and 2005 126 children 12 months of age were included with one blood-sample at one month after the 4th vaccination at 11 months of age (ISRCTN97785537); (2) between 2007 and 2009 214 children 4 years of age were included in whom blood was collected either before or 28 days after the preschool booster vaccination (ISRCTN65428640) [9]; (3) in 2010 75 children 9 years of age were included with paired blood samples before and one month after a second aP booster vaccination at 9 years of age (ISRCTN64117538) (figure 1).

In addition, a fourth cohort consisted of 56 children aged 2 to 17 years of age (median 7 years) who were vaccinated according to the national immunization program (NIP) and in whom high single serum anti-PT IgG levels (>100 EU/ml) and a positive polymerase chain reaction for *B. pertussis* in the nasopharyngeal or buccal swab was found, indicating a recent pertussis infection [11]. One blood sample was taken per child on average 43 days (range 7 - 119 days) after the first clinical symptoms of whooping cough.

All studies were conducted according to the Declaration of Helsinki, Good Clinical Practice Guidelines with the approval of the relevant ethics review committee. Written informed consent was obtained from parents or legal representatives before the start of the study

Vaccinations

All children had received either the Dutch whole-cell pertussis vaccine (DTwP-IPV-Hib, NVI, Bilthoven, the Netherlands) or an acellular pertussis vaccine (DTaP-IPV-Hib, Infanrix-IPV-Hib™, GlaxoSmithKline Biologicals S.A., Rixensart, Belgium) at 2, 3, 4 and 11 months of age according to NIP. The Dutch wP vaccine was replaced by an aP vaccine in 2005. Therefore, a subgroup of children 12 months of age had received 4 wP or 4 aP vaccines at 2, 3, 4 and 11 months of age and a third subgroup had received 3 wP vaccinations at 2, 3 and 4 months of age and one aP vaccination at 11 months of age as they were born in 2004, but received their fourth pertussis vaccination at 11 months of age in 2005. From 2001 onwards an additional preschool booster vaccination in children 4 years of age was implemented in the NIP. From 2001 until 2006 ACV-SB™ (acellular vaccine from Smithkline Biologicals) was administered at 4 years of age and from 2006 onwards Infanrix™ (from GSK) has replaced ACV-SB™. ACV-SB™ and Infanrix™ contained the same amounts of pertussis antigens; 25 µg of PT, 25 µg of FHA and 8 µg of Prn. For study purpose only, the children 9 years of age had received a second aP booster vaccination, Boostrix-IPV™ (GSK), containing 8 µg of PT, 8 µg of FHA and 2.5 µg of Prn. Of the 56 children who had been infected with *B. pertussis*, 52 children had been vaccinated according to the NIP (6/52 were aP-primed and 46/52 were wP-primed) and 4 children had not received any pertussis vaccination before infection.

Serological assays

For measurement of the IgG-subclasses, the validated MIA for the detection of IgG-subclasses specific for *Neisseria meningitidis* serogroup A and C polysaccharides was used with some modifications [13]; 50 µl of a 1/100 dilution of monoclonal mouse anti-human IgG1, IgG3 or IgG4 and a 1/200 dilution of monoclonal mouse anti-human IgG2 (all from Invitrogen, CA) was added to each well for 30 minutes before adding 50 µl of a 1/200 dilution of R-Phycoerythrin conjugated Goat anti-Mouse IgG (Jackson immunoresearch, UK). 8-fold serial dilutions of the reference serum were added on each plate and the reference-curves were parallel between the assays. Since the concentrations of the four IgG-subclasses against the pertussis antigens are unknown in both the FDA and the in-house reference serum, we presented the contribution of the mean fluorescent intensity (MFI) for each IgG-subclass as a percentage of the sum of MFIs of the 4 IgG-subclasses together. The concentrations of the total IgG levels against PT, FHA or Prn have previously been determined by a validated MIA [9, 12]. In this study, these total IgG levels were used to calculate the arbitrary concentration of each specific IgG-subclass per antigen and per sample by using the percentage of the specific IgG-subclasses as described above. This concentration of the IgG-subclass was expressed in arbitrary units (AU/ml). A value of 5 AU/ml was chosen as a minimal arbitrary level that indicated a positive response for each IgG-subclass.

For the measurement of serum IgE levels, we used the MIA [9, 12] with the following modifications. To prevent possible inter-immunoglobulin isotype competition, plasma samples were depleted of IgG by adding GullSORB (10:1 vol/vol) (meridian Bioscience Inc., Cincinnati, OH). In addition, 50 µl of a 1/100 dilution of Goat Anti-Human IgE with PE label (Epsilon, Fisher Scientific) was added to each well for 30 minutes.

Statistics

Anti-PT, anti-FHA and anti-Prn IgG levels as well as the levels of all subclasses were presented as geometric mean concentrations (GMCs). The distribution of the IgG-subclasses was presented as percentages with the mean and the standard deviation (±) calculated.

The Mann-Whitney-U-test was used for comparison between the different groups. A p-value of 0.05 was considered significant.

Results

IgG levels

The GMCs of the total IgG levels against PT, FHA and Prn in the different groups of children included in this study are summarized in table 1. In children who had received the aP vaccine at 2, 3, 4 and 11 months of age, significantly higher anti-pertussis IgG levels were shown at 1, 4 and 9 years of age as compared to Dutch wP-primed children of the same ages. In general, the IgG level against the pertussis vaccine antigens significantly increased with an increasing number of aP vaccinations received. After both wP and aP infant vaccinations, anti-PT, anti-FHA and anti-Prn antibodies had significantly declined at 4 years compared with 12 months of age. However in wP-primed children, similar low IgG-PT levels were found at 12

months and 4 years of age before the preschool booster vaccination, whereas only a small significant increase remained after this booster till the age of 9 years before the second aP booster vaccination. One-month after the booster at 4 or at 9 years of age significantly higher IgG levels were measured compared with pre-booster levels. In children who had been infected with *B. pertussis* GMCs for anti-PT, anti-FHA and anti-Prn were high and were 173.7 EU/ml, 180.5 EU/ml and 43.4 EU/ml respectively.

Table 1 Geometric mean concentrations of anti-PT, anti-FHA and anti-Prn total IgG levels (EU/ml) in the healthy vaccinated groups of children

Age	vaccination history	IgG-PT		IgG-FHA		IgG-Prn	
		pre	+1 month	pre	+1 month	pre	+1 month
12 months	3wP + wP	n.a.	4.8	n.a.	23.0	n.a.	41.3
	3wP + aP	n.a.	51.1*	n.a.	101.5*	n.a.	119.0*
	3aP + aP	n.a.	131.2*	n.a.	446.3*	n.a.	401.4*
4 years	4wP (+ aP)	4.5	60.7	8.8	194.7	3.1	186.7
	4aP (+ aP)	7.7*	192.3*	16.1*	518.0*	23.5*	1273.5*
9 years	4wP + aP + aP	8.1	124.8	41.1	350.0	13.0	357.1

* significant higher compared with the corresponding group of 4wP or 3wP +aP primed children
n.a. not available

Distribution of the IgG-subclasses

For each antigen, the sum of the MFI-values of the four IgG-subclasses correlated well with the original MFI-values of the total IgG responses as represented for one group of children (n=61) in figure 2. Figure 3 presents the contribution of each IgG-subclass to the sum of the four IgG-subclasses calculated per individual and now presented as mean percentages in the healthy vaccinated groups and in the infected children. IgG1 proved to be the predominant subclass in all groups, except for PT-antibodies in the pre-booster groups at 4 and 9 years of age. In these children IgG2 contributed to about a half of the total IgG levels (56% and 35% in wP and 41%in aP primed children, respectively).

However, the total anti-PT IgG levels in these groups were low (table 1). In aP-primed children the contribution of IgG4 to the sum of the 4 IgG-subclasses increased with an increasing number of aP vaccinations. After the fifth aP vaccination at 4 years of age the highest per-

centages of IgG4 were found for PT and FHA, but not for Prn. In general, IgG3 hardly contributed to the total IgG levels to any antigen. Similar to the healthy vaccinated children, the predominant subclass in infected children was IgG1 with low percentages of IgG2, 3 and 4 for all three pertussis antigens.

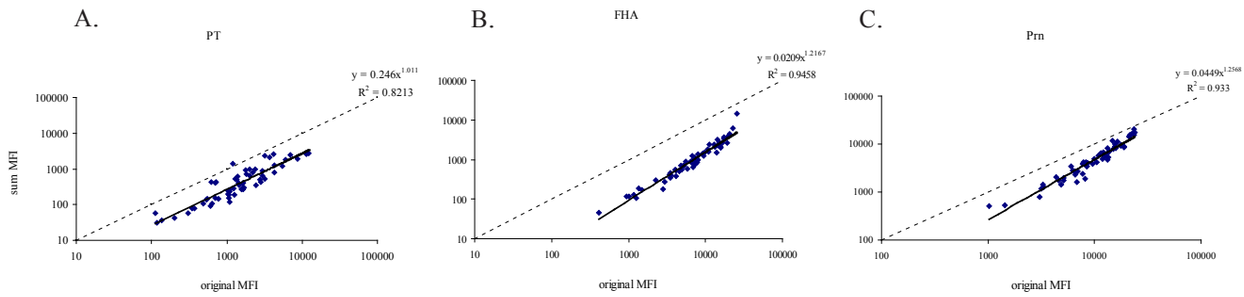


Fig. 2. Comparison of the sum of the mean fluorescent intensities (MFIs) for the ln (natural logarithm) of each IgG-subclass and the ln MFI of the total IgG against PT (A), FHA (B) or Prn (C). Linear regression and correlation coefficients (R2 values) are presented.

Table 2 Percentages of children with a positive immune response defined as a level above 5 AU/ml against PT, FHA and Prn for each IgG-subclass. Mean percentages per group of children are presented.

Age	Vaccination history	PT %				FHA %				Prn %			
		IgG1	IgG2	IgG3	IgG4	IgG1	IgG2	IgG3	IgG4	IgG1	IgG2	IgG3	IgG4
1 year	3wP +wP	43	0	0	0	95	0	0	5	100	5	5	0
	3wP +aP	100	5	13	5	100	5	33	5	100	3	5	0
	3aP +aP	100	4	2	29	100	27	35	91	100	2	4	47
4 years	4wP	8	41	0	0	46	7	0	3	16	5	3	0
	4wP +aP	88	63	8	6	100	27	15	33	98	17	15	13
	4aP	39	30	0	0	85	2	0	15	89	3	3	15
	4aP +aP	100	50	0	73	100	63	16	98	100	40	8	88
9 years	4wP +aP	40	19	0	0	93	5	1	7	69	5	4	3
	4wP +aP +aP	100	16	8	8	100	12	8	61	100	4	19	23
2-10 y.	infected	95	29	21	30	98	21	18	38	82	7	9	7

Positive immune responses of the IgG-subclasses against the pertussis antigens

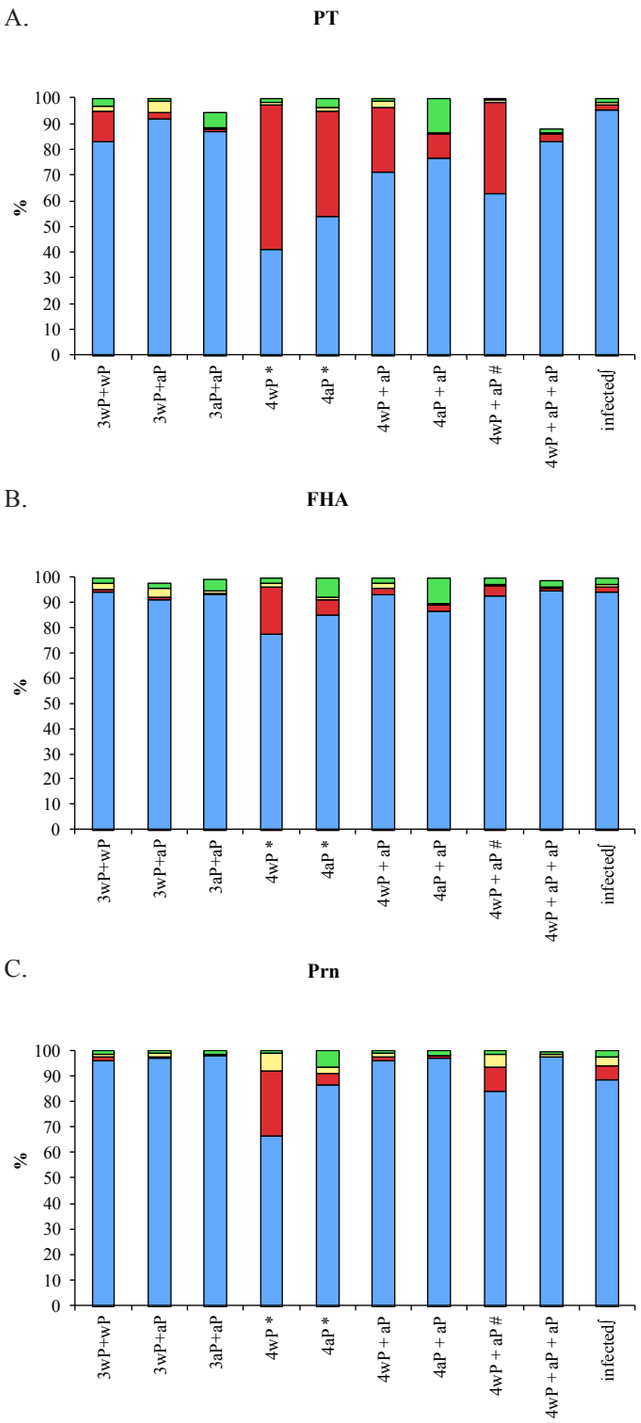
Using 5 AU/ml as an arbitrary cut-off value for a positive immune response in this study, the percentage of children with anti-PT, anti-FHA and anti-Prn levels for each IgG-subclass above this cut-off are presented in table 2.

In the majority of the children a positive IgG1 response against PT was measured. However, in wP-primed children 12 months of age and in wP- and aP-primed children 4 and 9 years of age before the booster, the IgG1-PT-specific immune response was positive in less than 40%. In almost all children positive IgG1 levels against FHA and Prn were found, except in wP-primed children at 4 years of age before the booster. In general, just a few children showed positive IgG2 and IgG3 responses against the three vaccine antigens, although around 50% of the children in the groups of wP (for PT) and aP (for PT, FHA and Prn) primed children 4 years of age after the booster showed a positive IgG2 response. Remarkably, in the majority of the aP-primed children a positive immune response for IgG4 against PT, FHA and Prn was observed one month after the aP booster at 12 months (30%, 90% and 50% respectively) and at 4 years of age (70%, 95% and 90% respectively). In contrast, in less than 10% of the children who were primed with the wP vaccine, positive IgG4 responses to any antigen were shown. Most infected children showed positive IgG1 responses to all antigens, but only modest IgG2, IgG3 and IgG4 responses.

Correlations between IgG4 and IgE levels

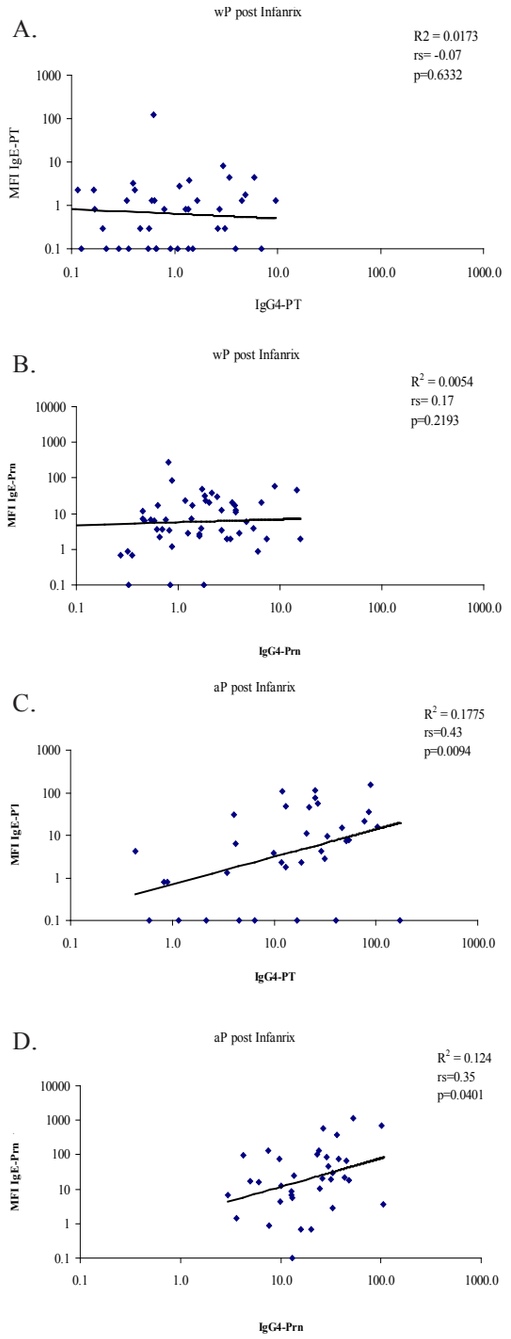
We compared IgG4 responses with total IgE levels and with PT- and Prn-specific IgE levels in the healthy vaccinated children 4 years of age. After the fifth consecutive aP booster vaccination in aP-primed children, IgE levels specific for PT and Prn significantly correlated with anti-PT and anti-Prn IgG4 levels, whereas no correlations were found after the aP booster vaccination in wP-primed children (figure 4). For PT, only 23% of the aP-primed children did not show an IgE-PT response, while positive IgG4-PT levels were observed. For Prn, all children except one showed both IgE-Prn and IgG4-Prn levels. Pre-booster IgE levels against PT and Prn did not correlate with the corresponding IgG4 levels in both wP- and aP-primed children most likely due to the very low values. Moreover, no correlations between total serum IgE levels and specific anti-PT or anti-Prn IgG4 levels were found (data not shown).

Fig. 3. Distribution of the IgG-subclasses against **(A)** PT **(B)** FHA and **(C)** Prn, presented as mean percentages in blue for IgG1, in red for IgG2, in yellow for IgG3 and in green for IgG4 and given for each vaccination group and the infected group of children.



All data are presented one month after the booster at the respectively ages (12 months, 4 years and 9 years), except for the groups indicated with the superscripts: *pre-booster group 4 years of age, # pre-booster group 9 years of age and ∫ the infected group

Fig. 4. Correlations between IgG4 levels (logarithmic x-axis) and MFI of IgE-values (logarithmic y-axis) specific for PT (A and C) and Prn (B and D) in wP- and aP-primed children 4 years of age at day 28 after the preschool booster vaccination



Discussion

The evaluation of IgG-subclass profiles after vaccination and natural infection gives insight into the still incompletely resolved immunological mechanisms that provide protection against whooping cough. In this study we demonstrate a different IgG-subclass distribution in children who had received the Dutch whole-cell pertussis vaccine in their first year of life. In general in both wP and aP primed children, IgG1 levels to the pertussis antigens were much higher than those of the three other IgG-subclasses and hardly any pertussis-specific IgG2 and IgG3 antibodies are produced in children irrespective of the vaccine used for priming. Remarkably, the level of IgG4 antibodies against pertussis seemed to increase with the number of aP vaccines received previously, which was significantly enhanced after the fifth consecutive aP vaccination in children 4 years of age. Moreover, these IgG4 responses were found to correlate well with IgE responses specific for PT and Prn. Interestingly, in children who were infected with *Bordetella pertussis*, the IgG-subclass distribution did not differ from healthy wP or aP vaccinated children.

Our data suggest that the vaccine used for priming in the first year of life influences the distribution of the IgG-subclasses against the pertussis antigens in a secondary immune response either after vaccination or natural infection with *B. pertussis*. Previous studies have shown that the high concentrations of PT, FHA and Prn in most pediatric aP vaccines elicit a Th2 type or mixed Th1/Th2 immune response [14, 15]. The wP vaccine contains less purified antigens in lower concentrations and more substances like LPS capable of inducing a Th1 associated immune response [16-18]. The pronounced IgG4-responses that correlated with PT- and Prn-specific IgE levels after a fifth consecutive aP vaccination as shown in this study provide further evidence for Th2-skewing of the immune system induced by aP vaccinations in children [3]. Preliminary results in the same group of children 4 years of age underline this Th2-skewing by also showing higher Th2 cytokine responses three years after the infant aP immunizations compared with Dutch wP vaccinations (Schure et al, submitted).

Information about IgG-subclass distributions against the pertussis vaccine antigens is scarce. Only two previous studies investigated the IgG-subclass distribution after pertussis vaccination and infection and have focused only on PT [19, 20]. In line with our results, IgG1 was the predominant subclass after both pertussis vaccination and infection in these studies. Normally, IgG4 only marginally contributes to the total IgG level (<5%), which illustrates the remarkable high contribution of IgG4 to the total IgG pool in aP primed children in our study (14% for anti-PT IgG4). This is in agreement with the findings of Giammanco et al [19] who also showed high IgG4 responses in children previously immunized with similar aP vaccines. Additionally they found elevated IgG2 levels to PT in these aP primed children, while our IgG2 levels to PT were generally low. However, we did find that IgG2 responses contributed for about 50% to the total IgG level against PT before booster vaccination in both Dutch wP and aP primed children 4 years of age and in wP primed children 9 years of age. Since the pre-booster total anti-PT IgG levels were very low, we suggest that this relative large IgG2 contribution is rather due to a decrease in IgG1 levels instead of an increase in IgG2.

Although previous studies [19-21] showed an increased anti-PT IgG3-response in naturally infected individuals, we hardly found any IgG3 response in either infected or in healthy vaccinated children. Since the majority of the infected children in our study had received pertussis vaccinations according to the NIP, this priming by vaccination might have influenced the IgG-subclass distribution after subsequent infection with *B. pertussis* leading to low IgG3 levels and predominant IgG1 responses. In contrast, in the study of Giammanco et al [19] high IgG3 responses were also measured in convalescent sera of children who had received three wP vaccinations in their first year of life. Since these children were younger (about 2 years old) than the infected children in our study than the infected children in our study (median 7 years old), age could have attributed to the differences in IgG-subclass responses as reported for anti-measles responses [22]. However, our data did not confirm age-dependency in the IgG-subclass responses specific for pertussis.

We showed differences in the distribution of the four IgG-subclasses after Dutch wP and aP vaccinations in children based on mean fluorescent intensities (MFIs), because the concentrations of the four IgG-subclasses against the pertussis antigens in international reference sera are not available. Since MFI values of the sum of the four IgG-subclasses correlated very well with the MFI value of the total IgG values, the distribution of the IgG-subclasses presented in this manner seems reliable. Moreover, similar MFI curves of the reference serum in each assay were found. Finally, (in contrast to ELISAs), the MIA is a highly accurate assay even in the lower limit of quantification, which made it possible to determine even low levels of antigen specific IgG-subclasses.

In conclusion, the pertussis vaccine used for priming seems to be important for the subsequently IgG-subclass distribution upon re-challenge either by vaccination or natural infection. In both healthy Dutch wP and aP primed children as well as in infected children, IgG1 was the predominant IgG-subclass and IgG2 and IgG3 antibodies scarcely contributed to the IgG response. The pronounced IgG4 response after five aP vaccinations correlated with an increased pertussis-specific IgE responses, which implies a different triggering of the immune system and Th2 polarization of T-cells. Since nowadays only aP vaccines are administered in children in most high-income countries, it is important to evaluate the clinical consequences of a high Th2 skewed immune response.

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Differential T- and B-Cell Responses to Pertussis in Acellular Vaccine-Primed versus Whole-Cell Vaccine-Primed Children 2 Years after Preschool Acellular Booster Vaccination

6

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Abstract

This study investigated the long-term cellular and humoral immunity against pertussis after booster vaccination of 4 years old children who had been vaccinated at 2,3,4 and 11 months of age with either a whole-cell (wP) or an acellular (aP) vaccine. Immune responses have been evaluated till 2 years after the preschool aP booster vaccination.

In a cross-sectional study (ISRCTN65428640) blood samples were taken from wP- and aP-primed children prebooster and 1 month and 2 years post-booster. Pertussis vaccine antigen-specific IgG levels, antibody avidities and IgG subclasses as well as T-cell cytokines were measured by bead-based fluorescent multiplex immunoassays. Numbers of pertussis-specific memory B-cells and IFN-γ producing T-cells were quantified by ELISPOT-assays.

Even 2 years after booster vaccination memory B-cells were still present and higher levels of pertussis-specific antibodies than prebooster were found in aP-primed children and to a lesser degree also in wP-primed children. The antibody levels mainly consisted of the IgG1 subclass but also showed an elevated IgG4 portion, primarily in the aP-primed children. The prebooster antibody avidity indices for pertussis toxin and pertactin in aP-primed children were already high and remained stable after 2 years whereas those in wP-primed children increased. All measured prebooster T-cell responses in aP-primed children were already high and remained at similar levels or even decreased during 2 years post-booster vaccination, whereas those in wP-primed children increased.

Since the Dutch wP vaccine has been replaced by aP vaccines, the induction of B-cell and T-cell memory immune responses has been enhanced, but antibody levels still wane after five aP vaccinations. Based on these long-term immune responses, the Dutch pertussis vaccination schedule might be optimized, and we discuss here several options.

Introduction

Despite a high vaccination coverage in young children since the 1940-50s, whooping cough is reemerging in high-income countries. In the Netherlands, this reemergence was noticed from 1996 onwards. Since then every 2-3 years peak incidences were observed which were most evident in children 4-5 years of age who have been vaccinated with whole cell pertussis vaccine (wP) at 2, 3, 4 and 11 months of age (1). However, the vaccine efficacy of the Dutch wP vaccine was not optimal due to low concentration of and low antibody responses to PT, FHA and Prn (2-4). Therefore, in 2001 an aP preschool booster vaccination at 4 years of age has been implemented, which shifted the age of the highest pertussis incidence towards 9 years and older (5). From 2005 onwards all primary wP vaccinations have been replaced by acellular pertussis (aP) vaccines. However, despite the implementation of aP vaccines in the industrialized world since the 1990's, this switch did not put a halt to the pertussis reemergence. Nowadays in 2012, a new pertussis incidence peak in the Netherlands is observed in adolescents and adults, who can infect not fully vaccinated newborns with high risk of severe disease and even mortality.

The immune mechanisms important for the protection against pertussis in humans still remain elusive. Protection against pertussis is probably multifactorial (6) and suggested to be mediated by both humoral (7, 8) and cell-mediated immunity (9-13). High levels of antibodies against pertussis indicate previous infection or recent vaccination and probably are associated with protection against pertussis (8, 14). In general, higher antibody levels have been observed after switching from wP to aP vaccinations, (2, 15). These antibody responses consist of different subclasses with IgG1 as the dominant subtype, followed by IgG2, IgG3 and IgG4 (16). The induction of IgG subclasses is regulated by T-cell cytokine production and is influenced by the nature and dose of the vaccine antigens as well as the age of the vaccinees (17).

Previously higher memory B-cell responses and a higher avidity of pertussis-specific antibodies in aP-primed children as compared to wP-primed children at 4 years of age were reported (3, 18), indicating a more robust humoral immune response over time after infant vaccination with aP vaccines. Additionally, the aP vaccination induced higher Th1 and Th2 T-cell responses compared with wP vaccination three years after the primary vaccination series (19).

For optimal vaccination strategies, it is important to evaluate the longevity of the pertussis-specific immune response. The aim of this study is to evaluate the long-term antibody production, memory B-cell and T-cell immune responses in children 6 years of age, 2 years after an aP preschool booster vaccination. The children had previously been vaccinated during infancy with either the Dutch wP or an aP vaccine.

Material and methods

Study population

The children described in this study represent a subset from a larger cross-sectional study (ISRCTN65428640) performed between 2007 and 2009 in the Netherlands that investi-

gated the immunity to *B. pertussis* in children 3-9 years of age. In this study, single blood samples (8-15 ml) were collected by venipuncture in two groups of wP-primed children, 4 years of age (prebooster n=61, 28 days post-booster n= 52) and one group 6 years of age (2 years post-booster n= 63) and three corresponding groups of aP-primed children of 4 and 6 years of age (prebooster n=61, 28 days n=40 and 2 years post-booster n=61, respectively) for antibody determination (Fig 1). In a randomly selected subset of these groups of children, by using samples varying in PBMC numbers from high to low values, also memory B-cells (n=11 to 19 per group) and T-cell responses (n=5 to 15) were determined. Among all groups males and females were equally divided. For each child both parents had signed an informed consent. This study was conducted according to the Declaration of Helsinki, Good Clinical Practice Guidelines with the approval of the ethics review committee (STEG-METC, Almere, the Netherlands).

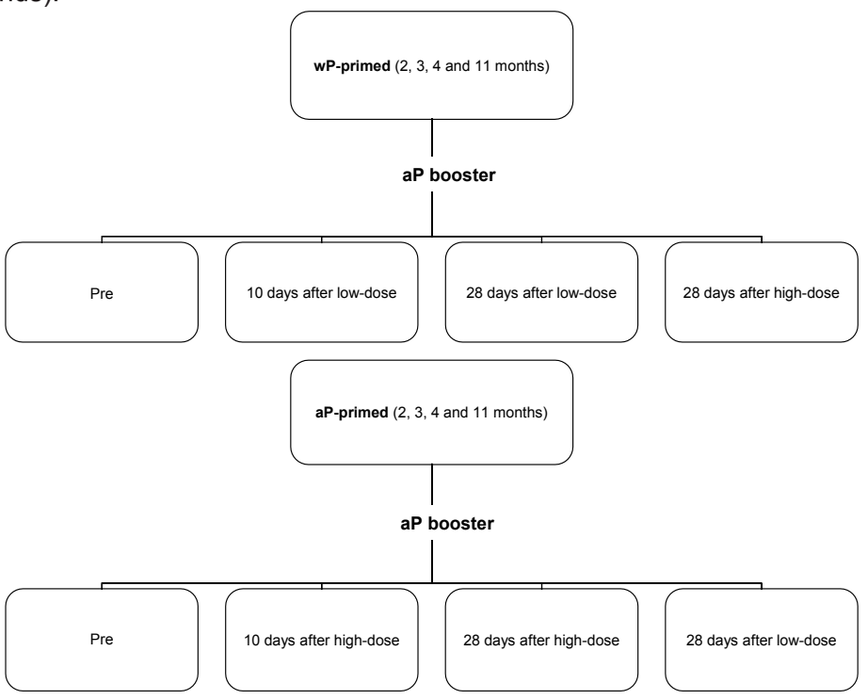

Fig.1. Different groups of children were used in this study: wP-primed and aP-primed children at 2, 3, 4 and 11 months of age received a booster vaccine at 4 years age with either low-dose or high-dose. Groups of children were studied before booster and at 10 and 28 days after booster. Numbers of individuals used varied in the different assays as indicated.

Vaccines

At the time of this study, all children had received either the Dutch whole-cell pertussis vaccine (DTwP-IPV-Hib, NVI, Bilthoven, the Netherlands) or the acellular pertussis vaccine (DTaP-IPV-Hib), Infanrix-IPV-Hib™ (GlaxoSmithKline Biologicals S.A., Rixensart, Belgium) at 2, 3, 4 and 11 months of age. Children who had received the Pediacel vaccine at 11 months of age were excluded from this study. At 4 years of age all children have been administered an additional preschool booster vaccination with Infanrix-IPV™ (GlaxoSmithKline Biologicals). Infanrix-IPV™ contained 25 µg pertussis toxin (PT), 25 µg filamentous heamagglutinin (FHA) and 8 µg pertactin (Prn).

Serological assays

Plasma IgG levels directed against PT, FHA and Prn were detected in all samples by using the fluorescent bead-based multiplex immunoassay against PT, FHA (both from Kaketsuken, Kutamoto, Japan) and recombinant Prn (20) as previously described (3, 21). The in-house reference was calibrated against the FDA-human pertussis antiserum lot 3 (for PT and FHA) and lot 4 (for Prn). Although a new international WHO reference is available results were expressed in EU/ml to keep this study in line with our earlier published data. An arbitrarily level of > 20 EU/ml for the PT antigen was defined as protective (22, 23).

For pertussis specific IgG-subclasses, the modified multiplex immuno-assay (MIA) was used as previously described (24). The contribution of the mean fluorescent intensity (MFI) for each IgG-subclass was assessed as a percentage from the sum of MFIs of the 4 IgG-subclasses together.

The avidity of PT- and Prn-antibodies was measured in plasma with the MIA as described previously (3). For FHA the avidity assay with thiocyanate leads to unacceptable high background levels. Avidity index (AI) was expressed as a percentage of the remaining IgG levels in the presence of ammonium thiocyanate in comparison with those in PBS, in which the avidity was set at 100 %.

B-cell ELISPOT

PBMCs were isolated from 4 ml vacutainer cell-preparation tubes, washed and stored at −135 °C and plasma's were frozen at −20 °C until further testing, subsequently B-cells were stimulated and antigen-specific ELISPOT-assays for PT, FHA and Prn were performed as described, using the same antigens as for the serological assays (25). The numbers of antigen-specific memory B-cells were determined per 10^5 B-cells. Mean spot values of non-coated wells served as negative controls and were subtracted. For determining the geometric mean number per group, the lower limit of quantification was set at a value of 0.1. Fresh and frozen cells have been compared previously and showed no differences in memory B-cell numbers (25).

IFN-γ ELISPOT and cytokines in T-cell culture supernatants

IFN-γ-ELIspot assays were performed as described (19). In short, $3x10^5$ PBMCs depleted from B cells were stimulated with the pertussis-antigens as mentioned earlier, i.e., PT (inactivated for 10 minutes at 80 °C), native FHA and recombinant Prn for 5 days at 37 °C and 5% CO_2. Pokeweed mitogen (PWM), was used as a positive control stimulus for every sample and individual data were included in the data analysis, when upon PWM stimulation more than 200 IFN-γ producing cells per 10.000 PBMCs were found. Spots were developed using biotin-labeled anti-IFN-γ, streptavidin and BCIP/NBT and were counted using Immunospot analyzer software (CTL-Europe GmbH, Bonn, Germany).

Mean spot values of non-stimulated served as negative controls and were subtracted from the antigen-stimulated cells per sample. Fresh and frozen cells have been compared previously and showed no differences in numbers of IFN-γ-producing cells (26).

Cell culture supernatants were stored at -80 º C till further use. By using Biorad cytokine assay kits (Bio-Rad Laboratories, Hercules, CA) IFN-γ, IL-10, IL-5 and IL-17 concentrations were determined according to manufacturer's procedure. Some samples have been excluded from the data analysis if the number of counted beads was too low making the measurement unreliable (27, 28).

Statistical methods

Results were expressed in geometric mean concentrations (GMC) with 95% confidence intervals (CI) or otherwise indicated. The Mann-Whitney test was used to determine significant differences between two groups differing in either age or priming. $P<0.05$ is considered significant different.

Results

Levels of post preschool booster IgG and subclasses of IgG after comparing wP- and aP-primed children

At 4 years of age prebooster and 28 days post-booster as well as 2 years post-booster at 6 years of age, the GMCs of pertussis-specific total IgG, IgG1 and IgG4 were significantly higher for all three antigens in aP-primed children compared with wP-primed children (table 1). Additionally, PT- and Prn-specific IgG3 levels were significantly higher prebooster and FHA- and Prn-specific IgG2 levels were higher at both 28 days and 2 years after preschool booster vaccination.

Following aP booster vaccination, all pertussis specific total IgG and subclass levels increased after 28 days compared with prebooster levels and subsequently decreased over the next 2 years in both wP- and aP-primed children. However, total IgG as well as the IgG1 and IgG4 levels still significantly exceeded the prebooster levels for all 3 antigens after 2 years. At age 6 years, significantly more (35/61) ($p=0.0024$) aP-primed children still had PT-specific antibody levels above the arbitrarily protective level of 20 EU/ml as compared to 19/63 of the wP-primed children. IgG1 was the most prevalent subclass for all three pertussis antigens in all children, especially post-booster vaccination. IgG4 increased also prominently 28 days post-booster in aP-primed children for all three pertussis antigens and remained higher at 6 years of age as compared to wP-primed children.

Memory B-cell responses

We found significantly higher numbers of Prn-specific memory B-cells in aP-primed as compared to wP-children already at 4 years prebooster, as well as 28 days and 2 years after the booster vaccination, while for PT these were only significantly higher at 28 days post-booster (Fig 2).

For wP-primed children, pertussis-specific B-cell numbers increased at both 28 days and 2 years post-booster for all antigens as compared to prebooster numbers, except for PT at 28 days post-booster. The numbers of FHA-specific memory B-cell responses in wP-primed children dropped significantly between 28 days and 2 years post-booster vaccination.

Table 1: Levels of total IgG and IgG-subclasses (EU/ml) before and after a preschool aP booster vaccination

		IgGt		IgG1		IgG2		IgG3		IgG4	
		wP	aP	wP	aP	wP	aP	wP	aP	wP	aP
prebooster	**PT**	4.5	7.7*	1.3	3.2*	1.7	1.7	0.02	0.05*	0.02	0.07*
(wP n=61)	**FHA**	8.8	16*	6.2	14*	0.46	0.36	0.08	0.10	0.09	0.60*
(aP n=61)	**Prn**	3.1	24*	1.8	20*	0.28	0.36	0.07	0.18*	0.02	0.67*
28 days post-booster	**PT**	61	187*	38	140*	5.5	4.6	0.65	0.56	0.40	14*
(wP n=52)	**FHA**	195	521*	181	450*	2.0	8.2*	1.6	2.2	3.2	36*
(aP n=40)	**Prn**	187	1253*	178	1215*	1.2	3.4*	1.3	1.2	1.3	18*
2 years post-booster	**PT**	11#	26*#	5.1#	16*#	1.7	2.7	0.03	0.04	0.05#	0.38*#
(wP n=63)	**FHA**	45#	81*#	42#	75*#	0.16‡	0.38*	0.10	0.08	0.97#	2.7*#
(aP n=61)	**Prn**	19#	110*#	17#	103*#	0.19	0.44*	0.11	0.14	0.27#	2.9*#

Data are expressed as geometric mean values (EU/ml) specific for PT, FHA or Prn of children who have been primed with either wP or aP.
All values 28 days post-booster were significant increased ($p<0.05$) compared to prebooster and those 2 years post-booster were significant decreased ($p>0.05$) compared to 28 days post-booster vaccination.
* Significant increased levels in aP-primed children compared to wP-primed children $p<0.05$.
# Significant increased values in children 2 years post-booster vaccination compared to prebooster levels $p<0.05$.
‡ Significant decreased levels in children 2 years post-booster vaccination compared to prebooster $p<0.05$.

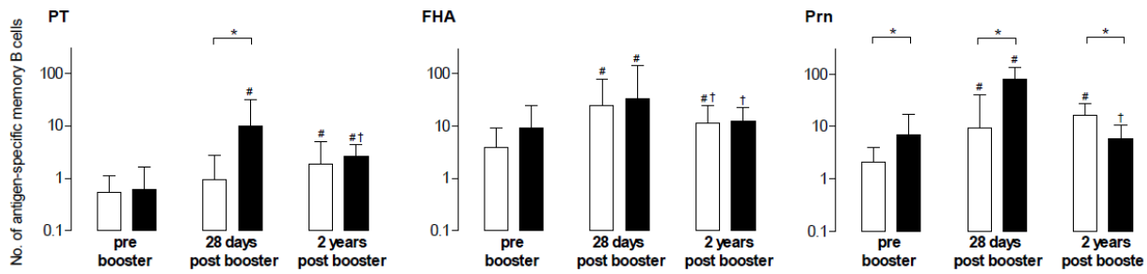

Fig. 2. Numbers (no.) of memory B-cells per 10^5 PBMCs specific for PT, FHA and Prn of wP-primed children (white bars) and aP-primed children (black bars) were represented prebooster and at 28 days and 2 years post-booster vaccination. Bars indicate geometric mean values with 95% CI.
* Significant different numbers in aP-primed children compared to wP-primed children $p<0.05$.
# Significant increased numbers post-booster compared to prebooster $p<0.05$.
† Significant decreased numbers 2 years post-booster compared to those at 28 days post-booster vaccination $p<0.05$.
n=11-19 per group.

In aP-primed children, the numbers of memory B-cells were enhanced at 28 days for all three antigens, and subsequently showed a significant decline at 2 years post-booster vaccination. At that time point only the PT-specific numbers of memory B-cells still exceeded prebooster numbers.

The geomean percentage of total IgG producing cells showed no differences between groups; in wP-vaccinated children it was 13.9%, 17.5%, and 14.7% and in aP-vaccinated children 17.4%, 16.3% and 15.7% for respectively prebooster, 28 days post booster and 2 years post booster vaccination.

Avidity indices of the PT- and Prn-specific IgG antibodies

The avidity indices of the antibodies specific for both PT and Prn were higher in aP-primed compared to wP-primed children at the 3 time points, but that of PT had become similar in children 6 years of age (Fig. 3). The aP preschool booster significantly increased the avidity indices in wP-primed children at 28 days post-booster and remained significantly higher up till 2 years post-booster. In aP-primed children, with already higher prebooster indices, the avidity pattern of PT resembled the memory B-cell response, showing a significant increase at 28 days but followed by a significant decrease to prebooster values after 2 years. In contrast, the Prn-specific avidity indices were also significantly increased at 28 days post-booster, but remained high up to 2 years post-booster in aP-primed children.

The numbers of pertussis-specific IFN-γ producing cells

Already before the preschool booster, higher numbers of IFN-γ producing cells were found in aP-primed children as compared to wP-primed children (Table 2) (19). In wP-primed children, the number of IFN-γ producing cells increased upon booster vaccination and still exceeded those prebooster for all three pertussis antigens after 2 years. In contrast, in aP-primed children the number of IFN-γ producing cells remained high during the first 28 days after booster. But at 2 years post-booster they had declined to lower numbers than prebooster values for all three pertussis antigens and even tended to be lower compared with wP-primed children, although this was not significant.

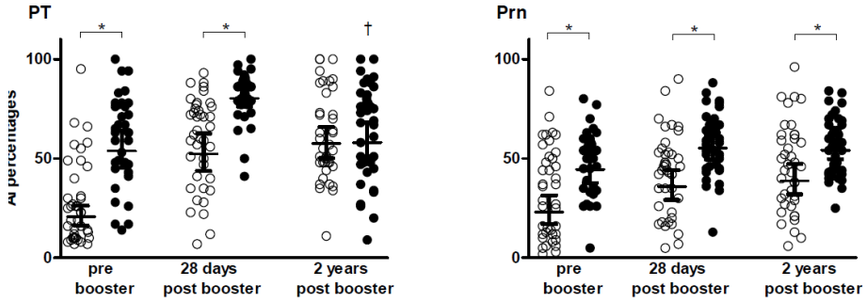

Fig. 3. Affinity indices of IgG antibodies to PT or Prn at prebooster vaccination and 28 days and 2 years post-booster in wP-primed (○) or aP-primed (●) children, expressed in percentages. All groups consisted of 40 samples, except the aP-primed children prebooster group (n=35). Horizontal bars indicate geometric mean values with 95% CI.
* Significant increased values in aP- compared to wP-primed children p<0.05.
† Significant decreased values 2 years post-booster compared to 28 days post-booster vaccination p<0.05.

Table 2: The numbers of IFN-γ producing cells 5 days after stimulation with PT, FHA or Prn

	PT			FHA			Prn		
	n	GMC	95% CI	n	GMC	95% CI	n	GMC	95% CI
wP prebooster	15	0.5	0.2 - 1.6	14	4.4	1.2 - 16	12	1.0	0.2 - 3.8
wP 28 days post-booster	12	5.3#	1.1 - 27	10	15.1	2.9 - 79	11	7.3	1.4 - 39
wP 2 years post-booster	15	9.9 #	2.2 - 45	15	51 #	16 - 163	13	13 #	2.4 - 74
aP prebooster	13	18	3.6 - 91	13	92	22 - 374	13	27	6.9 - 108
aP 28 days post-booster	11	7.0	1.2 - 41	11	46	10 - 215	11	6.0	1.2 - 31
aP 2 years post-booster	15	3.6‡	1.1 - 12	15	44‡	21 - 94	13	5.7‡	2.1 - 15

Significant increase post-booster compared to prebooster vaccination p<0.05.
‡ Significant decrease post-booster compared to prebooster vaccination p<0.05.

The numbers of pertussis-specific IFN-γ producing cells

Already before the preschool booster, higher numbers of IFN-γ producing cells were found in aP-primed children as compared to wP-primed children (Table 2) (19). In wP-primed children, the number of IFN-γ producing cells increased upon booster vaccination and still exceeded those prebooster for all three pertussis antigens after 2 years. In contrast, in aP-primed children the number of IFN-γ producing cells remained high during the first 28 days after booster. But at 2 years post-booster they had declined to lower numbers than prebooster values for all three pertussis antigens and even tended to be lower compared with wP-primed children, although this was not significant.

Cytokine responses in cell culture supernatants

PBMCs depleted from B-cells from children 4 and 6 years of age contained high percentages of CD3+ T-cells (72-85% of total) after stimulation for 5 days. These cells of aP-primed children 4 years of age showed prebooster significantly higher IFN-γ, IL-5 and IL-17 responses for all three antigens except for PT-specific IFN-γ and Il-17 levels and higher FHA-specific IL-10 as compared to wP-primed children (Fig. 4). In aP-primed children, only the Prn-specific IL-10 response still was significantly higher at 28 days post-booster vaccination; at 2 years post-booster the FHA-specific IL-10 was higher, whereas the FHA-specific IL-17 was lower than in wP-primed children.

In wP-primed children the aP booster vaccination resulted in increased IFN-γ, IL-5, IL-17 and IL-10 responses for all three pertussis antigen at 28 days post-booster, which was only significant for Prn-specific IFN-γ and IL-5 and FHA-specific IL-5. Two years post-booster in wP-primed children, all cytokine responses still exceeded the prebooster values and showed significantly increased responses for IFN-γ, IL-5 and IL-17 responses specific for all three pertussis antigens except for Prn-specific IL-5 and IL-17 responses. Additionally, the FHA-specific IL-10 values were significantly higher 2 years post-booster vaccination compared to prebooster values.

Remarkably, in aP-primed children, almost all pertussis-specific cytokine responses remained similar at 28 days post-booster compared to prebooster values, and the FHA-specific IFN-γ and IL-17 responses were even decreased. At 2 years post-booster, the IFN-γ responses increased, which was significant for PT and FHA. In contrast, IL-5 and IL-10 responses did not change and FHA-specific IL-17 responses still was significantly decreased, compared to prebooster vaccination.

Although FHA is not strictly *Bordetella*-specific, T-cell cytokine responses after stimulation with FHA purified from *Bordetella pertussis* showed about the same pattern as found for the strictly pertussis-specific PT and *Bordetella*-specific Prn emphasizing the general pattern of these specific T-cell results.

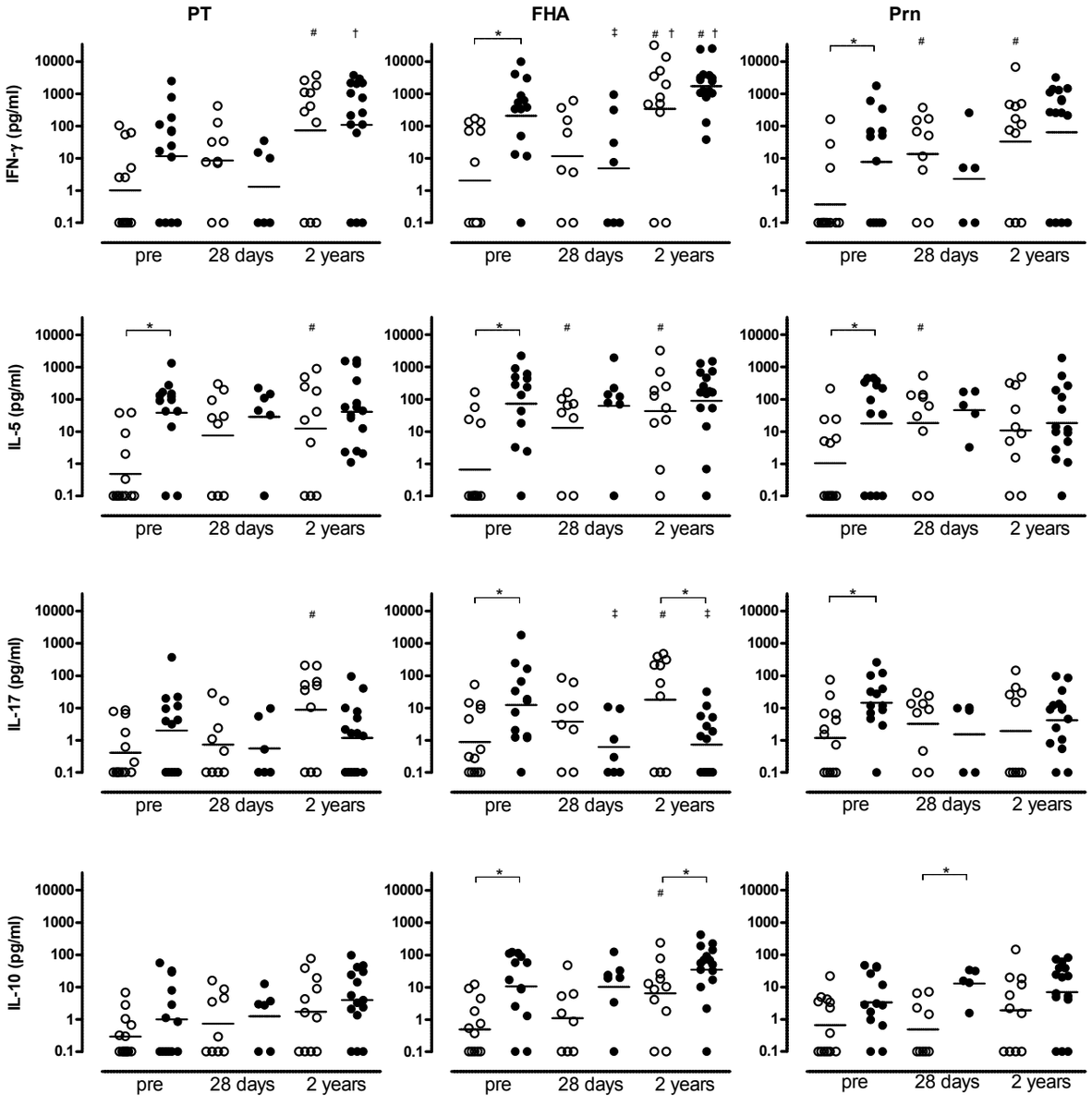

Fig. 4. Th1, Th2, Th17 and IL-10 T-cell cytokine responses in culture supernatants of PBMCs stimulated with PT, FHA or Prn of wP- (○) or aP- (●) primed children pre booster (n=12-15), 28 days (n=5-9) and 2 years post booster vaccination (n=10-15). Horizontal bars represent geometric mean concentration with 95% CI.

* Significant different values in aP- compared to wP-primed children p<0.05.

Significant increased values post-booster compared to prebooster vaccination p<0.05.

‡ Significant decreased values post-booster compared to prebooster vaccination p<0.05.

† Significant increased values 2 years post-booster compared to 28 days post-booster vaccination p>0.05.

Discussion

Although the acellular pertussis vaccines have been used in infants for some decades, and a considerable number of countries has introduced a preschool aP booster vaccination, long-term immunological data post-booster are limited. This study investigated the long-term humoral and cellular immune responses in children 6 years of age, who received 2 years earlier the preschool aP booster and who have been vaccinated during infancy with either wP or aP vaccines.

In this study we showed that the preschool aP booster at 4 years resulted in significantly higher pertussis specific IgG antibody levels in aP-primed children than in wP-primed children that remained higher for at least two years post-booster in particular for pertactin. Antibodies mainly consisted of the IgG1 subclass but primarily aP-primed children also showed elevated IgG4 antibody levels. The avidity of antibody responses was also higher in aP-primed children both pre- and post-booster. In wP-primed children, the numbers of pertussis-specific memory B-cells were increased, in line with higher IgG antibody levels and avidity indices at 2 years post-booster vaccination. Noteworthy, the avidity of PT antibodies had become similar in 6 years old children in both aP- and wP-primed children. Importantly, all prebooster T-cell responses in aP-primed children were already high and had not really changed after 2 years post-booster, whereas the T-cell responses in wP-primed children increased over that period. Together, these data indicated that the aP preschool booster improved pertussis immunity until 2 years after the booster immunization and that aP-primed children might have an advantage over wP-primed infants of 6 years of age. However, recent studies in the US have shown a high incidence of pertussis already observed in aP vaccinated children from 9 years onward, indicating that protection against pertussis by aP vaccines is relatively short-lived and antibody levels wane substantially within 4 to 5 years, even after a fifth aP vaccination (29, 30). It is conceivable that the pertussis-specific immune responses of the Dutch aP-primed children also will wane quickly within 5 years after the preschool booster vaccination and that these children become more vulnerable to pertussis as well [28, 29].

We earlier reported that 3 years after both wP or aP primary immunizations in the first year of life only low antibody levels to the pertussis vaccine antigens were found (3) suggesting a fast decay of these levels after both vaccine types as previous studies already have shown (2, 31, 32). Although we cannot exclude that the findings for wP-primed children at 4-6 years of age are influenced by the use of the non-optimal Dutch wP vaccine, the vaccination schedule and used vaccines of the aP-primed children in our study are comparable with other studies. The increased antibody levels that gradually decline over 2 years after the preschool booster in our study are in line with the observations of Meyer et al, who also showed a sharp decay in antibody levels at 3.5 years after a 5th aP vaccination at preschool age (33). Overall, their data on FHA and Prn-specific IgG levels at 3.5-year post-booster are rather comparable to our data at 2 years post-booster and the difference in PT antibody levels is likely due to the difference in time after booster vaccination. Our IgG values shortly after the 5th aP preschool booster vaccine were at least a 3-fold higher compared to the data of Sänger et al., but in that study

a reduced antigen-dose booster vaccine was used (34). Also after an adolescent booster antibody levels show the same decay as found after a preschool booster (35). Although antibody levels rapidly wane, the avidity indices of Prn-specific antibodies in aP- compared to wP-vaccinated children contributed to a higher strength of binding of antibodies, which might indicate a better protection. However, this does not count for PT-specific antibodies, since their avidity indices in aP-primed children seem to decline already shortly after booster vaccination, The avidity of FHA-specific antibodies measurements have not been measured, since FHA is not specific for *B. pertussis* alone and FHA antibodies show cross-reactivity with FHA proteins present in other bacteria (36).

Although IgG1 was the predominant subclass for all pertussis antigens in both groups of children, higher IgG4 levels were present post-booster especially in children who had been aP-primed (24). The pertussis vaccine used for priming seems to determine the IgG-subclass composition elicited after a secondary antibody response upon booster vaccination. In several other studies, IgG1 was also found to be the predominant subclass after pertussis vaccination and IgG4 only marginally contributed to the total IgG level (16, 24). The more pronounced anti-pertussis IgG4 response in aP-primed children, which was also observed by Giammanco et al. (16), might reflect a more Th2-skewing of the immune response after aP vaccination (37). Previous studies have shown that high concentrations of PT, FHA and Prn in most pediatric aP vaccines elicit a Th2 type or mixed Th1/Th2 immune response (10, 38). The wP vaccine contains lower concentrations of less purified antigens and more additional substances like LPS that are capable of inducing a more Th1 associated immune response (10, 11). Although the higher IgG4 responses correlated with PT- and Prn-specific IgE levels (24), we did observe only slightly higher Th2 responses as compared to Th1 responses one month after the fifth consecutive aP vaccination (19). Also the T-cell data 2 years post-booster do not show more Th2-skewing. In this study the samples from the children at the different timepoints are not longitudinally and some sample sizes per group in the T-cell assays are relatively low. Therefore, the interpretations of the T-cell responses over time should be done with care but surely are indicative for follow-up studies based on these results.

We have indications that booster responses induced by vaccination in combination with natural boosting with pertussis have resulted in increased T-cell responses in wP-primed children 6 and 9 years of age old (39, 40). However in this study the number of possible undiagnosed cases on basis of elevated PT antibody levels was similar in wP-primed and aP-primed children.

We have shown that, in the currently available aP vaccines, pertactin is the most immunogenic antigen. Recently, a number of *B. pertussis* strains not expressing the vaccine antigen Prn have been isolated from pertussis patients in a number of countries where the aP vaccines have been implemented for some time now (41). We might speculate that the high antibody levels induced by the aP vaccines, especially against Prn, have triggered the bacteria to escape from this immune pressure. Until now, these escape mutants have not been more virulent than the wild-type pertussis strains; however, we must continue the surveillance of

these strains carefully. Protection against these pertussis strains is now dependent on the immune responses primarily against PT, with FHA not being specific, making the antibody spectrum of the current acellular vaccines narrower.

To summarize, since the wP vaccine has been replaced by aP vaccines, the induction of B-cell and T-cell memory responses have been improved resulting in higher circulating antibody levels with better avidity. However, based on the already present high T-cell immune responses before the preschool booster, the dose of the aP vaccine and the vaccination intervals may need to be reconsidered in order to improve immune responses. The still high T-cell responses at 4 years of age combined with the reported local hypersensitivity after the fifth aP vaccine suggest that the preschool booster vaccination might be shifted to a later age to induce better long-term immune responses (19). Also, since Meyer et al. (33) reported that a reduced-antigen dose aP vaccine was as immunogenic as high-dose preschool booster aP vaccines and at least as well tolerated, the choice between low-dose and high-dose preschool boosters should be taken carefully. Evaluation of long-term humoral and cellular immune responses after vaccination against pertussis will be important for future decisions concerning pertussis vaccination schedules for adolescents and adults as well. On the short run, improved vaccination schedules could stimulate the immune system of children better and protect longer against pertussis. Our data suggest that postponing of the boosters at 11 months and 4 years to a later age (15-18 months and 6 years respectively) would be possible. On the long run, perhaps new generation aP vaccines are needed to further improve the protection against pertussis for newborns, in older age groups and even the whole population.

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Conflict of interest

There is no conflict of interest.

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Summarizing discussion

Summarizing discussion

Since 1996 the incidence of pertussis started to increase again in the Netherlands. Since then every 2-3 years epidemic cycles are seen in the Netherlands. Annually, the pertussis incidence peaks in the third quarter of the year in the Netherlands, as in most European countries and the USA. This resurgence of pertussis might be due to increased awareness and reporting, improved diagnosis, suboptimal vaccines, antigenic divergence and waning immunity. Waning immunity and strain variation are considered the most important causes of this resurgence.

After the pertussis resurgence in 1996, several changes in the national vaccination schedule have been implemented. The potency of the wP vaccine has been elevated from 4 to 7 IU/HD in 1997 and in 1999 the primary schedule for newborns at 3, 4, 5 months of age was accelerated to 2, 3 and 4 months of age. From 2001 onwards an additional acellular booster vaccination was implemented at 4 years of age and finally in 2005 the whole cell vaccine (wP) administered in the first year of life was replaced by an acellular vaccine(aP). The current Dutch pertussis vaccination schedule consists of four primary doses of the combined DTaP-IPV/Hib/HepB vaccine administered at 2, 3, 4 and 11 months of age and an additional preschool booster dose of an acellular vaccine (aP) at the age of 4 years.

The principal aim of the studies described in this thesis was to investigate the effects of either wP or aP vaccinations on the pertussis-specific T-cell immunity in groups of children of 4 to 9 years of age. This is important since it may learn us apart from antibody levels, whether different vaccines also affect the T-cell immunity. In this thesis we primarily compared T-cell responses of wP- and aP-primed children in infancy before, shortly after and on the long-term (years) after the aP preschool booster vaccination at 4 years of age. Furthermore, in several groups of children of 4 to 6 years of age, we characterized the T-cells phenotypically and studied B-cell immune responses. In the Netherlands, as in other countries, we recently experienced in 2012 a steep increase in pertussis notifications with the peak incidence in the spring. This is remarkable since the peak incidence usually is noticed in the autumn, and in older children from 8 years of age up. For this reason, we investigated the effects of an extra preadolescent pertussis booster vaccination at 9 years of age on the pertussis-specific T-cell responses.

In this chapter, the main findings of this thesis are summarized and discussed, followed by recommendations for future pertussis vaccination strategies.

Increased pertussis-specific T-cell memory responses in (older) children

In chapter 2, we demonstrated that pertussis-specific Th1 memory immunity in wP-primed children (in the first year of life,) gradually had increased with age in children 6 and 9 years of age, thus after 2 to 5 years following the aP booster vaccination at 4 years of age. The fact that T-cell responses at 9 years of age before the extra aP booster vaccination were also higher than those directly after the preschool booster at 4 years of age implies that natural boosting upon contact with *Bordetella pertussis* during childhood has stimulated the T-cell

responses. These enhanced T-cell responses upon natural infection are due to the circulation of *Bordetella* in the population. It is clear that *Bordetella pertussis* still is endemic, as is shown by monitoring the incidence of pertussis [1] and by antibody levels found in two large cross-sectional national serosurveillance studies performed in 1995/6 and 2006/7 in persons aged 10 to 80 years. Using the anti-PT-IgG level > 50 EU/ml as indication for a recent pertussis infection [2], a more than twofold increase in the numbers of persons recently infected with pertussis was observed in 2007 compared to 1996 (from 4% to 9%).

Next to the higher memory T-cell immunity in the 9 years old children we also observed that these children had higher percentages of pertussis-specific effector-memory T-cells (CCR7-CD45RA-) than 4-year-old children before booster vaccination (**chapter 2**). Moreover, in 9-year-old children more Th1-cytokines were produced by higher numbers of effector-memory T-cells than found in children 4 years of age. Also higher T-cell responses were observed at 1 month after an extra booster vaccination at 9 years of age than at 1 month after the preschool booster vaccination at 4 years of age. Altogether the results in this study might reflect a higher quality of the T-cell response in 9 years-old compared to the 4 years old children. Moreover, T-cells of older children have the ability to produce multiple cytokines simultaneously which reflects a qualitatively better immune response compared to younger children [3]. Apart from natural boosting, maturation of the immune system during age will influence the pertussis-specific T-cell immunity [4]. The maturation together with repeated stimulation of the immune response by multiple encounters with pertussis antigens due to vaccination and natural infection will end up into a better T-cell immunity to pertussis later on in life.

Pertussis-specific T-cell immunity due to a second pre-adolescent booster vaccination after wP-priming

After implementation of the preschool booster in the Netherlands the high number of pertussis notifications found in children of 3-4 years of age in 2001 had shifted to children 11-12 years of age in 2011. In the recent epidemic in Netherlands in 2011-2012 this was followed by an upsurge of pertussis cases especially in the group of children starting from 8 years up [5]. Although severe disease and complications due to whooping cough are rare in this group of children and adolescents, they are one of the major sources of infection for the children most at risk, i.e. infants who have not (fully) received the immunizations against pertussis [6]. To indirectly protect these infants several countries like Austria, Australia, Canada, France, Germany, Finland, the United Kingdom and the United States have introduced adolescent booster vaccinations [7-9].

Since Dutch children 9 years of age receive a DT-IPV and MMR vaccine as a booster simultaneously according to the Dutch NIP, a change of DT-IPV into DTaP-IPV at 9 years of age could be introduced relatively easily without adding an extra injection. Moreover, clinical studies have shown a low reactogenicity of these reduced-dose acellular vaccines used for adolescent vaccinations [9-14]. We already have shown that B-cell memory responses and antibody levels did increase after the extra booster vaccination at 9 years of age. We however found that the extra pertussis booster vaccination of children 9 years of age, who had been wP-primed at infancy and had been administered a preschool aP booster vaccination at 4

years, only minimally enhanced the T-cell immunity as was shown by only slightly higher Th1 and Th2 T-cell cytokine responses upon the aP booster (**chapter 2**). There is only one other study describing enhanced T-cell responses shortly after booster vaccination in adolescents 10-14 years of age, who had been either wP- or aP-primed at infancy [15]. In our study however, almost all T-cell responses were already high before the booster vaccination at 9 years of age and had enhanced significantly during the 5-year period between the two booster vaccinations. We did find higher T-cell responses at 1 month after an extra booster vaccination at in wP-primed children 9 years of age than at 1 month after the preschool booster vaccination in wP-primed children 4 years of age, but the question is how relevant this is. A certain level of T-cell memory immunity might be enough to provide T-cell help to activate B-cells and promote antibody production which was shown to rise. But T-cells itself may not need to be further up regulated by vaccination. After the extra booster vaccination at 9 years of age [16] the immune response might induce a better protection against pertussis in teenagers, despite the fact that T-cell immunity did not show much improvement, but was already on a high level. In Finland the incidence of pertussis has decreased both in teenagers as well as in the whole population after the implementation of a pertussis booster vaccination in children 14 years of age and no upsurge of the 2012 epidemic was observed [8]. This suggests that an adolescent booster vaccination might be beneficial to better prevent pertussis. However, data over a longer period of time are crucial. In time, the adolescent booster may also shift the peak of cases to young adults, which is a risk for newborn babies since these young adults will be the parents of those babies.

Effect of wP- and aP-priming on T-cell immunity

In general, the effects of a prime-boost combination in the first year of life are important in infant vaccination strategies for the establishment of early immunity that can then be boosted after further exposure [17].

Despite waning IgG antibody levels in children 4 years of age, three years after the priming vaccinations, T-cell responses had remained present in both wP and aP primed children. Both groups of children showed a mixed Th1/Th2 pertussis-specific cytokine production before the booster vaccination at the age of 4 years. Importantly, the infant vaccinations with high-dose acellular pertussis vaccines resulted in high pertussis antigen-specific Th1, Th2, and Th17 responses (**chapter 3**), which were higher than found in wP-primed children. This was also shown in **chapter 4**, where we demonstrated by FACS analysis detailed phenotypical and functional differences in the T-cells between wP- and aP-primed children 4 years of age. In general, in aP-primed children the pertussis-specific CD3+CD4+ and CD3+CD4- (CD8) T-cells showed the capacity to better proliferate compared to the T-cells of wP-primed children. In aP-primed children the pertussis-specific T-cells showed a higher proportion of effector memory T-cells and terminally differentiated T-cells compared to those cells of wP-primed children. Although similar proportions of Th1 cytokines were produced in both groups of children, the total cytokine production is higher in aP-primed children due to the presence of more effector memory cells. This is in line with the total T-cell cytokine responses described in **chapter 3**.

We found similar Th1 responses in our aP primed Dutch children when compared with Swedish aP-primed 4- to 6-year-old children just before the preschool booster [18]. We found slightly higher Th2 levels in the Dutch children compared to the Swedish children, but our data resemble the data of 4- to 6-year-old French aP-vaccinated children [19]. This already illustrates the fact that comparison of cell-mediated immune responses against pertussis between wP and aP vaccinated children is highly complicated since different vaccines, various vaccination schedules, different age groups and different exposure rates will confound the results [18-23].

Importantly, these T-cell responses at 4 years of age most likely have been induced by the high-dose aP vaccines administered early in life, since aP-primed children showed much higher T-cell responses than wP-primed children. The immature immune system of young infants is very sensitive to stimulation. Therefore, especially the priming vaccinations will determine the memory immune responses to a similar vaccine or pathogen later on in life. We believe that the high doses of proteins in the aP vaccines administered repeatedly within a short period of time induce high T-cell responses, which are maintained even up to 3 years later. In order to improve the immune response induced by aP vaccines, the vaccine dose and vaccination schedules might be reconsidered and investigated in future studies.

Effect of wP- and aP-priming on T-cell immunity after a preschool aP booster vaccination

Surprisingly, the already high levels of all T-cell cytokines in aP-primed children prevaccination remained elevated after the fifth aP vaccination at 4 years of age. So, we did not find a typical T-cell memory response shown by an increase in cytokine production shortly after a fifth high-dose aP vaccine, which is in contrast to the findings in wP-primed children (**chapter 3**). The similarly high T-cell levels in aP-primed children still assisted the T-cell-dependent recall antibody production upon an aP booster, since we found significantly increased post booster antibody levels with a good avidity, as described in **chapter 6** [24].

Previous studies from other groups have shown that wP vaccines rather prime for Th1 immune responses, whereas aP vaccines may lead to more Th2 cytokine production [22, 25]. However, we did observe just slightly higher Th2 responses than Th1 responses after the 5th aP vaccination at 4 years of age. The Th1 responses we have found after this fifth aP vaccination were similar to those described in the study of Ryan et al, after a 4th aP vaccination of children of the same age [18]. They found however, an increase in Th2 responses after aP vaccine while we did not found such an increase after a 5th aP booster vaccine. The post-booster increase in these Swedish children might be explained by the much lower preschool Th2 response at 4 years of age in comparison with children of the same age before the preschool booster vaccination in our study. This discrepancy between their and our study probably results from their lack of an additional booster given after the primary series in the first year of life, at 11 months of age.

After the fifth aP vaccination, at 4 years of age, the number of children reporting various side effects has doubled compared to an aP booster in wP-primed children. The local adverse effects range from non-tender redness (<5 cm) to severe swelling (>5 cm) at the injection site [26]. These symptoms occur within a few days after vaccination and disappear again within a few

days [27]. Moreover, the aP-primed children showed slightly increased pertussis-specific IgE levels after both a low-dose and a high-dose booster vaccination (**chapter 3**). This finding is in agreement with other studies [18, 28, 29], but the clinical relevance of these increased IgE responses is not clear. Since antihistamine was not effective in treating the adverse effects, the time course of the reaction reflects the possibility of a delayed-type hypersensitivity reaction caused by T-cells and macrophages [30]. A correlation between local adverse effects and high Th2 responses has been established [29]. Possibly these responses also induce higher pertussis-specific IgE levels [31, 32]. A correlation of PT- and Prn-specific IgE and IgG4 was found after the fifth aP vaccination (**chapter 5**). Th2 cytokines play a role in the isotype switching of antibody production and B-cells in an environment with Th2 cytokines tend to produce IgE and IgG4 antibodies [33]. Moreover, it is hypothesized that a more Th2 cytokine profile underlies the pathogenesis of allergic diseases [34, 35]. So, five high-dose aP vaccinations in children induce high B-cell memory immune responses, elevated IgE antibody levels together with a pronounced IgG4-response to the pertussis vaccine antigens (**chapter 5**). We, like others [31], proposed already in the former paragraph that high-dose aP vaccines administered repeatedly at 2, 3, and 4 months of age followed by a booster 7 months later, initiate both Th1 and Th2 responses which are maintained till even 3 years later. We now speculate that the preschool high-dose aP booster additive to ongoing T-cell immunity may explain the changes in antibody switching and adverse reactions.

Recently, a case-control study has started by the RIVM and the Netherlands Pharmocovigilance Centre Lareb to specifically recruit children with (severe) local adverse effects after the high-dose aP preschool booster vaccination. Possible immunological differences in children with and without adverse effects to the 5th aP pertussis vaccination in the Netherlands will be investigated.

Immune responses after a preschool booster vaccination with either high-dose or a low-dose.

In **chapter 3**, we also focused more on pertussis-specific T-cell cytokine responses shortly after a preschool booster with either a high-dose or a low-dose aP booster vaccine. In general, the T-cell responses in wP-primed children were increased after both an aP low-dose booster or a high-dose booster vaccination. Moreover, these responses were almost similar after a high-dose booster or a low-dose booster vaccination. In contrast, the already high T-cell responses in aP-primed children at 4 years of age before the booster vaccination did not further increase regardless of a high- or low-dose booster, and the Th1 cytokine responses even tended to decline slightly after a high-dose booster. Furthermore, after a low-dose preschool booster, the T-cell responses of aP-primed children were higher than those in wP-primed children. When we focus on T-cell immunity, we might speculate that a low-dose booster is as effective as a high-dose booster. Moreover, the B-cell immunity in aP-primed children after a low dose booster tended to be higher than after a high-dose booster vaccination [24], which suggests that a low-dose is sufficient or even better than a high dose booster at preschool age. In addition, the aP-primed children in this study showed increased pertussis-specific IgG4-spe-

cific responses after a high-dose booster that is associated with Th2 responses (**chapter 5**). Very high antibody levels against pertactin were found in aP-primed children after a high dose booster vaccine, which might be of benefit but at the same time pertussis strains deficient for pertactin are circulating. Although antibodies against pertactin have been shown to be protective [36-38], the clinical relevance of very high antibody levels is unknown. In short, the effects of high or low dose booster vaccines as well as that of postponing the 5th aP vaccination needs further study and clinical follow-up. Our results are based on just small groups of children, and next to antibodies and B-cell immunity, research on cellular T-cell immunity is needed to be able to propose the optimal vaccine dosage and vaccination schedule to the Health Council and policy makers.

Waning immunity after preschool booster vaccination in wP- and aP-primed children

In **chapter 6**, we investigated the long-term humoral and cellular immune responses in children 6 years of age at 2 years after the preschool aP booster in both wP- and aP-primed children. We showed that the preschool aP booster at 4 years resulted in significantly higher pertussis-specific IgG antibody levels in aP-primed children than in wP-primed children immediately after the booster and antibody levels remained higher for at least two years post-booster until 6 years of age, in particular for pertactin. The avidity of antibody responses was higher in aP-primed children immediately post booster compared to wP-primed children, but differences no longer were present after 2 years in 6-year old children. In wP-primed children, the numbers of pertussis-specific memory B-cells were found increased at 2 years post-booster vaccination compared to prebooster numbers, in line with higher IgG antibody levels and avidity indices. Importantly, all prebooster T-cell responses in aP-primed children were already high and remained so after 2 years post-booster, whereas the T-cell responses in wP-primed children still had increased over that period.

Together these data indicated that the aP preschool booster enhanced pertussis immunity until 2 years after the booster immunisation both in aP- and wP-primed children and that aP-primed children might have an advantage over wP-primed infants of 6 years of age. However, in the recent incidence peak of pertussis in Europe, USA and Australia in children from 8 years up [39-41], it appeared that priming with aP vaccines in the first year of life induces a shorter immune protection later on in childhood compared to priming with an wP vaccine. The priming and boosting combination may thus prove critical for long-term protection and need to be better elucidated.

Future recommendations

In general

The principle aim of pertussis vaccination is to protect the young infants most at risk. The recent increase in the incidence of pertussis among all age groups is particularly alarming because the bacteria can be transmitted to neonates too young to be fully vaccinated and who are the most endangered by pertussis. To achieve improved protection of these vulnerable infants, approaches to alter the Dutch vaccination programme should be considered.

All possible options have been carefully reviewed in an RIVM/CIB report offered as advice to the Dutch Health Council [5]. Here we would like to discuss the pertussis vaccination recommendations that have been the focus of the studies in this thesis, in view of the T-cell immune responses during time after pertussis vaccinations.

Preschool booster

The aP preschool booster vaccination is essential in providing protection against pertussis in school children as was shown by the shift of the peak age of clinical pertussis to a later age [42]. However, at 4 years of age aP-primed children still showed high T-cell responses even three years after the 4th vaccination at 11 months of age. Shortly after the 5th aP vaccination we did not find differences in T-cell responses between a high-dose and a low dose booster vaccine. Moreover, a more Th-2 cytokine profile in combination with IgG4 and IgE responses as well as a rise in the incidence of local side effects was seen in 4 year old children after the 5th high-dose aP vaccination. The fifth high dose response did neither contribute to enhanced T-cell proliferation which was already high before the booster nor to influence the T-cell phenotype and functionality.

These findings suggest that the preschool aP booster with a high dose vaccine could be replaced by a low-dose booster vaccine. When it is decided to change the booster vaccine, it is of the utmost importance to monitor the immune responses in school children and to evaluate the long-term effects of a low-dose preschool booster in aP-primed children.

A second possibility is to consider the possible benefits of postponing an aP booster from 4 years of age till 6 years of age. Although an exact time point for the preschool booster as 5th aP vaccination is difficult to determine this should be further investigated. Several other countries have implemented an aP booster at 6 years of age even as a 4th aP vaccination without any signs of an increase in the incidence of pertussis before that age indicating that the postponement is a good possibility. Moreover, better memory immunity is induced when children are vaccinated at a later age when the immune system is more mature [43]. In view of the still high T-cell responses at 4 years of age, it would be worthwhile to reconsider the current primary vaccination schedule of 2-3-4 and 11 months of age in future studies.

Adolescent booster vaccination

We have shown that an adolescent booster vaccination might be beneficial to better prevent pertussis in children who had been primed with the Dutch wP vaccine. Also, a change of DT-IPV into DTP-IPV at 9 years of age could be introduced relatively easily without adding an extra injection. However, nowadays, the Dutch children of 9 years of age have been primed with the aP vaccine which primes the immune system differently compared to wP vaccines. At present, immune responses after a 6th aP vaccine are currently investigated in a new study. The results from this study will contribute to the discussion whether an aP booster vaccination in aP-primed adolescents has to be recommended for this moment. Important in this discussion is that it recently became clear during the 2012 epidemic in the USA that the duration of protection of the 5th aP booster at 6 years and even the 6th booster vaccination at 11 years is

limited to approximately 2-3 years in aP-primed children [41, 44]. Children, who were primed with wP seemed better protected against pertussis [41]. In this light, the strategy of subsequent aP booster vaccinations seems not the way forward and other approaches need to be considered.

Other approaches

Newborns and unvaccinated infants represent the real high risk groups for fatal pertussis disease. In the CIB report one of the major advises is to implement maternal vaccination, which possibly protects the newborn in the first months of life till the first vaccinations. Countries like the USA, UK and Belgium have already chosen to implement maternal vaccination. The first results from the UK are hopeful and maternal Tdap vaccination seems to result in higher pertussis antibody concentrations during the period between birth and the first vaccine dose in the neonate [45]. At the moment a study on maternal vaccination has just been started in the Netherlands.

The acellular vaccines currently used are protecting the children in their first years but seem not so effective in preventing pertussis in the long run. Changes in the current acellular vaccines have been proposed, like implementation of genetically detoxified PT instead of chemically detoxified PT which seems less immunogenic [46-49]. So, this genetically detoxified PT could induce higher PT-IgG levels which might be beneficial for an improved protection against pertussis. Still it is doubtful if such alterations in the acellular vaccines are sufficient to provide long term protection. For this purpose, possibly different kinds of pertussis vaccines have to be developed. In this line an important and promising approach is the development of a modified inactivated live vaccine (BpZE1) which can be administered intranasally, thereby having the capacity to induce musosal immunity [50, 51].

In conclusion

The protection against vaccine preventable diseases in the Netherlands by the national immunisation programme (NIP) is very effective in general. However, although case fatalities and disease rates have been drastically diminished after introduction of pertussis vaccinations in the Dutch NIP and luckily only few case-fatalities still occur, the overall protection to pertussis is less effective than to the other vaccines present in the NIP. The definitive solution for all pertussis problems that occurred during the last two decades has not been found yet. Nevertheless, evaluating cellular immune responses to pertussis vaccination and/or natural infection additive to antibody levels provides important information for recommendations about new vaccination schedules. However, new and improved vaccines with longer lasting immunity seem the road forward to eradicate this disease.

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Samenvatting

Nederlandse samenvatting

Kinkhoest is een infectieziekte die wordt veroorzaakt door de bacterie *Bordetella pertussis*. De ziekte begint vaak als een heftige verkoudheid en zal na een week of twee overgaan in een typische hoest. Het hoesten kan zelfs enkele maanden aanhouden. Kinkhoest komt aan haar naam omdat bij peuters en kleuters zware hoestaanvallen optreden met een 'kinkende' hoest. Na een lange gierende inhalatie kan het kind weer lucht happen. Kinkhoest is vooral gevaarlijk voor baby's onder de 6 maanden omdat die nog niet (volledig) zijn gevaccineerd. Pasgeborenen en jonge baby's kunnen bij een infectie het slijm vaak niet zelf ophoesten en stoppen soms met ademen. Bij deze kinderen kan dan ook hersenschade ontstaan door gebrek aan zuurstof of ze kunnen zelfs overlijden

Ernstige kinkhoest kan worden voorkomen door te vaccineren. In Nederland is men in 1957 gestart met het massaal vaccineren tegen kinkhoest. Voor de introductie van vaccinatie werden jaarlijks 150.000 mensen ziek en stierven er bijvoorbeeld 400 mensen in het jaar 1940. Na introductie van de vaccinatie daalde de sterfte naar bijna nul in 1960.

In de jaren negentig echter kwam kinkhoest weer opzetten in Nederland evenals in de rest van de wereld, ook in landen waar wordt gevaccineerd. Sindsdien zien we in Nederland elke 2-3 jaar een epidemie van kinkhoest. In 2012 hebben we de grootste kinkhoest epidemie gehad sinds de start van het vaccinatie tijdperk, waarin meer dan 1000 ziektegevallen per maand werden gemeld en 3 ongevaccineerde jonge baby's zijn overleden.

In de loop van de tijd zijn er verschillende vaccins gemaakt, enerzijds op basis van hele gedode bacteriën (het cellulaire vaccin (wK)) en anderzijds op basis van enkele belangrijke gezuiverde eiwitten van de bacterie (het acellulaire vaccin (aK)). Het belangrijkste eiwit van kinkhoest is pertussis toxine (PT); dit toxine wordt uitgescheiden door de bacterie en veroorzaakt de symptomen van kinkhoest. Daarnaast zijn er belangrijke eiwitten zoals filamenteus heamagglutinine (FHA), pertactine (Prn) en fimbriae (Fim) waarmee de bacterie aan het epitheel van de luchtwegen kan binden. In de acellulaire vaccins die vaak worden gebruikt, zitten de eiwitten PT, FHA en Prn, soms zijn ook de Fim eiwitten toegevoegd.

Omdat kinkhoest weer opkwam in de jaren 90, zijn er diverse veranderingen in het rijks vaccinatie programma (RVP) doorgevoerd, zoals een dosisverhoging van het cellulaire vaccin (wK) van 4 naar 7 eenheden gedode bacteriën in 1997 en een vervroeging van het schema van de DKTP vaccinaties van 3, 4 en 5 maanden naar 2, 3 en 4 maanden in 1999. Vanaf 2001 is er een extra acellulaire kinkhoestvaccinatie (aK) geïmplementeerd in het RVP op 4-jarige leeftijd, voordat kinderen naar school gaan (de zogenoemde boostervaccinatie). De belangrijkste verandering in het RVP op het gebied van de kinkhoestvaccinaties is dat vanaf 1 januari 2005 de 4 vaccinaties met het wK vaccin in het eerste levensjaar helemaal vervangen zijn door de aK vaccins.

De introductie van de aK booster vaccinatie op 4-jarige leeftijd in 2001 heeft de piek van het aantal kinkhoest gevallen die eerst bij 4-5 jaar oude kleuters optrad, verplaatst naar een groep kinderen ouder dan 8-9 jaar en adolescenten. Echter, ook jong volwassenen worden momenteel vaker besmet met kinkhoest. Gevaccineerde kinderen en volwassenen die ziek worden van kinkhoest laten wel een veel milder ziekteverloop zien in vergelijking met jonge (nog) niet gevaccineerde kinderen.

Optimale vaccins induceren langdurende immuniteit, maar het acellulaire kinkhoest vaccin induceert helaas slechts een kortdurende immuniteit van ongeveer 5 jaar. Maar ook het doormaken van een echte kinkhoestinfectie biedt vaak slechts 5-10 jaar bescherming. Na een infectie of vaccinatie worden in het bloed antistoffen aangemaakt. Die antistoffen zijn specifiek voor kinkhoesteiwitten en lijken geassocieerd met bescherming tegen de ziekte, hoewel er nog geen duidelijk criterium is vastgesteld welke antistoffen en welke concentraties daarvan echt nodig zijn voor bescherming. Na vaccinatie lijken vooral de antistoffen snel af te nemen in de loop van een paar jaar. Behalve antistoffen worden na een vaccinatie of infectie echter ook specifieke afweercellen geactiveerd, maar dit is tot nu toe weinig onderzocht.

De cellulaire afweer wordt gevormd door de T- en B-cellen. De (geheugen) B-cellen zorgen voor de (hernieuwde) aanmaak van antistoffen na een nieuw contact met de bacterie en de opbouw van geheugen. Er zijn verschillende types T-cellen die een meer of mindere actieve rol spelen bij de afweer en met elkaar communiceren door het uitscheiden van signaalmoleculen, de zogenoemde cytokines. In onze studies hebben we vooral naar de aanmaak van cytokines van T-helper cellen gekeken. Deze T-helper cellen worden op hun beurt weer onderverdeeld in subgroepen. Elke subgroep met net een andere functie produceert zijn eigen cytokines. Een speciaal type T-helper cellen (Th2) kunnen o.a. B-cellen stimuleren tot de aanmaak van antistoffen. Wel is het voor het immuunsysteem van belang dat de verschillende T-cel responsen elkaar een beetje in balans houden.

In het eerste levensjaar worden bijna alle kinderen ingeënt op 2, 3, 4 en 11 maanden met het DKTP vaccin volgens het Nederlandse RVP. Ons onderzoek richtte zich op de immunologische verschillen tussen kinderen die enerzijds met het oude cellulaire vaccin (wK) waren ingeënt (geboren voor 2005) en kinderen die met het nieuwe acellulaire vaccin (aK) waren ingeënt (vanaf 2005). In de hoofdstukken 3 t/m 6 hebben we deze groepen kinderen met elkaar vergeleken op de leeftijd van 4 tot 6 jaar. In hoofdstuk 2 hebben we kinderen vergeleken van verschillende leeftijden (4, 6 en 9 jaar) die allemaal nog met het wK vaccin zijn ingeënt in hun eerste levensjaar.

Resultaten

Een van de belangrijkste bevindingen was dat aK gevaccineerde kinderen op 4-jarige leeftijd duidelijk veel hogere T-cel responsen hebben t.o.v. van 4-jarige kinderen die nog met het wK vaccin waren gevaccineerd. De T-cel responsen zijn dus nog hoog 3 jaar na de laatste aK vaccinatie, terwijl ook in deze groep net als in de wK gevaccineerde kinderen de antistof niveaus wel fors waren afgenomen gedurende die 3 jaar. In deze 4-jarige kinderen hebben we ook in detail de verschillende T-cellen bestudeerd en gezien dat het aantal goed functionerende T-cellen ook hoger is in aK gevaccineerde kinderen dan in wK gevaccineerde kinderen.

Op 4-jarige leeftijd wordt een 5e DaKTP (difterie, acellulair kinkhoest, tetanus en polio) inenting gegeven (dit heet een boostervaccinatie) aan zowel aK als wK gevaccineerde kinderen. In de aK gevaccineerde kinderen hadden wij dus al hoge T-cel responsen gevonden voor de boostervaccinatie en deze waarden gingen niet verder omhoog op de korte termijn of

op de lange termijn (tot 2 jaar later) na de boostervaccinatie op 4-jarige leeftijd. Daarentegen hebben we in de wK kinderen gezien dat de T-cel responsen wel duidelijk omhoog gingen kort na de boostervaccinatie op 4 jaar en vervolgens ook hoog bleven of nog verder toenamen op de langere termijn. Zowel een lage als een hoge dosis van het kinkhoest boostervaccin stimuleerde de afweer en de dosis van het acellulaire boostervaccin was niet van invloed op de hoogte van de T-cel respons na de vaccinatie.

Na een boostervaccinatie worden snel nieuwe antistoffen door de B-cellen aangemaakt. We hebben gezien dat na de boostervaccinatie in aK gevaccineerde kinderen soms een ander type antistoffen wordt aangemaakt, welke geassocieerd wordt met een ander type T-cel respons (type 1 en type 2 responsen) na de aK vaccinatie. Ook de verhouding tussen de type 1 en 2 T-cel respons is iets veranderd na de boostervaccinatie in aK kinderen richting een type 2 respons.

Opvallend was ook dat na de 5e aK vaccinatie op 4-jarige leeftijd bij kinderen die geboren waren na 2005, meer kinderen een grote rode plek of zwelling op de injectieplaats ontwikkelden na de vaccinatie, dan kinderen die als zuigeling het wK vaccin hadden gekregen, ook al komt deze bijwerking nog steeds weinig voor. De lokale symptomen op de injectieplaats komen op binnen een dag en verdwijnen ook weer binnen enkele dagen. De veranderde T-cel responsen die we hebben gevonden na de boostervaccinatie op 4 jaar zouden verband kunnen houden met deze lokale reacties en dat wordt momenteel verder onderzocht.

Aangezien het aantal gevallen van kinkhoest juist in de adolescenten ook is toegenomen heeft een aantal landen om ons heen een extra kinkhoestvaccinatie ingevoerd rond de 10- tot 12-jarige leeftijd. In Nederland krijgen kinderen sowieso al op 9-jarige leeftijd een herhalingsvaccinatie tegen difterie, tetanus en polio (DTP) en bof, mazelen en rode hond. Het zou relatief eenvoudig zijn om dan ook tegen kinkhoest te vaccineren met een DKTP vaccin in plaats van het huidige DTP vaccin. Wij hebben onderzocht of een extra kinkhoestvaccinatie in 9-jarige Nederlandse kinderen effectief zou kunnen zijn, 5 jaar na de aK boostervaccinatie op 4-jarige leeftijd. In dit proefschrift hebben we alleen de T-cel responsen van de 9-jarige kinderen, die als baby met het wK waren gevaccineerd, kunnen bestuderen, omdat de 9-jarige aK kinderen nog niet geïncludeerd konden worden. Uiteindelijk is het de bedoeling dat de 9-jarige aK en wK kinderen weer met elkaar worden vergeleken.

De T-cel responsen in de 9-jarige wK kinderen laten een lichte stijging zien na de extra boostervaccinatie. Deze hogere T-cel responsen lijken voldoende om opnieuw de B-cellen te activeren en het niveau van antistoffen te laten stijgen. Collega's hebben namelijk aangetoond dat na een inenting bij 9-jarige wK kinderen, de antistoffen en het aantal geheugen B-cellen tegen kinkhoest stijgen. Maar het is nog onduidelijk of een inenting bij 9-jarigen echt bijdraagt aan een langdurige bescherming tegen kinkhoest op antistof en op cellulair niveau. Hiertoe moeten deze kinderen langdurig worden vervolgd. Vervolgonderzoek moet ook aantonen hoe dit zal lopen in de huidige generatie van aK gevaccineerde kinderen die nu 9 jaar zijn geworden.

Van de kinderen met een wK vaccinatie achtergrond, hebben we kinderen van 4, 6 en 9 jaar oud met elkaar vergeleken. Wij vonden dat de T-cel responsen tegen kinkhoest eiwitten toenamen met de leeftijd. De T-cel responsen in 9-jarige kinderen zijn hoger dan in 6-jarige kinderen, terwijl er tussen de twee tijdpunten geen vaccinatie moment is geweest. Deze T-cel responsen zijn dus niet door een vaccinatie gestimuleerd, maar mogelijk door natuurlijk contact met de kinkhoestbacterie. De circulatie van de kinkhoestbacterie in de bevolking is sinds eind jaren '90 hoog en veel mensen, waaronder kinderen, kunnen dan geïnfecteerd worden met de bacterie. Het overgrote deel van deze infecties bij oudere kinderen en volwassenen zal een subklinisch verloop hebben en de betrokken mensen zullen er weinig van merken of hoogstens een periode hoestklachten hebben, wat niet wordt herkend als kinkhoest. Grote bevolkingsstudies hebben laten zien, dat het aantal mensen dat geïnfecteerd is geweest met kinkhoest, is verdubbeld in ruim tien jaar (2007 tov 1996). Ook is het aantal geregistreerde ziektegevallen van kinkhoest in de loop van de jaren toegenomen.

Conclusies/Discussie

Uit de resultaten van dit proefschrift blijkt dat de eerste vier inentingen in het eerste levensjaar nog lang bepalend zijn voor de immuniteit tegen kinkhoest op latere leeftijd. Met name op jonge leeftijd is het naïeve immuunsysteem erg gevoelig voor de eerste stimulatie. Met de vier acellulaire inentingen bij zuigelingen worden binnen enkele maanden relatief hoge concentraties van specifieke kinkhoesteiwitten toegediend. Het cellulaire vaccin bestaande uit gedode hele bacteriën bevat lagere concentraties van die specifieke kinkhoesteiwitten, maar bestaat wel uit meer verschillende kinkhoesteiwitten en andere biologische bestanddelen. Het type vaccin, het aantal vaccinaties op de babyleeftijd en de tijd tussen de vaccinaties lijkt van belang voor de immuunrespons die opgebouwd wordt tegen een bacterie en dat blijft van invloed op de immuniteit op latere leeftijd.

Onze studies geven aan dat acellulair ingeënte kinderen hogere immuunresponsen hebben in vergelijking met cellulair ingeënte kinderen totdat ze een jaar of zes zijn. Maar het effect op de langere termijn is nog niet duidelijk. Naast de invloed van de verschillende vaccinaties in het eerste levensjaar op de aansturing van het immuunsysteem van kinderen, zullen de immuunresponsen in kinderen ook toenemen door natuurlijk contact met de bacterie, omdat die circuleert in de bevolking.

Het evalueren van cellulaire immuunresponsen na kinkhoest vaccinatie en/of natuurlijke infectie naast het bepalen van de specifieke antistofniveaus, geeft belangrijke informatie voor de richting van een mogelijke aanpassing van het bestaande vaccinatie schema. Zo kan men denken aan het meer spreiden van de vaccinaties in het eerste levensjaar en het uitstellen van de vaccinatie op 4 jaar tot bijvoorbeeld 6 jaar. Dit vraagt om onderzoek met de bestaande vaccins. Het blijft echter van groot belang om nieuwe vaccins te ontwikkelen die wèl een langdurende bescherming kunnen induceren om de ziekte ooit een halt toe te kunnen roepen.

Aanbevelingen

Met name jonge, ongevaccineerde baby's worden ernstig ziek van kinkhoest en moeten hiertegen worden beschermd. De toename van het aantal gevallen van kinkhoest de afgelopen decennia in veel leeftijdsgroepen van de bevolking is daarom riskant. Omdat de bacterie circuleert in de hele bevolking kan die door (groot)ouders, broers, zussen en andere sociale contacten worden overgedragen op de jonge kwetsbare baby's. Om deze circulatie van kinkhoest in de bevolking terug te dringen zouden er een aantal mogelijke veranderingen in het RVP verder onderzocht kunnen worden. In dit proefschrift hebben we laten zien dat het meten van T-cel immuunresponsen waardevol is om mee te nemen in die onderzoeken. Op basis van de T-cel responsen gemeten in de studies die beschreven zijn in dit proefschrift kunnen we alvast een paar aanbevelingen doen om verder te bestuderen.

De Kleuterprik

De 5e DKTP vaccinatie van kinderen op 4-jarige leeftijd is belangrijk voor de bescherming tegen kinkhoest. Echter 4-jarige kinderen, die als baby het acellulaire vaccin hebben gekregen, laten ruim 3 jaar na de laatste kinkhoestvaccinatie nog hoge T-cel responsen zien. Een optie zou kunnen zijn dat de 5e vaccinatie uitgesteld gaat worden naar de leeftijd van een jaar of zes. Het goed vervolgen van het effect van deze verandering op de korte en lange termijn is wel noodzakelijk, ook al weten we dat een dergelijk schema al in andere landen wordt toegepast.

Na de 5e acellulaire vaccinatie op de kleuterleeftijd gaat de T-cel respons niet verder omhoog, maar het type T-cel respons verschuift wel enigszins. De B-cel responsen stijgen wel, maar ook hier zien we veranderingen in de antistof types. Deze veranderingen in de T- en B-cel respons worden wel geassocieerd met een meer allergische reactie, waarbij het opvalt dat de lokale reactie van zwelling en roodheid van de arm na vaccinatie meer voorkomt bij 4-jarige kinderen kort na de 5e acellulaire vaccinatie ten opzichte van 4-jarige kinderen die als zuigeling het cellulaire vaccin hebben gekregen. Omdat we geen verschillen in T-cel responsen zagen na een vaccinatie met een hoge dosis of lage dosis acellulaire kinkhoest vaccin op 4 jaar, denken we dat deze 5e vaccinatie evengoed met een lage dosis vaccin gegeven zou kunnen worden. Daarbij is het wel van belang dat de dosis van PT, het belangrijkste vaccin eiwit, niet te laag mag zijn voor een goede bescherming.

Gezien het feit dat op 4-jarige leeftijd de T-cel responsen tegen kinkhoest bij acellulair gevaccineerde kinderen nog erg hoog waren, is het de vraag of het huidige vaccinatieschema tijdens het eerste levensjaar niet herzien zou moeten worden en of de vaccinatie niet beter uitgesteld kan worden naar 6 jaar.

Adolescente boostervaccinatie

Het invoeren van een extra kinkhoest inenting op 9-jarige leeftijd zou adolescenten wellicht beter kunnen beschermen tegen kinkhoest. Kinderen, die met wK gevaccineerd zijn geweest in het eerste levensjaar zijn ondertussen ouder dan 9 jaar. Een extra vaccinatie voor voor deze wK gevaccineerden op adolescente leeftijd zou effectief kunnen zijn, maar het is de

vraag of dit alsnog zou moeten plaatsvinden, aangezien dit niet kosten-effectief zal zijn. Of een extra DaKTP herhalingsvaccinatie in 9-jarige aK gevaccineerde kinderen een betere immuunrespons tegen kinkhoest zal induceren wordt momenteel onderzocht. De uitkomsten van die studie zijn belangrijk om te zien hoe het gesteld is met de cellulaire immuunrespons tegen kinkhoest 5 jaar na de 5e DaKTP vaccinatie op 4-jarige leeftijd. In de epidemie van 2012 bleek dat kinderen in de Verenigde Staten (VS), ondanks een 5e (op 6 jaar) en een 6e (op 11 jaar) acellulaire inenting, toch vrij snel na deze vaccinaties geïnfecteerd konden raken. De bescherming van deze acellulaire boostervaccinaties is voor een aantal kinderen waarschijnlijk beperkt tot 2-4 jaar. Er zijn aanwijzingen dat kinderen met de volledige cellulaire serie (wK) in het eerste levensjaar beter beschermd waren tijdens de epidemie van 2012 in de VS. Uitgaande van deze bevindingen lijkt het toevoegen van een extra acellulaire boostervaccinatie voor 9-jarigen in Nederland geen garantie te zijn voor een betere bescherming op de langere termijn.

Vaccineren in de zwangerschap

Om pasgeborenen te beschermen doet het RIVM, in een recent kinkhoestrapport aan de Gezondheidsraad, de aanbeveling om zwangere vrouwen te vaccineren tijden het laatste trimester van de zwangerschap. De pasgeborene wordt dan via antistoffen van de moeder, die zij tijdens de zwangerschap overdraagt, beter beschermd in de eerste kwetsbare maanden. Andere landen, zoals de VS, UK en België hebben deze aanbeveling al ingevoerd tijdens de laatste kinkhoestepidemie en de eerste resultaten van het onderzoek naar de overdracht van antistoffen aan de baby's zijn hoopgevend. Ook zal de moeder geen besmettingsbron voor haar baby zijn omdat ze zelf goed is beschermd. In Nederland wordt een onderzoek gestart naar de bescherming tegen kinkhoest door het vaccineren van zwangeren.

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About the author

Rose-Minke Schure was born on December 12th, 1983 in Goirle, the Netherlands. She attended high school at the Jacob Roeland Lyceum in Boxtel and completed her Gymnasium exam in 2002. In 2007, she graduated from the Utrecht University at the department of Biomedical Sciences, studying Biomedical Sciences with a specialization in Immunity and Infection. During this study she completed her first internship at Orthopaedic Research at the Utrecht Medical Center under supervision of Prof. Dr. W.J.A. Dhert, Dr. L. Creemers and Dr. D.J. Moojen. During this research project she studied the role of platelet rich plasma on orthopaedic implant related infections. Her final internship was at the National Institute of Public Health and the Environment (RIVM) in Bilthoven under the supervision of Dr. A.M. Buisman. This research project had the focus on T-cell immunity to pertussis. In 2007 she started as a technical research associate to evaluate T-cell immunity to pertussis. In 2008, she started her PhD project at this department under the supervision of Dr. A.M. Buisman, Dr. G.A.M. Berbers and Prof. Dr. E.A.M. Sanders. Her work resulted in several international publications, which are included in this thesis.

List of publications:

Schure, R. M., L. de Rond, K. Ozturk, L. Hendrikx, E. Sanders, G. Berbers, and A. M. Buisman. 2012. Pertussis Circulation Has Increased T-Cell Immunity during Childhood More than a Second Acellular Booster Vaccination in Dutch Children 9 Years of Age. PLoS One 7:e41928.

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