

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Attenuated *Bordetella pertussis* BPZE1 protects against allergic airway inflammation and contact dermatitis in mouse models

R. Li^{1,2,*}, C. Cheng^{2,3}, S. Z. Chong^{1,3}, A. R. F. Lim^{1,2}, Y. F. Goh^{2,3}, C. Locht^{4,5,6,7}, D. M. Kemeny^{1,2}, V. Angeli^{1,2}, W. S. F. Wong^{2,3} & S. Alonso^{1,2}

¹Department of Microbiology; ²Immunology programme; ³Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore; ⁴Inserm, U1019, Lille; ⁵CNRS UMR8204, Lille; ⁶Univ Lille Nord de France, Lille; ⁷Institut Pasteur de Lille, Lille, France

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Keywords

allergic airway inflammation; *Bordetella pertussis* BPZE1; contact hypersensitivity; inflammatory disease; pro-inflammatory cytokines.

Correspondence

S. Alonso, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, CeLS building #03-05N, 28 Medical Drive, 117597 Singapore, Singapore.
Tel.: +65 6516 3541
Fax: +65 6778 2684
E-mail: micas@nus.edu.sg

*Present address: Department of Microbiology and Microbial Engineering, School of Life Sciences, Fudan University, Shanghai, China.

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Abstract

Background: We previously reported that prior nasal administration of highly attenuated *Bordetella pertussis* BPZE1 provides effective and sustained protection against lethal challenge with influenza A viruses. The protective effect was mediated by suppressing the production of major pro-inflammatory mediators. To further explore the anti-inflammatory properties of BPZE1, we investigated the effect of BPZE1 nasal pretreatment on two mouse models of allergic disease, allergic airway inflammation, and contact hypersensitivity (CHS).

Methods: Allergic reactions were induced in mice nasally pretreated with live attenuated BPZE1 bacteria using the ovalbumin (OVA)-induced allergic airway inflammation and dinitrochlorobenzene (DNCB)-induced CHS models.

Results: Prior BPZE1 nasal treatment suppressed OVA-induced lung inflammation and inflammatory cell recruitment and significantly reduced IgE levels and cytokine production. Similarly, BPZE1 nasal pretreatment markedly inhibited ear swelling, skin inflammation, and production of pro-inflammatory cytokines in the DNCB-induced CHS model. For both models, we showed that BPZE1 pretreatment does not affect the sensitization phase. Upon challenge, BPZE1 pretreatment selectively reduced the level of cytokines whose production is increased and did not affect the basal level of other cytokines. Together, our observations suggest that BPZE1 pretreatment specifically targets those cytokine-producing effector cells that are recruited and involved in the inflammatory reaction.

Conclusion: Our study demonstrates the broad anti-inflammatory properties of the attenuated *B. pertussis* BPZE1 vaccine candidate and supports its development as a promising agent to prevent and/or treat allergic diseases.

We previously reported that prior nasal administration of the highly attenuated *Bordetella pertussis* strain BPZE1 provides effective and sustained protection against lethal challenge with influenza A viruses (1) and that the protective mechanism(s) do not involve cross-reactive *B. pertussis*-specific antibodies or T cells. Instead, prior nasal BPZE1 treatment prevented virus-induced lung inflammation and death by suppressing the production of major pro-inflammatory mediators. To further explore the nonspecific anti-inflammatory properties of BPZE1, we investigated in the present study the effect of BPZE1 nasal pretreatment on two

inflammation-induced pathologies, namely allergic asthma and contact hypersensitivity (CHS).

Asthma is a chronic inflammatory lung disease characterized by intermittent airflow obstruction, airway hyperreactivity (AHR), mucus hypersecretion, enhanced IgE responses, and infiltration of inflammatory cells - mainly eosinophils into the airways (2). In recent years, the incidence of asthma has increased dramatically, with the greatest prevalence observed in developed countries (3). Although altered Th2/Th1 balance with a Th2-dominant immune response has been shown to be important in the development of asthma, the

mechanism underlying the pathogenesis of asthma remains to be fully deciphered (4).

Allergic contact dermatitis (ACD) caused by reactive haptens and metal ions is a form of delayed type hypersensitivity and is one of the most common skin diseases worldwide (5). Contact hypersensitivity (CHS) is a recognized mouse model for studying human ACD and is an epidermal T cell-mediated inflammatory response to low molecular weight haptens (6, 7).

According to the hygiene hypothesis, frequent exposure to pathogens triggers a certain degree of protective immunity against atopic diseases (8). However, conflicting observations have been reported regarding the protective vs detrimental role of the preexposure to viral or bacterial microorganisms against allergic diseases (9–13), underscoring that the effect of encounter with pathogenic and nonpathogenic bacteria on shaping the host immune response is complex and remains poorly understood. Furthermore, despite intensive studies on the mechanisms and optimal medical management, current therapeutic approaches that tackle these inflammatory disorders are largely ameliorative rather than curative and can cause unexpected side effects (14). In addition, many asthmatic patients develop resistance to treatment and/or progressive pulmonary dysfunction (14). Thus, there is a strong medical need for better therapy to treat allergic diseases.

In this work, we evaluated the ability of nasal pretreatment with live BPZE1 to protect against ovalbumin (OVA)-induced allergic airway inflammation and dinitrochlorobenzene (DNCB)-induced CHS in preclinical murine models. In addition to address the potential of BPZE1 nasal treatment as a novel prophylactic and therapeutic intervention against allergic diseases, the relative protection against a Th2- vs Th1-dominated inflammatory response may help further characterize the BPZE1-induced protective mechanisms.

Materials and methods

Bacterial strains and growth conditions

Attenuated *B. pertussis* BPZE1 is a streptomycin-resistant *B. pertussis* Tohama I derivative, lacking dermonecrotic toxin, producing inactivated pertussis toxin and background levels of tracheal cytotoxin (15). Virulent *B. pertussis* BPSM (16) and BPZE1 were cultured as described previously (1).

Animal experiments

Female BALB/c mice (6–8 weeks old) were purchased from CARE Centre (Singapore). Mice were kept under specific pathogen-free conditions in individual ventilated cages. All animal experiments were approved and carried out according to the institutional guidelines set by the Animal Care and Use Committee of National University of Singapore.

Briefly, lightly anesthetized mice were intranasally (in.) administered once or twice at a 4-week interval with 5×10^6 colony-forming units (CFU) of live attenuated BPZE1 or BPSM bacteria as previously described (1). Six weeks after one BPZE1 or BPSM dose, or 2 weeks after the 2nd dose (day 42), after complete bacterial clearance from the lungs

(data not shown), sensitization and challenge were performed as follows: For the allergic airway inflammation model, OVA sensitization consisted of two intraperitoneal (ip.) injections of 0.1 ml of 200 µg/ml OVA with aluminum hydroxide at a 2-week interval. OVA aerosol challenge was performed 1 week after the last OVA ip. injection for three consecutive days as described previously (17). Positive control mice received phosphate-buffered saline (PBS) in. instead of bacteria followed by OVA sensitization and challenge. Negative control mice were OVA-sensitized but challenged with PBS.

In the DNCB-induced CHS model, mice were sensitized by applying 50 µl of 1% w/v DNCB (Sigma, St. Louis, MO, USA) dissolved in acetone and olive oil (4:1; v/v) on their shaved back. Five days after sensitization (day 47), 25 µl/side of 1% DNCB was applied on both sides of both ears. Negative control mice were sensitized and challenged with acetone and olive oil (4:1; v/v) without DNCB. Ear thickness was monitored daily after challenge using a caliper.

Cellular infiltrates in bronchoalveolar lavage fluids

Mice were euthanized 24 h after the last aerosol OVA challenge (day 66), and bronchoalveolar lavage fluids (BALFs) were harvested as described previously (17). BALFs were centrifuged, and the supernatants were stored at -80°C for cytokine detection. The cell pellets were resuspended, spotted onto a glass slide using a Cytospin device (Thermo Shandon, Thermo Fisher Scientific, Waltham, MA, USA), and stained using a modified Wright staining procedure as described previously (17). A total of 500 cells were examined for each slide. Counts were performed on blinded samples. Eight mice per group were individually assessed.

Antibody and cytokine detection

The serum levels of total IgE and OVA-specific IgE, IgG1, and IgG2a were determined by ELISA as described previously (18). Cytokine levels were measured in the BALFs supernatants (allergic airway inflammation model) or ear homogenates (CHS model) using a custom-made multiplex cytokine detection assay (BioPlex; BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Ear homogenates were prepared on ice upon addition of 200 µl RIPA buffer (Sigma) with protease inhibitor and mechanical homogenization using High Shear homogenizer (Omni International). After centrifugation at 9000 g and 4°C to remove the cellular debris, the supernatants were analyzed using a BioPlex instrument (BioRad).

In vitro restimulation experiment

Single-cell suspensions from auricular lymph nodes were prepared, and 2×10^6 cells/well were seeded in 96-well round-bottom plates (Nunc, Roskilde, Denmark) in 100 µl RPMI complete medium (RPMI 640 supplemented with 10% FCS, 5×10^{-5} M β -mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES, 200 U/ml penicillin, 200 µg/ml streptomycin). Cells were restimulated with 1 µg/ml of plate-bound anti-CD3 (cat# 553057) and 1 µg/ml soluble anti-CD28

(cat# 553294) (BD) After 24-h incubation, the supernatants were harvested for Interferon (IFN)- γ measurement using mouse IFN- γ ELISA Ready-SET-Go!® detection kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The cells were pulsed with 0.4 μ Ci [^{3} H] thymidine in 20 μ l RPMI complete medium. After 18-h incubation, cells were harvested, washed, and the incorporated radioactivity was measured in a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer, Waltham, MA, USA). Each sample was assayed in triplicate.

Histological analyses

The lungs or ears were harvested from euthanized mice, fixed in 4% formalin in PBS, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E), and examined under an inverted light microscope at $\times 100$ and $\times 400$ magnifications. Alternatively, lung sections were stained with periodic acid-fluorescence Schiff stain (PAFS) and examined for mucus production as described previously (19).

Statistical analysis

Unless otherwise stated, bars represent mean $\pm$ SEM, and averages were compared using a bidirectional unpaired Student's *t* test with a 5% significance level at $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$. For DNCB-induced CHS model, ear thickness data were analyzed by 2-way ANOVA. Values shown are the mean $\pm$ SEM.

Results

Intranasal pretreatment with BPZE1 provides long-term protection against OVA-induced allergic asthma

Adult BALB/c mice were intranasally (in.) administered either once or twice with live BPZE1 or PBS. After complete bacterial clearance from the lungs (data not shown), OVA sensitization and challenge were performed. In contrast to the sensitized but not challenged animals (OVA/saline), typical inflammation of the airway walls with marked infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues was observed with the sensitized and challenged animals (OVA/OVA) (Fig. 1A). Nasal pretreatment with BPZE1 (one or two doses) visibly reduced peribronchial inflammation (Fig. 1A, c,d,h,i), whereas enhanced pathology was observed in mice pretreated with virulent *B. pertussis* BPSM (Fig. 1A, e & f). PAFS staining revealed that OVA-induced mucus hypersecretion and goblet cell hyperplasia were noticeably reduced in the BPZE1 pretreated mice whereas preexposure to virulent BPSM did not result in reduction compared with the untreated challenged animals (OVA/OVA) (Fig. 1B). Together, these data indicate that BPZE1 nasal pretreatment reduces the pathological manifestations of OVA-induced allergic airway inflammation, whereas preinfection with its virulent counterpart BPSM does not. In addition, noticeably a single nasal administration of BPZE1 appeared to be at least as potent as two doses.

BPZE1 pretreatment suppresses OVA-induced inflammatory cell recruitment in the lungs

Examination of the inflammatory cell influx in the BALFs collected 24 h after OVA challenge showed a marked increase in total cell, eosinophil, macrophage, neutrophil, and lymphocyte counts in the sensitized and challenged (OVA/OVA) control animals compared with the nonchallenged (OVA/saline) group (Fig. 2A). BPSM pretreated animals displayed comparable cell counts to those measured in the OVA/OVA control group. In sharp contrast, the total cell, eosinophil, macrophage, neutrophil, and lymphocyte counts in the BALFs from BPZE1-treated mice were significantly decreased (Fig. 2A), demonstrating that a single nasal dose of BPZE1 significantly suppressed OVA-induced inflammatory cell recruitment in the lungs.

BPZE1 pretreatment reduces serum IgE production

ELISA data indicated a marked elevation in total serum IgE, OVA-specific IgE, and OVA-specific IgG1 levels, but not in the OVA-specific IgG2a level in the OVA/OVA mice compared with the OVA/saline group (Fig. 2B). Pretreatment with BPSM gave antibody levels comparable to those measured in the OVA/OVA group. In contrast, the total serum and OVA-specific IgE levels were strongly reduced in the BPZE1-treated mice whereas BPZE1 pretreatment had no effect on the serum levels of OVA-specific IgG2a and IgG1 (Fig. 2B). Thus, in. pretreatment with BPZE1 did not modulate the serum OVA-specific IgG responses, but specifically suppressed the production of total and antigen-specific IgE.

BPZE1 pretreatment reduces OVA-induced inflammatory cytokine production in BALFs

The levels of Th1 (IL-1 β , IL-2, and IFN- γ) and Th2 (IL-4, IL-5, and IL-13) cytokines, as well as IL-10, were determined in the BALFs. As expected and as reported previously (9, 12, 20, 21), OVA sensitization and challenge triggered a significant increase in IL-4, IL-5, IL-13, IL-10, IL-1 β , and IL-2 levels in the BALFs compared with saline aerosol controls (Fig. 3A,B,D–G), whereas the level of IFN- γ remained unchanged (Fig. 3C). BPZE1 pretreatment significantly reduced the production of all the cytokines tested except IFN- γ , suggesting that BPZE1 pretreatment affects the production of major Th2 and Th1 pro-inflammatory cytokines involved in the pathogenesis of allergic airway inflammation.

BPZE1 in pretreatment inhibits the progression of DNCB-induced CHS

The anti-inflammatory effect of BPZE1 was further investigated in the DNCB-induced ear swelling mouse model of CHS, a Th1-dominated allergic contact dermatitis. Mice were pretreated in. with BPZE1 either once or twice, and after complete bacterial clearance from the lungs, they were sensitized and challenged with DNCB. The protective efficacy of BPZE1

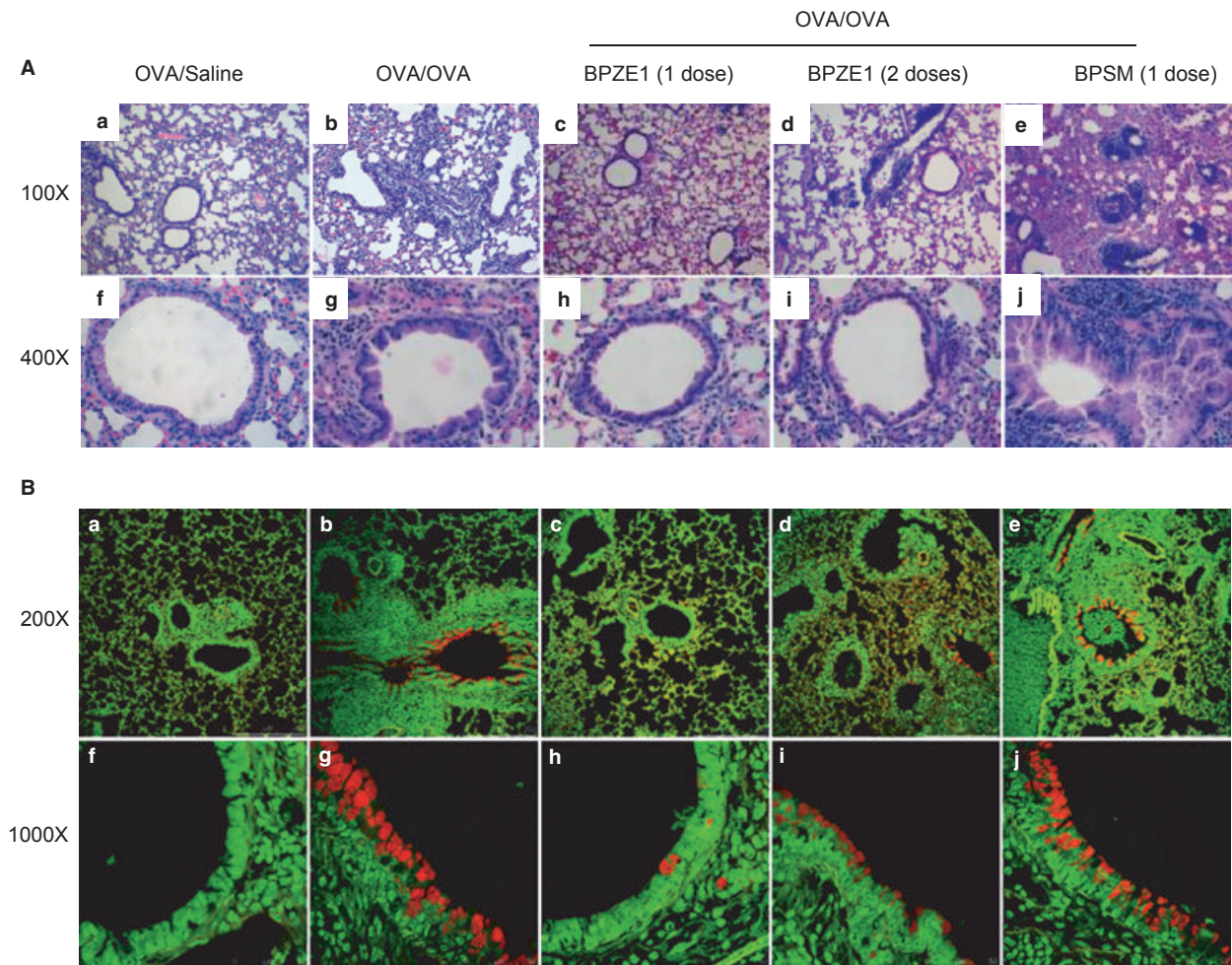


Figure 1 Effects of BPZE1 pretreatment on OVA-induced airway pathology. Airway inflammation was analyzed by hematoxylin and eosin staining (A) and periodic acid-fluorescence Schiff stain (PAFS) (B). Representative morphological changes of fixed lung sections from OVA/Saline (a and f), OVA/OVA (b and g), BPZE1 (1

dose) + OVA/OVA (c and h), BPZE1 (2 doses) + OVA/OVA (d and i), and BPSM (1 dose) + OVA/OVA (e and j) were viewed under 100 $\times$ (panel A a–e) and 400 $\times$ (panel A f–j) magnification for H&E staining, 200 $\times$ (panel B a–e) and 1000 $\times$ (panel B f–j) magnification for PAFS.

pretreatment against ear swelling was evaluated by measuring the ear thickness daily after DNCB challenge. Exposure to DNCB resulted in a marked increase in skin thickness 24 h after challenge, which was sustained for up to 4 days (Fig. 4). Unlike in the OVA-induced allergic airway inflammation model, a single dose of BPZE1 did not significantly affect the ear thickness upon DNCB challenge over the course of the experiment, whereas two doses of BPZE1 significantly inhibited ear swelling in the DNCB-challenged mice (Fig. 4A).

Histological analyses of the ear skin collected 48 h after DNCB challenge showed vascular congestion and significant swelling with characteristic tissue edema and pronounced inflammatory infiltrate in the DNCB-challenged mice (Fig. 4B a & b). Similar observations were made with mice pretreated once with BPZE1 (Fig. 4B c). However, two doses of BPZE1 markedly reduced ear swelling and inflammation, with reduced edema and cellular infiltration compared to the non-BPZE1-treated animals (Fig. 4B d).

BPZE1 pretreatment downregulates the production of pro-inflammatory cytokines induced by DNCB

Examination of the cytokine profile in the ear homogenates from the different mouse groups showed that DNCB challenge triggered a marked increase in pro-inflammatory cytokines IL-1 β , IL-2, IL-17, IL-6, TNF- α and IL-4 (Fig. 5). Consistent with the reduced inflammation and edema observed by histology (Fig. 4B), two doses of BPZE1 resulted in significantly reduced levels of all the pro-inflammatory cytokines measured (Fig. 5). In contrast, the levels of IL-10 remained unchanged for all the groups (Fig. 5).

BPZE1 pretreatment does not affect the sensitization phase

To test whether BPZE1 pretreatment affects the sensitization phase in both inflammation models, BPZE1

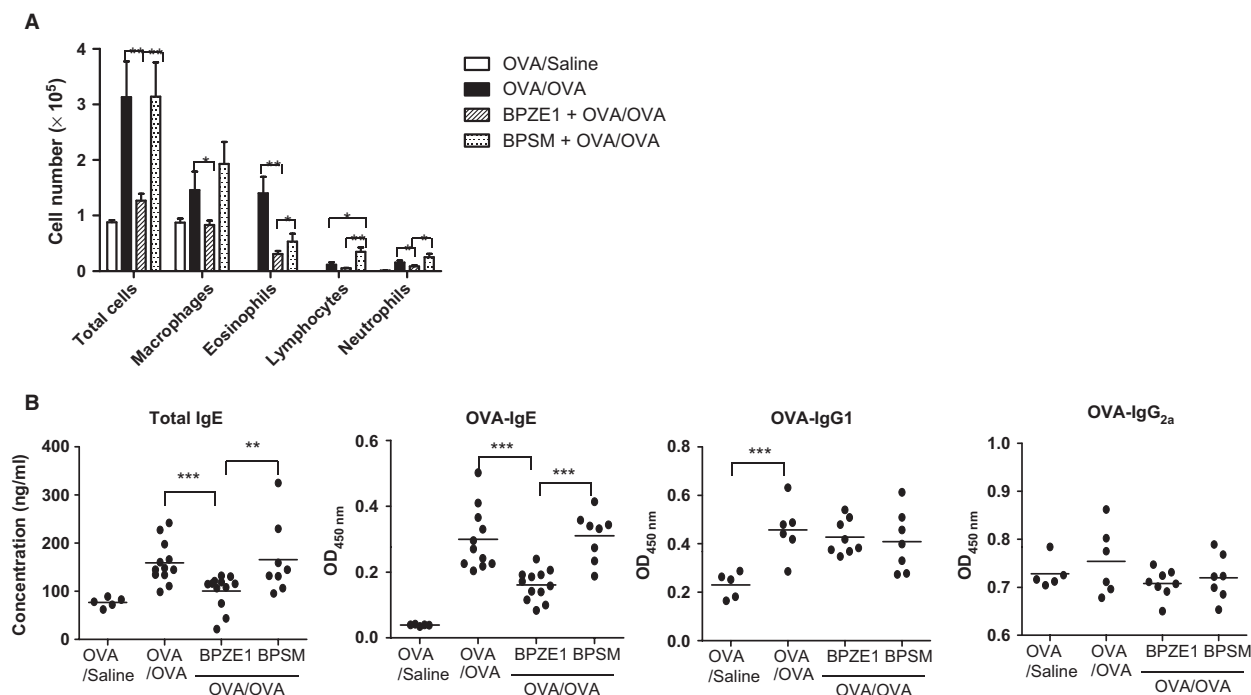


Figure 2 Effects of BPZE1 pretreatment on the cellular infiltration in bronchoalveolar lavage fluids and OVA-specific antibody responses upon OVA challenge. (A) Inflammatory cell counts in the BALFs were obtained from the different mouse groups 24 h after the last saline ($n = 6$) or OVA ($n = 8$) aerosol challenge. Differential cell counts were performed blindly on a minimum of 500 cells/slide to identify eosin-

ophils, macrophages, neutrophils, and lymphocytes. $*P \leq 0.05$, $**P \leq 0.01$. (B) Mouse serum from the different groups ($n = 5$ – 12 mice per group) was collected 24 h after the last saline or OVA aerosol challenge. The levels of total IgE, OVA-specific IgE, IgG1, and IgG_{2a} were determined by ELISA on individual sera diluted 1/5 (A–B), 1/300 000 (C), or 1/10 000 (D). $**P \leq 0.01$, $***P \leq 0.001$.

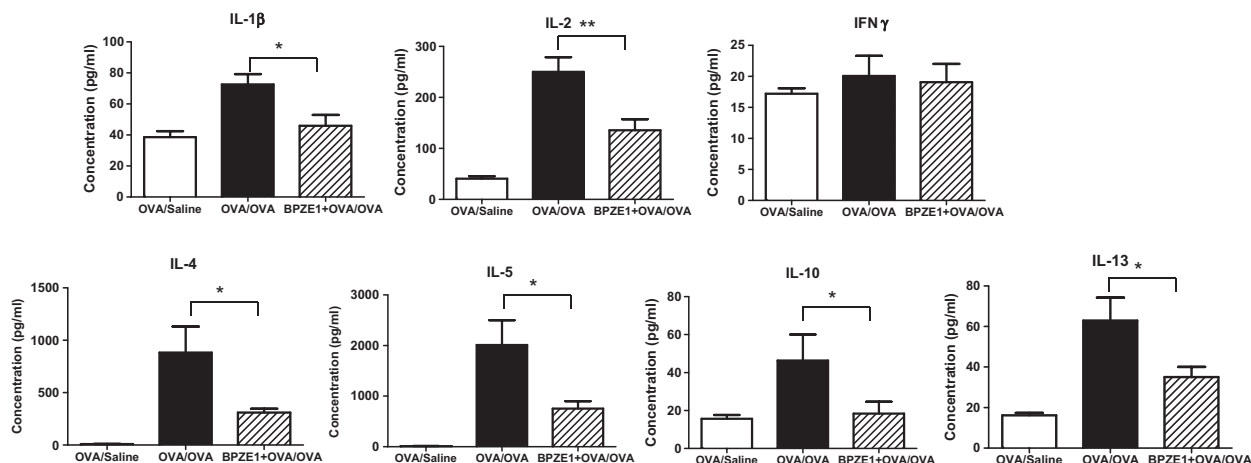


Figure 3 Effects of BPZE1 pretreatment on the local cytokine production in the OVA-induced airway inflammation model. BALFs from OVA/Saline, OVA/OVA, BPZE1 + OVA/OVA groups ($n = 6$ mice per group) were collected 24 h after the last saline or OVA

aerosol challenge. The levels of cytokines (as indicated) were determined by multiplex assay. Values shown are the mean $\pm$ SEM. $*P \leq 0.05$, $**P \leq 0.01$.

pretreated or untreated mice were subjected to OVA or DNCB sensitization. One week post-OVA sensitization, the OVA-specific antibody responses were measured and

comparable levels of OVA-specific IgE and IgG levels were observed in both BPZE1 pretreated and untreated animals (Fig. 6). Similarly, 3 days post-DNCB sensitization,

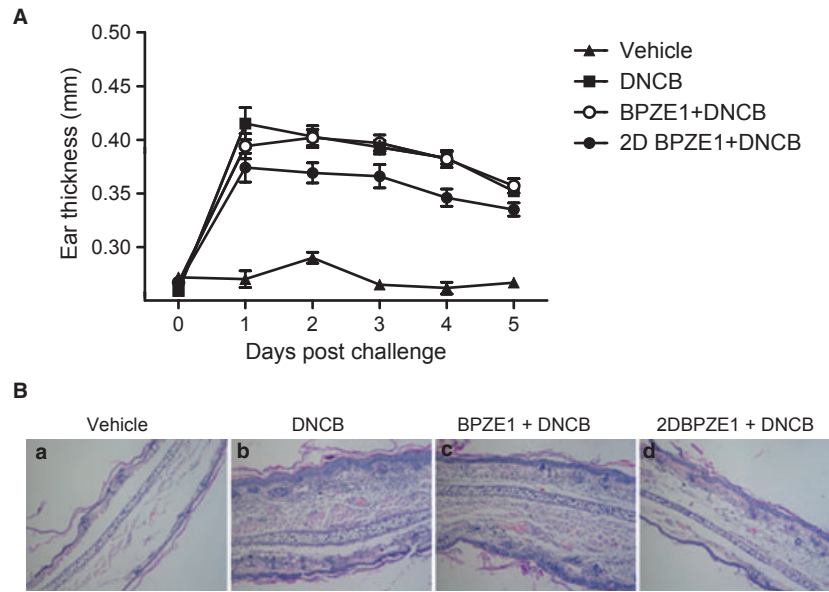


Figure 4 Effect of BPZE1 pretreatment on ear thickness (A) and histology (B) in the DNCB-induced CHS mouse model. Mice were treated with BPZE1 once or twice before DNCB sensitization and challenge as indicated in Fig. 1 (C & D). (A) Ear thickness was measured daily using a caliper ($n = 5$ mice per group). Data were

analyzed by two-way ANOVA. Significant differences were observed between BPZE1 (2 doses)-treated group and BPZE1 (1 dose)-treated group/untreated group after DNCB challenge. Values shown are the mean $\pm$ SEM. (B) H&E staining of ear sections. Observations were made at 100 $\times$ magnification.

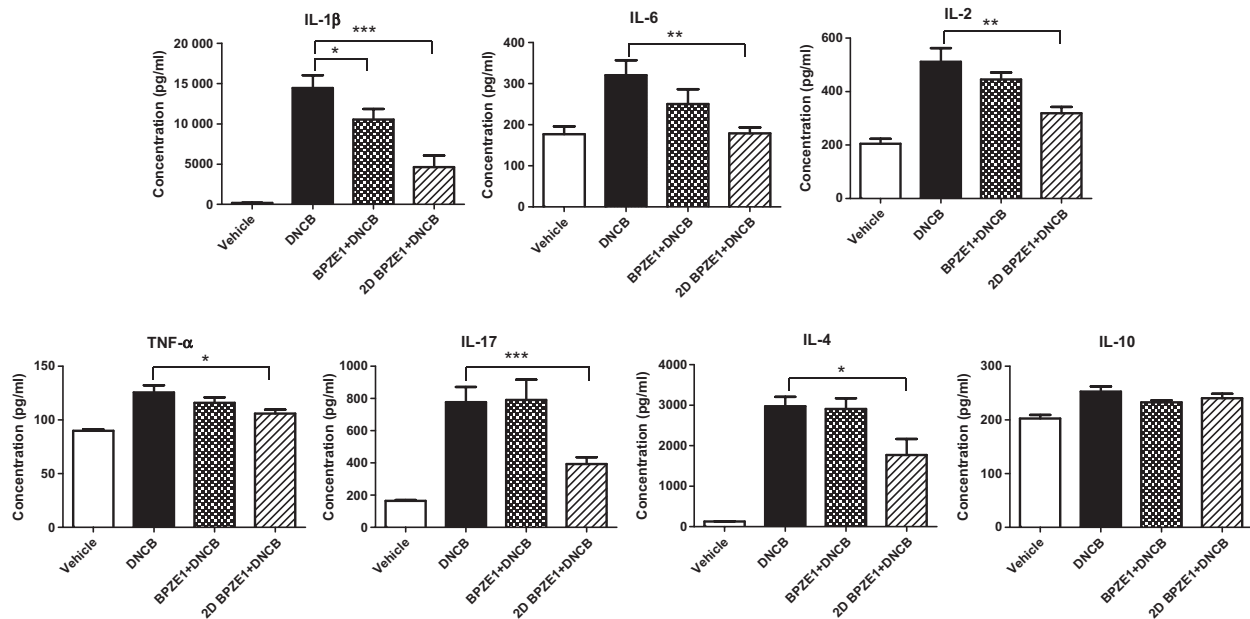


Figure 5 Effects of BPZE1 pretreatment on local cytokine production in the DNCB-induced CHS model. Two days after DNCB or vehicle challenge, ears from BPZE1 pretreated or untreated mice were collected and homogenized, and cytokines production was

determined in the individual homogenates by multiplex assay ($n = 5$ mice per group). Values shown are the mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

the auricular lymph nodes were harvested and the total cell counts, T-cell proliferation, and IFN γ production upon *in vitro* restimulation were found comparable in both BPZE1 pretreated and untreated animal groups (Fig. 6).

Therefore, these results strongly support that BPZE1 pretreatment does not affect the sensitization phase but instead impact on the effector cells that are recruited upon challenge.

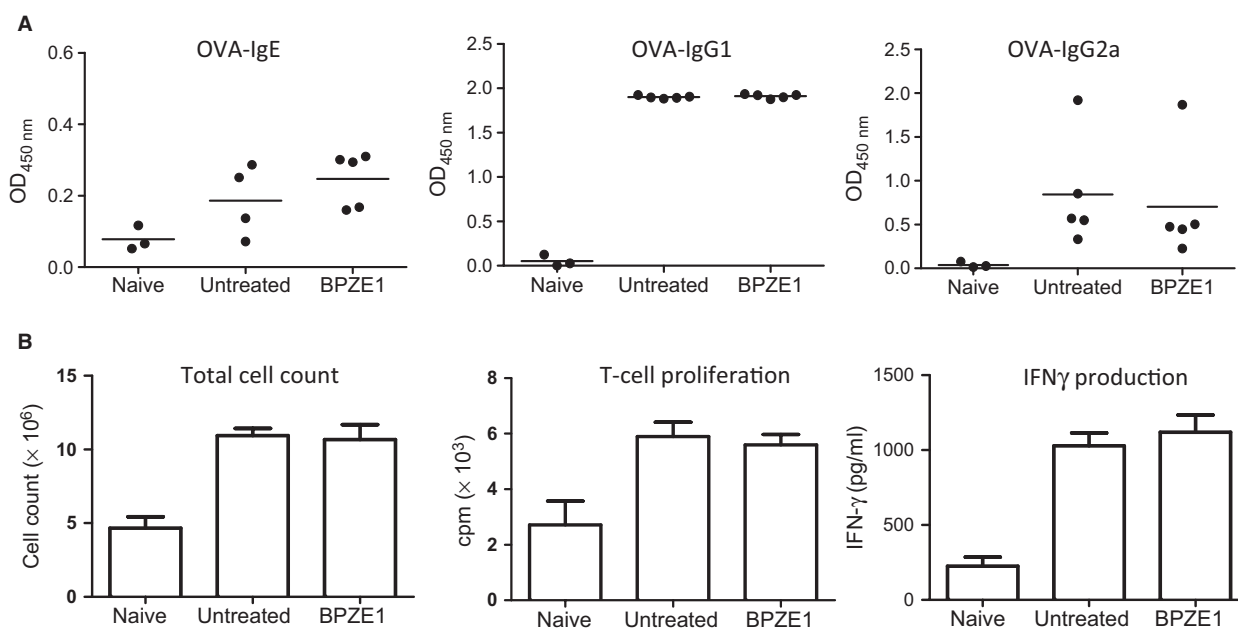


Figure 6 Effect of BPZE1 pretreatment on the sensitization phase. (A) Mice were nasally pretreated with BPZE1 (1 dose) or left untreated and OVA-sensitized. Serum from naïve ($n = 3$), untreated ($n = 4$), and BPZE1-treated ($n = 4$) groups was collected 1 week postsensitization. The levels of OVA-specific IgE, IgG1, and IgG2a were determined by ELISA on individual sera diluted 1/5 (A) or 1/

100 (B-C). (B) Mouse groups ($n = 5$) were nasally pretreated with BPZE1 (2 doses) or left untreated and subjected to DNCB sensitization. Their auricular LNs were harvested 3 days postsensitization for total cell count, T-cell proliferation upon restimulation with anti-CD3/CD28 antibodies, and IFN γ production in the culture supernatants as indicated. Each individual sample was assayed in triplicates.

Discussion

The highly attenuated *B. pertussis* strain BPZE1 was originally engineered as a live nasal pertussis vaccine against whooping cough (15, 22, 23) and has as such recently completed a phase I clinical trial in humans (<http://www.child-innovac.org>). Although highly attenuated, BPZE1 still retains the ability to colonize the respiratory tract efficiently (15).

We have recently reported that in. pretreatment with live BPZE1 protects against influenza virus-induced pneumonitis, by dampening the cytokine storm (1). Uniquely for a nonspecific anti-inflammatory agent, the protective effects manifested after the bacteria were cleared from the mouse lungs, and the protective effect could still be observed when influenza challenge was performed 12 weeks after BPZE1 treatment (1). Furthermore, a second dose of BPZE1 enhanced the protective efficacy and shortened the time necessary to trigger the protective mechanisms (1), indicating a booster effect. These observations suggested that the mechanisms responsible for the BPZE1-mediated anti-inflammatory effect are likely different from those responsible for the short-term transient protective effects conferred by a number of previously reported anti-inflammatory agents (17, 18, 24, 25).

Here, we demonstrate that prior in. treatment with BPZE1 effectively prevents allergic inflammation in two different murine allergic inflammatory disease models, namely OVA-induced airway inflammation and DNCB-induced CHS, underscoring the broad anti-inflammatory potential of

BPZE1. More importantly, this result indicates that the protective effect of in. pretreatment with BPZE1 is not restricted to the site of administration and multiplication of the bacteria, that is, the respiratory tract, but is also observed at a distant mucosa, that is, the skin.

In. pretreatment with BPZE1 effectively reduced lung inflammation, cellular infiltration, and IgE and cytokine production in the OVA-induced airway inflammation mouse model. In sharp contrast to BPZE1 treatment, preadministration of the virulent counterpart BPSM did not prevent the allergic airway inflammation reaction. These findings confirm and extend previous studies in which OVA sensitization was initiated during the bacterial colonization (9, 12), thus demonstrating here a long-term protective effect of BPZE1.

In addition, a protective effect was also found in the DNCB-induced CHS model. As protection was observed with both Th2- (OVA-induced airway inflammation) and Th1- (DNCB-induced CHS) dominated allergic models, we conclude that it is mediated by a broad anti-inflammatory mechanism through the suppression of major inflammatory cytokines involved in the pathogenesis of inflammatory diseases.

Nevertheless, a number of differences between both models can be highlighted. In particular, whereas a single dose of BPZE1 protected efficiently in the OVA-induced airway inflammation model, two doses were necessary to protect in the DNCB-induced CHS model. Likewise, two doses of BPZE1 increased the protective efficacy against influenza

viruses (1). The difference in the dosage requirement may be due to the difference in the nature of the cellular immune effectors responsible for the pathogenesis in the different disease models studied. Our observations would suggest that BPZE1 is more effective in suppressing Th2-mediated than Th1-dominated inflammatory reactions. Furthermore, interestingly, we found that BPZE1 pretreatment specifically reduced the level of cytokines involved in the inflammatory reaction but did not affect the level of other cytokines, implying that the BPZE1-induced effector mechanisms specifically target the immune effector cells that are recruited and involved in the inflammation reaction. The precise cellular and molecular mechanisms underlying the long-term broad anti-inflammatory effect of BPZE1 are yet to be deciphered. The simplistic idea that tilting the immune response toward a Th1 phenotype to suppress a Th2-mediated pathology, as proposed for the protective role of BCG vaccination against allergic inflammation, for example (26), may not apply here.

In recent years, there has been accumulating evidence indicating that a pathogen can shape the host immune response to a secondary unrelated pathogen or allergen even long after elimination of the first pathogen and in the absence of cross-reactive immunity (11, 27–29). Bystander activation of unrelated memory T cells was shown to be retained in the lungs long after resolution of respiratory viral infection (30, 31), which may modify the local cytokine balance and influence immunity and pathology to subsequent infections. Furthermore, instead of returning to its preinfection state after multiple waves of inflammation, as has been assumed for long, the innate immune system may actually undergo long-lasting

changes that influence subsequent challenges, such as the number of dendritic cells and their T-cell-priming ability (32, 33), or the sustained desensitization of lung sentinel cells to Toll-like receptor ligands (34). It is thus tempting to speculate that preexposure to BPZE1 modifies the lung environment, which would result in the suppression of inflammatory reactions induced subsequently.

In conclusion, this study provides strong support for the development of BPZE1 as a novel promising prophylactic and therapeutic agent to prevent and/or treat allergic diseases.

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Authors contributions

LR performed the experiments, analyzed the data, and wrote the article; CC, SZC, AL, and FYG performed the experiments; CL, DMK, VA, and WSWF helped interpret the data and critically read the article; SA conceptualized the experiments, analyzed the data, and wrote the article.

Conflict of interests

All the authors declare that they have no conflict of interest.

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