

**Elevated Immune Response Among Children 4 Years Of Age With Pronounced Local
Adverse Events After The Fifth Dtap Vaccination**

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Background: In the Netherlands, acellular pertussis vaccines replaced the more reactogenic whole-cell pertussis vaccines. This replacement in the primary immunization schedule of infants coincided with a significant increase in pronounced local adverse events (AEs) in 4 years old children shortly after the administration of a fifth diphtheria, tetanus, acellular pertussis and inactivated polio (DTaP-IPV) vaccine. The objective of this study was to investigate possible differences in vaccine antigen-specific immune responses between children with and without a pronounced local AE after the fifth DTaP-IPV vaccination.

Methods: Blood was sampled in two groups of four year olds: a case group reporting pronounced local swelling and/or erythema up to extensive limb swelling at the injection site (n=30) and a control group (n=30). PBMCs were stimulated with individual vaccine antigens. Plasma antigen-specific IgG, IgG-subclass and total IgE concentrations, and T-cell cytokine (IFN- γ , IL-13, IL-17 and IL-10) production by stimulated PBMCs were determined by multiplex bead-based-fluorescent multiplex immunoassays.

Results: In children with AEs, significantly higher total IgE and vaccine antigen-specific IgG and IgG4 responses as well as levels of the Th2 cytokine IL-13 were found after pertussis, tetanus and diphtheria stimulation compared with controls.

Conclusions: Children with pronounced local reactions show higher humoral and cellular immune responses. Acellular vaccines are known to skew towards more Th2 responses. The pronounced local AEs may be associated with more Th2-skewing after the fifth DTaP-IPV vaccination, but other biologic factors may also impact the occurrence of these pronounced local reactions.

Keywords: Pronounced adverse events, Acellular booster vaccination, Immune response, Children, Th2-skewing

Introduction

Whooping cough is reemerging world-wide despite widespread adaptations of vaccination schemes. Since the early 1990s, whole cell pertussis (wP) vaccines have been replaced by acellular pertussis (aP) vaccines¹⁻³. In 2005, the Dutch wP combination vaccine used in the primary vaccination series for infants at age 2, 3, 4 and 11 months, was replaced by an aP combination vaccine. Three years later, children who have been primed with aP vaccines in infancy received a fifth aP combination vaccine at four years of age. Since then, an increase in adverse events (AEs) shortly after the administration of the diphtheria, tetanus, acellular pertussis and inactivated polio (DTaP-IPV) preschool booster vaccine was observed. Significantly more pronounced local and systemic AEs were reported in aP-primed children compared to wP-primed children⁴.

The underlying immunologic mechanisms for these heightened AEs in aP-primed children are not well understood. Previous studies revealed higher numbers of pertussis-specific T-helper 2 (Th2) cells indicative for Th2-skewing in aP-primed children compared with wP-primed children^{5,6}. Profound Th2-skewing after aP vaccination was also shown in the baboon model when compared with wP vaccination⁷. Besides pertussis-specific T-cell differences, significantly higher levels of pertussis-specific IgG, IgG4 and IgE antibodies were described in aP-primed children compared with wP-primed children^{6,8,9}. In general, IgG4, IgE and Th2-responses are associated with allergic inflammatory diseases such as asthma and atopic dermatitis^{10,11}.

Pre-existing high tetanus-specific Th2 cytokine responses have been correlated with increased risk of local AEs¹² and pertussis toxin specific-IgE antibodies before and after DTaP booster vaccination were significantly higher in children with AEs compared to children without

them¹³. The differences in AEs between aP- and wP-priming suggest that the occurrence of AEs in aP-primed children could be related to pertussis-specific humoral and cellular immune responses. Few studies have reported on immune responses in children with AEs after a DTaP booster vaccination at preschool age. We therefore performed a study in four years old children with and without a pronounced local AE. Blood samples were collected shortly after a fifth DTaP-IPV vaccination to investigate a possible association between immune responses and AEs. Vaccine antigen-specific humoral and cellular immune responses were compared between the two groups.

Materials and Methods

Study population and design

Adverse events are requested to be reported to the Dutch Pharmacovigilance Centre (Lareb). Parents of four years old children who spontaneously reported local swelling and/or erythema of ≥ 5 cm's at the injection site within 48 hours after the booster vaccination were invited to participate in the study by Lareb (case-group). If parents were willing to participate, the study nurse made an appointment for a home visit 10 days (± 3 days) after the booster vaccination. A blood sample (8 ml) and a questionnaire including a few questions regarding atopic background of the children were collected. Parents of four year olds without pronounced AEs (< 1 cm) were invited to participate by an information letter (control-group). The same study procedures after the booster vaccination applied for the control-group. All children were vaccinated according to the Dutch national immunization program. Children were excluded in case of other vaccinations within a month before DTaP-IPV booster vaccination, and in case of comorbidity or fever $\geq 38^{\circ}\text{C}$ at the day of blood sampling.

This phase-IV case-control study was approved by the Medical research Ethics Committees United (MEC-U, Nieuwegein, the Netherlands). Written informed consent from both parents or legal representatives was obtained.

Vaccines

At four years of age, children received a pediatric combination vaccine (DTaP-IPV), i.e. Infanrix-IPVTM (GlaxoSmithKline Biologicals (GSK), Rixensart, Belgium). In infancy, children had received four pediatric DTaP-IPV combination vaccines at 2, 3, 4 and 11 months of age (Infanrix-IPV+HibTM, Infanrix-hexaTM (both GSK) or PediacelTM (Sanofi Pasteur, Lille, France)).

Blood samples

Peripheral blood was collected in vacutainer cell preparation tubes containing sodium citrate (BD Biosciences, CA) and PBMCs were isolated within 18 hours, washed, counted and stored at -135°C as described earlier¹⁴ and plasma's were stored at -20°C.

Serologic analysis

Plasma IgG antibody concentrations against diphtheria toxoid (Dd), tetanus toxin (TT), and the three pertussis vaccine antigens pertussis toxin (PT), filamentous hemagglutinin (FHA) and pertactin (Prn) (DTaP antigens) were determined using the fluorescent-bead-based multiplex immunoassay (MIA) as described^{15,16}. The in-house pertussis reference sample was calibrated to the WHO International Standard (Pertussis Antiserum 1st international standard, 06/140, NIBSC) to express IgG concentrations in international units (IU) per ml. As a control, plasma IgG antibody levels against mumps, measles and rubella (MMR) were quantified using MIA as described¹⁷.

The four IgG-subclasses against DTaP vaccine antigens were measured as reported⁹. Blanc mean fluorescent intensity (MFI) values were subtracted from all samples. Individual IgG-

subclass levels were first expressed as MFI values and subsequently each IgG-subclass was expressed as a percentage of the sum of MFI of all four IgG-subclasses together. Samples without a detectable response were given a value of 1 MFI.

DTaP antigen-specific IgE was determined according to the IgG MIA described above, by using mouse anti-human IgE-PE (eBioscience, CA) as secondary antibody and expressed as MFI. For quantification of total IgE, Bio-Plex Pro™ Human IgE Isotyping Assay (Bio-Rad Laboratories, CA) was used according to manufacturers' instructions.

T-cell stimulation

PBMCs were stimulated according to Schure *et al.*⁶. In short, 3.0×10^5 viable cells per well were cultured in AIMV medium (Gibco Invitrogen, NY) containing 5% heat-inactivated human AB serum (Harlan Laboratories, Leicestershire, UK) and stimulated with 2 µg/ml heat-inactivated PT or FHA (Kaketsuken, Kumamoto, Japan), 4 µg/ml recombinant Prn¹⁸, 6.67 Lf/ml tetanus toxoid (Td) (Netherlands Vaccine Institute (NVI), Bilthoven, The Netherlands), 10 Lf/ml Dd (NVI), or 4.4 µg/ml lectin (LC) (Pokeweed mitogen, Sigma-Aldrich, MO) as a positive control. Non-stimulated cells served as negative controls. Cells were incubated for 5 days at 37°C with 5% CO₂ in 96 wells round-bottom culture plates, supernatants were collected and stored at -80°C until cytokine analysis.

Cytokine analysis

Cytokines interleukin-10 (IL-10), IL-13, IL-17 and interferon-gamma (IFN-γ) were quantified with MIA as described^{19,20}. Cytokine concentration of non-stimulated cells were subtracted from all samples. Values below the detection limit were set at half of the lowest quantifiable concentration for each cytokine.

Statistical analysis

Geometric mean concentrations (GMCs) and corresponding 95% confidence intervals (CIs) of vaccine antigen-specific antibody and cytokine responses were calculated. Percentages of IgG-subclasses were described as mean with standard deviations (SD). Normal distribution of GMCs and mean percentages of log transformed data was checked prior to analysis. Differences between case and control-group were tested with Mann-Whitney-U test. Differences in the ratio of IFN- γ /IL-13 within the groups were tested with Wilcoxon Signed Rank test. GMC's and corresponding 95% CIs were calculated using GraphPad Prism 6 (GraphPad Software), statistical analysis was performed using SPSS statistics 22 (IBM). A P-value ≤ 0.05 was considered statistically significant. Due to changes in the immunization program regarding vaccine manufacturer, participants have been divided in subgroups based on primary vaccines, for sub-analysis (**table 1**).

A linear-mixed-model analysis was performed to explore possible differences between the two study groups regarding the response to all vaccine antigens together per participant. This model consists of three parts, first, the response variable, which are the IgG4 MFI values, IFN- γ or IL-13 cytokine concentrations. Secondly, the so-called fixed effect variables (the explanatory variables), here a variable indicating whether a participant belongs to the case or control-group (our variable of interest), and a variable indicating one of the five DTaP vaccine antigens, because vaccine responses will differ between the antigens. Thirdly, the so-called random effect variable, here the participant ID, which is included in the model as a random intercept.

Results

Study population

Between March 2012 and September 2013, 63 children were enrolled in the study. During the inclusion period, 290 eligible children with pronounced local AEs were reported to Lareb and were invited to participate, of which 10% accepted to join the study. From 60 children, i.e. 30 cases and 30 controls, sufficient plasma was available and from 59 enough PBMCs. Of the cases, 16 children showed inflammation of the whole upper limb and adjacent body parts (ELS, extensive limb swelling), and 14 children had injection site inflammation (ISI) with at least two inflammation symptoms (swelling, redness, heat and/ or pain) (see supplementary **figure S1**, Supplemental Digital Content 1, <http://links.lww.com/INF/C715> for an example of an AE).

There were no differences in mean age and gender between the two groups (**table 1**). A small but significant difference existed in the sampling window between the case and the control-group (mean days 10.4 ± 2.0 versus 9.0 ± 1.8 , respectively). Therefore, children were also divided into subgroups excluding those sampled at day 13 (seven children in the case-group, none in the controls) which resulted in a similar sampling window between these subgroups. Since subgroup-analysis excluding children sampled at day 13 resulted in similar findings, humoral and cellular immune responses were described for the entire study population.

Humoral immune responses

The GMC of non-antigen-specific total IgE (**table 2** and supplementary **figure S2**, Supplemental Digital Content 2, <http://links.lww.com/INF/C716>) of the case-group (284.2 ng/ml) was significantly higher than that of the controls (109.8 ng/ml; $P = 0.029$). Antigen-specific IgE levels were also determined but were below the detection limit for the majority of children (data not shown).

Overall, the GMCs of DTaP antigen-specific IgG responses upon the preschool booster vaccination tended to be higher in the case-group compared to the controls, reaching significance only for diphtheria-specific IgG ($P=0.01$) (**figure 1** and **table 2**). As a control, IgG antibodies to the measles, mumps and rubella (MMR) vaccine antigens, which all children had received at 14 months of age, were measured. No differences in MMR-IgG GMCs between the case and control-group were observed (**table 2** and supplementary **figure S3**, Supplemental Digital Content 3, <http://links.lww.com/INF/C717>).

Subsequently, IgG-subclass responses showed that DTaP antigen-specific IgG2 and IgG4 MFI values were significantly higher in the case-group compared to the controls. In concordance with total vaccine antigen-specific IgG, IgG1 MFI values were only significantly different between the two groups for diphtheria (**table 3**). No differences in IgG3 MFI values were found between the groups. Notably, within the case-group and the controls, the antigen specific MFI values for the IgG subclasses varied considerably between the individuals. In line with results of a previous study, the sum of the MFI values of the IgG-subclasses correlated well with the MFI values of antigen-specific total IgG⁹.

The predominant IgG-subclass for all five vaccine antigens in both groups was IgG1, with mean values ranging from 86.2% in the case-group to 95.4% in the controls (**figure 2a** and **table 3**). After IgG1, IgG4 contributed most to the IgG levels, up to 13%. IgG2 and IgG3 hardly contributed to the antigen-specific IgG levels, respectively ranging from 0.5% to 4.7% and 0.2% to 1.2%. The contribution of IgG1 to total IgG was significantly lower in the case-group compared to the controls for the pertussis antigens PT, FHA and Prn and for tetanus (**table 3**). In particular, the relative contribution of IgG4 specific for FHA, Prn and tetanus was significantly higher in the case-group compared to the controls. Also, most of the IgG2 contributions were

significantly higher in the case-group, but these percentages were (very) low (**table 3**). Next to the overall antibody levels, several children in both groups showed high proportions of antigen-specific IgG4 varying from around 20% up to even 72% (**figure 2b**). More specific, 17/30 children (57%) in the case-group and 7/29 (23%) in the controls showed a proportion of IgG4 of $\geq 10\%$ for one or more antigen. Interestingly, three children in the case-group and two in the control-group showed $\geq 10\%$ IgG4 antibodies for all five vaccine antigens. Overall, children with ELS tended to show higher humoral responses than children with ISI, reaching significance for PT and Prn specific IgG4.

The response to all five vaccine antigens together was analyzed with a linear-mixed-model for IgG4 MFI values. Comparing the case and the control-group, the combined IgG4 response to the five DTaP antigens resulted in a GMC ratio of 4.06 (95% CI: 1.84-8.86; p-value < 0.001). This corroborates the difference in IgG4 response between the two groups even further.

Since a difference was observed between the case and the control-group in vaccines during the primary series, a sub-analysis was performed including just the participants primed with Pediacel vaccines (see table 1). Overall, the sub-analysis resulted in similar differences in humoral immune responses between the case and the control-group compared to the analysis of the entire study population, although for some parameters significance was lost due to low numbers in these subgroups, (data not shown).

Cellular vaccine antigen-specific cytokine responses

Thelper cytokines IFN- γ (Th1), IL-13 (Th2), IL-17 (Th17) and IL-10 (T-regulatory) were evaluated after antigen stimulation. IFN- γ was higher in the case-group compared to the controls for FHA, Prn and diphtheria, although not significant (see supplementary **figure S4**, Supplemental Digital Content 4, <http://links.lww.com/INF/C718>). Furthermore, no difference

was found between the two groups for the combined results of the DTaP antigens using the linear-mixed-model. IL-13 values specific for Prn, diphtheria and tetanus, as well as Prn-specific IL-10 values, were significantly higher in the case-group compared with the controls. Antigen-specific IL-17 responses were not significantly different between the two groups.

Variation between individuals within the groups proved high for all different cytokine levels, up to a 1000 fold. While some children did not have detectable antigen-specific IFN- γ or IL-13 responses, high levels of them were produced by PBMCs stimulated with lectin, indicating that all children were able to induce Th1- and Th2-responses. Furthermore, all children showed IL-10 and IL-17 responses to at least one of the antigens. After lectin stimulation, no significant differences between the two groups were observed for any of the cytokines supporting that differences between groups were antigen-specific (see supplementary **figure S4**, Supplemental Digital Content 4, <http://links.lww.com/INF/C718>).

Comparing IFN- γ and IL-13 concentrations within the two groups, IFN- γ was significantly lower than IL-13 for all five DTaP vaccine antigens (all P-value's < 0.01). This resulted in IFN- γ /IL-13 ratios <1 for all antigens in both groups, which is indicative for Th2-skewing (**figure 3**). This low IFN- γ /IL-13 ratio was most pronounced for diphtheria (ratio of 0.06 for the case-group and 0.04 for the controls) but without differences between the two groups. In contrast, aspecific lectin stimulation induced significantly more IFN- γ than IL-13 (with IFN- γ /IL-13 ratio of 13.5 for the case-group and 12.1 for the controls), showing that the Th2-skewing was specific for the antigens in the preschool DTaP booster vaccination. Similar results were found in the subgroups based on vaccination background showing significance just for tetanus-specific IL-13.

Discussion

This study showed elevated immune responses to the DTaP vaccine antigens in children with a pronounced local adverse event (AE) within 48 hours upon a fifth DTaP-IPV vaccination at four years of age compared to children without an AE (controls), though with high individual variation. Children with AEs showed significantly higher levels of total IgE, diphtheria-specific IgG, IgG4 for all DTaP antigens and IL-13 for Prn, diphtheria and tetanus. Th2-skewing was observed for all DTaP vaccine antigens in both groups.

Two previous studies found no differences in total antigen-specific IgG responses between children with and without AEs after vaccination^{12,21}. In contrast to our study, only tetanus-specific IgG was analysed¹² or the allocation of subgroups was based on AEs after the primary vaccinations rather than the occurrence of AEs after the booster vaccination²¹. However, the significantly higher diphtheria-IgG levels in our study were in agreement with results of Edelman *et al.*¹³. This similarity was remarkable since their study was conducted in two years old children sampled one month after their fourth DTaP vaccination.

The most remarkable finding in our study was the high values of all vaccine antigen-specific IgG4 responses in the case-group. In general, protein antigens predominantly elicit an IgG1-subclass response, with less IgG3 and IgG4 and rarely IgG2²²⁻²⁴. Surprisingly, we found high proportions of DTaP antigen-specific IgG4, with a prevalence of even 11% tetanus-specific IgG4 antibodies in the case-group. This is in contrast to previous studies, where a very low prevalence of tetanus-specific IgG4 was reported^{23,25}. With regard to pertussis-specific IgG-subclass responses, so far only three studies were conducted in aP-primed children^{9,26,27}. The results of these studies were consistent with our study showing higher IgG4 levels after repeated exposure to vaccine antigens^{23,25}. IgG4 responses are associated with chronic antigenic

stimulations²⁸, suggesting that the high amounts of purified antigens in the DTaP vaccines given within a relatively short period (at 2, 3, 4 and 11 months of age) initiate this response.

Interestingly, a sixth DTaP booster vaccination in pre-adolescents also results in at least similar high IgG4 production (van der Lee *et al.*, manuscript in preparation). Since class-switching to IgG4 is dependent on the Th2 cytokines IL-4 and IL-13, similar to IgE class-switching, IgG4 is often associated with Th2-skewed responses²². IgG4 antibodies are suggested to have anti-inflammatory properties as the appearance of IgG4 seems to be related to symptom reduction in allergic patients^{22,29}. This might suggest that an AE following DTaP vaccination is associated with higher total IgE and IL-13 concentrations and that IgG4 may not contribute to clinically observed AEs in these children. The determination of IgG4 responses could be relatively easily implemented in more vaccine immunogenicity studies to be able to evaluate Th2-skewing of vaccine responses.

Studies investigating atopic diseases have reported significantly lower Th1/Th2 ratios in supernatants of lectin-stimulated PBMCs in atopic children compared to non-atopic children¹¹. In contrast, we observed a high Th1/Th2 ratio in both groups upon lectin-stimulation suggesting that the higher IgE levels ten days post-vaccination may be related to vaccine antigens and not to the already established Th2-skewing in the case-group. However, it is also reported that plasma Th2cytokine levels in 4-6 years old aP-primed children were higher before and 35 days after aP-booster vaccination in atopic children compared to non-atopic children, all without AEs³⁰. We did not find more atopic children in our case-group based on questionnaires, but the numbers of children in our groups were small. However, the higher total IgE values in children with an AE suggests a more atopic background. Atopic children could be at increased risk for AEs after a fifth DTaP vaccination but this needs to be further studied^{10,11,28,31}. Interestingly, swelling of the

injection site is also seen after repeated administration of influenza vaccines in children and adults³².

Recently, Scheifele *et al.* reported a doubled incidence of erythema at the injection site in children with detectable diphtheria-specific cell-mediated immunity (CMI) prior to a DTaP booster vaccination, compared to children without diphtheria-specific CMI³³. This suggests that pre-existing high levels of vaccine antigen-specific Th2-cells and the Th2-skewed immune response to DTaP vaccination influence the susceptibility to local AEs post-booster vaccination. We found significantly higher levels of the Th2 cytokine IL-13 for Prn, diphtheria and tetanus in the case-group, which is in line with others showing this just for tetanus¹². The high Th2-responses to the vaccine antigens in aP primed children might be due to the absence of bacterial components in the aP combination vaccines, which were present in wP vaccines. The lack of these components could be the cause of a more Th2-skewed response, not just specific for the pertussis antigens, but also for the diphtheria and tetanus antigens^{34,35}. Our findings of Th2-skewing were vaccine antigen-specific since aspecific stimulation with lectin induced Th1-dominated responses as expected, and the IFN- γ /IL-13 ratio below 1 was only found for the vaccine antigens. Although human studies are scarce, we and others previously reported mixed Th1/Th2 profiles in aP-primed children, with a tendency to Th2-skewing^{5,36}.

Immune responses in relation to AEs have been studied for one or some of the DTaP vaccine antigens, mostly one month post-booster vaccination in 4-6 year olds^{12,33}. A strength of our study was the ability to investigate a broad range of vaccine antigen-specific humoral and cellular immune responses in relation to AEs already ten days after the four years old DTaP booster vaccination, when immune responses are already at its peak^{6,8,37}. This short time interval between vaccination and sampling enabled us to establish differences between groups that would

otherwise have remained unobserved. Although there was a significant difference in sampling window between the two groups, a sub-analysis was performed excluding children sampled on day 13, which resulted in a similar sampling window between the two groups. Moreover, results of the sub-analysis regarding differences in immune responses between the two groups were comparable with the analysis performed on all children. Furthermore, de Voer *et al.* demonstrated that meningococcal serogroup C (MenC) specific IgG concentrations were similar 10 and 17 days after MenC conjugate booster vaccination, indicating that variation in sampling interval from 10 to 13 days post-vaccination may not be critical as the IgG levels seem not to increase any further³⁸.

One of the limitations of this study was that pre-vaccination samples were not included. However, the occurrence of pronounced AEs is too low and doesn't allow the enrolment of many children for longitudinal sampling and also resulted in small group numbers in this study. Despite the small groups, we do see interesting differences between children with and without pronounced AEs. Another limitation is the different priming with three aP-combination vaccines. However, we re-analyzed all findings in subgroups with participants only vaccinated with Infanrix or Pediacel combinations. Although numbers were low and the power limited, similar differences as described were observed in these subgroups. This indicates that the differences observed between the case and the control-group in humoral immune responses after the pre-school booster vaccination were most likely not driven by a difference in priming vaccines.

As a control, MMR antibody levels, induced after MMR-vaccination at 14 months of age, were determined to explore if the antibody levels for non-DTaP vaccine antigens between both groups were similar. From a population-based serosurveillance study performed in 2006/2007, it was clear that the decay of MMR antibody levels was only minimal between 14 months and 4

years. We found no differences in MMR antibody levels between the two groups indicating that other vaccines induced comparable immune responses in both groups³⁹.

In summary, it was previously suggested that the AEs after the DTaP preschool booster vaccination were most likely due to the repeated administration of the aP vaccine antigens, since the increase in AEs was mainly seen after introduction of aP-combination vaccines in the primary vaccination schedule. We now demonstrated high immune responses in children with pronounced AEs not only to the pertussis vaccine antigens, but also to diphtheria and tetanus, and Th2-skewing for all antigens in all participants. The variation in the individual immune responses of children with an AE indicates that these events are associated with an accumulation of high Th2-skewed responses in combination with an additional biologic factor. This needs to be evaluated in further studies. On the long run, new DTaP vaccines that would elicit a more Th1-skewed response might reduce the risk of these rare local, but pronounced, reactions.

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Figure 1. Geometric mean IgG concentration with 95% confidence interval of pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (Prn), diphtheria toxoid (Dd) and tetanus toxin (TT) in plasma of children in the case-group (red circles) and the control-group (blue circles) around day 10 post-booster vaccination. For each antigen, each dot represents one participant. * = significant difference between case and control-group ($P \leq 0.05$).

Figure 2. a: Distribution of IgG-subclasses against PT, FHA, Prn, Dd and TT, presented as mean percentages for IgG1 (blue), IgG2 (red), IgG3 (yellow) and IgG4 (green). b: Mean IgG4-percentages specific for PT, FHA, Prn, Dd and TT in the case-group (red circles) and the control-group (blue circles). For each antigen, each dot represents one participant. Significant difference between case and control-group: * = $P \leq 0.05$; ** = $P \leq 0.01$

Figure 3. The geometric means of IFN- γ / IL-13 ratio with 95% confidence intervals for PBMCs stimulated with DTaP vaccine antigens and lectin (LC) for the case-group (red circles) and the control-group (blue circles) around day 10 post-booster vaccination. Individuals with undetectable cytokine concentration for both IFN- γ and IL-13 were excluded. For each antigen, each dot represents one participant.

List of Supplemental Digital Content

Supplemental Digital Content 1: Picture of an example of a local adverse event after the fifth DTaP vaccination

Legend: Example of an adverse event after the fifth pediatric DTaP-IPV vaccination at four years of age. (David et al. 2010 Nederlands Tijdschrift voor Geneeskunde)

Supplemental Digital Content 2: Figure that illustrates total IgE antibody concentrations of the case- and the control-group

Legend: Geometric mean concentration with 95% confidence interval for total IgE for the case group (red circles) and the control group (blue circles). * = $P \leq 0.05$

Supplemental Digital Content 3: Figure that illustrates IgG antibody concentrations of mumps, measles and rubella of the case- and the control-group

Legend: Measles, Mumps and Rubella specific geometric mean IgG concentrations with 95% confidence intervals for the case group (red circles) and the control group (blue circles) in children four years of age.

Supplemental Digital Content 4: Figure that illustrates all cytokine responses after DTaP antigen stimulation of PBMC's of the case- and the control-group

Legend: Geometric mean cytokine concentrations with 95% confidence intervals for Th1 (a, IFN- γ), Th2 (b, IL-13), Th17 (c, IL-17) and regulatory (d, IL-10) in supernatants of PBMCs of the case-group (red circles) and the control-group (blue circles) around day 10 post-booster vaccination. Stimulated with: pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (Prn), diphtheria toxoid (Dd), tetanus toxoid (Td), and pokeweed mitogen lectin (LC). N=25-30. Significant difference between case- and control-group: * = $P \leq 0.05$; ** = $P \leq 0.01$

Table 1. Characteristics of the case and control-groups.

Group	Number	Male	Age mean	Window mean	Priming vaccines*		
		no (%)	years ($\pm$ SD)	days ($\pm$ SD)	Infanrix ^a	Pediacel ^b	Mix ^c
Case	30	12 (40)	3.9 (0.2)	10.4 (2.0)*	7	11	12
Control	30	16 (53)	3.8 (0.1)	9.0 (1.8)	4	22	4

a. Number of children who received Infanrix-IPV-Hib (hexa) at 2, 3, 4, 11 months of age containing 25 μ g PT, 25 μ g FHA, 8 μ g Prn, $\geq$ 30 IU diphtheria toxoid, $\geq$ 40 IU tetanus toxoid, respectively 40, 8 and 32 D-antigen of inactivated poliovirus (IPV) serotype 1, 2 and 3, 10 μ g of *Haemophilus influenzae* type b polysaccharide conjugated to tetanus toxoid (approximately 25 μ g), (10 μ g of Hepatitis-B), 0.5 mg of Al(OH)₃ and 0.32 mg AlPO₄.

b. Number of children who received Pediacel at 2, 3, 4, 11 months of age containing 20 μ g PT, 20 μ g FHA, 3 μ g Prn, 5 μ g Fimbriae type 2 and type 3, $\geq$ 30 IU diphtheria toxoid, $\geq$ 40 IU tetanus toxoid, respectively 40, 8 and 32 D-antigen of inactivated poliovirus serotype 1, 2 and 3, 10 μ g of *Haemophilus influenzae* type b polysaccharide conjugated to tetanus toxoid (20 μ g), and 0.33 mg AlPO₄.

c. Number of children who received a combination of Infanrix-IPV-Hib (hexa) and Pediacel vaccines at 2, 3, 4, 11 months of age.

* = significant difference between case and control-group ($P \leq 0.05$)

Table 2. Specific IgG geometric mean concentrations (GMC) with the corresponding 95% confidence intervals (95% CI) for pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (Prn), diphtheria toxoid (Dd), tetanus toxin (TT) (the five DTaP vaccine antigens), measles, mumps and rubella (the MMR vaccine antigens) and total IgE around day 10 post DTaP-IPV booster.

	Antigen	Case group	Control group	P-value^b
GMC IgG IU/ml (95% CI)	PT	76.9 (51.6-114.5)	63.4 (43.7-92.1)	0.487
	FHA	266.6 (199.7-356)	173.3 (119.1-252.1)	0.067
	Prn	590.5 (384.2-907.5)	358.6 (202.3-635.5)	0.231
	Dd	7.1 (5.6-9.1)	3.8 (2.5-5.6)	0.010
	TT	9.5 (7.0-12.8)	7.1 (5.1-10.0)	0.574
	Measles	1.8 (1.3-2.4)	1.7 (1.2-2.2)	0.745
	Mumps^a	166.2 (115.4-239.3)	116.9 (80.7-169.5)	0.690
	Rubella	76.3 (55.0-105.7)	80.2 (59.8-107.7)	0.145
GMC Total IgE ng/ml (95% CI)		284.2 (138.2-584.4)	109.8 (50.6-238.4)	0.029

a. IgG concentration for mumps presented in RIVM Units (RU)/ml

b. P-values of the case-group versus the control-group, statistically significant when P-value $\leq$ 0.05 (bold values)

Table 3. DTaP vaccine antigen-specific IgG-subclasses.

Subclass	Antigen	Geometric mean MFI x10 ⁵ (95% CI) ^a			Mean percentage (± SD) ^b		
		Case group	Control group	P-value ^c	Case group	Control group	P-value ^c
IgG1	PT	8.7 (5.7-13.2)	7.5 (5.1-10.9)	0.559	87.8 (± 14.9)	94.4 (± 5.9)	0.022
	FHA	58.6 (44.2-77.5)	39.8 (27.4-57.7)	0.132	88.6 (± 11.1)	93.6 (± 5.3)	0.041
	Prn	235.1 (160.2-344.9)	164.8 (96.6-281.2)	0.469	87.5 (± 16.6)	95.4 (± 7.9)	0.029
	Dd	246.4 (194.7-311.9)	139.1 (93.0-208.1)	0.021	86.2 (± 16.4)	90.9 (± 12.8)	0.135
	TT	183.3 (133.1-252.4)	151.9 (107.8-213.9)	0.525	87.3 (± 18.2)	92.1 (± 15.3)	0.015
IgG2	PT	0.12 (0.07-0.21)	0.04 (0.02-0.06)	0.010	4.7 (± 9.9)	1.5 (± 2.6)	0.023
	FHA	0.76 (0.52-1.13)	0.29 (0.16-0.55)	0.011	1.7 (± 1.7)	1.2 (± 1.8)	0.025
	Prn	0.81 (0.53-1.22)	0.23 (0.14-0.38)	0.001	0.9 (± 1.6)	0.5 (± 0.8)	0.084
	Dd	1.10 (0.82-1.47)	0.45 (0.32-0.65)	<0.001	0.6 (± 0.8)	0.5 (± 0.5)	0.329
	TT	0.75 (0.49-1.15)	0.24 (0.14-0.42)	0.001	0.9 (± 1.3)	0.6 (± 1.1)	0.040
IgG3	PT	0.03 (0.02-0.06)	0.02 (0.01-0.04)	0.574	0.8 (± 1.6)	0.6 (± 0.9)	0.941
	FHA	0.36 (0.23-0.57)	0.32 (0.22-0.47)	0.941	1.0 (± 1.4)	1.2 (± 1.7)	0.086
	Prn	0.33 (0.13-0.82)	0.20 (0.07-0.61)	0.679	0.4 (± 0.8)	0.6 (± 1.1)	0.941
	Dd	0.27 (0.19-0.38)	0.25 (0.17-0.36)	0.701	0.2 (± 0.2)	0.3 (± 0.4)	0.046
	TT	0.48 (0.32-0.73)	0.49 (0.35-0.69)	0.888	0.5 (± 0.6)	0.5 (± 0.5)	0.515
IgG4	PT	0.3 (0.2-0.6)	0.1 (0.1-0.2)	0.016	6.7 (± 9.8)	3.5 (± 4.5)	0.081
	FHA	2.7 (1.5-4.8)	0.6 (0.3-1.3)	0.002	8.7 (± 10.8)	4.0 (± 4.8)	0.018
	Prn	7.5 (3.3-16.7)	1.5 (0.7-3.2)	0.004	11.1 (± 15.9)	3.5 (± 7.3)	0.024
	Dd	17.0 (9.4-30.7)	5.2 (3.3-8.3)	0.002	13.0 (± 16.4)	8.4 (± 12.7)	0.128
	TT	6.8 (3.6-12.8)	1.2 (0.5-2.7)	0.001	11.3 (± 17.9)	6.8 (± 14.4)	0.003

a. Geometric means of mean fluorescent intensity (MFI) with 95% confidence intervals (95% CI). MFI values were corrected for measurement dilution.

b. Individual MFI values of each IgG-subclass was expressed as a percentage of the sum of MFI of all four IgG-subclasses together and depicted as mean percentages with standard deviation (SD)

c. P-values of the case-group versus the control-group, statistically significant when P-value ≤ 0.05 (bold values)

Figure 1

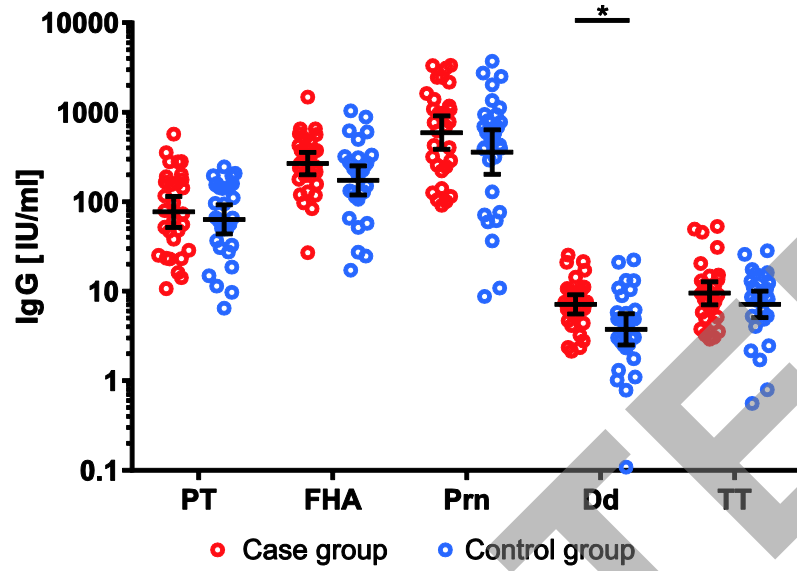


Figure 2

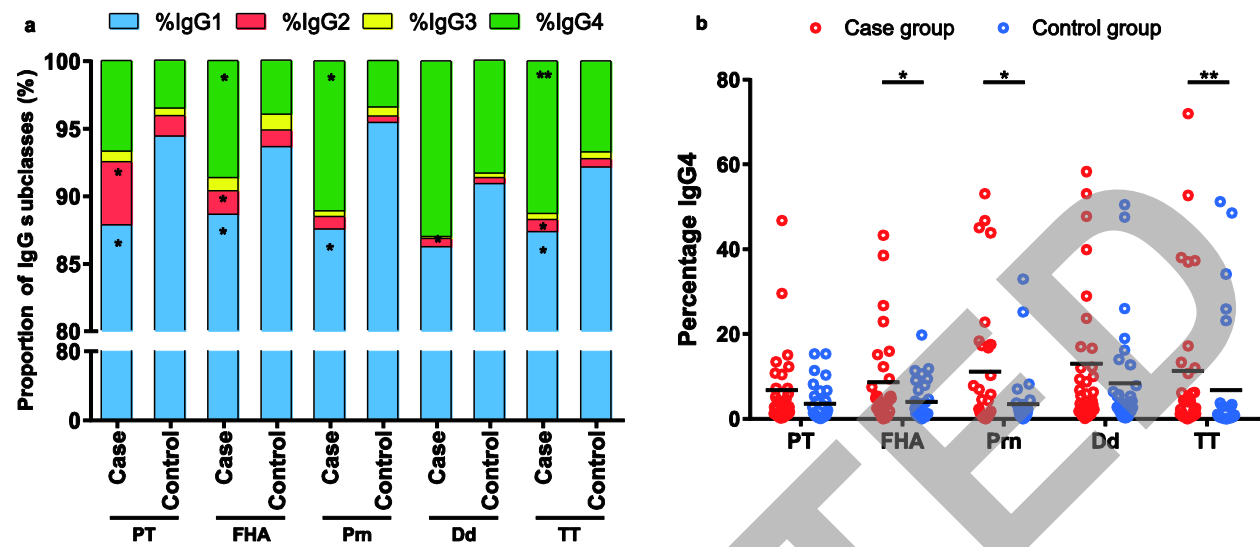


Figure 3

