

A Live, Attenuated *Bordetella pertussis* Vaccine Provides Long-Term Protection against Virulent Challenge in a Murine Model[∇]

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Despite successful mass vaccination programs, whooping cough remains a significant cause of neonatal mortality. Immunity induced by current vaccines wanes in adolescence, requiring additional immunizations to prevent resurgence. There is a need for a new generation of vaccines capable of conferring long-lasting immunity from birth. Recently, a live, attenuated whooping cough vaccine, BPZE1, has been developed. Here, an established murine immunization model was used to examine the induction and longevity of immunological memory. In this predictive model, BPZE1 conferred a level of protection against virulent bacterial challenge comparable to that conferred by recovery from prior infection, up to 1 year after immunization. One year after immunization with BPZE1, a pertussis-specific persistent response, with high levels of gamma interferon (IFN- γ), could be detected from spleen cells restimulated with inactivated *Bordetella pertussis*. BPZE1 induced low levels of interleukin-17 (IL-17) and no IL-10 or IL-5. BPZE1 immunization induced long-lasting, efficacious memory B-cell and specific antibody responses dominated by IgG2a, which were boosted by subsequent challenge. Finally, the antibody induced by BPZE1 was functionally relevant and could clear a virulent *B. pertussis* infection in antibody-deficient mice following passive transfer. This study suggests that BPZE1 is capable of conferring a high level of long-lived effective protection against virulent *B. pertussis*.

Bordetella pertussis is a Gram-negative pathogenic bacterium that is the causative agent of whooping cough, a disease that remains a significant cause of neonatal and infant morbidity and mortality. Whooping cough results in 300,000 deaths annually, with debilitated lung function, bronchiectasis, febrile convulsions, and neurological sequelae associated with infection (7, 47). The current prevalence of disease is remarkable considering the incorporation of pertussis vaccines into mass immunization programs (9). The survival of natural infection by *B. pertussis* is associated with long-lived and efficacious immunity (12, 46), and recent analyses suggest that such immunity persists for more than 30 years in humans (45). Whole-cell *B. pertussis* vaccines (Pw) developed in the 1940s confer highly effective immunity, but public acceptance has been poor due to reports of a suboptimal reactogenicity profile (2, 8). These issues led to the development of acellular *B. pertussis* vaccines (Pa) consisting of recombinant, detoxified, purified virulence factors of *B. pertussis* and usually are formulated with tetanus and diphtheria toxoids (DTaP) and other antigens.

It is now clear, from mass immunization campaigns, that immunity conferred by Pa is less persistent than that induced by Pw (17) or immunity induced by natural infection (12, 46). Pa-induced immunity wanes during a 10- to 15-year period (36), an observation that has necessitated the introduction of booster immunization in adolescents to prevent the establishment of an adolescent reservoir of infection (9). When this situation is accompanied by the late conferral of protection (6 months) imposed by the requirement for three infant doses, a situation is created that allows the transmission of *B. pertussis*

to an unprotected and highly susceptible infant population. This may explain the intransigence of whooping cough as a public health crisis despite the development of effective vaccines (9).

Immunity to *B. pertussis* is complex. While single correlates of protection have been proposed (42, 43), protective immunity requires both a strong cellular and antibody response (22). The reasons for this are open to debate but appear to go beyond the contribution of CD4 populations to the antibody response (22, 25). Pw and Pa appear to protect through slightly different mechanisms, with Pw or natural infection inducing Th1 or Th1/Th17 type responses, respectively, whereas Pa typically induce more Th2-like immunity in humans (39, 40). It has been suggested that CD4 populations and, in particular, Th1 cells contribute directly to the elimination of bacteria (25, 34). This has been clarified by mechanistic studies demonstrating that intracellular *B. pertussis* corrupts the sentinel functions of macrophages and dendritic cells (5), and that gamma interferon (IFN- γ) counters this effect, supporting earlier observations that IFN- γ responses were beneficial to the protective response against *B. pertussis* (25, 26).

The requirements for improved neonatal protection and more persistent long-term immunity (15, 28) have led to the development of a live, attenuated *B. pertussis* candidate vaccine, BPZE1, which is appropriate for single neonatal immunization (30). BPZE1 retains the capacity to colonize but lacks pathogenicity through the attenuation of the *B. pertussis* virulence factors pertussis toxin (PT), dermonecrotic toxin (DNT), and tracheal cytotoxin (TCT). Here, PT has been genetically attenuated to ablate the classical enzymatic activity while remaining highly immunogenic (30). This was achieved by two alterations, at Arg-9 to Lys and Glu-129 to Gly, in subunit 1 to render PT enzymatically inactive. In BPZE1 the DNT gene has been completely deleted, while TCT has been attenuated by

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the transgenic expression of the *Escherichia coli* transporter protein AmpG, which restored regular peptidoglycan metabolism with a consequent loss of TCT production. Previously, we and others have shown BPZE1 to be safe and efficacious in adult and neonate animal models, including immunocompromised mice (41). Consequently, BPZE1 is a candidate for phase I human clinical trials. However, the nature and persistence of long-term immunity following BPZE1 immunization has yet to be characterized.

There is a long tradition of using murine models in support of pertussis vaccine development (20). Here, an established murine aerosol challenge model of *B. pertussis* immunization that correlates with vaccine performance in human clinical trials (33, 35) was used to examine the induction of immunological memory and the persistence of protection. Serum antibody responses and the profile of cell-mediated immunity to BPZE1 were investigated at extended periods after immunization. BPZE1 induced Th1 responses and functionally effective antibody. It primed immune memory and conferred long-lasting protection. This study suggests that BPZE1 offers a viable alternative to acellular pertussis immunization. It confers a level of protection comparable to that of previous exposure to virulent *B. pertussis*, supporting the translation of BPZE1 to human trials.

MATERIALS AND METHODS

Mice. Adult female (6- to 8-week-old) BALB/c, C57BL/6, or μ MT mice were used under the guidelines of the Irish Department of Health, and all procedures were approved by the research ethics committee of the National University of Ireland Maynooth.

***B. pertussis* strains and growth conditions.** *B. pertussis* Tohama I derivatives BPSM and BPZE1 have been described previously (29, 30). In BPZE1, the expression of other virulence factors, such as filamentous hemagglutinin (FHA), adenylate cyclase-hemolysin (ACT), or pertactin (PRN), was not significantly different from that of the parental strain (C. Loch, personal communication). *B. pertussis* strains were grown at 37°C for 48 to 72 h on Bordet-Gengou agar (Gibco) supplemented with 15% defibrinated horse blood. Colonies then were transferred to Stainer and Scholte liquid medium containing streptomycin (30 μ g/ml). Cultures were grown to mid-log phase and were maintained carefully to prevent phase modulation.

Aerosol infection. Respiratory infection was initiated by aerosol infection with the various *B. pertussis* strains according to established protocols (26). Bacteria from mid-log-phase cultures (2×10^{10} CFU/ml) were delivered by nebulizer, during a 20-min period, to mice housed in an exposure chamber such that a colonization of between 5 to 7×10^5 CFU/lung was achieved. This dose was chosen for consistency with previous reports using this system (26) and with previous studies examining the effect of dose on the aerosol challenge model (4). Control mice were challenged with a sham aerosol of 1% (wt/vol) casein solution in sterile physiological saline. For long-term cytokine and antibody studies, sera or spleens were harvested 1 year postinfection.

Antibody passive transfer studies. C57BL/6 mice were challenged, as described above, via aerosol with BPZE1. On day 40 postinfection, whole blood was collected from the submandibular vein (BD Biosciences, Oxford, United Kingdom). Whole blood was allowed to clot and then was centrifuged for 5 min at $900 \times g$, and serum was removed. B-cell-deficient μ MT mice were challenged via aerosol with virulent BPSM. On days 7 and 42 postinfection, μ MT mice received 50 μ l of serum intravenously (i.v.) (via the tail vein) as previously described (35), and the bacterial burden was monitored.

Determination of *B. pertussis*-specific antibody. Endpoint titers of *B. pertussis*-specific antibody were measured from murine sera in a 96-well plate format (4). Enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Roskilde, Denmark) were coated overnight with heat-inactivated virulent *B. pertussis*. This was selected as the antigen mixture to be consistent with previous studies and to allow the assessment of the immune response directed against whole virulent bacteria (as in exposure) and not simply that against selected virulence factors. Plates then were blocked and washed before incubation with 10-fold dilutions of murine sera. Following 2 h of incubation and washing, biotinylated IgG, IgG1, IgG2a,

IgG2b, or IgG3 detection antibody (BD, Oxford, England) was added for 1 h before repeat washes and the addition of streptavidin-horseradish peroxidase (HRP) (GE Healthcare, Uppsala, Sweden). Following a 30-min incubation, plates were washed and IgG binding detected by the HRP substrate TMB (Sigma-Aldrich, Wicklow, Ireland), and the reaction was stopped using 1 M H_2SO_4 . Absorbance was measured at 450 nm. The endpoint titer was calculated as the dilution at which the regression line of absorbance intersected a line 2 standard deviations greater than the threshold generated by control naïve samples (16). All detection reagents used in this study had been previously optimized and validated.

Measurement of IgG⁺ B cells by ELISpot assay. Enzyme-linked immunospot (ELISpot) assay was used to detect the number of IgG⁺ B cells in murine spleen homogenates. A multiscreen HA 96-well plate (Millipore, Cork, Ireland) was coated with heat-inactivated *B. pertussis* overnight at 4°C. Following incubation, plates were washed with sterile phosphate-buffered saline (PBS) and blocked with complete RPMI medium for 1 h before the addition of diluted splenocytes and incubation at 37°C in a 5% CO_2 atmosphere for 24 h. Following incubation, cells were removed, the plate was washed gently in PBS, and *B. pertussis*-specific IgG-positive cells were detected by incubation with 1 μ g/ml biotinylated anti-mouse IgG antibody (Biosera, Sussex, England) using streptavidin-HRP and TMB substrate. Color development was stopped by extensive washing with tap water, the plate then was left to air dry, and the total spot count was determined by microscopy under $\times 10$ magnification. Controls included similar preparations from age-matched naïve mice.

Cytokine measurement. Cytokine concentrations were determined by a multiplexed bead array (FlowCytomix, Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. Briefly, cytokine standards, buffer control, or sample were added to beads coated with analyte-specific antibodies with a biotin-conjugated antibody mix and incubated for 2 h in the dark at room temperature. Following incubation, 1 ml 1% (vol/vol) bovine serum albumin (BSA)-PBS buffer was added to each tube, and the beads were centrifuged at $200 \times g$ for 5 min. Following centrifugation, supernatant was discarded, leaving 100 μ l, and streptavidin-phycoerythrin was added to each tube before 1 h of incubation at room temperature. After this incubation, beads were washed twice in fluorescence-activated cell sorting (FACS) buffer and centrifuged as described above. Beads then were resuspended in 1 ml buffer, and cytokine concentrations were determined by flow cytometry calibrated against standards of known concentrations.

Statistical methods. All results are expressed as the means \pm standard errors of the means (SEM). A Student's *t* test was used to determine significance between the groups. A value of $P < 0.05$ was considered significant. Analyses and graphical representations were performed using Graph-Pad Prism software (Graphpad, San Diego, CA).

RESULTS

A live, attenuated pertussis vaccine, BPZE1, confers long-term immune protection in a predictive murine model. The murine respiratory aerosol challenge model of protection (26, 35) was used to examine the ability of a live, attenuated vaccine candidate, BPZE1, to confer long-lasting immunity against virulent *B. pertussis*. The aerosol challenge model not only closely mimics natural infection, but protection induced by aerosol challenge in mice previously has correlated strongly with estimated vaccine efficacy in infants (33, 35). Mice were challenged, by aerosol, with attenuated BPZE1, virulent *B. pertussis* BPSM, or a sham inoculum of 1% casein (wt/vol) in PBS, and they were housed long term under isolator conditions. One year postchallenge, mice received an aerosol challenge of virulent *B. pertussis* BPSM. Lungs were harvested and bacterial loads determined at days 0, 7, 10, and 14 postinfection. Naïve mice showed a typical course of primary infection and had no protection against virulent *B. pertussis* infection (Fig. 1). These data also indicated that the potential immunological experience of the mice regarding environmental antigen in the year between setup and challenge did not confer any cross-protection. As previously demonstrated, there was a significant reduction in bacterial carriage in the lungs of mice

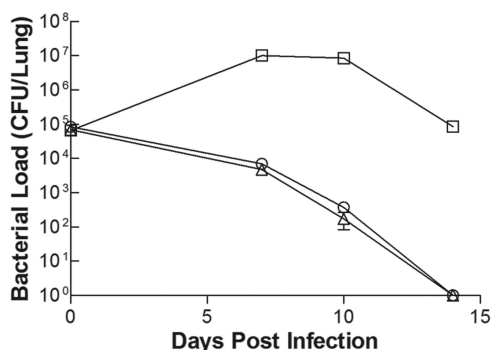


FIG. 1. BPZE1 induced long-term protection against virulent *B. pertussis*. Mice were challenged or immunized with virulent BPSM (triangles), attenuated BPZE1 (circles), or a sham immunization control (squares). One year later, mice were challenged with virulent BPSM. Results are representative of two experiments expressed as mean (\pm SEM) CFU per lung detected in triplicate from five mice per group at each time point. Error bars that are not visible are masked by the data point symbol.

previously challenged with virulent *B. pertussis* BPSM (Fig. 1). This was evidenced by a significant reduction of bacterial burden at days 7 ($P < 0.0001$), 10 ($P < 0.0001$), and 14 ($P < 0.0001$) and confirmed the capacity of prior *B. pertussis* infection to induce long-term protection. Mice immunized with live, attenuated BPZE1 were protected 1 year after immunization (Fig. 1). These mice showed reduced bacterial burden compared to that of naive mice and a level of protection not significantly different from that observed following prior viru-

lent BPSM exposure at day 7 ($P = 0.0605$), 10 ($P = 0.2099$), or 14 ($P = 0.5734$) postinfection. Thus, live, attenuated BPZE1 confers long-term protection at least comparable to that induced by virulent challenge.

Immunization with BPZE1 induces a pertussis-specific persistent Th1 response. Previous reports had suggested that Th1-associated cytokines were beneficial for the optimal clearance of and protection against *B. pertussis* (4, 25, 40). However, to date, nothing is known about the nature of the long-term cytokine response following BPZE1 vaccination. To examine the nature of the memory response, mice were challenged, as described above, with either BPSM or BPZE1 and then challenged 1 year later with virulent BPSM. Splenocytes from before and after the second challenge were harvested from groups of mice, and IFN- γ , IL-5, IL-10, and IL-17 recall responses to inactivated *B. pertussis* were measured.

Prior to a secondary virulent challenge, control mice showed no *B. pertussis*-specific cell-mediated immunity, as expected (Fig. 2). Virulent BPSM or attenuated BPZE1 challenge induced strong *B. pertussis*-specific IFN- γ recall responses but little or no IL-5 response, indicating the induction and persistence of a strong Th1 response (Fig. 2A and B). However, recall in these mice was not immunologically identical; mice exposed to virulent *B. pertussis* displayed high IL-17 responses to antigen restimulation, whereas BPZE1 priming resulted in detectable but significantly lower ($P = 0.0036$) IL-17 responses, suggesting that the attenuation to BPZE1 directly or indirectly (41) altered the ability to induce a Th17 response. Negligible IL-10 was induced by either BPSM or BPZE1.

Primed animals also were rechallenged after 1 year by aro-

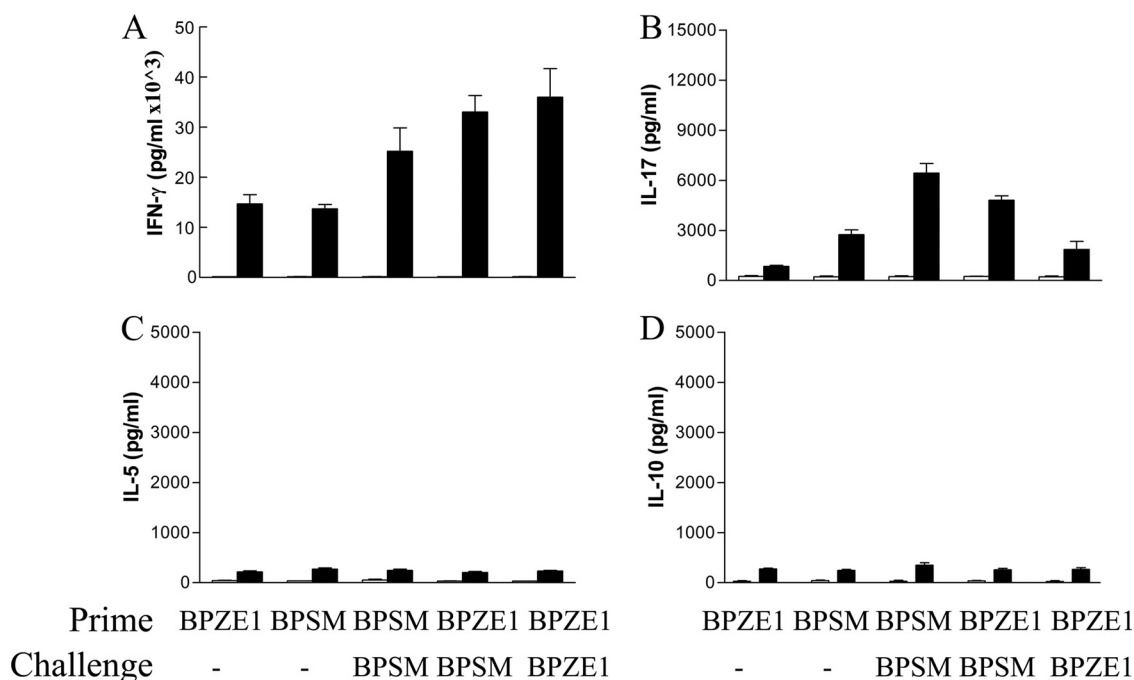


FIG. 2. BPZE1-primed mice developed a long-lived cell-mediated immune response with a cytokine profile comparable to that of virulent challenge. Mice were primed by BPZE1 immunization or BPSM challenge. One year later, primed mice were challenged with a casein control (-), virulent BPSM, or attenuated BPZE1. Splenocytes were harvested and restimulated *in vitro* with heat-inactivated BPSM (closed) or medium control (open bars). Splenocytes from naive mice failed to respond to antigens at levels above those in medium controls (data not shown). Results are expressed as mean (\pm SEM) analyte concentrations (in pg/ml) ($n = 5$) from triplicate determinations.

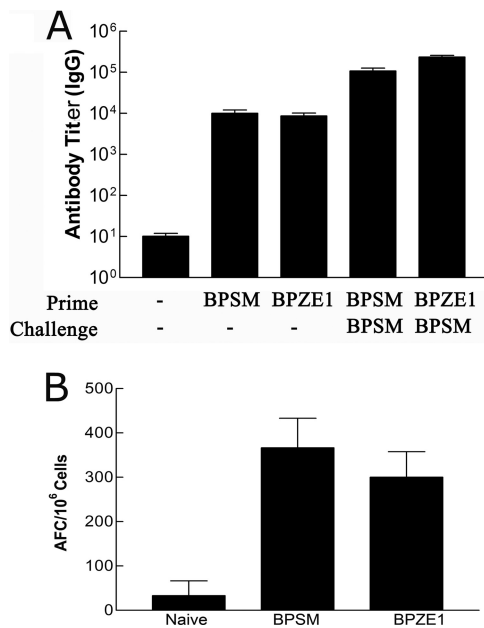


FIG. 3. BPZE1 challenge induced a long-term specific antibody response comparable to that of virulent challenge. (A) *B. pertussis*-specific serum IgG levels were measured 1 year after BPSM or BPZE1 priming and again following virulent BPSM challenge by ELISA using heat-inactivated whole bacteria as the antigen. Results are expressed as mean (\pm SEM) endpoint titers ($n = 5$ for triplicate determinations). (B) Splenocytes were isolated from BPZE1, BPSM, or sham-challenged naive mice 1 year postpriming to measure the induction of long-term *B. pertussis*-specific IgG⁺ memory B cells by ELISpot. Results are expressed as mean antibody-forming cells per 10⁶ splenocytes for triplicate determinations.

sol with either virulent BPSM or BPZE1 to examine the anamnestic response. BPSM-primed and -re-challenged mice produced elevated IFN- γ (Fig. 2A) and IL-17 (Fig. 2B) but not IL-5 (Fig. 2C) or IL-10 (Fig. 2D) in response to *in vitro* restimulation by *B. pertussis*. A second challenge with BPZE1, 1 year after the initial priming, resulted in boosted IFN- γ (Fig. 2A) but not IL-5 (Fig. 2C), IL-17 ($P = 0.1079$), or IL-10 ($P = 0.0571$) response.

Mice primed by BPZE1 and then re-challenged 1 year later by BPSM (a situation analogous to immunization and exposure) showed boosted IFN- γ responses but no IL-5 or IL-10 response, demonstrating the persistence of the initial Th1-like phenotype (Fig. 2). IL-17 levels were significantly enhanced only when mice were exposed to virulent BPSM, not BPZE1, and this was similar to the primary Th17 response evoked by BPSM. Thus, BPZE1 induced a stable, long-lasting Th1-like immunity and primed IL-17 responses in animals exposed to virulent bacteria.

BPZE1 immunization induced long-lasting, efficacious B-cell and antibody responses. Murine models have been used extensively to probe the longevity of the vaccine-induced immunity to *B. pertussis* (20, 32, 33, 35). To characterize the nature and longevity of the antibody response to BPZE1 immunization, sera and splenic B-cell responses were examined from long-term-immunized mice. Antibody responses to heat-inactivated *B. pertussis* were measured in an attempt to measure the total immune response directed against whole virulent

bacteria (as in exposure) and not simply that against selected virulence factors; parallel studies have been performed using single antigens to compare the BPZE1 response to that of acellular vaccines (14). *B. pertussis*-specific IgG⁺ memory B cells were quantified by ELISpot assay, and the titer of antibody responses were quantitatively and qualitatively characterized. High serum levels of specific IgG were found 1 year after BPSM or BPZE1 priming, with no antibody detected in control animals (Fig. 3A). A boost in IgG was noted following a second challenge with virulent BPSM. Thus, BPZE1 induced long-lasting antibody responses to virulent BPSM, but intriguingly, IgG responses to BPSM were significantly higher ($P = 0.0128$) in BPZE1-primed mice than in BPSM-primed mice (Fig. 3), supporting previous data suggesting that during natural infection by virulent *B. pertussis* virulence, factors such as PT and DNT suppress adaptive immunity (19). Both virulent BPSM and attenuated BPZE1 resulted in murine antibody responses dominated by IgG2a (Table 1), mirroring the induction of IFN- γ seen in Fig. 2. No *B. pertussis*-specific IgE was detected following BPZE1 immunization.

Antibody titer is only one index of the persistence of memory, and indeed the presence of memory B cells may be more important in vaccine-induced immunity. ELISpot therefore was used to measure pertussis-specific IgG⁺ memory B cells from the spleens of long-term-challenged mice. Both BPSM challenge and BPZE1 vaccination induced high numbers of IgG⁺ memory B cells (Fig. 3). No significant difference ($P = 0.4918$) was seen between mice that received BPZE1 or those that received virulent BPSM (Fig. 3B). Taken together, these results demonstrated that BPZE1 induced a long-lasting memory B-cell response and corresponding specific antibody similar or superior in both quality and quantity to that induced by virulent infection.

Antibody induced by BPZE1 can clear virulent *B. pertussis* following passive transfer. Antibody titers determined by ELISA or cellular responses by ELISpot do not indicate the quality of the antibody response and, in particular, do not predict whether the antibody is functionally relevant or neutralizing. It has been shown previously that antibody, passively transferred from mice vaccinated with whole-cell or acellular vaccines, is capable of reducing bacterial loads in infected mice lacking conventional Ig responses (35). To determine whether BPZE1-induced antibody was functionally effective against virulent *B. pertussis*, μ MT mice, which lack functional antibody

TABLE 1. *B. pertussis*-specific IgG induced by priming and/or challenge

Prime/challenge	Antibody titer ^a				
	Naïve	BPSM	BPZE1	BPSM-BPSM	BPZE1-BPSM
IgG1	1/10	1/250	1/500	1/1,000	1/1,000
IgG2a	1/10	1/7,500	1/6,000	1/20,000	1/50,000
IgG2b	1/10	1/500	1/200	1/5,000	1/5,000
IgG3	1/10	1/1,000	1/1,000	1/2,000	1/500

^a IgG subclass endpoint titers were determined 1 year following the aerosol administration of 1% casein (wt/vol) (naïve), virulent BPSM, or vaccine strain BPZE1. To determine if primed mice experienced boosted antibody levels following repeat exposure, serum antibody levels were measured from mice primed with BPSM (BPSM-BPSM) or BPZE1 (BPZE1-BPSM) who received a second BPSM challenge 1 year following priming. Results are expressed as mean endpoint titers ($n = 5$).

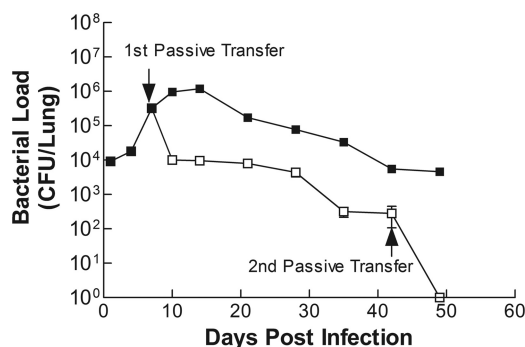


FIG. 4. BPZE1-induced antibody is functionally effective against virulent *B. pertussis*. To assess the functional efficacy of antibody induced by BPZE1 vaccination, μ MT mice were challenged with virulent BPSM. At days 7 and 28 postinfection, mice received the passive transfer of different sera by i.v. injection. Sera were derived from control naive mice (closed squares) or from mice immunized with attenuated BPZE1 (open squares). Results are expressed as mean (\pm SEM) CFU per lung, detected in triplicate from five mice per group per time point. Error bars that are not visible are masked by the data point symbol.

(21), were challenged with BPSM. At days 7 and 21 postinfection, mice were intravenously injected with sterile PBS or diluted sera raised against BPZE1.

A chronic persistent infection was observed in the control μ MT mice, which is consistent with previous findings (26) showing antibody to be essential for sterilizing protection against *B. pertussis* (Fig. 4). Mice that received passive immunization with serum from BPZE1-challenged mice successfully cleared infection. Following the initial passive transfer, serum-treated mice exhibited reductions of approximately 2 log in bacterial load. Complete clearance subsequently was observed following a second passive transfer to these mice (Fig. 4). These findings indicate that antibody evoked by BPZE1 immunization is functional against virulent *B. pertussis* and affords protection against virulent bacteria.

DISCUSSION

This study examines the longevity of protection induced by the live, attenuated candidate vaccine BPZE1. In prime-boost experiments, BPZE1 primed long-lasting *B. pertussis*-specific IgG responses. This antibody response was accompanied by strong antigen-specific IFN- γ responses from restimulated splenocyte cultures. Such antibody was predominantly of the IgG2a subclass and conferred protection by passive transfer to infected immunodeficient mice. Taken together, these data confirm that BPZE1 conferred a long-lasting protective immune response against virulent *B. pertussis* challenge.

Immunity to Pa vaccines wanes in adolescence (15, 36). While infection in adolescents and adults often results in asymptomatic infection (37), these populations serve as a reservoir for infection, increasing the potential transmission of the disease to neonates and infants (15). When Pa coverage is persistently high in adolescent populations, herd immunity controls the disease (6). These observations highlight three issues: the importance of vaccine coverage of the target populations, the need for *B. pertussis* vaccines that are capable of conferring long-term protection, and the desirability of pertus-

sis vaccines suitable for neonates. While neither vaccine- nor infection-induced immunity to *B. pertussis* can be said to be life long, immunity conferred by infection has been estimated to last at least 20 years, whereas Pa-induced immunity may wane after 12 years in humans, although little difference is seen between two- and three-component Pa in the longer term (45, 46). The long-term protection afforded by BPZE1 will require human clinical trials; nevertheless, this study demonstrated that BPZE1 could induce long-term protection in a murine model comparable to prior virulent infection. In very recent parallel studies, the longevity of the BPZE1-induced protection by aerosol challenge (herein) was similar to that seen in the alternative intranasal delivery model (14), where BPZE1 was shown to confer significantly better protection against virulent challenge than Pa at 9 and 12 months postvaccination. However, further studies directly comparing the efficacies of BPZE1 and Pa in this model are required. The virulent infection of mice and humans induces either Th1 or mixed Th1/Th17 responses (40), whereas Pw induces Th1 immunity (12, 38, 40). In contrast, a less polarized or even a Th2-dominant response has been noted when Pa has been employed (23, 39). Previously, the induction of a Th1 response has been associated with optimal immunity against pertussis (40), and suboptimal Pa can be improved when IL-12 is used as an adjuvant in mice (23). BPZE1 induces strong IFN- γ responses that are detectable 1 year after priming. There has been speculation as to why IFN- γ has a role in the containment and clearance of *B. pertussis* (3, 22). This may be related to observations showing that virulent *B. pertussis* subverts sentinel antigen-presenting cell functions (5). Immunization with attenuated BPZE1 (or challenge with BPSM) resulted in high levels of IFN- γ but low levels of IL-5. This is in keeping with previous reports of the immunity induced by virulent infection (32, 46) and Pw immunization (40). IL-17 was induced by BPZE1 but was more evident following BPSM infection. This suggests that the presence of PT, DNT, and/or TCT contributes to IL-17 induction in natural infection. Enzymatically active pertussis toxin induces the production of IL-17 (1), and so, reduced IL-17 responses to BPZE1 could be a product of PT attenuation. Recently, Th17 responses to *B. pertussis* have been linked to adenylate cyclase (CyaA) (11). As CyaA is fully functional in both BPSM and BPZE1, our results suggest redundancy in this system, possibly linked to intracellular cyclic AMP. A second challenge with virulent BPSM resulted in a Th1/Th17 response regardless of the priming antigen. Overall, a key finding of this study is that BPZE1 primes a persistent cell-mediated immune response against *B. pertussis*. The Th1/Th17 profile induced by BPZE1 may be important in the neonatal context, as Th1 responses typically are weak in human neonates (27, 44) and during early life (10). In terms of pertussis immunity, priming this type of response can support long-lived memory and protection even when antibody responses have waned (24).

While cell-mediated responses are important in vaccine-induced immunity to *B. pertussis*, the antibody response induced by immunization is central to clearance and protection (26, 35). BPZE1 induced a long-lived memory B-cell response, as evidenced by ELISpot assay, and long-lasting, high-titer antibody that is detectable in serum. Patterns of IgG subclass induction in mice are not predictive of human immunity, and indeed there is little evidence to suggest that various patterns

of human IgG have any influence on pertussis immunity. However, this study showed that the antibody evoked by BPZE1 was effective at neutralizing virulent infection *in vivo* and to passively confer sterilizing immunity. Thus, BPZE1 promotes an antibody response of functional efficacy. It is important that no *B. pertussis*-specific IgE was detected following BPZE1 immunization in this study, in line with recent data showing that BPZE1 had a pronounced antiatopic effect in murine allergy models (19). This study also made an intriguing observation of increased antibody production following challenge with BPZE1 compared to that with the virulent strain BPSM. Previously, a *B. pertussis* strain lacking functional PT was shown to induce higher antibody levels in mice than wild-type strains (30), indicating that our findings are associated with the attenuation of an immunosuppressive effect of PT.

Defining correlates of protection against *B. pertussis* has been notoriously difficult. However, priming immunological memory is, undoubtedly, a key aspect in the induction of protection through pertussis immunization. In this regard, the ELISpot data here are important, as BPZE1 not only evokes persistent antibody responses but also induces strong immunological priming in the B-cell compartment (Fig. 3), which is boosted on encounter with virulent bacteria. Previous preclinical studies have shown that BPZE1 is genetically stable (13, 31), safe in neonatal and immunocompromised mouse models (41), and a feasible vehicle for vaccination with heterologous antigens (18). This study demonstrates that live, attenuated BPZE1 confers protection against virulent *B. pertussis* challenge, consisting of a Th1 memory response, high-titer *B. pertussis*-specific serum antibody, and the priming of a pertussis-specific memory B-cell response that persists 1 year following vaccination. Taken together, these data strongly suggest that BPZE1 is a viable candidate for translation to human use as a novel live, attenuated pertussis vaccine.

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