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Persistence of T-cell immune response induced by two acellular pertussis vaccines in children five years after primary vaccination

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SUMMARY

The resurgence of pertussis suggests the need for greater efforts to understand the long-lasting protective responses induced by vaccination. In this paper we dissect the persistence of T memory responses induced by primary vaccination with two different acellular pertussis (aP) vaccines, hexavalent Hexavac® vaccine (Hexavac) (Sanofi Pasteur MSD) and Infanrix hexa® (Infanrix) (Glaxo-SmithKline Biologicals). We evaluated magnitude and duration of T-cell responses to pertussis toxin (PT) by measuring T-cell proliferation, cytokines (IL-2 and IFNγ) production and memory subsets in two groups of children 5 years after primary vaccination. Some of the enrolled children received only primary vaccination, while others had the pre-school boost dose. Positive T-cell responses to PT were detected in 36% of children. Percentage of responsive children, T-cell proliferation and CD4IL-2$^+$ cells were significantly higher in the children primed with Hexavac than in those who received Infanrix vaccine. No major effects of the boost on PT-specific proliferation were observed. Overall, our data documented a persistence of T-cell memory against PT in a minor fraction of children 5 years after primary vaccination. The different responses induced by Hexavac and Infanrix vaccine could rely on differences in PT inactivation process or excipients/adjuvants formulations.

KEY WORDS: Pertussis resurgence, Acellular pertussis vaccine, T-cell responses; Proliferation, Cytokines, Memory T-cell subsets.

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INTRODUCTION

Several potential factors, possibly acting cooperatively, have been identified to explain pertussis resurgence in countries with high vaccination coverage. These include genetic changes in circulating *B. pertussis*, and increased recognition and reporting of pertussis by the application of new, more sensitive, laboratory diagnostic tests (Burns *et al.*, 2014). Nonetheless, age-related waning of protective immunity conferred by the acellular pertussis (aP) vaccines has emerged as a major contributing factor (Burns *et al.*, 2014; Koepke *et al.*, 2014; Klein *et al.*, 2012; Ausiello and Cassone, 2014; Mills *et al.*, 2014).

Several lines of evidence suggest that the duration of protection provided by aP vaccines is lower than that provided by whole-cell pertussis (wP) vaccines (Burns *et al.*, 2014; Koepke *et al.*, 2014; Klein *et al.*, 2012; Ausiello and Cassone, 2014). Furthermore, the rate of vaccine failure gradually increases with the length of

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the interval from the last dose of the aP vaccine (Koepke *et al.*, 2014; Klein *et al.*, 2012; Ausiello and Cassone, 2014; Cherry *et al.*, 1998; Edelman *et al.*, 2007; Edwards, 2014; Storsaeter *et al.*, 1998). Recent data indicate that protection wanes some 3-5 years after aP pertussis vaccination in infancy (Klein *et al.*, 2012; Gustafsson *et al.*, 2006; McGirr and Fisman, 2015). A possible explanation for pertussis resurgence derives from evidence gathered in a baboon infection model, showing that aP vaccine does not protect against pertussis infection, although symptoms are prevented (Warfel *et al.*, 2014).

Despite major efforts, the correlates of protection in pertussis are still elusive (Burns *et al.*, 2014; Vaughan *et al.*, 2014; Plotkin, 2013). Past (Mills, 2001; Crowcroft and Pebody, 2006; Leef *et al*, 2000; Ausiello *et al.*, 1997; Mascart *et al*, 2007) and recent (Schure *et al.*, 2012[a]; Schure *et al.*, 2012[b]; Smits *et al.*, 2013) studies demonstrated the importance of T-cell-mediated immune mechanisms involving individual T-cell populations. However, the effective duration of T memory immune responses induced by vaccination remains undetermined nor it is known to what extent natural or vaccination boosters influence the persistence of memory immune responses.

In a previous study we analyzed serological and B memory responses induced by the primary vaccination with two different aP vaccines, hexavalent Hexavac vaccine (Hexavac) (Sanofi Pasteur MSD) and Infanrix-hexa (Infanrix) (GlaxoSmithKline), in the age group of 6-7 year-old children, five years after the completion of primary vaccination (Carollo *et al.*, 2014). This age group was chosen because, at this age, aP vaccine-induced protection is supposed to wane (Klein *et al.*, 2012). Here we compared the magnitude and duration of memory T-cell responses in the same cohort. Some of the enrolled children (34.7%) received only primary vaccination, while others (65.3%) received the pre-school boost dose, thus we could analyze the impact of primary vaccination on the pre-school booster dose of pertussis vaccine.

The Hexavac vaccine is no longer marketed, based on a EMEA decision taken in 2005 which discontinued the vaccine following the identification of a decreased immunogenicity of the hepatitis B component (Zanetti *et al.*, 2010, Carollo *et al.*, 2013). However, other vaccines, such as Pediacel and Tetravac, (both from Sanofi-Pasteur MSD), with equal PT antigen inactivation and adjuvant composition, are still widely used.

MATERIAL AND METHODS

Study population, vaccine information and sample collection procedures

This study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Ethical Committee of the Bambino Gesù Research Hospital, Rome, Italy, and the children's parents or legal guardians provided written informed consent.

Ninety-seven 6-7 year-old children (mean age: 6.69) were included in the study as detailed in the parallel study focused on serological and B memory responses (Carollo *et al.*, 2014). Sixty-two children received Hexavac and 35 children received Infanrix as primary vaccination between 2002 and 2003. Table 1 reports details of the vaccine composition (European Medicines Agency, HEXAVAC; European Medicines Agency, INFANRIX). The vaccine was administered at 3, 5 and 11 months of age, according to the Italian immunization schedule (Zanetti *et al.*, 2010). All children had a properly completed primary vaccination schedule. Sixty-two children (37 and 25 from Hexavac and Infanrix vaccinees, respectively) received Boostrix® (GlaxoSmithKline) as pre-school booster before immune monitoring. Monitoring of T-cell immunity was performed five years after the primary vaccination. Two children with IgG-PT value above 100 EU/ml, considered indicative of a recent *B. pertussis* infection (Versteegh, *et al.*, 2005; Guiso *et al.*, 2011] were not included in the study, both children showed PT-specific T-cell response. Serology data of this cohort of children are published in (Carollo *et al.*, 2014).

PT specific T-cell proliferation, IFNγ secretion, intracellular IFNγ and IL-2 production and memory subset phenotypic analysis

Peripheral blood mononuclear cells (PBMC) were isolated as described in (Carollo *et al.*, 2012) and freshly seeded at a concentration of $1x10^6$/ml in the presence of PT [5 µg/ml, Novar-

TABLE 1 - *Vaccines' composition.*

Vaccine	*Dose (mL)*	*Antigen*	*PT-detoxification*	*Adjuvant*	*Excipient*
Hexavalent Hexavac® (Sanofi Pasteur MSD) European Medicines Agency. Hexavac	0.5	PT toxoid 25 µg FHA 25 µg	glutaraldheyde	aluminum hydroxide (no more than 0.3 mg)	di-sodium/ potassium phosphate; sodium carbonate bicarbonate, tromethamol, saccarose
Infanrix hexa® (Glaxo-SmithKline Biologicals) European Medicines Agency. Infanrix Hexa	0.5	PT toxoid 25 µg FHA 25 µg Prn 8 µg	glutaraldehyde and formaldehyde	aluminum hydroxide (no more than 0.625 mg)	sodium chloride and polysorbate 80 (Tween 80)
Boostrix® (Glaxo-SmithKline Biologicals)	0.5	PT toxoid 25 µg FHA 8 µg Prn 2.5 µg	glutaraldehyde and formaldehyde	aluminum hydroxide (not more than 0.625 mg)	sodium chloride and polysorbate 80 (Tween 80)

tis (Siena, Italy] or negative (medium) or positive [Staphylococcus Enterotoxin B (0.5 µg/ml) (Sigma)] controls (Carollo *et al*., 2012). Before its use PT was heat inactivated (96°C/1 h) to avoid any mitogenicity, as described in (Carollo *et al*., 2012). Cells were cultured at 37°C in a 5% CO_2 incubator for 6 days. Proliferation was measured by enumerating blast cells identified by forward and side cell-scatter, using flow-cytometry analysis and gate strategy as described in (Carollo *et al*., 2012). Data are expressed as percentage of PT-stimulated blasts subtracted by the percentage of blasts in un-stimulated cultures.

Supernatants were collected after 6 days of cultures to measure IFNγ by specific ELISA (Quantikine, R&D Systems, Minneapolis, MN). The lower detection limit was 8.0 pg/ml. Optical density was measured using a 3550-UV Microplate Reader (BioRad, Philadelphia, PA, USA) according to the manufacturer's instructions.

Memory subsets frequency, performed in PT responsive children, and intracellular cytokine analysis were performed after 6 days of culture both in CD4 and CD8 subsets (Carollo *et al*., 2013; Carollo *et al*., 2012). Cells were simultaneously stained for extracellular markers (CD4, CD8, CD45RA, CCR7) and intracellular cytokines (IL-2 and IFNγ), using mouse anti-human CD4-PerCP-Cy5.5, CD8-APC-Cy™7, CD45RA-PE-Cy™7, IFNγ Alexa Fluor® 647, IL-2-FITC, and rat-anti-human CCR7-PE (clone CD197). Appropriate isotype matched controls were run in parallel. All monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA, USA). Cell acquisition was performed using FACSCanto flow cytometer (Becton Dickinson), following the gating strategy shown in reference (Carollo *et al*., 2012). For each analysis, 50,000 events were acquired in the CD4 cell gate. The data were analyzed using the FlowJo software (Tree Star, Ashland, OR, USA). Data are expressed as percentage of PT-stimulated cytokine positive cells subtracted by the percentage of positive cells in unstimulated cultures.

Definitions, data presentation and statistical analyses

Based on arbitrary criteria a subject was considered PT responsive when PT-induced proliferation, subtracted from the percentage of blasts in unstimulated cultures, was higher than 8% and simultaneously the PT-CD4-IFNγ-positive cells stimulation index (SI, stimulated/none) was higher than ≥2 (Smits *et al*., 2013). The cut-off for blast proliferation was determined considering the Gaussian curves of PT-blast proliferation data subtracted from the unstimulated blast data. We found a gap in the distribution for a value equal to 8.

Data were recorded in a computerized database and were analyzed using the GraphPad Prism version 4.00 for Windows (Graphpad Software, SanDiego, CA, USA www.graphpad.com) and the IBM SPSS statistics version 21 (Chicago, IL, USA). To compare differences of continuous variables between groups or within groups two-sided Mann Whitney Test or Wilcoxon paired-samples test were performed, respec-

tively. The Fisher exact test was used for categorical variables (Smits *et al*., 2013). To study the association between time from the boost and the T-cell parameters, a linear regression model was applied and the Pearson correlation coefficient was calculated. $P<0.05$ was considered statistically significant.

RESULTS

Pertussis specific T-cell proliferation and IL-2 production

T-cell immune responses to PT, the only aP antigen specific for *B. pertussis* (Mattoo and Cherry, 2005) were analyzed. Figure 1 shows PT-specific T-cell responses in the two cohorts of children who received a primary vaccination (primed) with either Hexavac or Infanrix vaccine five years earlier. PT-specific proliferation of T-cells was significantly higher in the children primed with Hexavac than in those who received Infanrix (Figure 1A), and the difference between the two vaccines persisted, though at a lower level, even among those recipients of a booster dose at pre-school age (Figure 1B).

No major effects of the boost on PT-specific proliferation were observed. In Hexavac recipients higher levels of proliferation in children without the booster were found as compared

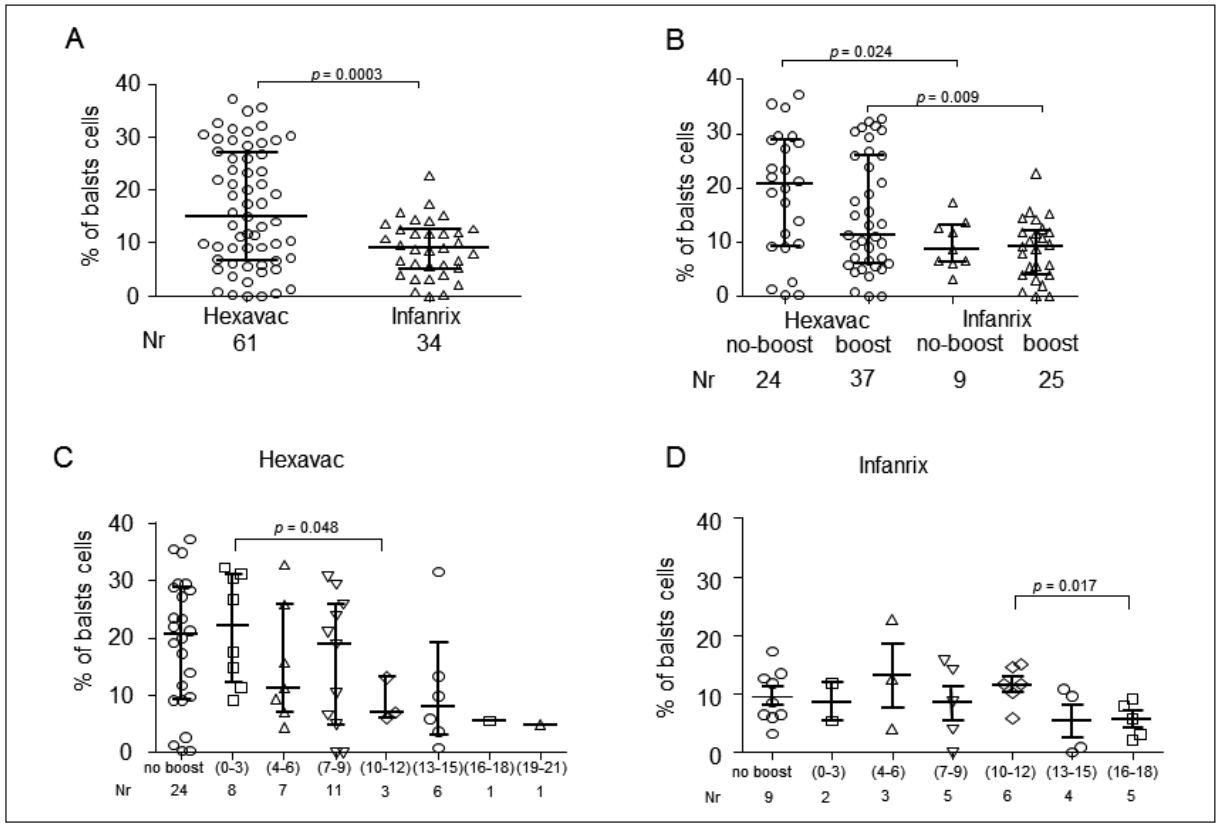

FIGURE 1 - *Pertussis toxin (PT)-specific T blast proliferation in Hexavac or Infanrix vaccine recipients. PBMC unstimulated or stimulated with PT (5 μg/ml) were cultured in 5% CO2 for 6 days. Cells were harvested and proliferation measured, as percentage of proliferating T blasts, as described in Methods. Data are expressed as percentage of T blast cells (median with interquartile range). Panel A: groups of children primed 5 years before with Hexavac or Infanrix vaccines. Panel B: the same groups of children considering if they had or not received the pre-school boost dose before the performance of the assay. Panels C (Hexavac) and D (Infanrix): analysis of post-boost persistence of PT blast proliferation. PT blast proliferation was plotted taking in consideration the time frame elapsed from the boost and the proliferation assessment (Months since the boost, indicated - as 3 months interval - in parenthesis in the x-axis). The number (Nr) of children is indicated below the x-axis. Statistical significant differences are indicated.*

to children tested after the pre-school booster dose. In Infanrix-primed children these levels were similar (Figure 1B). This study was not planned to evaluate the effects of the boost and in the boosted children group the time elapsed between the boost and the blood sampling was variable from few days to almost 2 years. When time from the boost was taken in consideration, a slight increase in blast proliferation was found in the children primed with Hexavac and tested in the 0-3 months interval after the boost with respect to children without the booster. Then a tendency to a decrease of PT-specific proliferation was found, which reach statistical significance (Pearson correlation coefficient r=-0.384, *P*=0,002) (Figure 1C). In the Infarix primed children, few children were tested in proximity of the boost, so no information on this point is available. The already low level of proliferation did not show an evident decrease with the time passed from the boost in this group of vaccinees (Figure 1D).

The analysis of IL-2-positive cells, measured by intracellular staining, was in agreement with the proliferation results. The percentages of IL-2-positive cells were in general low (Figure 2), but a significant higher ability to secrete IL-2 in PT stimulated cultures in Hexavac- vs. Infanrix-primed children in CD4 cells was found (Figure 2A). Similar results were found

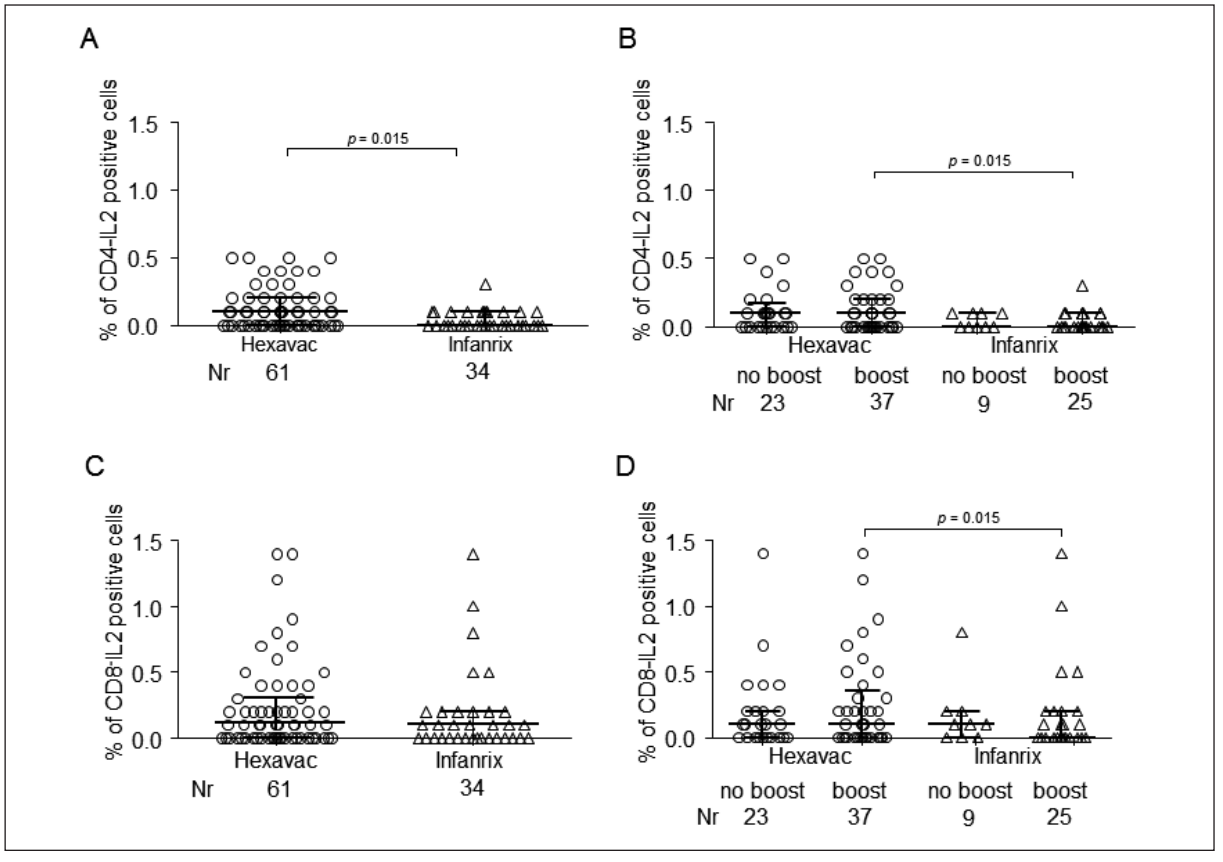

FIGURE 2 - *Pertussis toxin (PT)-specific production of IL-2 measured in CD4 and CD8 cells in Hexavac or Infanrix vaccine recipients. PBMC unstimulated or stimulated with PT(5 µg/ml) were cultured in a 5% CO2 incubator for 6 days. IL-2 were measured by intracellular staining, as described in Methods. Values from PT-stimulated cultures were subtracted of values from un-stimulated cultures and expressed as percentage of positive cells (median with interquartile range). Percentage of IL-2-positive cells in CD4 and CD8 are shown in panels A and C, respectively, in groups of children primed 5 years before with Hexavac or Infanrix vaccines. Panels B (CD4) and D (CD8) shows the analysis of persistence of IL-2-positive cells in the same groups of children considering if they had or not received the pre-school boost dose before the performance of the assay. The number (Nr) of children in each group is indicated below the x-axis. Statistical significant differences are indicated.*

in the boosted group both in CD4 (Figure 2B) and CD8 (Figure 2D) cells.

Pertussis-specific IFNγ production

Figure 3A shows the IFNγ levels in the culture supernatants of PT-stimulated PBMC in Hexavac and Infanrix primary vaccination recipients. No differences in IFNγ levels were found in the two groups of vaccinees. The children tested after the boost showed an increase of IFNγ values, particularly in the Infanrix group, but without reaching statistical significance (Figure 3B). In Hexavac-primed children, the level of IFNγ tended to increase in the first months after the booster and then decreased. Statistical significance was reached comparing the IFNγ level at 0-3 versus 13-15 months after the boost (Figure 3C). In the Infanrix-primed children a scattered post-boost PT-specific IFNγ response was observed (Figure 3D). Similar levels of IFNγ-positive cells measured by intracellular staining were found in the two vaccinee groups both in CD4 and CD8 cells (Figure 4A and B, respectively). When IFNγ-positive cells were measured in PBMC from children tested in proximity of the boost (0-3 months), a tendency to a higher percentage of positive cells in boosted with respect to children without the boost was found both in CD4 (Figure 4C) and CD8 cells (Figure 4E) in Hexavac-primed children.

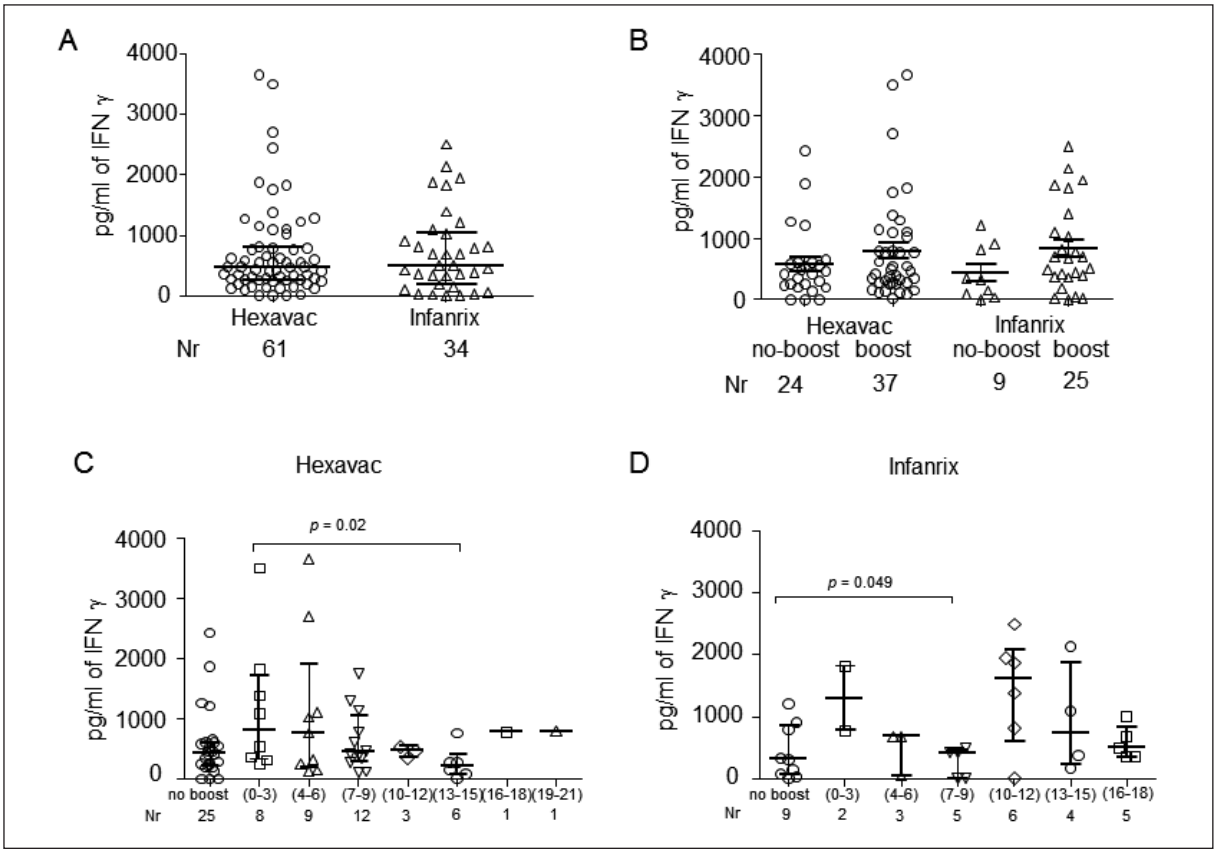

FIGURE 3 - *Pertussis toxin (PT)-specific IFNγ secretion in Hexavac or Infanrix vaccine recipients. PBMC unstimulated or stimulated with PT (5μg/ml) were cultured in 5% CO2 incubator for 6 days. Supernatants were harvested and IFNγ measured by ELISA. Values from PT-stimulated cultures were subtracted of values from unstimulated cultures and expressed as pg/ml (median with interquartile range). Panel A: groups of children primed 5 years before with Hexavac or Infanrix vaccines. Panel B: the same groups of children considering if they had or not received the pre-school boost dose before the performance of the assay. Panels C (Hexavac) and D (Infanrix): analysis of persistence of PT-specific IFNg levels. IFNg levels (pg/ml) were plotted taking in consideration the time frame elapsed from the boost and IFNg assessment (3 months interval) (x-axis). The number (Nr) of children is indicated below the x-axis. Statistical significant differences are indicated.*

To establish the levels of PT responsiveness in children 5 years after primary vaccination we considered simultaneous evidence of PT-specific T-cell proliferation and CD4IFNγ production. As shown in Table 2, using these criteria we found an overall rate of responders equal to 36.8% (35/95). Responsiveness was 54.5% (18/33) in children without the boost, significantly higher compared to children tested after the booster [27.4% (17/62)] (Fisher'exact test P=0.014). When considering the two different vaccines, a statistically significant higher proportion of responsive children was found in the Hexavac [45.9% (28/61)] vs. Infanrix [20.6% (7/34)-primed group (Fisher'exact test P=0.016). In the group of children without the booster, a significant higher rate of responder was found in the Hexavac [66.7% (16/24)] vs Infanrix [22.2% (2/9)]-primed group (Fisher'exact test P=0.047). Similarly, in children tested after the

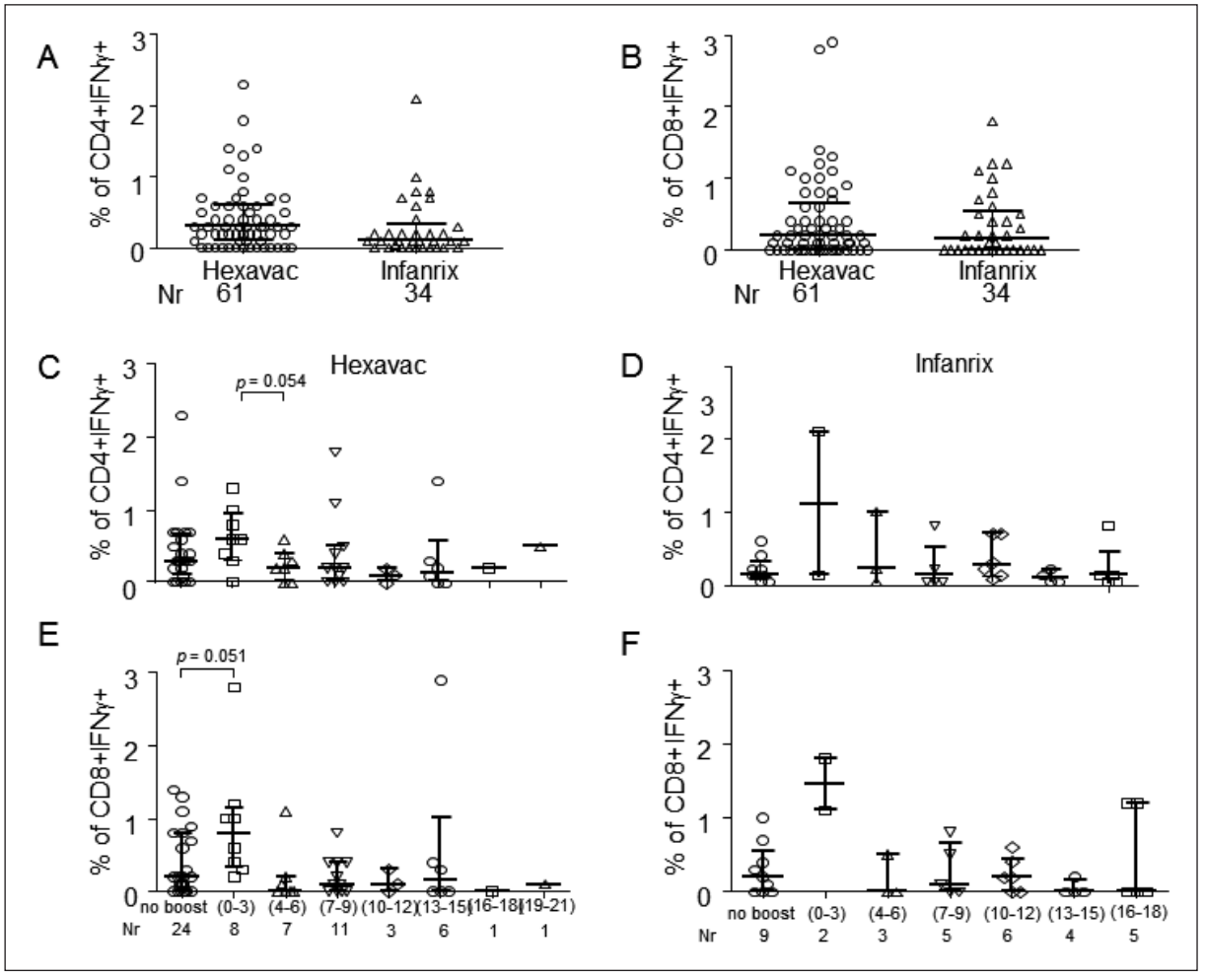

FIGURE 4 - *Pertussis toxin (PT)-specific production of IFNγ measured in CD4 and CD8 cells in Hexavac or Infanrix vaccine recipients. PBMC unstimulated or stimulated with PT(5 μg/ml) were cultured in a 5% CO2 for 6 days. IFNγ were measured by intracellular staining, as described in Methods. Values from PT-stimulated cultures were subtracted of values from unstimulated cultures and expressed as percentage of positive cells (median with interquartile range). Panels A and B: the percentage of IFNγ-positive cells in CD4 and CD8, respectively, in groups of children primed 5 years earlier with Hexavac or Infanrix vaccines. Panels C, E (Hexavac) and D, F (Infanrix): analysis of persistence of IFNγ-positive cells in CD4 or CD8 subsets, plotted taking in consideration the time frame elapsed from the boost and the IFNg assessment (3 months interval) (x-axis). The number (Nr) of children is indicated below the x-axis. Statistical significant differences are indicated.*

TABLE 2 - *Percentage of PT positive children (PT-Blasts ≥8% and PT-CD4-IFNγ positive cells SI ≥2).*

	All children	*Without the boost*	*Boosted [Boostrix]*
All children	36.8(35/95)	54.5(18/33)[a]	27.4 (17/62)[a]
Hexavac	45.9 (28/61)[b]	66.7(16/24)[c,d]	32.4(12/37)[c]
Infanrix	20.6(7/34)[b]	22.2(2/9)[d]	20.0(5/25)

Fisher's exact test [a]p=0.014; [b]p=0.016; [c]p=0.017; [d]p=0.047.

booster, a higher proportion of responsive children was found in the Hexavac [32.4% (12/37)] vs. Infanrix [20.0% (5/25)]-primed group.

T memory subsets in pertussis toxin responsive children

Memory subsets frequency was performed in PT responsive children. Due to a failure of the anti-CCR7 mAb batch in its capacity of cell staining, we could evaluate only 16 out of 28 Hexavac responders. All 7 responsive Infanrix recipients were evaluated. We measured the frequency of T central memory (cm) ($CCR7^+CD45RA^-$), which have the capacity to proliferate; T effector memory (em) ($CCR7^-$ $CD45RA^-$) that differentiate in response to antigenic stimulation in T effector (e) ($CCR7^-CD45RA^+$), the most differentiated T-cells; T naive or stem cell memory (n/scm) ($CCR7^+CD45RA^+$) cells, a T-cell population that includes a recently described memory subset characterized for self-renewal, multipotent ability and increased proliferative capacities (Appay *et al.*, 2008; Gattinoni *et al.*, 2011; Wyndham-Thomas *et al.*, 2014).

Figure 5 shows the CD4 and CD8 memory populations in PT-stimulated and untreated cell cultures. No differences in the frequency of memory populations were evident between the two groups of vaccinees.

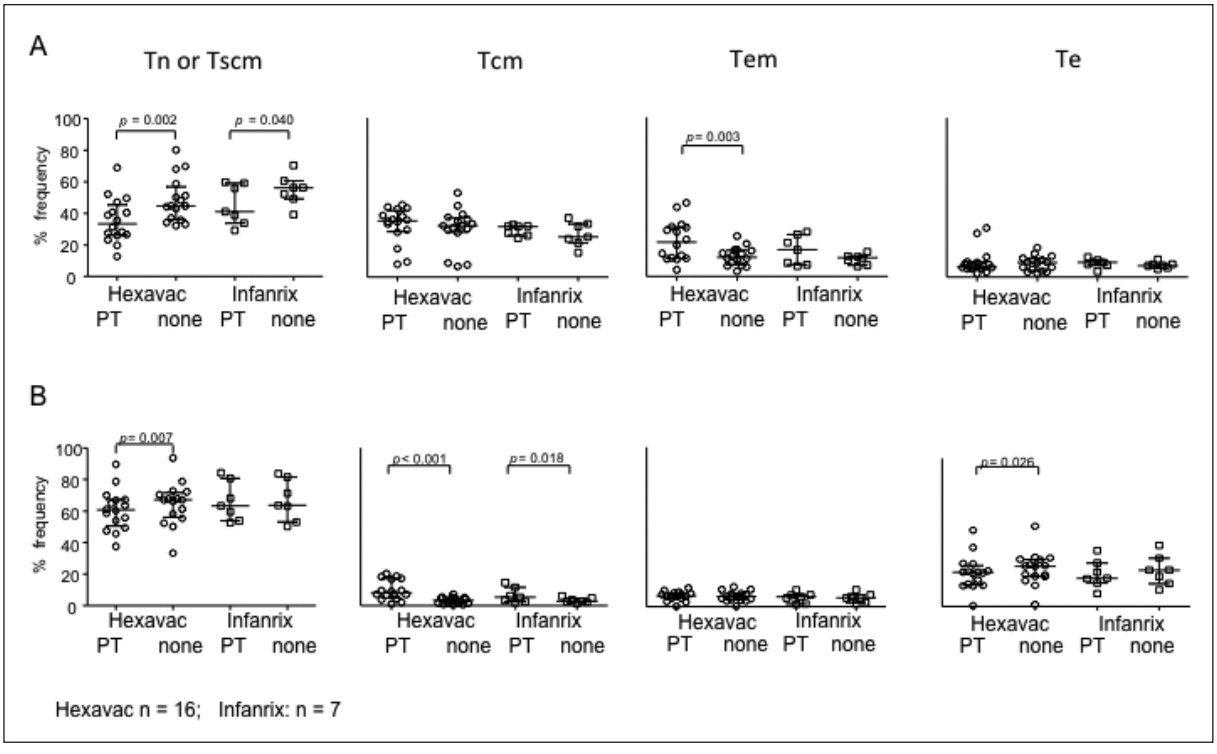

FIGURE 5 - *Frequency of CD4 and CD8 memory subsets in PT-stimulated PBMC from responsive Hexavac and Infanrix vaccine recipients. The frequency of memory subsets defined as naive or stem cell memory (Tn or Tscm, CCR7+ CD45RA+), central memory (Tcm, CCR7+ CD45RA-), effector memory (Tem- CCR7- CD45RA-), effector (Te- CCR7- CD45RA+) cells in CD4 (Panel A) and CD8 (Panel B) stimulated with PT (5 μg/ml) or unstimulated (none) PBMC cultured in a 5% CO2 for 6 days are shown. Data are expressed as median with interquartile range. Responsive children are defined as: PT-Blasts (stimulated - none) ≥8% and PT-CD4IFNγ-positive cells (stimulated/ none) SI ≥2. The number (Nr) of children in each group and statistical significant differences are indicated.*

In CD4 cells, PT stimulation induced a significant decrease of Tn/Tscm frequencies, both in Hexavac and Infanrix-primed children and an increase in Tem cells, which reach the significance in Hexavac-primed children (Figure 5A). In CD8 cells, the frequency of Tcm in both groups of vaccinees was significantly increased by PT stimulation and the frequency of Tn/Tscm and Te cells was significantly lower in PT-stimulated versus unstimulated PBMC from Hexavac-primed children (Figure 5B).

DISCUSSION

Recent epidemiological studies in several countries with high pertussis vaccination coverage suggest that the resurgence of pertussis involves a more rapid waning of aP vaccine compared to wP vaccine-induced immunity (Burns *et al.*, 2014; Koepke *et al.*, 2014; Klein *et al.*, 2012; Ausiello and Cassone, 2014). In this study, five years after primary vaccination, we found a positive T-cell response, evaluated in terms of proliferation and IFNγCD4-positive cells, in 36.8% of vaccinees. Furthermore, we detected differences in the T specific responses to PT, potentially impacting on immunity waning, between children completing primary vaccination with two different combined aP vaccines (Hexavac and Infanrix). Importantly, these differences were maintained even after a booster dose at a pre-school age. More specifically, we found that PBMC from children primed five years before with Hexavac vaccine showed a higher capacity to proliferate and to induce specific IL-2CD4-positive cells respect to PBMC from Infanrix-primed children. In addition, the percentage of responsive children was significantly higher [45.9% vs. 20.6%] in Hexavac vs. Infanrix vaccines.

Differences in the capacity to induce protective immunity may depend on differences in the formulation of aP vaccines. The main difference between the two vaccines is the presence of the pertactin antigen in the Infanrix and not in the Hexavac vaccine. However, we found differences in immunity to PT antigen, present in the same amount in the two vaccines. In Infanrix, PT is inactivated by glutaraldehyde and formaldehyde treatment, while in Hexavac only by glutaraldehyde. It is conceivable that the milder inactivation in Hexavac vaccine may be responsible for a better T epitope preservation and an induction of a more sustained proliferative response. In a previous study performed during the clinical trial of aP vaccines, an aP vaccine containing PT genetically inactivated, with better preserved T epitopes (diTommaso *et al.*, 1994), was able to induce a significant higher T-cell-positive responses (83%) vs. an aP vaccine containing chemically inactivated PT (55%) (Cassone *et al.*, 1997).

Differences in the capacity to induce protective responses due to differences in aP vaccine components were already reported (Vermeulen *et al.*, 2013; Morel *et al.*, 2011). In agreement with our data, Vermeulen and colleagues reported that Infanrix-vaccinated preterm children had a persistently lower specific Th1-type immune response, resulting in lower antigen-specific IFNγ/IL-5 ratios, as compared to Tetravac (Sanofi Pasteur MSD) vaccinees (Vermeulen *et al.*, 2013). Also in this case PT present in the Tetravac vaccine was inactivated by glutaraldehyde. In a recent study (Morel *et al.*, 2011), immunization of mice with Infanrix and Pediacel (Sanofi-Pasteur MSD) resulted in similar protection against *B. pertussis* infection, but the levels of Ab to vaccinal antigens were different, due to differences in the adjuvants. In our case, both vaccines are formulated using antigens adsorbed onto aluminum hydroxide, however, in Infanrix the content of aluminum hydroxide is higher than in Hexavac (0.5 mg versus 0.3 mg).

It is not clear if these differences may affect the antigenic power of the vaccines, but in a parallel study performed in the same cohort of children (Carollo *et al.*, 2014), a longer persistence of IgG-PT and IgG-pertactin levels after the preschool boost was observed in Infanrix-primed with respect to Hexavac-primed children. Analysis of concordance between IgGPT responders (IgGPT ≥20) and T-cell responders [PT-Blasts (stimulated - none) ≥8% and PT-CD4-IFNγ-positive cells stimulation index was higher than ≥2] in our cohort of children did not disclose any concordance. Hence, we could stress that the two vaccines behave differently in terms of B and T-cell response induction and there was no evidence that B and T-cell responses are in any way correlated.

The capacity to induce a differential immune response is not confined to the pertussis vaccine component. In recent studies, Infanrix vaccine priming demonstrated a greater ability to induce an antibody response for the hepatitis component, than the Hexavac vaccine (Zanetti *et al.*, 2010) even if the memory B and T-cell responses were fully comparable (Carollo *et al.*, 2013, Rosado *et al.*, 2011).

In the absence of pertussis correlates of protection it is not possible to appreciate the importance of this dichotomous (T and B) response as observed in the capacity to induce a protective specific response of the two aP vaccines. When considered together, in the absence of efficacy data, it is difficult to draw conclusions on the effectiveness of aP vaccines. Thus, the relevance of T-cell responses to PT, as opposed to B-cell responses is still an open question.

The analysis of memory subsets in responsive children did not disclose any differences between Hexavac and Infanrix-primed groups, suggesting that similar memory subsets are activated in responsive children, irrespective of the vaccine used as primary vaccination.

Our results confirm the data of Sharma and Pichichero, 2012) and our previous data (Smits *et al.*, 2013) showing that pertussis-specific T-cell responses in infants after aP primary vaccination were mainly restricted to Tcm and Tem subsets. PT antigen stimulation increased Tem reducing the levels of Tn/Tscm in CD4 subsets. In CD8 cells a limited expansion of Tcm was observed.

The present study confirms that pertussis-specific T memory cells are induced by PT stimulation and may contribute to protection against pertussis (deRond *et al.*, 2015; Rieber *et al.*, 2011; Dirix *et al.*, 2012]. No specific effect of the boost was observed in the frequency of memory subsets expansion (not shown) in agreement with previous data (Schure *et al.*, 2012[a]; deRond *et al.*, 2015). The comparison between the non-boosted and boosted children was made in different subjects. We found a higher level of PT-specific proliferation in children tested before as compared to children tested after the preschool booster dose, in the Hexavac group (Figure 1B). Similar results were obtained also when the filamentous hemagglutinin antigen was analyzed (data not shown). Even when the data were analyzed in the proportion of responsive children we were not able to measure a boost effect either in Hexavac or Infanrix-primed children.

An interpretation is that the proliferation response had already vanished when the post-boost test was performed, the time elapsed between the boost and the blood sampling being extremely variable in our study. Indeed, in children assayed at 0 to 3/6 month time intervals after the boost, both the proliferative response and the production of IFNγ were slightly higher than in non-boosted children. Nevertheless, a rapid decrease of T proliferative response was observed particularly in children primed with the Hexavac vaccine.

It is not easy to compare our data with those obtained by other studies, because the experimental conditions and the vaccine preparations under study were different. Several studies have pointed to the importance of booster immunizations in enhancing T-cell responses to pertussis antigens (Tran Minh *et al.*, 1999; Edelman *et al.*, 2004; Rieber *et al.*, 2008). More recently, Vermeulen and colleagues reported in preterm infants that the aP booster administered between 13 and 16 months had no major effect on antigen-induced cytokine production but it allowed significant immune responses to be maintained (Vermeulen *et al.*, 2013). Schure and colleagues (Schure *et al.*, 2012[b]) showed that in children primed with aP vaccine an increase in cytokine production was missed after boost vaccination, in contrast to wP-vaccinated children. The same research group (Schure *et al.*, 2012[a]) reported that upon a second aP booster vaccination in children at 9 years of age T-cell responses were already high and could not increase after the boost. The authors' conclusion suggested that the enhancement of T-cell immunity during the 5 years following the booster at 4 years of age is probably caused by natural boosting, due to the high circulation of pertussis.

The decrease of T recall capacity with the time passed from the boost is evident in our data, especially for Hexavac-primed children and is apparently in contrast with our previous studies, where we did not find this rapid decrease of T-cell responses (Ausiello *et al.*, 1999). However, we demonstrated that vaccination-induced T-cell response could wane by 4 years of age

and can be naturally boosted by symptomless clinical infection by *B. pertussis*. This might explain, at least in part, the persistence of protection against pertussis in aP vaccine recipients despite a substantial waning of both Ab and T responses induced by the primary immunization (Ausiello *et al*., 1999, Cassone *et al*., 2000).
Overall, the data indicate that T memory response to PT persists only in a fraction of children 5 years after primary vaccination. Importantly, the children receiving the Hexavac aP vaccine showed a higher T-cell response to PT than the recipients of the Infanrix aP vaccine.
These data reveal potential differences in long-term protection between the two aP vaccines and solicit careful attention to even minimal differences in aP vaccine composition that may influence the specific induction of immune response and consequently effective protection capacity of a vaccine.
The lack of a group of subjects vaccinated with wP vaccine is a limitation of our study when trying to draw conclusions on the significance of waning PT-specific T-cell immunity and the resurgence of pertussis. It is likely that T-cell responses against antigens other than PT are responsible for the enhanced protection elicited by wP vaccine and that efforts should be made to ameliorate the aP vaccines and/or vaccination strategies. Adjuvant optimization, and the inclusion of new antigens can all be envisaged to improve vaccine efficacy (Fedele *et al*., 2015).

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