

Inhibition of murine allergic airway disease by *Bordetella pertussis*

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SUMMARY

Whole-cell pertussis vaccines have been shown to selectively induce T helper 1 (Th1)-type responses in human and animals. In this study, we investigated whether whole-cell *B. pertussis* could inhibit allergic airway reactions in a murine model of asthma induced by ovalbumin (OVA). Systemic administration of whole-cell *B. pertussis* strongly inhibited allergic airway reactions such as eosinophil recruitment into the airway, lung inflammation, and airway hyperresponsiveness to methacholine. The inhibitory effect of whole-cell *B. pertussis* was mediated by chromosomal DNA and pretreatment of DNA with CpG methylase or DNase I resulted in a loss of the inhibitory effect. Treatment of animals with *B. pertussis* DNA significantly decreased the Th2 cytokine (interleukins IL-4 and IL-5) concentrations in the airways without increasing Th1 cytokines. These results suggest that *B. pertussis* DNA containing unmethylated CpG appears to be responsible for the inhibitory effect of whole cell *B. pertussis* on the allergic airway reactions through inhibition of the Th2 response.

Keywords Allergy, animal models, Th cell response, vaccines

INTRODUCTION

Asthma is an inflammatory disease characterized by bronchial hyperresponsiveness that can proceed to life-threatening airway obstruction. The T helper 2 (Th2)-type cytokines interleukins (IL)-4, IL-5, and IL-13, produced by activated CD4⁺ T cells play a central role in the pathogenesis of asthma^{1,2} by controlling the key process of immunoglobulin E (IgE) production, growth of mast cells and the differentiation and activation of mast cells and eosinophils.^{3–5} In contrast, Th1 cytokines such as interferon- γ (IFN- γ) and IL-12, which down-regulate Th2 responses, inhibit the development of allergic lung inflammation.^{6,7} Thus, interventions that inhibit Th2 cytokines by enhancing Th1 cytokine production, may be useful in the

treatment of allergic asthma. CpG-oligodeoxynucleotide (ODN) is a potent adjuvant of Th1-like responses characterized by production of IL-12 and IFN- γ .^{8,9} Consequently, immunomodulatory protocols employing CpG-ODN have recently been applied to murine models of allergic airway diseases.^{10–12}

Bordetella pertussis is a pathogenic organism that causes whooping cough. Widespread use of standardized vaccine has resulted in a dramatic decline in the incidence of the disease. Recently, increasing evidence suggests that whole-cell pertussis vaccines, similar to natural infection with *B. pertussis*, selectively induce Th1-type responses in infants^{13,14} and mice.^{15,16} This suggests that whole-cell pertussis vaccines or natural infection with *B. pertussis* might be associated with decreased incidence of allergic asthma. However, little experimental evidence exists to support this view.

In this study, we investigated whether administration of heat-killed *B. pertussis* could inhibit murine allergic airway reactions induced by with ovalbumin (OVA). We found that *B. pertussis* profoundly inhibited allergic airway reactions in the preventive and therapeutic manners and the inhibitory effect of *B. pertussis* appeared to be mediated by chromosomal DNA containing unmethylated CpG motifs.

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Abbreviations: ODN, oligodeoxynucleotides; OVA, ovalbumin; BAL, bronchoalveolar lavage; AHR, airway hyperresponsiveness; Mch, methacholine.

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MATERIALS AND METHODS

Animals

Male C57BL/6 mice were obtained from the Korean Institute of Chemistry Technology (Daejeon, Korea) and were kept in our animal facility for at least 2 weeks before use. All mice were at 8–10 weeks of age.

Reagents and antibodies

Chicken egg albumin (OVA), pertussis toxin (PT), lipopolysaccharide (LPS) and filamentous haemagglutinin (FHA) were purchased from Sigma (St. Louis, MO) and aluminium hydroxide adjuvant (Imject^R Alum) was from Pierce (Rockford, IL). CpG methylase and DNase I were purchased from New England BioLabs (Beverly, MA) and *B. pertussis* strain (ATCC 10380) was provided by the National Institute of Health (Seoul, Korea). *B. pertussis* was grown on Bordet-Gengou agar (BD Bioscience, Sparks, MD) containing 15% sheep blood at 37°. *B. pertussis* was inactivated by incubation of cells at 80° for 30 min.¹⁷ The heat-killed *B. pertussis* (2×10^9) were moderately sonicated in phosphate-buffered saline (PBS) and centrifugated for 20 min at 15000 g to separate the cytoplasmic and nuclear fractions.

Purification of *B. pertussis* DNA

The chromosomal DNA was purified by using genomic DNA extraction kit for gram-negative bacteria (iNtRON Biotech, Kyungki-Do, South Korea) according to the manufacturer's instruction. Briefly, cells were harvested in PBS on ice and lysated with G buffer containing 200 µg/ml of RNase and 40 µg/ml of proteinase K by incubation at 65° for 15 min. After addition of binding buffer, cell lysates were loaded on a G-spin column and washed with washing buffer. *B. pertussis* DNA was obtained by passing elution buffer through column and used for experiments. The endotoxin level in *B. pertussis* DNA contained less than 0.05 units/ml when tested by a *Limulus amoebocyte* lysate assay kit (E-Toxate^R kit from Sigma). The treatment of *B. pertussis* DNA with M.SssI methylase (New England Biolabs, Beverly, MA) or DNase I (Roche Diagnostics, Mannheim, Germany) was performed at 37° for 2 or 16 hr, respectively, according to the manufacturer's instructions.

Immunization and challenge

Mice were immunized intraperitoneally (i.p.) with 20 µg of OVA plus 1.0 mg aluminium hydroxide adjuvant on days 0 and 14. Mice were challenged via the airway with OVA (50 mg/ml of saline) each day from days 21–23 on consecutive days. Control mice were exposed to aerosolized saline. Aerosolization was performed for 20 min by placing the mice in a chamber (15 × 25 × 15 cm) connected to the ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan). The heat-killed whole-cell *B. pertussis* was administered i.p. on day 0 (the day of 1st immunization with OVA), 14 (the day of 2nd immunization) or 20 (one day before the first airway challenge), and the cellular components or purified *B. pertussis* DNA were injected i.p. on day 20.

Cytokine assays

The levels of cytokines in bronchoalveolar lavage (BAL) fluid were determined by enzyme-linked immunosorbent assay (ELISA). ELISA kits from R & D Systems (Minneapolis, MN) were employed for the measurement of IL-4 and IL-5, and a kit from Endogen (Woburn, MA) was employed for the measurement of IL-12 and IFN-γ.

Determination of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was measured at 3 days after the last OVA challenge in mice in an unrestrained conscious state, according to the method of Hamelmann *et al.*¹⁸ Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine (Mch) in increasing concentrations (from 2.5 to 50 mg/ml) was then nebulized through an inlet of the main chamber for 3 min, readings were taken and were averaged for 3 min after each nebulization. The bronchopulmonary resistances is expressed as enhanced pause (Penh), calculated as: $[\text{expiratory time } T_e] / [\text{relaxation time } (RT) - 1] \times [\text{peak expiratory flow } (PEF) / \text{peak inspiratory flow } (PIF)]$, according to the manufacturer's protocol. Results are expressed as the percentage increase of Penh following challenge with each concentration of Mch, where the baseline Penh (after saline challenge) is expressed as 100%.

Histological studies

The lungs were removed 60 hr after the last challenge and fixed in 10% formalin, embedded in paraffin, sectioned, and stained with haematoxylin and eosin for examination by light microscope.

Bronchoalveolar lavage

Mice were anaesthetized and the trachea was cannulated while gently massaging the thorax. Lungs were lavaged with 0.7 ml of PBS. The BAL fluid samples collected and the number of cells in a 50 µl aliquot were determined using a haemocytometer. The remaining sample was centrifuged, and the supernatant was stored at -70° until assay of cytokines. The pellet was resuspended in PBS and cytospin preparations of BAL cells were stained with Diff-Quik (International reagents Corp., Kobe, Japan). The different cell types were enumerated based on morphology and staining profile.

RESULTS

Whole-cell *B. pertussis* inhibits airway eosinophilia and lung inflammation

Two days after the last airway OVA challenge, the number of total cells and eosinophils in the BAL fluid increased 36- and 46-fold, respectively, compared to those of saline-challenged group (Fig. 1a). Administration of varying doses of heat-killed whole-cell *B. pertussis* on day 0 (the day of 1st immunization with OVA) significantly reduced airway infiltration of cells in a dose dependent manner. At 2×10^{10} *B. pertussis*/mouse, a significant reduction in the number of

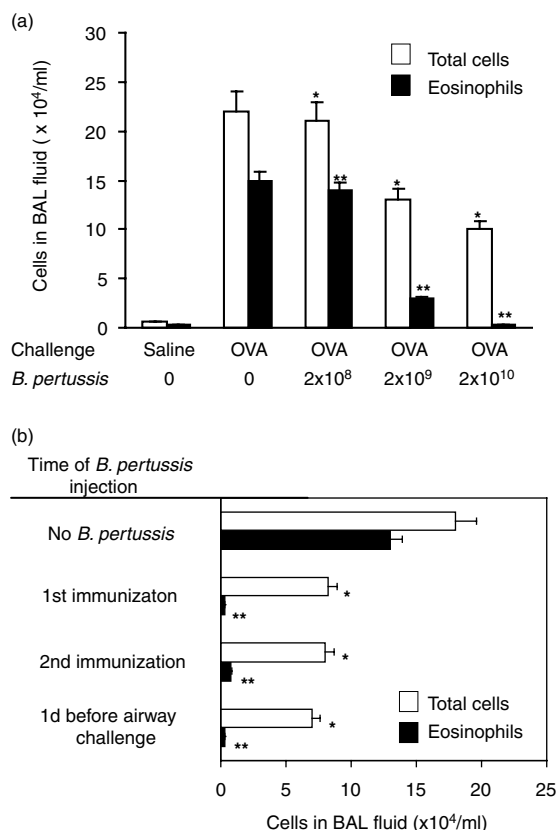


Figure 1. Whole-cell *B. pertussis* inhibits airway eosinophilia. Mice were immunized i.p. with 20 μg of OVA at days 0 and 14 and challenged via the airway with either OVA (50 mg/ml of saline) or saline on days 21 through 23. (a) Varying doses of heat-killed *B. pertussis* were administered i.p. 1 day before the first airway challenge. (b) heat-killed *B. pertussis* (2×10^{10} cells) was administered at the time indicated. BAL cells were collected 2 days after the last airway challenge. Results for all panels are expressed as the mean $\pm$ SEM of three separate experiments ($n = 4\text{--}6$ for each time point). * $P < 0.05$; ** $P < 0.01$ versus control; Mann-Whitney *U*-test.

total cells and eosinophils (Fig. 1a) was observed. Administration of *B. pertussis* on day 14 (the day of 2nd immunization) or 20 (1 day before the first airway challenge) also inhibited recruitment of inflammatory cells into the airway to a similar degree (Fig. 1b). Results of histological examination of lung tissues paralleled the cell numbers in BAL fluid. A marked influx of inflammatory cells into the airway and around blood vessels and airway luminal narrowing by secreted mucus were observed in the OVA-treated group (Fig. 2). Administration of *B. pertussis* resulted in a marked inhibition of the cellular infiltration of the surrounding airways and pulmonary blood vessels as well as improvement of luminal narrowing in the airway (Fig. 2).

Whole-cell *B. pertussis* inhibits allergen-induced airway hyperresponsiveness (AHR) to methacholine

AHR was assessed as a percent increase of Penh in response to increasing doses of Mch. In mice, the OVA-inhalation

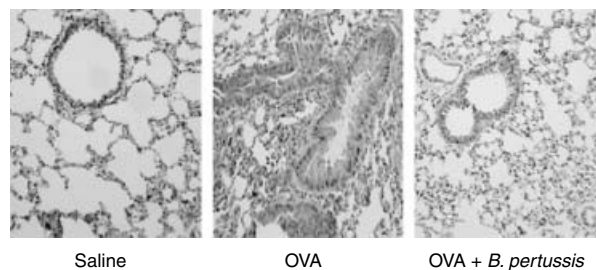


Figure 2. Whole-cell *B. pertussis* inhibits lung inflammation. Mice were sensitized and challenged as described in Fig. 1. Sections were obtained from the lungs of mice receiving saline, an OVA-challenge, and an OVA-challenge and heat-killed *B. pertussis* (2×10^{10} cells) 1 day before the first airway challenge. Lungs were removed 2 days after the last airway challenge. Sections were stained by haematoxylin and eosin staining ($\times 100$).

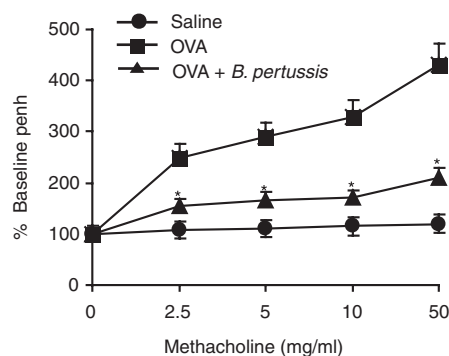


Figure 3. Whole-cell *B. pertussis* inhibits AHR to aerosolized methacholine. Mice were sensitized and challenged as described in Fig. 1. Heat-killed *B. pertussis* (2×10^{10} cells) were administered i.p. 1 day before the first airway challenge. Airway responsiveness was measured 2 days after the last airway challenge. Results for all panels are expressed as the mean $\pm$ SEM of three separate experiments ($n = 3$ for each time point). * $P < 0.05$ versus asthma control; Mann-Whitney *U*-test.

dose-response curve of percent Penh was shifted to the left compared with saline-treated mice (Fig. 3). In addition, the percent of Penh produced following Mch (from 2.5 mg/ml to 50 mg/ml) administration increased significantly in the OVA-inhalation mice compared with the saline-treated. In response to increasing doses of Mch, *B. pertussis*-treated mice showed a dose-response curve in the percent of Penh shifted to the right in a dose-dependent manner compared with OVA-treated mice (Fig. 3).

B. pertussis DNA is responsible for the inhibiting the asthmatic reactions

It is well known that bacterial DNA containing the unmethylated CpG dinucleotides (CpG motif) inhibits inflammatory processes associated with asthma.^{10–12} Therefore, we prepared the nuclear fraction to examine whether the DNA has such an inhibitory activity, and then

compared the nuclear fraction's activity with the cytoplasmic fraction's activity. The nuclear, but not cytoplasmic, fraction, inhibited the airway eosinophilia (Fig. 4a). We next purified chromosomal DNA of *B. pertussis*, as described in Materials and Methods, and 8.9 μg of DNA

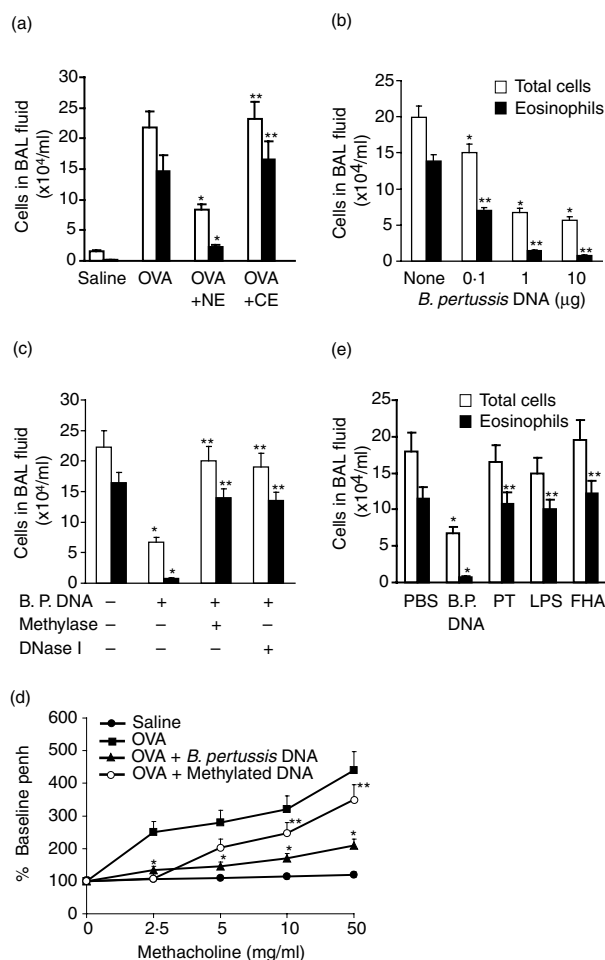


Figure 4. *B. pertussis* DNA inhibits the allergic airway inflammatory processes. Mice were sensitized and challenged as described in Fig. 1. (a) The nuclear fractions or cytoplasmic fractions from heat-killed *B. pertussis* (2×10^9 cells) and (b) Varying doses of *B. pertussis* DNA were administered i.p. 1 day before the first airway challenge. BAL cells were collected 2 days after the last airway challenge. * $P < 0.05$; ** $P < 0.01$ versus control. (c) CpG methylase prevents the inhibitory ability of *B. pertussis* DNA on the allergic airway reactions. Ten μg of DNA was mixed with 10 U of CpG methylase for 16 hr or DNase I for 2 hr at 37°. One μg of *B. pertussis* DNA were administered i.p. 1 day before the first airway challenge. (d) Airway responsiveness was measured 2 days after the last airway challenge. * $P < 0.01$ versus control ** $P < 0.05$ versus *B. pertussis* DNA-treated group. (e) PT, LPS, and FHA were inactivated as described in the Materials and Methods, and 1 μg of cellular components were administered i.p. 1 day before the first airway challenge. * $P < 0.01$; ** $P < 0.05$ versus control. Results for all panels are expressed as the mean $\pm$ SEM of three to five separate experiments ($n = 4$) and statistical significance was assessed by Mann-Whitney *U*-test.

was obtained from 2×10^{10} cells. The recovery rate of DNA by extraction kit was approximately 90%. We thus used chromosomal DNA in the range of 0.1–10 μg /mouse. Administration of chromosomal DNA of *B. pertussis* (at a concentration as low as 1 μg) inhibited airway eosinophilia and AHR by more than 80–85% (Fig. 4b). To analyse whether the inhibition induced by chromosomal DNA was mediated by unmethylated CpG motif, the chromosomal DNA was treated with CpG methylase or DNase I as control. Pretreatment with either CpG methylase or DNase I abrogated the ability of *B. pertussis* chromosomal DNA to prevent the influx of inflammatory cells into the airway (Fig. 4c) and AHR (Fig. 4d). These results suggest that the unmethylated CpG motif in *B. pertussis* DNA is responsible for the inhibitory effect of the bacteria on the airway allergic reactions. We next examined the influence of other cellular components rather than DNA in the inhibition of airway eosinophilia. These cellular components, such as PT, LPS, and FHA, failed to attenuate the airway eosinophilia (Fig. 4e). These results suggest that *B. pertussis* DNA may be a major component responsible for whole *B. pertussis*-induced suppression of allergic airway reactions.

B. pertussis DNA down-regulates Th2 responses in the airways

To determine if pretreatments with *B. pertussis* DNA could affect cytokine secretion in the BAL fluid, ELISA for Th1 cytokines (IL-12 and IFN- γ) and Th2 cytokines (IL-4 and IL-5) was performed. Cytokine levels of BAL fluid were measured 12 hr after the last airway challenge. Sensitization and challenge with OVA increased Th2 cytokine levels in BAL fluid, but Th1 cytokines were not detected (Fig. 5). Administration of *B. pertussis* DNA significantly reduced the concentration of IL-4 and IL-5 by 60–70%. However, *B. pertussis* DNA failed to increase the levels of Th1 cytokines (Fig. 5). These findings

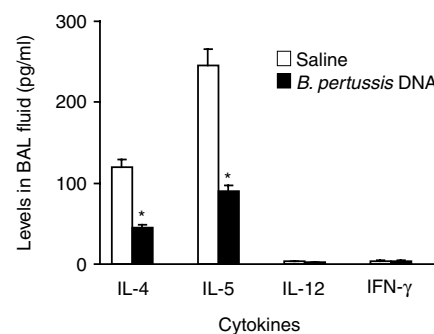


Figure 5. Assessment of Th1 and Th2 responses in the airway by measuring cytokine levels in BAL fluid. BAL fluid was collected 12 hr after the last airway challenge and analyzed for IL-4, IL-5, IL-12, and IFN- γ contents by ELISA. *B. pertussis* DNA was administered as described in Fig. 4(b). Results for all panels are expressed as the mean $\pm$ SEM of three separate experiments ($n = 4$ for each time point). * $P < 0.05$ versus control; Mann-Whitney *U*-test.

suggest that *B. pertussis* DNA could inhibit allergic airway reactions through inhibiting the recruitment of Th2 cells into the airway rather than enhancement of a Th1 response.

DISCUSSION

This study is the first to provide experimental evidence demonstrating that heat-killed *B. pertussis* inhibits OVA-induced allergic responses in a murine model of asthma. The heat-killed *B. pertussis* profoundly inhibited the allergic reactions such as eosinophil recruitment into the airway, lung inflammation, and airway hyperresponsiveness to methacholine.

Bacterial, but not vertebrate, DNA contains unmethylated CpG dinucleotides in particular base contexts, which activates macrophages and dendritic cells to secrete IL-12 and IFN- γ -secreting Th1 cells.^{8,9} Consequently, immunomodulatory protocols employing CpG-ODN have recently been applied to murine models of allergic airway diseases. It has been reported that CpG-ODN (treatment at the time of antigen sensitization or before antigen challenge) has a prophylactic effect on antigen-induced airway eosinophilia and AHR by inducing a Th1 response.^{10–12} In this study, the findings that loss of the inhibitory effect of *B. pertussis* DNA on the allergic airway reactions by pretreatment of DNA with CpG methylase suggest that the unmethylated CpG motif in *B. pertussis* DNA appeared to be responsible for the inhibitory effect of the bacteria on the allergic airway reactions through Toll-like receptor (TLR)-9 pathway, as reported in the studies using bacterial DNA.¹⁹

Many investigators studying CpG ODN effects have reported that it inhibits Th2 responses and augments Th1 responses. In this study, we have observed that administration of *B. pertussis* DNA results in the down-regulation of the Th2 cytokines, IL-4 and IL-5, in BAL fluid, while we could not observe the significant increase in the levels of Th1 cytokines, IL-12 or IFN- γ , in BAL fluids by *B. pertussis* DNA pretreatment. It is not certain that *B. pertussis* DNA inhibited Th2 response without augmenting Th1 response. It may be because of the facts that: (i) *B. pertussis* DNA contains larger amount of K-type CpG-ODN which elicits weak IFN- γ production than type B which elicits strong Th1 cytokine production;²⁰ (ii) although Th1 cytokine were not detected in BAL fluid by ELISA, even the undetectable small amount of Th1 cytokines can exert their biological activities; or (iii) CpG-ODN can directly down-regulate Th2 responses, suggested by other investigators.^{12,21} Further studies are required to address the question.

As asthma and allergic diseases are disorders with a predominant Th2 immune response, tilting the immune response towards a Th1 phenotype or by suppressing the Th2 immune response by infection with intracellular pathogens^{22,23} may reduce the development of atopic allergy. Recent studies have suggested an inverse relationship between allergic disorders and infections with intracellular pathogens including tuberculosis, measles, and hepatitis

A.^{24,25} Studies in animal models support the view regarding a protective effect of BCG vaccination early in life. Administration of BCG vaccination to Th2-susceptible newborn mice blocked allergic inflammation.²⁶ In a similar study, Tukenmez and coworkers have shown that pre-immunization with *Mycobacterium bovis* and *M. vaccae* in newborn ovalbumin-sensitized mice inhibits IgE production in response to aerosolized allergen.²⁷

Recently, a dichotomy between immunostimulatory sequence (ISS or CpG motifs)-ODN-based immunomodulation and vaccination has been reported.²⁸ The immunomodulation of ISS-ODN rapidly inhibits the effector arm of the allergic reactions in an allergen-independent time-limited manner (4–6 weeks), whereas adaptive immune response to ISS-ODN-based vaccines develop slowly, are allergen specific, and are long lived (1 year). Based on the findings, our data showing an inhibition of asthmatic reactions by administration of *B. pertussis* not only simultaneously with immunization but also 1 day prior to the OVA challenge appears to be achieved by allergen-dependent vaccine activity and allergen-independent immunomodulatory activity of *B. pertussis*, respectively.

Pathogen recognition receptors, including the TLRs, allow the innate immune system to detect conserved patterns of molecules on pathogens and to respond in the first line of defence against infection by producing inflammatory cytokines.²⁹ Binding of pathogens to TLRs on DCs results in their maturation, characterized by up-regulation of MHC class II, CD80, CD86, and CD40, but also activation of proinflammatory cytokine production, including TNF- α and IL-12. The mature DCs migrate from the tissue to the lymph nodes, where they present antigen to naive T cells. CpG motifs in bacterial DNA bind TLR9 and stimulate IL-12 production by innate cells, such as DCs, direct the induction of Th1 cells.³⁰ Thus, it is likely that the pathway involving TLR9 plays a role in

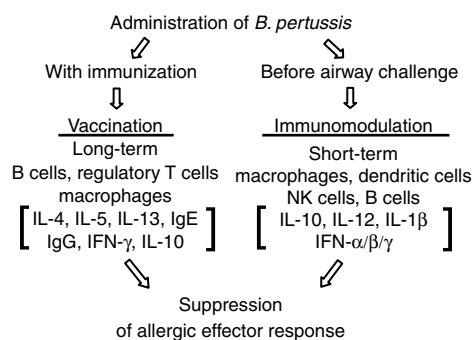


Figure 6. Hypothetical model of *B. pertussis*-based vaccination and immunomodulation in the inhibition of allergic response. Adaptive immune response to *B. pertussis*-based vaccines develop slowly, are allergen specific, and are long lived (1 year), whereas *B. pertussis*-based immunomodulation rapidly stimulate innate response, leading to the production of antiallergic and Th1-promoting cytokines. These two types of the inhibitory mechanisms were originally postulated by Horner *et al.*²⁸

B. pertussis DNA-mediated inhibition of allergic airway reactions in this study.

Based on the dichotomy, we proposed a hypothetical scheme explaining *B. pertussis*-induced inhibition of allergic airway reactions in Fig. 6. Given the results from the present study demonstrating that *B. pertussis* profoundly inhibits allergic airway reactions in a murine model supports the view that *B. pertussis* infection may result in the induction of a predominant Th1 immune response. This response could reduce the development of asthma later in life. This study also provides evidence that *B. pertussis* may offer a new therapeutic approach to allergic airway diseases.

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