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Identification of Pertussis-Specific Effector Memory T Cells in Preschool Children

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Whooping cough remains a problem despite vaccination, and worldwide resurgence of pertussis is evident. Since cellular immunity plays a role in long-term protection against pertussis, we studied pertussis-specific T-cell responses. Around the time of the preschool acellular pertussis (aP) booster dose at 4 years of age, T-cell memory responses were compared in children who were primed during infancy with either a whole-cell pertussis (wP) or an aP vaccine. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with pertussis vaccine antigens for 5 days. T cells were characterized by flow-based analysis of carboxyfluorescein succinimidyl ester (CFSE) dilution and CD4, CD3, CD45RA, CCR7, gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) expression. Before the aP preschool booster vaccination, both the proliferated pertussis toxin (PT)-specific $CD4^+$ and $CD8^+$ T-cell fractions ($CFSE^{dim}$) were higher in aP- than in wP-primed children. Post-booster vaccination, more pertussis-specific $CD4^+$ effector memory cells ($CD45RA^-$ $CCR7^-$) were induced in aP-primed children than in those primed with wP. The booster vaccination did not appear to significantly affect the T-cell memory subsets and functionality in aP-primed or wP-primed children. Although the percentages of Th1 cytokine-producing cells were alike in aP- and wP-primed children pre-booster vaccination, aP-primed children produced more Th1 cytokines due to higher numbers of proliferated pertussis-specific effector memory cells. At present, 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 those in wP-primed children. The booster at 4 years of age is therefore questionable; this may be postponed to 6 years of age.

Whooping cough remains a worldwide problem in high-income countries despite high pertussis vaccination coverage. Already since the 1990s, acellular pertussis (aP) vaccines have been implemented in the immunization programs to replace whole-cell pertussis (wP) vaccines in many countries. In the past 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 have been observed since 1996 (4, 5). Since 2001, preschool children in The Netherlands are 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 than in 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 $CD45RA^+$ $CCR7^+$ naive T cells. The $CD45RA^-$ $CCR7^+$ cells 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

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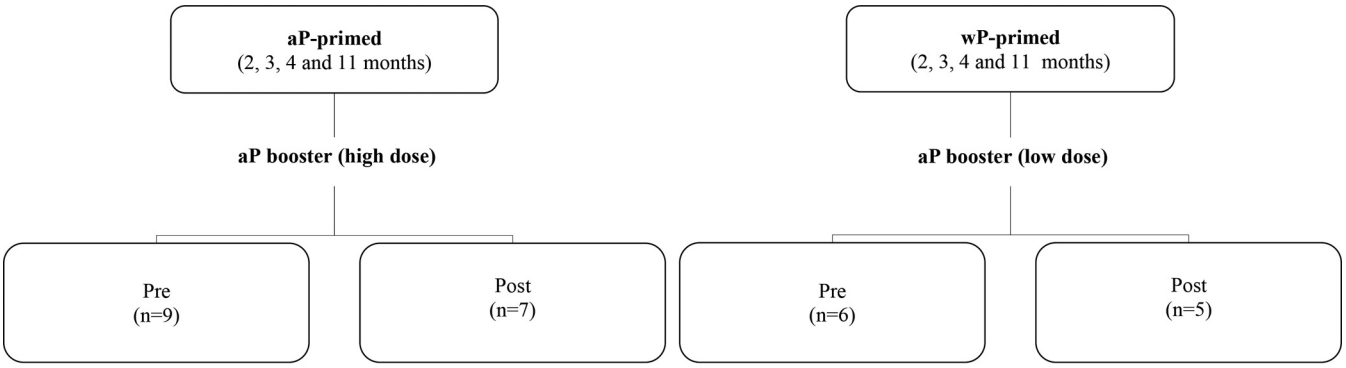


FIG 1 Four different groups of children were used in this study. Children primed with either aP or wP vaccine at 2, 3, 4, and 11 months of age received an aP booster vaccine at 4 years of age. The aP-primed group received a high-dose vaccine and the wP-primed group received a low-dose vaccine. The groups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

most differentiated T cells, still capable of producing cytokines (16–18).

The aim of the present study was to improve the insight in the immunological memory 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 (carboxyfluorescein succinimidyl ester [CFSE]), the memory markers CD45RA and CCR7, and the intracellular Th1-type cytokines gamma interferon (IFN-γ) and tumor necrosis factor alpha (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.

MATERIALS AND METHODS

Study population. In this study, T cells of children 4 years of age were analyzed. 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., before and 10 days after booster vaccination (Fig. 1). This study was conducted according to the Declaration of Helsinki and good clinical practice guidelines, with the approval of the relevant ethics review committee. Written informed consent was obtained from both parents or from legal representatives.

Vaccines. All aP-primed children had received diphtheria–tetanus–acellular-pertussis vaccine (DTaP)-inactivate polio vaccine (IPV)-*Haemophilus influenzae* type b (Hib) (Infanrix-IPV-Hib; GlaxoSmithKline Biologicals S.A., Rixensart, Belgium), containing 25 μg pertussis toxin (PT), 25 μg filamentous hemagglutinin (FHA), and 8 μg pertactin (Prn) (high-dose vaccine), at 2, 3, 4, and 11 months of age according to the Dutch National Immunization 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 types 2 and 3 (Fig. 1). During the inclusion period of this study, children received randomly either a low-dose or high-dose preschool booster vaccine. Due to a temporarily shortage in supply of the high-dose booster vaccine, we had to include wP-primed children who had received a low-dose vaccine, Triaxis.

Eight-color FACS analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from blood as described earlier and frozen (19). After thawing, 1×10^6 PBMCs were stained with 5 μM carboxylfluorescein succinimidyl ester (CFSE) (Sigma Chemicals, St. Louis, MO) for 10 min in the dark at 4°C to measure proliferation of cells. After the PBMCs were washed, cells were cultured in AIMV medium (Gibco Invitrogen, Grand Island, NY) containing 5% human AB serum (Harlan Laboratories, Leicestershire, United Kingdom). 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). Nonstimulated cells (NS) and cells stimulated with 1 μg/ml pokeweed mitogen (PWM) (Sigma) 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 fluorescence-activated cell sorting (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 h before further intracellular cytokine staining. Cells were collected, washed, and stained for dead cell discrimination by Aqua amine-reactive dye (Invitrogen, Paisley, Scotland, United Kingdom) and for the cell surface markers allophycocyanin (APC)-H7-labeled CD4 (BD), phycoerythrin (PE)-Cy7-labeled CD45RA (BD), and PE-labeled CCR7 (R&D Systems, Minneapolis, MN). Subsequently, cells were resuspended in a Cytofix/Cytoperm Plus kit (BD) and stained for V450-labeled CD3 (BD), PerCP-Cy5.5-labeled TNF-α (BioLegend, San Diego, CA), and APC-labeled IFN-γ (BD). After being washed, the cells were analyzed using the FACSCanto cytometer (BD) in combination with Diva software (version 5.2 BD) and FlowJo software (Mac version 9.3.2; Tree Star, Ashland, OR). Proliferated ($CFSE^{dim}$) viable $CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$ T-cell populations were further divided in naive ($CCR7^+$ $CD45RA^+$), T_{CM} ($CCR7^+$ $CD45RA^-$), T_{EM} ($CCR7^-$ $CD45RA^-$), and T_{TD} ($CCR7^-$ $CD45RA^+$) subsets and analyzed for intracellular Th1 cytokine production as described recently (21, 22). The $CD3^+$ $CD4^+$ and $CD3^+$ $CD4^-$ T-cell populations are further defined as $CD4^+$ and $CD8^+$ T cells.

Flow cytometric data analysis. Flow cytometry standard format 3.0 files were exported, and data were evaluated using FlowJo software. The gating strategy is shown in Fig. 2. Dead cells were excluded if stained with

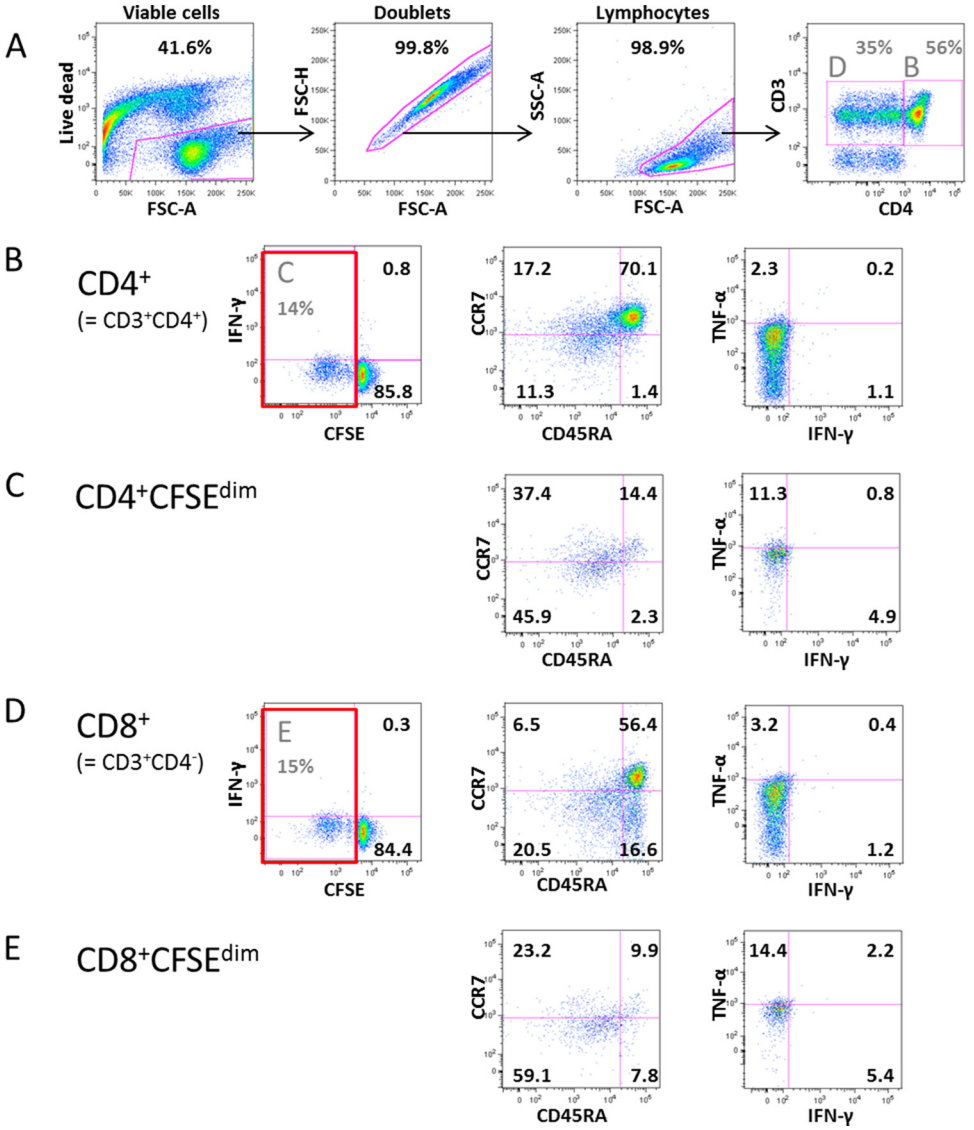


FIG 2 Example of the 8-color FACS analysis of T cells stimulated with pertussis antigen. (A) Viable cells were gated, doublet cells were excluded, and lymphocytes were selected using forward scatter (FSC-A) and side scatter (SSC-A), and subsequently $CD4^+$ and $CD8^+$ cells were gated. Total $CD4^+$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

Aqua amine-reactive dye as showed in Fig. 2A. Singlets were selected using forward-scatter area (FSC-A) and forward-scatter height (FSC-H) of viable cells, and lymphocytes were gated based on FSC-A/side-scatter area (SSC-A) parameters (Fig. 2A). Both $CD4^+$ and $CD8^+$ total 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 ($CD4^+$ $CFSE^{dim}$ and $CD8^+$ $CFSE^{dim}$) (Fig. 2C and E, respectively) were further analyzed for memory subset populations (CD45RA, CCR7) and intracellular cytokine-producing IFN-γ^+ TNF-α^-/IFN-γ^+ TNF-α^+/IFN-γ^- TNF-α^+ ($Th1^+$ cells) T cells. Using the FSC-A/SSC-A morphological parameters, we identified the blast region of the proliferated $CD4^+$ cells (Fig. 3A). Only samples showing both more than 1×10^4 lymphocytes and proliferation after positive control (PWM) stimulation were included in the data analysis. Because of the limited numbers of PBMCs available per sample, not all antigens could be tested in each sample. Pertussis antigen-specific stimulation is the mean from stimulation data from the three pertussis antigens (PT, FHA, and Prn) or even 2 antigens if numbers of PBMCs were limited.

Statistical methods. Results were expressed in medians (25th to 75th percentile) or means ± standard deviations of the mean (SD). The Mann-Whitney U test was used to determine differences between groups. *P* values of <0.05 were considered significantly different. Correlations were compared by linear regression and by calculating the correlation coefficients (R^2 values).

RESULTS

Proliferation of pertussis antigen-specific T cells. In general, 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, and the frequency was significantly elevated for PT pre-booster vaccination (Table 1). The total frequencies of PT-specific proliferating $CD4^+$ T cells of aP-primed children were significantly decreased post-booster vaccination compared to pre-booster vaccination. The FHA and Prn stimulation of T cells of aP-primed children resulted

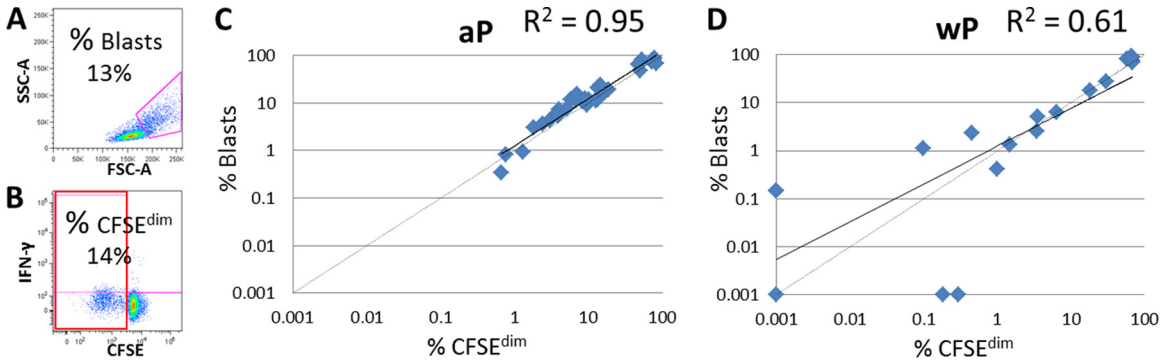


FIG 3 (A and B) Representative example of pertussis-specific T-cell proliferation determined by FACS analysis of the blast region of T cells using the morphological parameters FSC-A/SSC-A (A) or the $CFSE^{dim}$ frequencies of T cells (B). (C and D) Correlation of proliferated T cells identified by the blast region with proliferated T cells determined by the $CFSE^{dim}$ frequencies of aP (C)- and wP (D)-primed children 4 years of age before booster vaccination. Per sample, data from nonstimulated proliferated cells (NS) were subtracted from those from the pertussis-specific stimulated cells.

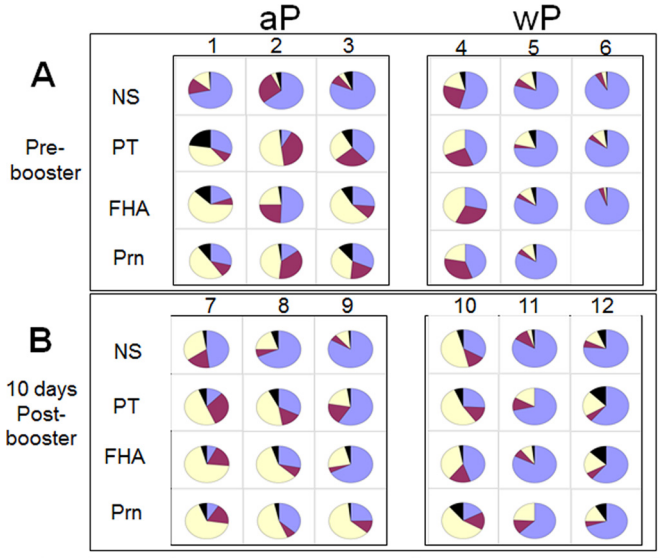
in comparable frequencies of proliferated $CD4^+$ and $CD8^+$ T cells before and after booster vaccination. In wP-primed children who were boosted with a low-dose aP vaccine at 4 years, all PT and FHA stimulations resulted in similar T-cell proliferation pre- and post-booster vaccination. The Prn-specific T cells showed a tendency to increase post-booster vaccination. The frequencies of proliferated $CD8^+$ T cells were highly comparable to those of $CD4^+$ T cells after pertussis stimulation at all time points in both groups (Table 1). Within a European collaboration, the T-cell proliferation has been determined by either identifying the blast region using the morphological parameters FSC-A/SSC-A or by staining for CFSE (21) (Fig. 3A and B). Pre-booster vaccination, a good correlation ($R^2 = 0.95$) 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 ($R^2 = 0.61$) (Fig. 3D). This was caused by the lower cell proliferation of some samples. The post-booster vaccination correlation of these two parameters was similar ($R^2 = 0.75$) for aP- and wP-primed children (data not shown).

Memory subset characterization of pertussis antigen-specific T cells. We determined the distribution of the memory subsets of proliferated $CD4^+$ $CFSE^{dim}$ T cells both pre-booster vaccination and at 10 days post-booster vaccination. The results of three representative cross-sectional sampled subjects 4 years of age primed with either aP or wP are illustrated in Fig. 4. Overall, the nonstimulated samples in all four different groups consisted of large amounts of naive T cells. Upon pertussis-specific stimulation, T cells of aP-primed children displayed higher proportions of T_{CM} and especially T_{EM} and T_{TD} than the control nonstimulated cells (Fig. 4A and B). In contrast, T-cell subsets of wP-primed children before the booster did not really change upon stimulation (Fig. 4A). The distributions of the T-cell memory subsets of either aP- or wP-primed children also remain similar upon booster vaccination (Fig. 4B). Overall, stimulation with the three different pertussis antigens resulted in a similar distribution of the subsets. The ratio of the proportion of T_{EM} after stimulation with pertussis antigen related to nonstimulated cells per individual is presented in Fig. 4C. In aP-primed children, the proportion of T_{EM} before the booster showed an increase up to13-fold, whereas post-booster vaccination, a 2- to 6-fold increase with less variation was observed. In wP-primed children, the proportion of T_{EM} before the booster at 4 years increased just up to 3-fold and was lower than that in aP-primed children. Post-booster vaccination, the

TABLE 1 Proliferation of $CD4^+$ and $CD8^+$ T cells determined by CFSE staining[a]

Antigen by cell type	Pre-booster vaccination				10 days post-booster vaccination			
	aP primed		wP primed		aP primed (Infanrix)		wP primed (Triaxis)	
	% of T cells (median [25th to 75th percentile])	No. of children	% of T cells (median [25th to 75th percentile])	No. of children	% of T cells (median [25th to 75th percentile])	No. of children	% of T cells (median [25th to 75th percentile])	No. of children
$CD4^+$								
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

[a] Children 4 years of age were vaccinated with either Infanrix (high-dose) or Triaxis (low-dose) aP booster vaccine. PBMCs were stimulated for 5 days with PT, FHA, or Prn. Per sample, the nonstimulated T-cell proliferation data were subtracted. *, significant difference between aP- and wP-primed children; #, significant difference between pre- and post-booster vaccination T cells of aP-primed children.



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

Pre-booster

10 days Post-booster

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

FIG 4 Memory subsets of proliferated ($CFSE^{dim}$) $CD4^+$ T cells of aP- and wP-primed children cross-sectionally sampled before (A) and 10 days after (B) booster vaccination are presented. The proportions of naive T cells ($CCR7^+$ $CD45RA^+$; purple), central memory T cells (T_{CM}) ($CCR7^+$ $CD45RA^-$; magenta), effector memory T cells (T_{EM}) ($CCR7^-$ $CD45RA^-$; yellow), and terminally differentiated T cells (T_{TD}) ($CCR7^-$ $CD45RA^+$; black) specific for PT, FHA, and Prn antigens are shown. (C) The ratio of the fraction of proliferated ($CFSE^{dim}$) T_{EM} (yellow in panels A and B) in antigen-stimulated cultures to individual nonstimulated cultures is shown.

proportion of T_{EM} of only one wP-primed child showed an increase up to 7-fold (Fig. 4C).

In aP-primed children, several differences between nonstimulated cells and cells with pertussis stimulation were detected. Pre-booster vaccination, the proportions of pertussis-specific naive T cells of these children were lower and the proportions of T_{EM} and T_{TD} were significantly higher than those in nonstimulated T cells (Fig. 5A). After booster vaccination in aP-primed children, the proportions of both T_{CM} and T_{EM} were significantly higher and the proportion of naive cells was still lower than those in the nonstimulated T cells (Fig. 5B). Within the aP-primed children, there were no significant differences in memory subsets between the pre- and post-booster vaccination samples.

In the pre-booster vaccination samples of wP-primed children, just the pertussis-specific T_{TD} levels were lower than those of nonstimulated T cells (Fig. 5A), although these proportions were very small. Post-booster vaccination, no significant differences were found between the T-cell distributions of these children and nonstimulated T cell distribution (Fig. 5B). Between wP-primed children, there was a significant rise in pertussis-specific T_{TD} post-booster compared to pre-booster vaccination, although the proportions are very low.

Comparing aP-primed children to wP-primed children pre-booster vaccination, the proportion of pertussis-specific naive T cells was lower in aP-primed children, whereas that of T_{TD} was higher (Fig. 5A). Post-booster vaccination, the proportion of the pertussis-specific naive T cells was lower and that of T_{EM} was significantly higher in aP-primed children than in wP-primed children (Fig. 5B).

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

Th1 intracellular cytokine production of pertussis antigen-specific $CD4^+$ $CFSE^{dim}$ and $CD8^+$ $CFSE^{dim}$ T cells. The T cells producing intracellular IFN-γ and TNF-α have been identified in aP- and wP-primed children before and 10 days after booster vaccination both in the proliferated ($CFSE^{dim}$) $CD4^+$ and $CD8^+$ T cells (Fig. 6). In both aP- and wP-primed children, the same proportions of $CD4^+$ $CFSE^{dim}$ IFN-γ^+ TNF-α^-/IFN-γ^+ TNF-α^+/IFN-γ^- TNF-α^+ ($Th1^+$ cells) T cells were found before the booster. Pre-booster vaccination, only a few aP-primed children had high proportions of both $CD4^+$ $CFSE^{dim}$ and $CD8^+$ $CFSE^{dim}$ Th1 cytokine-producing cells. Post-booster vaccination, a significantly higher proportion of $CD4^+$ $CFSE^{dim}$ $Th1^+$ cells was detected in aP-primed than in wP-primed children. Moreover, the proportion of Th1 cytokine-producing $CD4^+$ $CFSE^{dim}$ cells of aP-primed children post-booster vaccination displayed less variation than those of wP-primed children. In general, in the same individual, higher proportions of Th1 cytokine-producing $CD4^+$ $CFSE^{dim}$ cells than $CD8^+$ $CFSE^{dim}$ cells were found after pertussis antigen stimulation.

Notably, there were no significant differences in the proportions of $CD4^+$ or $CD8^+$ $CFSE^{dim}$ memory subsets producing Th1 cytokines between pre- and post-booster vaccination samples of both aP- and wP-primed children. The proliferated T cells that produce Th1 cytokines in aP-primed children were mainly $CD4^+$ T_{CM} (15% ± 14%), $CD4^+$ T_{EM} (17% ± 13%), $CD8^+$ T_{CM} (9% ± 15%), and $CD8^+$ T_{EM} (11% ± 12%). The memory subset proportions in wP-primed children are lower than those found in aP-primed children.

DISCUSSION

We investigated possible differences in memory T-cell subsets of proliferated $CD4^+$ and $CD8^+$ T cells specific for pertussis vaccine antigens in pre- and post-booster vaccination samples from children 4 years of age who were 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. The T cells of aP-primed children contained more effector memory T cells and terminally differentiated T cells than those of wP-primed children. Moreover, priming with four acellular infant vaccine doses in the first year of life resulted in a higher proportion of effector memory T cells already before the preschool booster than in children primed with wP at 4 years of age. Conversely, the proliferated T cells of wP-primed children compared to aP-primed children still had a significantly larger amount of naive T cells in pertussis-stimulated cultures, both pre- and post-booster vaccination.

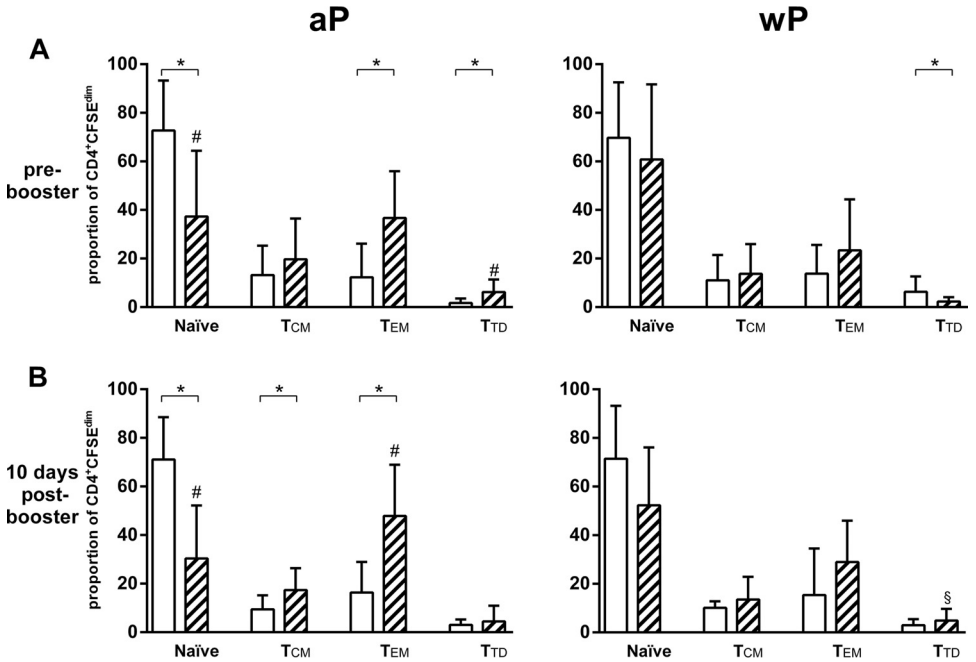


FIG 5 Memory subsets of proliferated ($CFSE^{dim}$) $CD4^+$ T cells of aP-primed (left) and wP-primed (right) children, before (A) and 10 days after (B) booster vaccination at 4 years of age. Data are presented as proportions of naive ($CCR7^+$ $CD45RA^+$), central memory (T_{CM}) ($CCR7^+$ $CD45RA^-$), effector memory (T_{EM}) ($CCR7^-$ $CD45RA^-$), and terminally differentiated (T_{TD}) ($CCR7^-$ $CD45RA^+$) T-cell subsets. Bars represent means and SD for nonstimulated cells (NS) (white bars) and pertussis-stimulated T cells (hatched bars). Pertussis stimulation is shown as the mean from the stimulation data for three pertussis antigens (PT, FHA, and Prn), aP-primed pre-booster vaccination ($n = 23$), aP-primed post-booster vaccination ($n = 19$), wP-primed pre-booster vaccination ($n = 13$), and wP-primed post-booster vaccination ($n = 13$). *, significant difference between nonstimulated and pertussis-stimulated cells; #, significant difference between aP- and wP-primed children; §, significant difference between pre- and post-booster vaccination T cells of wP-primed children.

Although the percentages of Th1 cytokine-excreting cells were similar in both groups of children, the total amount of the Th1 cytokine-positive cells was higher in aP-primed children than in wP-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 memory T-cell subsets and functionality of pertussis-specific T cells in either aP-primed or wP-primed children.

In this study, we focused on proliferated T cells, since these cells are involved mainly in the development of T_{CM}, T_{EM}, and T_{TD}, as agreed upon within a European collaboration network, Child-Innovac. Our results, showing a clear proliferation of $CD4^+$ and $CD8^+$ T cells of aP-primed children upon pertussis stimulation, are in agreement with studies that showed that $CD4^+$ and $CD8^+$ T cells are involved in the immune response against pertussis (13, 21, 23). Moreover, the observations that pertussis-specific T_{CM} were more prevalent in $CD4^+$ than in $CD8^+$ T cells and that the relative proportions of T_{CM} and T_{EM} do not change after a booster immunization are in line with other studies on memory T-cell subsets (16, 24). In general, we confirmed the presence of higher proportions of T_{TD} in non-

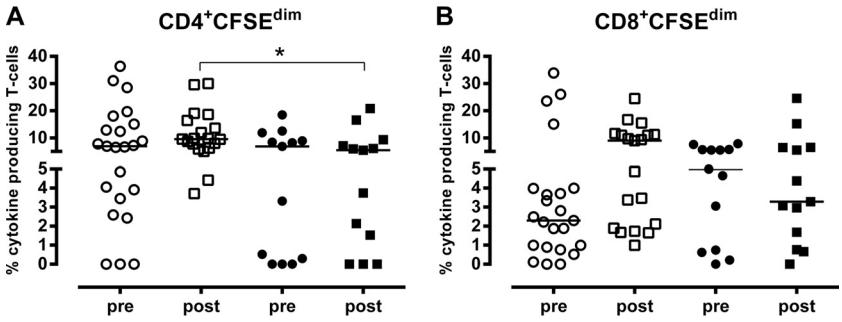


FIG 6 Percentages of IFN-γ^+ TNF-α^-/IFN-γ^+ TNF-α^+/IFN-γ^- TNF-α^+-producing $CD4^+$ $CFSE^{dim}$ (A) and $CD8^+$ $CFSE^{dim}$ (B) proliferated T cells of aP-primed (open symbols) and wP-primed (filled symbols) children, before (circles) and 10 days after (squares) booster vaccination at 4 years of age. Per sample, the nonstimulated data of cytokine-producing T cells were subtracted. Percentage of IFN-γ^+ TNF-α^-/IFN-γ^+ TNF-α^+/IFN-γ^- TNF-α^+-producing proliferated T cells is shown as the mean from the stimulation data for three pertussis antigens (PT, FHA, and Prn), aP-primed pre-booster vaccination ($n = 23$), aP-primed post-booster vaccination ($n = 19$), wP-primed pre-booster vaccination ($n = 13$), and wP-primed post-booster vaccination ($n = 13$). Horizontal lines indicate the median per group. *, $P \leq 0.05$.

stimulated $CD8^+$ T cells than in $CD4^+$ T cells both in wP- and aP-primed children (16, 24).

Pertussis-specific memory subsets have been studied in relation to the T-cell activation status after vaccination in other age groups (13, 14). Sharma and Pichichero found that pertussis-specific T-cell responses of infants who have received 3 doses of DTaP vaccine were restricted to $CD4^+$ T_{CM} and that adults had more fully differentiated pertussis-specific $CD4^+$ T cells than infants due to multiple vaccinations (14). We earlier have demonstrated that wP-primed children 9 years of age showed higher numbers of pertussis-specific T_{EM} than wP-primed 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- than in 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 reimmunization lies between 5 and 15 days after vaccination (27), the 10-day post-booster vaccination T-cell responses in the vaccinated groups of children were analyzed in this study.

Both $CD4^+$ and $CD8^+$ T cells of aP- and wP-primed children contain pertussis-specific $Th1^+$ cells before and 10 days after the preschool booster vaccination at 4 years of age. Some studies indicate that cytokine production is dependent on the kind of T-cell subset involved and that naive T cells can also produce the IFN-γ and TNF-α cytokines but at a lower level than T_{CM} and T_{EM} (16–18, 28). In this study, Th1 cytokines in the $CD4^+$ or $CD8^+$ $CFSE^{dim}$ T cells were produced mainly by the T_{CM} and T_{EM} memory subsets. Because of the limited numbers of cells available, we could not determine just the 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 pre-booster vaccination 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 vaccination and were enhanced in wP-primed children following the booster vaccination. This is in agreement with the high 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. All together, the higher numbers of pertussis-specific T_{EM} producing Th1 cytokines in aP-primed children than in wP-primed children leads to a higher total production of these cytokines.

Several limitations of this study need to be discussed. The aP- and wP-primed children were boosted with different aP (high-dose versus low-dose) vaccines. However, we did not find any difference in the induction of effector memory cells producing cytokines at 10 days post-booster vaccination compared to pre-booster vaccination. In addition, we previously showed that cytokine responses at 10 days were similar to those at 28 days post-booster vaccination. 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 the 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. The Dutch wP vaccine contained very small amounts of inactivated PT (29), which is in line with the weak antibody response to PT (7). Interestingly, however, the various recent peaks in pertussis disease in aP-vaccinated populations of Europe, the United States, and Australia indicate that priming with an aP vaccine in the first year of life induces a shorter immune protection later in childhood than priming with a wP vaccine (30–33). This recent knowledge stresses the impact of the pertussis vaccine used for neonates and substantiates the need for a better understanding of the immunogenicity induced by the widely used safe and efficacious aP vaccines and their duration of protection. In this study, we showed that effector memory responses to pertussis are present in aP-vaccinated children 4 years of age at just a few years after the former vaccination at 11 months of age. However, we speculate that this immune response is rather short-lived, and long-term memory might be induced better after wP vaccination. Now we are investigating immune responses in aP-primed children 9 years of age, at 5 years after the 5th aP booster vaccination at preschool age. These results will add to our knowledge on the more long-term immunogenicity of the aP vaccines.

Another limitation is that the method we used for identifying the memory subsets 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 and 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 enzyme-linked immunosorbent spot assay (ELISpot) method showed a higher sensitivity (34, 35) and is easier to handle than FACS analysis. 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 specific Th1 as well as Th2 and Th17 responses by measuring cytokines in the supernatant of pertussis-stimulated T cells, all of which were higher at pre-booster vaccination assessment in aP-primed than in wP-primed children (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 higher T-cell immunity already before the boosting. The 5th aP vaccination at age 4 years had no effect on the phenotype and functionality of these T cells. In the present national immunization program, infants are primed with four high-dose aP vaccines in the first year of life. Therefore, it is tempting to speculate that the highly differentiated pertussis-specific T cells in these aP-primed children cannot be boosted in an efficient way, although antibody levels and B-cell memory responses are enhanced after the aP booster (7, 12). Al-

though the implementation of an aP booster at 4 years of age for wP-primed children in The Netherlands has been successful in the protection of schoolchildren from pertussis, the fifth consecutive aP vaccination at 4 years of age is probably too early for aP-primed children and could be postponed to a later time point, contributing to a longer protection (7, 15). In addition, the dosage may have an impact, although we were not able to investigate this. Other countries have implemented an aP booster at 6 years of age for aP-primed children without an increase in clinical pertussis cases before that age, indicating that the postponement could be a good possibility (8, 36). Moreover, better memory immunity might be induced when children are vaccinated at a later age, when the immune system is more mature (37). 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 (1), 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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