# Long-term immunity against pertussis induced by a single nasal administration of live attenuated *B. pertussis* BPZE1

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## ARTICLE INFO

## ABSTRACT

Duration of vaccine-induced immunity plays a key role in the epidemiology and in the pattern of transmission of a vaccine-preventable disease. In the case of whooping cough, its re-emergence has been attributed, at least partly, to the waning of immunity conferred by current pertussis vaccines. We have recently developed a highly attenuated live vaccine, named BPZE1, which has been shown to be safe and to induce strong protective immunity against *Bordetella pertussis* infection in mice. In this study, we evaluated the long-term immunogenicity and protective efficacy induced by a single intranasal dose of BPZE1. Up to 1 year after immunization, BPZE1 showed significantly higher efficacy to protect adult and infant mice against *B. pertussis* infection than two administrations of an acellular pertussis vaccine (aPV). *B. pertussis*-specific antibodies were induced by live BPZE1 and by aPV, with increasing amounts during the first 6 months post-immunization before a progressive decline. Cell-mediated immunity was also measured 1 year after immunization and showed the presence of memory T cells in the spleen of BPZE1-immunized mice. Both cell-mediated and humoral immune responses were involved in the long-lasting protection induced by BPZE1, as demonstrated by adoptive transfer experiments to SCID mice. These data highlight the potential of the live attenuated BPZE1 candidate vaccine as part of a strategy to solve the problem of waning protective immunity against *B. pertussis* observed with the current aPV vaccines.

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## 1. Introduction

Pertussis or whooping cough is a respiratory disease caused by the Gram-negative bacterium *Bordetella pertussis* and remains one of the leading causes of infant mortality among vaccine-preventable diseases worldwide [1–3]. The progressive waning of vaccine-induced immunity conferred by commercially available acellular (aPV) and whole-cell (wcPV) pertussis vaccines is undoubtedly involved in the re-emergence of pertussis. Follow-up studies from clinical trials evaluating aPV-induced immune responses in children have indicated that 15–33 months after a complete course of vaccination, specific antibodies were almost undetectable [4–9], suggesting that booster vaccinations are needed to prolong the duration of immunity [4]. Studies in murine models designed to probe the longevity of vaccine-mediated

immunity also showed that serum antibody responses did not persist for more than 44 weeks following immunization with aPV or wcPV [10].

In contrast, naturally acquired immunity to *B. pertussis* has been proposed to be long-lasting (>30 years) [11]. Several parameters might explain these differences in the duration of immunity induced by bacterial infection and vaccination. While aPVs consist of two to five *B. pertussis* antigens, natural infection induces immune responses against a much wide range of antigens, some of which may contribute to the induction of long-lasting protective immunity. In addition, since *B. pertussis* is a strictly respiratory pathogen, it is likely that mucosal or local immunity in the respiratory tract plays an important role in the long-term protective immunity. None of the current pertussis vaccines target the mucosal immune compartment. These observations led us to hypothesise that a pertussis vaccine delivered by the nasal route and mimicking as closely as possible natural infection may perhaps induce long-lasting protective immunity.

In this study, we used a mouse intranasal (i.n.) challenge model to compare the long-term protection and the duration of immune

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responses induced by a single i.n. administration of a novel, live attenuated *B. pertussis* vaccine candidate, named BPZE1, with those obtained after two injections of a commercial aPV. BPZE1 is a genetically engineered *B. pertussis* strain obtained by removing or altering genes involved in the production of three major *B. pertussis* toxins, pertussis toxin (PTX), dermonecrotic toxin (DNT) and tracheal cytotoxin (TCT) [12]. Although highly attenuated, this strain induces strong B- and T-cell-mediated immunity against *B. pertussis* [12,13] and shows high stability in *in vitro* and *in vivo* studies [14]. By comparison with aPV, we show here that the i.n. administration of BPZE1 induces stronger and longer-lasting immunity against *B. pertussis* infection.

## 2. Materials and methods

### 2.1. Bordetella strains and growth conditions

*B. pertussis* BPSM [15] and BPZE1 [12] used in this study are streptomycin-resistant derivatives from *B. pertussis* Tohama I and were grown on Bordet-Gengou (BG) agar (Difco Laboratories, Detroit, MI, USA) supplemented with 1% glycerol, 20% defibrinated sheep blood, and 100 μg/ml streptomycin (Sigma). The bacteria were then resuspended in phosphate-buffered saline (PBS), at the desired density.

### 2.2. Study design, animal immunization, infection and serum transfer

Adult (8 weeks old) and infant (3 weeks old) Balb/C mice were intraperitonealy (i.p.) sedated with pentobarbital (CEVA Santé Animale – La Ballastière, France) and infected or immunized by pipetting 20 μl PBS containing approximately $1 \times 10^6$ colony-forming units (CFU) of *B. pertussis* BPSM or BPZE1 onto the tip of the nares [12,14]. For vaccination with aPV (Tetravac; Aventis-Pasteur, Lyon, France), mice were immunized i.p. with 20% of the human dose and boosted 1 month later using the same dose. Groups of four to five animals were sacrificed at the indicated time points after inoculation. The lungs were harvested, homogenized in PBS and plated in serial dilutions onto Bordet-Gengou agar. Colony-forming units (CFU) were counted after incubation at 37 °C for 3 days.

For adoptive/passive transfer experiments, 4–5 weeks old female SCID mice received i.p. $50 \times 10^6$ whole spleen cells or 100 μl of serum, as described earlier [13]. One day later, mice were infected with virulent *B. pertussis* BPSM, and protection was assessed on day 7 after challenge infection as described above.

### 2.3. Antibody determination

The levels of antibodies to PTX or filamentous hemagglutinin (FHA) in sera were determined by enzyme-linked immunosorbent assays (ELISA). F 96 Maxisorp Microtiters plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4 °C with 50 μl per well of 10 μg/ml FHA (purified from PTX-deficient *B. pertussis* BPRA [16]) or 5 μg/ml PTX (purified from FHA-deficient *B. pertussis* BPGR4 [17]) diluted in PBS. After washing with PBS, pooled serum samples, serially diluted in PBS containing 0.1% Tween plus 0.5% gelatin (PBS/Tw/g), were added to the coated plates and incubated for 2 h at 37 °C. The plates were then washed three times in PBS/Tw before addition of goat anti-mouse IgG–horseradish peroxidase (HRP) conjugate (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and incubation for 2 h at 37 °C. After four washes with PBS/Tw, 100 μl of HRP Substrate TMB solution (Interchim, Montluçon, France) was added, and plates were incubated for
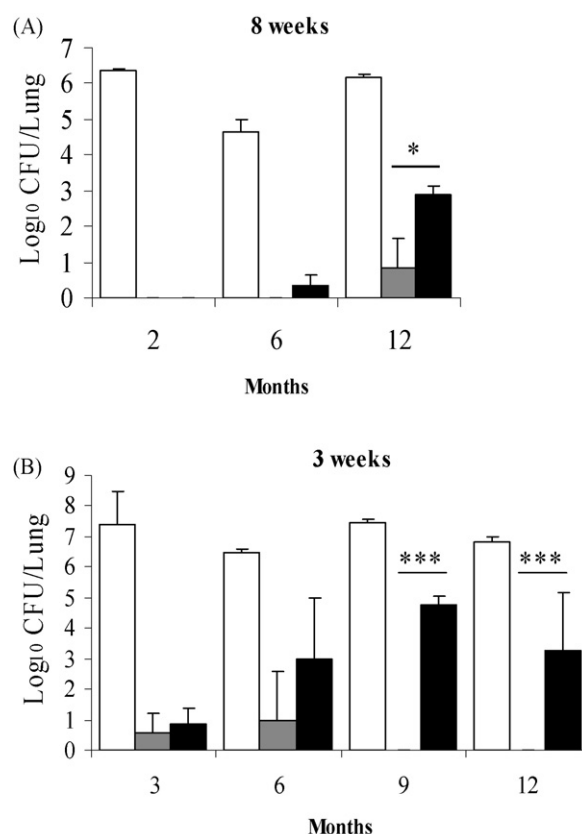


**Fig. 1.** Vaccine-induced long-term protection against *B. pertussis* infection. Eight (A) or 3 (B) weeks old BALB/c mice were immunized with BPZE1 (gray columns), or aPV (black columns), or received PBS only as control (white columns). At the indicated time points after vaccination, the mice were challenge, and protection was evaluated 7 days after challenge by CFU counting in the lungs. CFU ± SE from 4 mice per group are shown. *$P < 0.01$, ***$P < 0.0001$, live attenuated BPZE1 versus acellular aPV vaccine.

30 min at room temperature. The reaction was stopped by the addition of 50 μl of 1 M $H_3PO_4$. The optical density (OD) was measured with a Biokinetic reader EL/340 microplate (Bio-Tec[TM] Instruments Inc., Paris, France) at 450 nm, and a linear regression curve of log OD versus the serum dilution was plotted. Results were expressed in titers, reported as the reciprocal of the dilution giving an OD three times that of the conjugate control.

### 2.4. Cell-mediated immunity assessment

Spleen cells from individual mice were harvested at indicated time points after BPZE1 or aPV administration. To measure cytokine production by stimulated cells, $1 \times 10^6$ cells resuspended in RPMI were placed in U-shaped 96-well culture plates and stimulated with 10 μg/ml FHA, 5 μg/ml PTX or 2 μg/ml Concanavalin A (Con A) in serial dilutions. After 60 h, supernatants were collected from triplicate cultures and analyzed for IFN-γ and IL-5 production with ELISA kits (BD Biosciences, San Diego, USA) using the manufacturer's procedure. Proliferation was assessed by a [3H]-thymidine incorporation assay for the next 16 h.

### 2.5. Statistical analysis

The results were analyzed using the unpaired Student's *t*-test and the Kruskal–Wallis test followed by the Dunn post-test (GraphPad Prism Program) when appropriate. Differences were considered significant at $p \leq 0.05$.
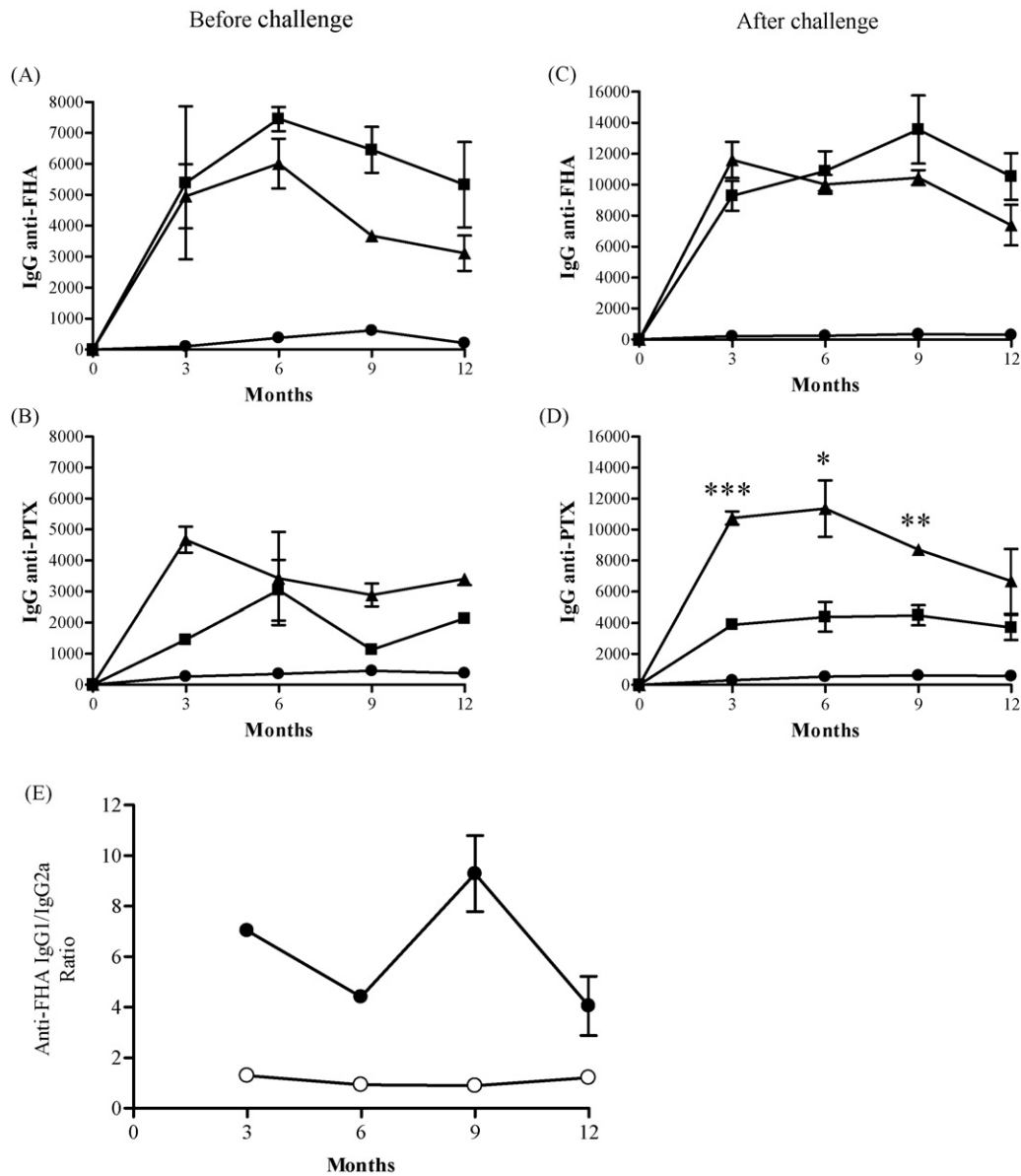
Before challenge

After challenge



**Fig. 2.** Persistence of antibody responses against *B. pertussis* antigens after vaccination. Three weeks old BALB/c mice were immunized with BPZE1 (squares) or aPV (triangles), or left un-immunized (dots), and at indicated time points, groups of 3 mice were bled for antibody determination against FHA (A), PTX (B). After bleeding, the mice were challenged with *B. pertussis* BPSM at the indicated time points and bled 1 week after challenge for determination of anti-FHA (C) and anti-PTX (D) antibodies. The ratio of FHA-specific IgG1/IgG2a after vaccination with BPZE1 (open circles) or aPV (closed circles), but before challenge over the duration of the study is also shown (E). $^*P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$, aPV-vaccinated versus BPZE1-immunized mice.

## 3. Results

### 3.1. BPZE1-induced long-term protection against virulent B. pertussis

We previously reported that a single i.n. administration of live BPZE1 induced full protection in adult (8 weeks old) and in infant (3 weeks old) mice, when challenged i.n. with virulent *B. pertussis* 8 weeks post-immunization, and that in infant mice, the protection was stronger than that induced by two i.p. administrations of aPV [12].

Now, we tested the long-term ability of BPZE1 and aPV vaccines to promote lung clearance of virulent *B. pertussis* in the same mouse i.n. challenge model. Adult or infant mice were immunized either i.n. with a single dose of BPZE1 or i.p. with two doses of aPV

and challenged with *B. pertussis* BPSM at different time intervals. As shown in Fig. 1A and B, independently of the age of mice at the time of the immunization, strong protection was conferred by BPZE1 immunization during the entire course of the experiment, as evidenced by the low to undetectable levels of virulent bacteria in the lungs of BPZE1-immunized mice 7 days after challenge. No or limited waning of protective immunity was observed up to 1 year after immunization with BPZE1. In contrast, the protection induced by aPV immunization waned over the time, as 1 year after immunization roughly $10^3$ CFU of virulent *B. pertussis* was still present in the lungs 7 days after challenge infection of adult mice vaccinated with aPV (Fig. 1A). The difference between the longevity of aPV-induced versus BPZE1-induced immunity against virulent *B. pertussis* challenge was even stronger when the mice were immunized at infancy (3 weeks old; Fig. 1B).

### 3.2. Long-term humoral immunity induced by BPZE1

Since BPZE1 is aimed to be used for vaccination during infancy, we next focused our study on the long-term humoral immune responses induced by pertussis vaccination in infant mice. Levels of serum antibodies against two major protective antigens, FHA and PTX, produced by *B. pertussis* BPZE1 and present in aPv, were measured 3, 6, 9, and 12 months after immunization. As shown in Fig. 2A, before challenge, both BPZE1 and aPV vaccination induced strong serum IgG responses against FHA, with a maximum production 6 months after immunization. These specific anti-FHA antibody responses remained high during the following 6 months despite a decrease, which was slightly faster in aPV-vaccinated mice, compared to mice immunized with BPZE1. Seven days after challenge given at each follow-up time points, mice from both the BPZE1- and the aPV-immunized groups had significantly higher serum anti-FHA antibody concentrations than before challenge (Fig. 2C). There was no statistical difference between groups in anamnestic anti-FHA antibodies.

We also compared the long-term production of antibodies against PTX. Before challenge infection with virulent *B. pertussis*, significant amounts of anti-PTX antibodies were measured in sera of mice immunized with either of the two vaccines, although they were somewhat higher in aPV-vaccinated than in BPZE1-vaccinated mice (Fig. 2B). Seven days after challenge, anamnestic anti-PTX responses were higher after aPV vaccination than after BPZE1 immunization at each time point tested (Fig. 2D). Twelve months after immunization, antibodies induced with aPV vaccine were slightly lower than at 3 months, but were still detectable at significant amounts, while antibodies induced by BPZE1 vaccination remained constant over the entire period (Fig. 2).

When the ratio of FHA-specific IgG1 to IgG2a was determined over the duration of the study (Fig. 2E), at each time point tested, immunization with BPZE1 resulted in a low IgG1/IgG2a ratio, suggesting that BPZE1 immunization skewed the immune response to a Th1 profile. In contrast, significantly higher IgG1/IgG2a ratios were observed after vaccination with aPV, indicating a Th2 type of immune response (Fig. 2E).

### 3.3. Adoptive transfer of serum from BPZE1-immunized mice induced protection against B. pertussis in SCID mice

To compare the role of BPZE1- and aPV-induced *B. pertussis* antibodies in the long-term protection, we used passive transfer experiments in female SCID mice, defective in T, B and natural killer (NK) cell populations. Sera (100 μl) from mice immunized with BPZE1 or aPV 3 or 12 months earlier were transferred into recipient SCID mice 24 h before *B. pertussis* BPSM challenge. Seven days after challenge, a significant reduction (4 log) in bacterial load was observed in animals which had received sera from BPZE1-immunized mice 3 or 12 months earlier, compared to control mice, which had received serum from non-immunized mice (Fig. 3), and there was no difference in the level of protection afforded by sera from mice that had been immunized either 3 or 12 months earlier. These data suggest that efficient protective antibodies are produced at least up to 12 months after BPZE1 immunization. In contrast, SCID mice who had received serum from animals vaccinated 3 months before with aPV were much less protected and showed only a 1.5 log reduction in CFUs compared to mice which had received naïve serum (Fig. 3). In addition, no significant protection was found when serum was transferred from mice aPV-vaccinated 12 months before. Since antibody levels against FHA and PTX were comparable in BPZE1- and aPV-vaccinated mice 12 months after immunization, these results suggest that antibodies specific to *B. pertussis* antigens other than FHA and PTX play an important role in protection against *B. pertussis* infection. However, alternatively, the IgG1
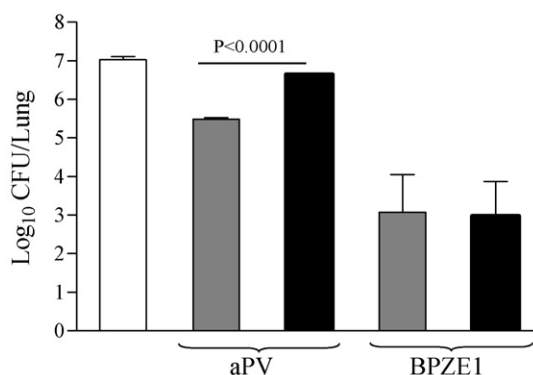


**Fig. 3.** Transfer of protection by serum from immunized mice. 100 μl of serum from non-immunized BALB/c mice (white column), or from BALB/c mice that had been immunized either 3 (gray columns) or 12 months (black columns) previously with aPV or BPZE1 were transferred i.p. to female SCID mice 24 h before they were i.n. infected with $1 \times 10^6$ CFU *B. pertussis* BPSM. The mice were sacrificed 7 days after challenge, and CFUs in the lungs were counted. Results are expressed as means of $\log_{10}$ CFU values per lung from 5 mice per group ($\pm$SE) and are representative of three independent experiments.

to IgG2a ratio may be involved in protective efficacy, as this ratio was substantially different between aPV- and BPZE1-vaccinated mice.

### 3.4. BPZE1 immunization-induced specific memory T cells

Induction of long-lived memory T cell responses after pertussis vaccination has been previously described both in mice and in humans [18–21]. To determine whether BPZE1 immunization induces long-lived memory T cell responses, spleen cells from immunized mice were isolated and stimulated *in vitro* with *B. pertussis* antigens (FHA and PTX) 12 months after immunization. As shown in Fig. 4A and B, *B. pertussis*-specific T-cell proliferative responses were detectable in BPZE1- and in aPV-immunized mice 12 months after immunization, suggesting that memory T cell responses to *B. pertussis* antigens persisted for at least up to 1 year. A cytokine analysis revealed that IFN-γ was produced in significant amounts in the supernatant of FHA-stimulated spleen cells from both BPZE1- and aPV-immunized mice, whereas PTX stimulation induced significant IFN-γ secretion only in the aPV-vaccinated group. IL-5 secretion was only produced by spleen cells from aPV-immunized mice after stimulation with either antigen (Fig. 4D and E). Thus, 12 months after immunization, BPZE1 promotes a Th1-type of immune response with a low level of IFN-γ and no IL-5 secretion, while aPV immunization induced a mixed Th1/Th2 type response with high levels of both IFN-γ and IL-5 (Fig. 4D and E).

### 3.5. Adoptive transfer of cell-mediated immunity from BPZE1-immunized mice to SCID mice

To evaluate the contribution of T cells in the long-term protection induced by BPZE1, spleen cells from BPZE1- and from aPV-immunized mice were isolated 1 year after immunization and transferred into SCID mice. One day after transfer, the mice were challenged, and 7 days post-infection, CFUs were counted in the lungs. BPZE1-immunized animals conferred protection against *B. pertussis* as indicated by approximately 100-fold reduction in bacterial load in the lungs of recipient SCID mice compared to non-transferred or mice transferred with cells from non-vaccinated donors (Fig. 5). The level of protection resulting from the adoptive transfer of splenocytes from aPV-vaccinated mice was low (less than 10-fold reduction in bacterial counts), but nevertheless significant. These results suggest that, 12 months after immunization,
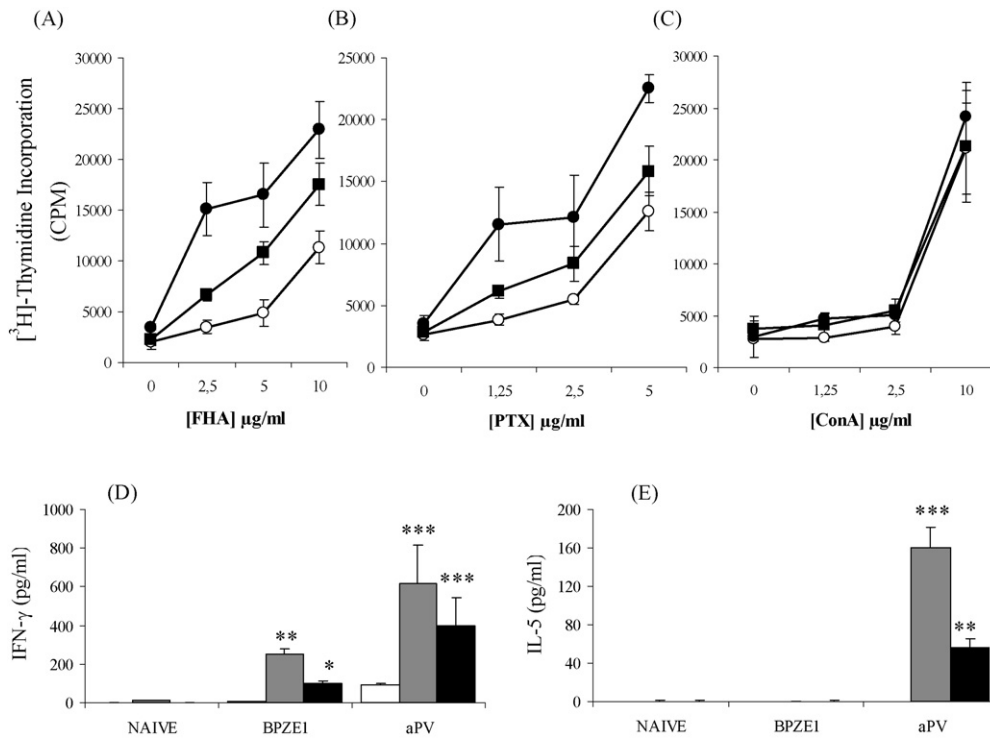
**Fig. 4.** Immunization with BPZE1 or aPV induces long-term memory T cells to *B. pertussis* antigens. Twelve months after immunization, spleen cells from BPZE1- (squares) and from aPV-immunized (black dots) mice were stimulated *in vitro* with FHA (A), heat-inactivated PTX (B) or concanavalin A (ConA, C) as positive control. Proliferation (A, B, C) was evaluated by [$^3$H]-thymidine incorporation. Open circles represent the proliferation results of T cells from non-immunized mice. Supernatants were harvested after 48 h stimulation with FHA (grey columns), PTX (black columns) or no antigen (white columns) and assessed for IFN-$\gamma$ (D) and IL-5 (E) secretion. Results are means ($\pm$SE) proliferation/cytokine concentrations for 4 mice/group. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$, vaccinated versus non-vaccinated mice.

cellular immunity still contributes to the protective mechanism induced by BPZE1 immunization (Fig. 5).

## 4. Discussion

Resurgence of whooping cough is observed worldwide both in infants and adults, despite high pertussis vaccine coverage, and has been attributed at least partly to the waning of vaccine-induced protective immunity over the time [22]. Increasing the longevity of vaccine-induced immunity against *B. pertussis* might therefore have a profound public health impact. Recently, a live, highly attenuated pertussis vaccine candidate, named BPZE, has



**Fig. 5.** Transfer of protection by spleen cells from immunized mice. 50 × 10$^6$ whole spleen cells from BPZE1- or from aPV-immunized mice harvested at 12 months post-immunization were transferred i.p. to female SCID mice 24 h before they were i.n. infected with 1 × 10$^6$ CFU *B. pertussis* BPSM. Non-transferred SCID mice (No transfer) and mice that had received spleen cells from naïve animals (NAÏVE) served as controls. The mice were sacrificed 7 days after challenge, and CFUs in the lungs were counted. Results are expressed as means of log$_{10}$ CFU values per lung from 5 mice per group ($\pm$SE) and are representative of three independent experiments. *$P < 0.01$; **$P < 0.001$; vaccinated versus negative controls.

been developed [12] to be administered early in life in order to protect young children before they receive the three-dose pertussis vaccine regimen at the age of 2–6 months. In the present study, we have evaluated the persistence of BPZE1-induced protective immunity. During 1 year following BPZE1 administration, we measured the immunogenicity and protective efficacy against *B. pertussis* infection, compared to that induced by a widely used aPV. Our hypothesis was that by mimicking natural infection with BPZE1, we may induce long-lasting protective immunity against *B. pertussis*, as a recent analysis using dynamic transmission models in human populations has predicted that immunity resulting from natural infection would last for more than 30 years [11].

To test this hypothesis, we used a murine i.n. infection model to compare the protective efficacy of BPZE1 to that of aPV over a period of 1 year. During this entire period, we found that BPZE1 conferred high levels of protection against *B. pertussis* infection, both in mice vaccinated during adulthood or during infancy. At early time points (3 months after vaccination), this protective efficacy was similar to that induced by aPV. However, later on, in particular 9 and 12 months after vaccination, BPZE1-induced protection was still strong, whereas aPV-induced immunity waned. The longevity of the BPZE1-induced protection was similar to that induced by infection with virulent *B. pertussis*, as shown in the study by Skerry and Mahon (submitted for publication). While BPZE1 is planned to be administered early in life and to be followed by the current three-dose pertussis vaccination regimen, between 2 and 6 months of age, long-term protection conferred by BPZE1 as demonstrated in this paper, suggests that a late booster administration with aPV in BPZE1-primed individuals would be an alternative to explore. On the other hand, BPZE1 could be also an option to vaccinate adults when their immunity to previous pertussis vaccines has waned, in order to prevent transmission of the infection to young children.
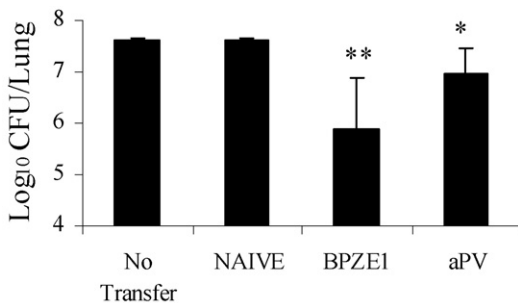
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Mechanisms of protective immunity against *B. pertussis* are only partially understood. However, both antibodies and T-cell-mediated immunity to *B. pertussis* antigens have been shown to contribute to protection against infection [19–21,23]. Recently, a strong correlation has been found between the vaccine dose of BPZE1 and the antibody titers against the *B. pertussis* antigens FHA and PTX, IFN-γ production by spleen cells upon stimulation with these antigens, and protection against subsequent challenge with virulent *B. pertussis* [24]. In addition, we have recently shown that BPZE1-mediated protection can be transferred to SCID mice by either antibodies or T cells, in particular CD4⁺ T cells [13].

Several parameters can modulate the long-term T and B cell memory and protective immune responses against microorganisms [25]. Among them, the abundance of antigen and the duration of antigen exposure during the clonal expansion of T cells are important criteria to shape the quantity and quality of immune memory. The ability of BPZE1 to colonize and to persist in the respiratory tract of mice for approximately 1 month [12] is therefore an asset to induce the highly efficient long-lasting protective immunity observed in this study. We found antibodies against FHA and PTX at high levels in sera both of BPZE1- and of aPV-immunized mice over the period of up to 1 year after vaccination, as well as anamnestic responses after challenge at 3, 6, 9 and 12 months after vaccination, while none of them was detected in the control mice. However, despite the relatively similar anti-FHA and anti-PTX antibody profiles, the protective effect of antibodies transferred from aPV-immunized mice to SCID mice was lower than that of antibodies transferred from BPZE1-vaccinated animals, and was almost negligible 1 year after vaccination. Thus, while BPZE1-induced *B. pertussis*-specific antibodies maintained their protective capacity for at least up to 1 year, a decrease or loss of protection was observed with aPV-induced *B. pertussis*-specific antibodies over time. Previous studies have highlighted the difficulties in correlating protection against pertussis to antibody levels [8,10,26–28]. This is thus consistent with the observations in the present study, also suggesting that the strength of protection conferred by pertussis vaccines is not correlated to the amount of anti-FHA and/or anti-PTX antibodies induced. This may either be due to the isotype differences between these antibodies, as the most striking difference between the aPV-induced and BPZE1-induced antibodies was observed in their isotype profile, or to the fact that BPZE1-induced antibodies not only recognize FHA and PTX, but also a large number of other antigens which may contribute to protection (e.g. [29]) against pertussis.

In addition to antibodies, cell-mediated immune responses, in particular Th1 cells producing IFN-γ, have been shown to play a major role in the protective immunity, especially in long-term protection, against pertussis, both in humans and in mice [13,18–21,23,30,31]. Furthermore, we recently reported the contribution of BPZE1-induced cell-mediated immunity in the clearance of *B. pertussis* in mice [13]. Here, we found that memory T cells were still present in the spleen of BPZE1-immunized mice 12 months after immunization, and that they produced significant levels of IFN-γ in response to FHA or PTX stimulation, although the IFN-γ response to PTX was low. No IL-5 was produced by memory T cells from BPZE1-immunized mice. These results are in line with our previous findings that BPZE1 produces low levels of mutated PTX [12]. In contrast, vaccination with aPV induced both FHA-specific and PTX-specific IFN-γ and IL-5 upon *in vitro* stimulation of T cells with these antigens, even 12 months after vaccination. Thus, whereas BPZE1 induced [12,13] and maintained a Th1 polarized response, with IFN-γ and no IL-5 secretion, aPV induced a mixed Th1/Th2 response characterized by high levels of both IFN-γ and IL-5. In that aspect, these results resemble those recently reported on long-term studies with wcPV and aPV in children, showing slightly lower levels of PTX- and FHA-specific IFN-γ and no or very

low levels of IL-5 secreted by PBMC from wcPV vaccinated children compared to high levels of IFN-γ and IL-5 produced by PBMC from children vaccinated with aPV [18,19]. Although at 12 months post-vaccination the levels of FHA-specific and of PTX-specific IFN-γ-producing T cells in BPZE1-vaccinated mice were lower than in aPV-vaccinated animals, adoptive transfer of the spleen cells from the BPZE1-immunized animals to SCID mice protected them with a higher efficacy than cells from the aPV-vaccinated mice. Again, these results suggest that memory T cells specific for *B. pertussis* antigens other than FHA and PTX may play a role in cell-mediated protective immunity and/or that the quality of these memory T cells might depend on the type of vaccines encountered initially. Alternatively, it cannot be excluded that the Th1 to Th2 ratio may play a role in protective immunity, and that the higher amounts of *B. pertussis* antigen-specific Th2 cytokines induced by aPV over BPZE1 may contribute to its lower protective efficacy.

In conclusion, in contrast to aPV-induced immunity, protection afforded by a single i.n. administration of the BPZE1 vaccine shows longevity over at least 1 year in a mouse i.n. challenge model, with no significant waning even when the mice were vaccinated in their infancy. Similar results were obtained in a mouse aerosol infection model (Skerry and Mahon, submitted for publication), where longevity of protection was comparable to that conferred by infection with virulent *B. pertussis*. However, since we have compared BPZE1 to only one aPV, we cannot ascertain that the conclusions drawn here can be generalized to all aPV. Currently, several aPV are available which differ from each other in antigen composition, ranging from 2 antigens (FHA and PTX), as in the aPV used here, to up to 5 different antigens, as well as in the amounts of antigen present. It may thus be interesting to compare different aPV for ability to induce long-term immunity. Nevertheless, considering that in humans natural infection induces immunity that is longer lasting than vaccination with the current vaccines [11,22], we speculate that administration of BPZE1 to children may also result in long-lasting protection. However, in order to evaluate the longevity of immune responses that contribute to protection, it may not be sufficient to study B- or T-cell responses to the most common antigens, such as PTX and FHA, but to consider other antigens as well, including those that are not present in the current aPV.

### Acknowledgements

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