B-cell responses after intranasal vaccination with the novel attenuated *Bordetella pertussis* vaccine strain BPZE1 in a randomized phase I clinical trial

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ARTICLE INFO

ABSTRACT

Despite high vaccination coverage, pertussis is still a global concern in infant morbidity and mortality, and improved pertussis vaccines are needed. A live attenuated *Bordetella pertussis* strain, named BPZE1, was designed as an intranasal vaccine candidate and has recently been tested in man in a phase I clinical trial. Here, we report the evaluation of the B-cell responses after vaccination with BPZE1. Forty-eight healthy males with no previous pertussis-vaccination were randomized into one of three dose-escalating groups or into a placebo group. Plasma blast- and memory B-cell responses were evaluated by ELISpot against three different pertussis antigens: pertussis toxin, filamentous haemagglutinin and pertactin. Seven out of the 36 subjects who had received the vaccine were colonized by BPZE1, and significant increases in the memory B-cell response were detected against all three tested antigens in the culture-positive subjects between days 0 and 28 post-vaccination. The culture-positive subjects also mounted a significant increase in the filamentous haemagglutinin-specific plasma blast response between days 7 and 14 post-vaccination. No response could be detected in the culture-negatives or in the placebo group post-vaccination. These data show that BPZE1 is immunogenic in humans and is therefore a promising candidate for a novel pertussis vaccine. This trial is registered at ClinicalTrials.gov (NCT01188512).

1. Introduction

Bordetella pertussis (*B. pertussis*) is the causative agent of whooping cough, a disease that despite high vaccination coverage still remains a major concern in infant mortality and morbidity. The introduction of pertussis vaccines greatly decreased the incidence of pertussis disease and mortality [1]. There are two types of available pertussis vaccines, whole-cell (Pw) and acellular (Pa). The first dose of the vaccine is given at the age of 2–3 months [2–4]. Infants

below four months are thus not optimally protected and are at risk for severe and fatal pertussis [5]. Improving the current immunization scheme so that young infants are offered protection is therefore important.

A natural pertussis infection induces a type I T-helper (Th1) cell response, and clearing of the primary infection depends on interferon gamma (IFN-γ) production [6,7]. Mouse studies have shown a protective role for B cells as well [8,9]. In children, Pw-vaccines are reported to induce a Th1-type profile like a natural infection, whereas Pa-vaccinated children are seen to induce a more Th1/Th2-mixed type of response [10,11]. Mielcarek et al. have developed a live attenuated *B. pertussis* vaccine strain named BPZE1 [12] with the long-term aim to administer it to infants at birth. This vaccine strain is attenuated by genetic removal of the dermonecrotic toxin and the tracheal cytotoxin as well as detoxification of the pertussis toxin (PT). These alterations have not affected the immunogenic properties [12], and the strain has been shown to be genetically

stable after both continuous *in vitro* and *in vivo* passages over at least one year [13]. It can colonize the respiratory tract and induce long-lasting memory B-cell responses, as well as T-cell mediated protective immunity against challenge in mice [12,14,15].

A recent randomized, placebo-controlled, double-blind, dose-escalating phase I clinical trial has shown that BPZE1 is safe in humans, able to transiently colonize the human nasopharynx and to induce antibody responses [16]. Here, we have evaluated B-cell responses after vaccination with BPZE1. Plasma blast- and memory B-cell responses were detected by ELISpot, and B-cell subsets were identified by flow cytometry.

2. Materials and methods

2.1. Ethical conductance of the study

The study was conducted according to the protocol ICH Good Clinical Practices standards, Declaration of Helsinki and applicable regulatory requirements as well as any related European and Swedish applicable laws and regulations. The trial was registered at ClinicalTrials.gov (NCT01188512) and approved by the Swedish Medical Product Agency and the regional ethical review board in Stockholm. All volunteers signed an informed consent form after receiving oral and written information in Swedish.

2.2. Vaccine strain BPZE1 and dose groups

The clinical BPZE1 lots were produced by Innogenetics (Ghent, Belgium) as a suspension in phosphate-buffered saline (PBS) containing 5% saccharose. Three doses of BPZE1 were tested, 10^3 colony forming units (cfu), 10^5 cfu and 10^7 cfu, as described earlier [16]. The placebo group received diluent only.

2.3. Subjects

The forty-eight healthy males (born between 1979 and 1991) recruited to the BPZE1 phase I clinical trial [16] were included for B-cell response evaluation. No subjects had previously received any pertussis vaccination as they were born during a time period without any national pertussis vaccination. Due to the circulation of pertussis in the population no subject was considered naïve meaning that all had pertussis-specific antibodies pre-vaccination. Subjects with any additional pertussis vaccination or a clinical pertussis during the preceding 10 years were excluded. Subclinical infections were excluded by including only subject with serum anti-PT Ig levels of ≤20 IU/ml. More inclusion- and exclusion criteria as well as study protocol are published in detail elsewhere [16]. Blood samples were collected from all subjects pre-vaccination (day 0) and at days 7, 14, 28 and month 5–6 post-vaccination.

2.4. Colonization of BPZE1

After vaccination, all subjects were tested for bacterial shedding as described in [16]. Seven subjects were positive for BPZE1 colonization at different time points. The positive cultures were sampled between day 4 and day 28, and bacterial shedding was generally found around day 11 post-vaccination. No shedding was detected after day 28 post-vaccination.

2.5. Antigens

PT (lot 042) and filamentous hemagglutinin [FHA] (lot 039) were obtained from Kaketsuken, Japan. Pertactin [PRN] (lot 180805 RS) was kindly provided by Dr. Buisman at RIVM, the Netherlands. Tetanus Toxoid (TTd), lot 59-5, was obtained from SSI, Denmark.

2.6. Purification, cryopreservation and thawing of PBMC

Peripheral blood mononuclear cells (PBMC) were purified from whole blood collected in BD Vacutainer® CPT tubes with sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA) and separated according to the manufacturer's instruction. Cryopreservation and thawing were performed as previously described [17] but using freezing medium with 90% Fetal Calf Serum (Gibco Invitrogen, Paisley, UK) and 10% Dimethyl Sulphoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA).

2.7. B-cell ELISpot method

For the plasma blast analysis (days 7 and 14) fresh samples were used and 38 subjects (of which 6 were culture positive) were included. 10 subjects (low $n = 3$, medium $n = 5$ and high $n = 2$ [of which 1 was culture positive]) did not have available samples for days 7 and 14 post-vaccination. Frozen samples were used for the memory B-cell analysis (days 0, 28 and 150–180) and the analyses included all subjects in the medium and the high dose groups ($n = 32$) as well as placebo subjects ($n = 8$). All 7 culture positive subjects were also included. The inclusion of subjects (group wise and colonization status) is stated in Table 1. All antigens included in the ELISpot-analysis were used at a coating concentration of 0.5 µg/well. A subject was considered a vaccine responder to an antigen if ≥50 antigen-specific antibody secreting cells (ASC)/10^6 PBMC were detected and at least a 100% increase in spot number/10^6 PMBC at any following time point compared to day 0. This method has previously been described ([18], for specific details see supplementary method S1).

2.8. Flow cytometry

For flow cytometry analyses isolated PBMCs were washed, plated at $1–2 \times 10^6$ cells per sample and stained using direct fluorochrome-conjugated antibodies in different combinations: PerCp-Cy5.5 anti-CD19 (clone HIB19), PE-Cy7 anti-CD10 (HI10a), V450 anti-CD27 (MT271), PE anti-CD21 (B-ly4), FITC anti-IgG (G18-145), PE anti-IgG (G18-145) and FITC anti-IgD (IA6-2) all from BD biosciences. APC anti-FCRL4 (413D12) was from BioLegend. LIVE/DEAD Fixable Near-IR kit (Invitrogen) was used to exclude the dead cells from analyses. Cells were washed three times before being fixed in 1% formalin. All antibodies were used in the concentrations determined after titration experiments. Matched

Table 1
Number of subjects included in the study as well as in the plasma blast and memory B-cell ELISpot analysis.

Dose group	All subjects			Plasma blast ELISpot			Memory B-cell ELISpot		
	Culture negatives	Culture positives	Total	Culture negatives	Culture positives	Total	Culture negatives	Culture positives	Total
Low	11	1	12	8	1	9	0	(1[a])	(1[a])
Medium	11	1	12	6	1	7	11	1	12
High	7	5	12	6	4	10	7	5	12
Placebo	12	0	12	12	0	12	8	0	8

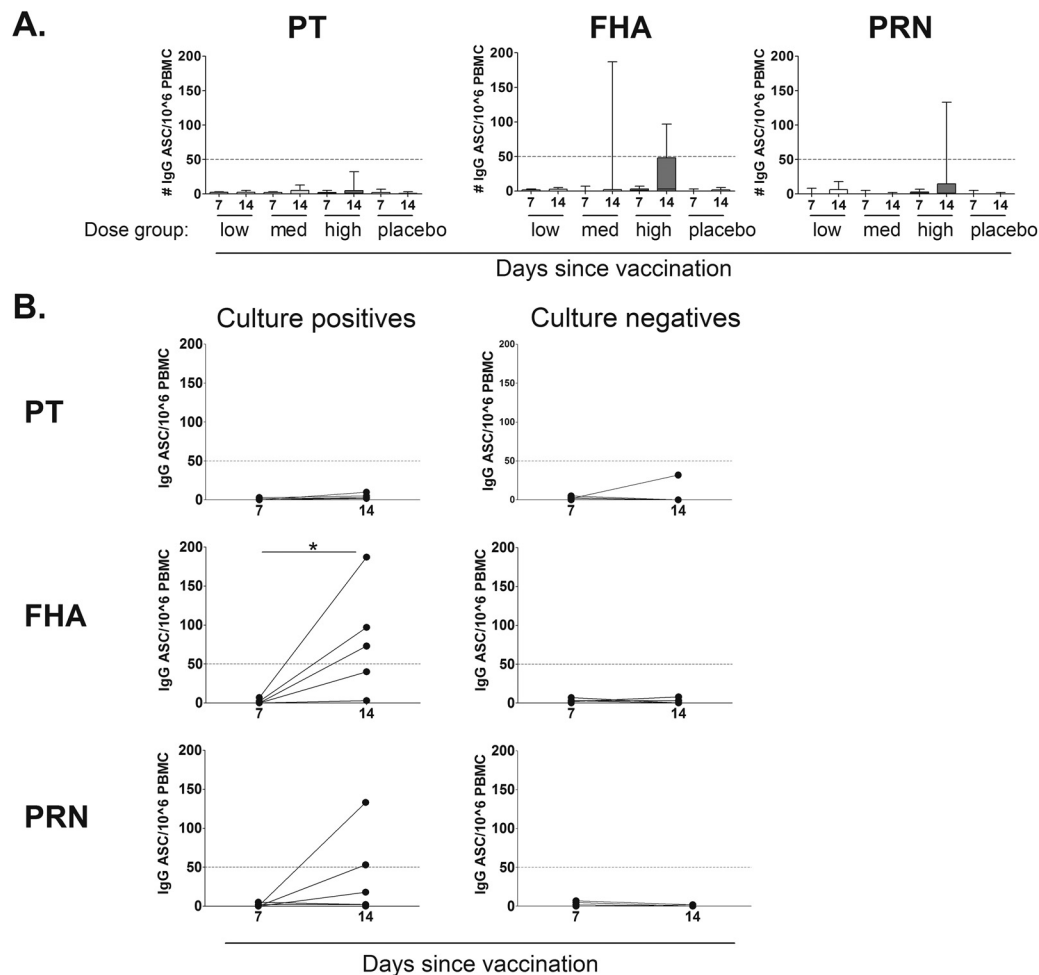[a] Only included in the culture positive analysis (Fig. 2B).

Fig. 1. Plasma blast responses against BPZE1. (A) 38 subjects were included in the plasma blast analysis (low $n = 9$, medium $n = 7$, high $n = 10$, placebo $n = 12$). No vaccine-induced increase of plasma blasts was seen between groups and time points compared to placebo. (B) Six of the culture positive subjects were analyzed and compared to culture negatives. A significant increase could be detected against FHA ($p = 0.0355$) between days 7 and 14 in the culture positive subjects with three subjects responding against the antigen. Two of these subjects were also responding in PRN. No vaccine-responders were detected in the culture negative subjects. Only results reaching statistical significance are marked in the figure. PT: pertussis toxin, FHA: filamentous hemagglutinin, PRN: pertactin, med: medium dose group.

isotype controls were used to set up the gates. Fluorescence intensities were measured with Cyan ADP (Beckman Coulter) and data was analyzed using FlowJo, version 9.4.11 (Tree star). All samples used had previously been frozen.

The peripheral whole B-cell population was gated out as CD19$^+$ cells after exclusion of dead cells. Whole B cells were further subdivided into various B-cell subsets using multi-color flow cytometry panels. Immature Transitional CD19$^+$CD10$^+$, Naive CD19$^+$CD10$^-$CD21$^+$CD27$^-$, Activated Memory CD19$^+$CD10$^-$CD21$^-$CD27$^+$, Resting Memory CD19$^+$CD10$^-$CD21$^+$CD27$^+$, Tissue Like Memory CD19$^+$CD10$^-$CD21$^-$CD27$^-$B cells, switched memory B cells CD19$^+$CD27$^+$IgD$^-$, Un-switched Memory B cells CD19$^+$CD27$^+$IgD$^+$, Naive CD19$^+$CD27$^-$IgD$^+$ and double negative B cells CD19$^+$CD27$^-$IgD$^-$. The expression of IgG and FCRL4 was studied on all B-cell subsets.

2.9. Statistics

All data were considered non-parametric, and p-values <0.05 were considered statistically significant. Comparisons between two time points were done with Wilcoxon matched-pairs signed rank test. Comparisons between two or more groups were done with one-way ANOVA, Kruskal–Wallis test with Dunn post-test. For

comparison within one group at different time-points one-way ANOVA with Friedman test and Dunn post-test were done. All statistical analyses were performed using GraphPad Prism (Graphpad Software Inc., San Diego, USA).

3. Results

3.1. Plasma blast responses against BPZE1

When all 38 included subjects were considered, no significant increase in the antigen-specific plasma blast response was detected between dose groups or between time points (Fig. 1a). However, when the culture-positive subjects were analyzed, a significant increase ($p = 0.0355$) between days 7 and 14 could be detected against FHA (Fig. 1b). Two of the FHA-responders also responded to PRN. No vaccine-responders were detected in the culture negative group (Fig. 1b), or was any response seen against the control antigen TTd (data not shown).

3.2. Memory B-cell responses against BPZE1

There was no significant increase in antigen-specific responses between time points or dose groups. However, in the high dose group a response was seen at day 28 against all antigens, but did not
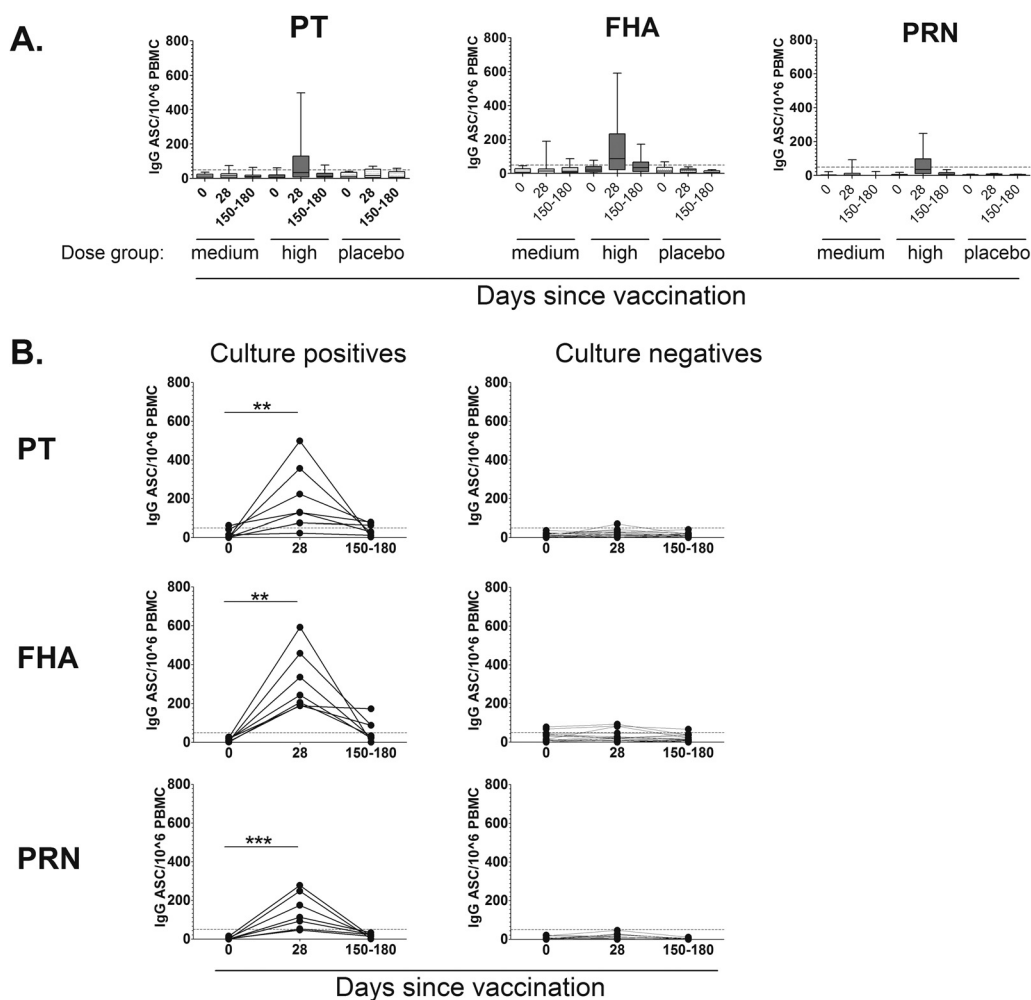
Fig. 2. Memory B-cell responses against BPZE1. (A) All subjects in the medium and high dose group were included in the memory B-cell analysis (medium and high $n = 12$ each, placebo $n = 8$). No significant increase in antigen-specific memory B cells could be detected in the two groups between time points or compared to placebo. (B) The culture positive subjects ($n = 7$) showed significant increases of antigen-specific memory B cells ($p < 0.05$) between day 0 and 28. All subjects responded against all antigens, except one who did not mount any PT-specific response. At day 150–180 this increase had declined, some subjects were back to background levels whereas others had maintained higher levels of antigen-specific memory B cells compared to day 0. No vaccine-responders were detected in the culture negative subjects. Only results reaching statistical significance are marked in the figure. PT: pertussis toxin, FHA: filamentous hemagglutinin, PRN: pertactin.

reach statistical significance (Fig. 2a). The seven culture-positive subjects had significant increases ($p < 0.05$) in antigen-specific memory B cells between days 0 and 28 (Fig. 2b). All subjects responded against all antigens, except one who only had FHA- and PRN-specific responses. Between days 28 and 150–180 after vaccination the numbers of antigen-specific memory B cells had declined. Some subjects were back to background levels, whereas others had maintained higher levels of antigen-specific memory B cells compared to day 0. One subject had maintained the level of FHA-specific memory B cells between days 28 and 150–180. No vaccine-responders were seen in the culture-negative group (Fig. 2b) or against the control antigen TTd (data not shown).

3.3. Flow cytometric analysis of B-cell subsets

For an in-depth evaluation of the memory B-cell response two panels were included in the flow cytometric analysis. Panel I identified different memory B-cell subpopulations (activated, resting and tissue-like) and panel II identified IgG-switched memory B cells. Detection and analysis were performed for 12 subjects (4 culture positives, 4 culture negatives and 4 placebos). Not all subjects had samples available for all time points. No differences

were found between the culture positives, culture negatives or placebo when antibody isotype-switch was evaluated (IgD$^{+/−}$ and IgG$^{+/−}$), data not shown. However, there was an increase in the culture-positive group at days 7 and 14 of the activated memory B cells, as well as the tissue-like memory B cells (fig. 3). This was not seen in the naïve and resting memory B-cell subpopulations, nor did the FcLR4 staining differ between the groups (data not shown).

3.4. Comparison of serum IgG levels and B-cell responses

The number of responding subjects was insufficient for a thorough correlation analysis. Therefore, a more general comparison of the B-cell responses detected was made. The serological response (as detected by ELISA, reported in detail in Ref. [16]), the plasma blast response and the memory B-cell response were compared in all seven culture-positive subjects (Fig. 4). As expected, the cellular response had declined in blood at day 150–180, whereas the serological response was maintained. There were minor exceptions where subjects differed between their cellular and humoral responses, but in general the subjects responded similarly in the antigen-specific responses detected by both ELISpot and ELISA.
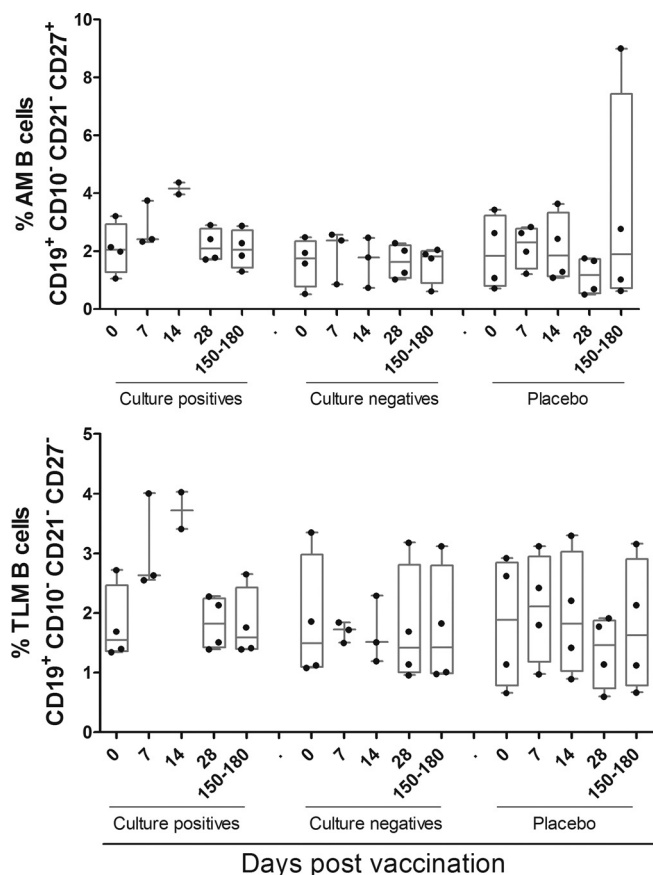
Fig. 3. Flow cytometric analysis of B-cell subsets. 12 subjects were included in an analysis of the BPZE1 vaccination influence on different B-cell subsets. Not all subjects had available samples at all time points (days 0, 28 and 150–180 $n = 12$, day 7 $n = 10$, day 14 $n = 9$). The culture positive subjects had an increase in the activated memory B-cell response (CD19$^+$CD10$^-$CD21$^-$CD27$^+$) as well as the tissue-like memory B-cell response (CD19$^+$CD10$^-$CD21$^-$CD27$^-$) at days 7 and 14. This increase was not maintained at days 28 and 150–180 or seen in the culture negative subjects or placebo. No statistical significance was reached in this evaluation. AM: activated memory, TLM: tissue-like memory.

4. Discussion

The novel, live attenuated pertussis vaccine candidate, BPZE1, was tested for the first time in man and showed to be safe and able to induce serological responses [16]. In this study, we evaluated the B-cell responses evoked by BPZE1 during the same trial. In total 48 subjects were recruited to the study. Out of the 36 subjects that received the vaccine 7 were colonized by BPZE1 and mounted a response against the vaccine-related antigens. Since it was a first-in-man study, the dosages used in this study were based on studies in mice [19]. An optimization of the doses may perhaps lead to a better vaccine take.

The results obtained in this study are considered exploratory due to the novelty of the vaccine. The BPZE1-vaccine is not yet optimized and the results obtained from the clinical trial will serve as guidance to continued development. Therefore, no comparison with other pertussis vaccines is made in this study. Also, the vast differences in study populations, vaccination and administration routes in this study compared to other published pertussis-vaccine studies impedes an accurate comparison.

The low detection of plasma blast responses suggests that an optimization regarding the sampling time points should be considered in future studies. The BPZE1-vaccine immunogenicity is dependent on bacterial colonization and it is likely that the colonization period delays the response compared to a parenterally administrated vaccine [20]. Adjusting the sampling time point could therefore enable a better detection of the BPZE1-induced plasma blast response.

Nevertheless, all colonized subjects mounted strong pertussis-specific memory B-cell responses between days 0 and 28 as detected in blood. These responses had declined at month 5–6, but despite suboptimal vaccine dosages, some subjects had maintained higher memory B-cell responses compared to day 0. Using peripheral blood to analyze the long-term presence of memory B-cell populations is not optimal, as memory B cells home to secondary lymphoid organs and are only seen circulating in low frequencies [21,22]. Studies in mice have shown that between days 28 and 40 following primary vaccination the frequencies of memory B cells are similar in the spleen and the circulation [23]. This indicates that the response detected in blood at day 28 in our study is a more accurate estimation of the true number of pertussis-specific memory B cells than the response detected at month 5–6. Similar kinetics with peak levels one month after vaccination, followed by declining levels of memory B cells in blood are reported in other studies, both for an intranasal Norwalk-vaccine [24] as well as parenterally administered diphtheria and pertussis vaccines [25–27].

We combined two different flow cytometry based phenotypical panels in order to analyze in depth the changes in frequency and, to some extent, the phenotype of memory and naive B-cell compartments after vaccination in the peripheral blood. Staining for CD10, CD21 and CD27 on B cells enabled the identification of four different subsets (naïve, resting memory, activated memory and tissue-like memory), whereas CD27 and IgD staining allowed for the identification of switched memory B cells. Each subset of the B cells has been shown to have a different phenotype, indicating a different function in the immune response. Their activity following vaccination were therefore of interest to investigate. In this limited analysis of the different memory B-cell subpopulations we detected an increase in the activated memory B cells and the tissue-like memory for a few culture positive subjects, indicating active memory B-cell subsets following BPZE1 vaccination. Tissue-like memory cells have previously only been identified as an exhausted B cell subset in HIV-patients, and their role here is unclear [28]. The antigen-specificity of the B cells was not investigated by flow cytometry but as strong pertussis-responses were detected in the other evaluations it is most likely induced by the vaccine.

In the last years there has been a resurgence of pertussis cases and infant deaths in countries with high vaccination coverage [29–31], emphasizing the need for a different vaccine approach to provide protection for the most susceptible infants. Studies have shown that a primary dose of a Pw-vaccine reduces the risk of pertussis compared to a primary dose of a Pa-vaccine [30–32], and the live attenuated BPZE1 vaccine may be a promising priming candidate in that context. It has been shown to protect infant mice against virulent *B. pertussis* challenge [12] and to provide long-term immunity, substantially longer than Pa [33]. Complementing the current pertussis immunization program with a birth-dose of BPZE1 in the future could therefore offer a better protection for the vulnerable infants. However, due to the immaturity of the infant immune system, especially with respect to IFN-γ producing CD4$^+$ T cells [34,35], extensive studies of the BPZE1 safety and efficacy in declining age groups must be performed before a birth dose of BPZE1 is implemented. In this regard it is, however, interesting to note that very young infants are able to induce a strong *B. pertussis*-specific IFN-γ producing CD4$^+$ T cell response upon natural infection, in contrast to vaccination with Pa [6].

In conclusion, the novel attenuated pertussis vaccine strain BPZE1 was able to induce pertussis-specific B-cell responses in colonized subjects. Nasopharyngeal colonization of BPZE1 was, however, crucial for the induction of B-cells responses. With
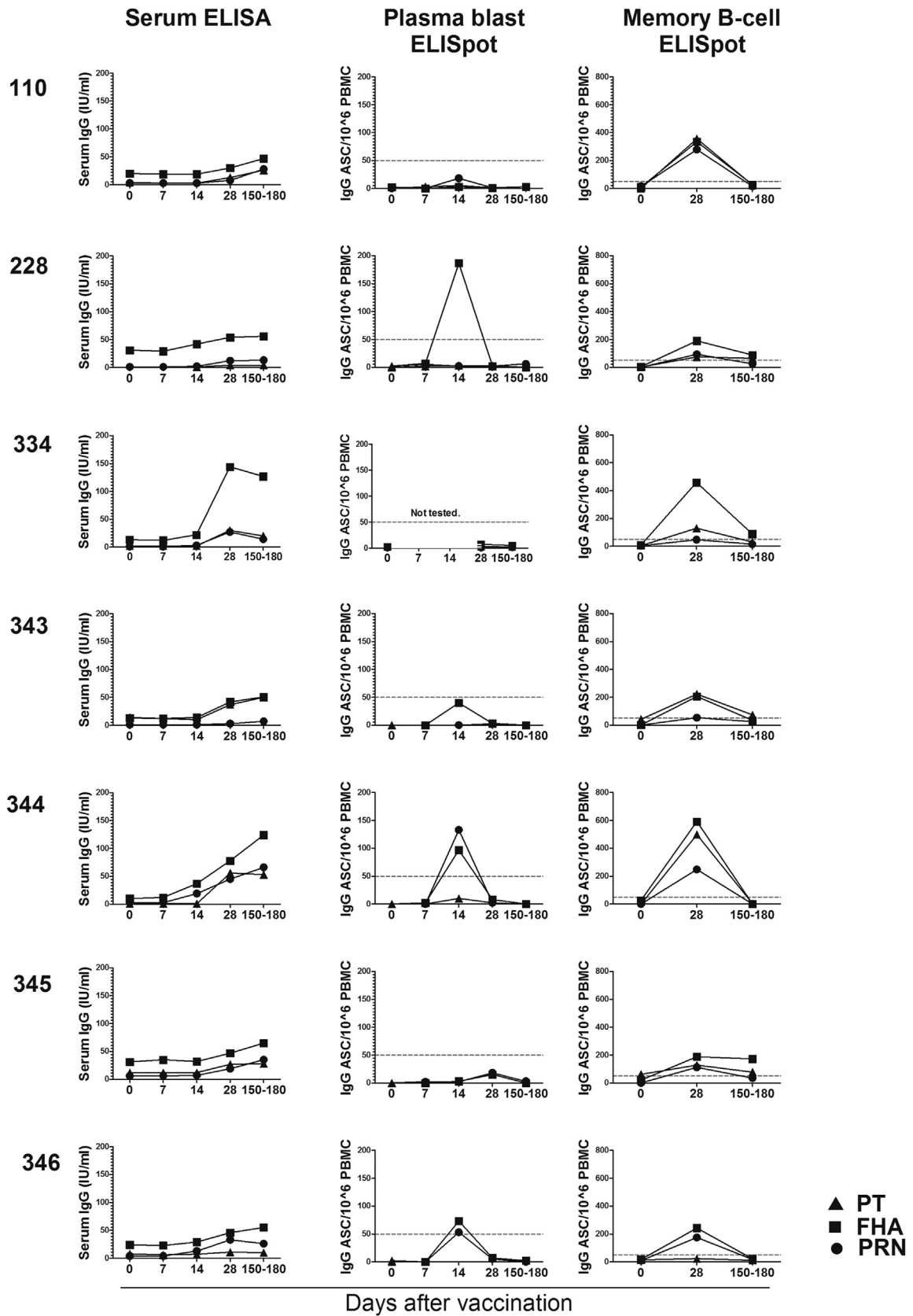
Fig. 4. Comparison of serum IgG levels and B-cell responses. The humoral and cellular response detected in the sevens subjects that had been colonized by BPZE1 were compared. The numbers on the left hand side (110–346) are the subjects' identification number in the study. No major differences were seen in the responses and the subjects responded against the same antigens in both the serological as well as the cell mediated response. The serological response was maintained at day 150–180 whereas the memory B-cell response was no longer detectable in peripheral blood. PT: pertussis toxin, FHA: filamentous hemagglutinin, PRN: pertactin.

optimization, the BPZE1 is a promising candidate to supplement the current pertussis vaccination schedule and thereby provide protection against pertussis disease.

Conflict of interest: CL and NM are inventors of patent applications on BPZE1. None of them have currently been out-licensed for commercial purposes. There are no further patents, products in development or marketed products to declare. The other authors declare no conflict of interest.

Contributors: Conceived and designed the experiments: MJ, RT, SA, FC. Performed the experiments: MJ, SA, ML, LW. Analyzed the data: MJ, ML, SA, FC. Contributed materials: NM, CL. Wrote the paper: MJ, RT, CL, SA. All authors have read and approved the final version of this article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.04.048.

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