

Whole-cell pertussis vaccine protects against *Bordetella pertussis* exacerbation of allergic asthma

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Received 23 August 2004; received in revised form 1 October 2004; accepted 4 October 2004

Abstract

The prevalence of asthma and allergic disease has increased in many countries and there has been speculation that immunization promotes allergic sensitization. *Bordetella pertussis* infection exacerbates allergic asthmatic responses. We investigated whether whole-cell pertussis vaccine (Pw) enhanced or prevented *B. pertussis* induced exacerbation of allergic asthma. Groups of mice were immunized with Pw, infected with *B. pertussis* and/or sensitized to ovalbumin. Immunological, pathological and physiological changes were measured to assess the impact of Pw immunization on immune deviation and airway function. Pw immunization modulated ovalbumin-specific serum IgE production, and reduced local and systemic IL-13 and other cytokine responses to sensitizing allergen. Histopathological examination revealed Pw immunization reduced the severity of airway pathology and decreased bronchial hyperreactivity to methacholine exposure. Pw does not enhance airway IL-13 and consequently does not enhance but protects against the exacerbation of allergic responses. We find no evidence of Pw contributing to allergic asthma, but rather provide evidence of a mechanism whereby whole-cell pertussis vaccination has a protective role.

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Keywords: *Bordetella pertussis*; Vaccine; Asthma; IL-13; Allergen

1. Introduction

Asthma is a chronic disease of the respiratory tract of increasing prevalence in developed societies [1]. The current understanding of allergic asthma is that it results from a breakdown in the normal tolerance to inhaled antigens, associated with Th2 cytokine production [2,3]. The inflammatory response in asthma is tightly associated with airway hyperresponsiveness, increased mucus production and an infiltration of the bronchial mucosa with CD4+ T-cells [4]. There is evidence of an altered local T-cell response in favour of Th2 cytokine release (IL-4, IL-5 and IL-13) resulting in B-cell isotype switching to IgE, recruitment of eosinophils, basophils and mast cells and production of inflammatory mediators [5].

The murine OVA model of airway hyperresponsiveness exhibits many of the features of human asthma, including airway hyperreactivity, inflammation and increased serum IgE levels [6,7]. Th2 cells secreting IL-4, IL-5, and IL-13 play a central role in initiating and sustaining the asthmatic response in this model [8]. While Th2 cells promote airway inflammation in asthma, it has been proposed that Th1 cells protect against allergic disease by antagonizing Th2 activity. Infectious diseases that induce Th1 type responses, might hamper the development of allergen-specific Th2 cells and prevent allergy [9].

Epidemiological and clinical studies have suggested a link between the relative absence of infectious diseases and the increase in allergic disorders [10,11]; this is referred to as the 'hygiene hypothesis'. It predicts that infections prevent the induction of allergen-specific Th2 cells through antagonism or the induction of regulatory T-cells, particularly during neona-

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tal and early childhood development [10,12]. However, there are data that confound this interpretation, increased IFN- γ is seen in asthmatic patients compared with normal subjects [3,13]. Allergen-specific Th1 cells also fail to counteract airway hyperresponsiveness in murine models [14]. Furthermore, several studies have suggested that viral/bacterial infections do not protect but exacerbate disease. Respiratory syncytial virus, commonly associated with lower lung infections in infancy, is known to exacerbate asthma [15,16], as does *Bordetella pertussis* [17]. Consequently, competing interpretations for the pathogenesis of asthma have been proposed [18,19].

B. pertussis is a Gram-negative bacterium and the causative agent of pertussis or “whooping cough”, a respiratory disease that remains a significant cause of morbidity and mortality in infants worldwide. It is a highly contagious disease, and can occur at any age, though severe illness is more common in young un-immunized children. *B. pertussis* infection induces Th1 responses [20,21] and can be modelled by respiratory challenge of mice, which correlates well to responses in humans [22].

There has been speculation about the possible promotion of allergy by common childhood vaccinations [23,24]. A substantial proportion of children predisposed to allergy and asthma may not be fully immunized because of public apprehension surrounding immunization [25]. A number of studies have analysed the prevalence of allergic sensitization and atopic disease in relation to immunization [24,26]. Gruber et al. found that children with higher immunization coverage seemed to acquire transient protection against development of atopy in the first years of life [26]. In contrast, Hurwitz and Morgenstern suggested that diphtheria/pertussis/tetanus (DTP) immunization appeared to be associated with an increased risk of subsequent asthma or other allergies [24].

Two different types of pertussis vaccine have been employed in infant immunization programmes. The whole-cell pertussis vaccine (Pw) consists of heat/formalin inactivated virulent whole bacteria whereas the pertussis acellular vaccine (Pa) is composed of purified components of the bacteria (Pa), typically including inactivated pertussis toxin. Pw immunization has a high efficacy and is associated with the induction of antigen-specific Th1 cells [21,27,28], but has been associated with reactogenicity. In contrast Pa immunization induces a mixed Th1/Th2 response in children and in murine models, but has reduced reactogenicity [29]. It has been suggested that promotion of allergy may occur directly, by administering potentially pro-allergic vaccines, or indirectly, by hindering the Th1-promoting effect of infectious agents. Pertussis vaccination acts as an adjuvant for antigen-specific responses in laboratory animals [30]; active pertussis toxin, is known to enhance immunoglobulin E (IgE) formation in animal models [31] and has been linked with a shift toward Th2-like cytokines in humans [32,33].

Infection with *B. pertussis* modulates allergen priming and the severity of airway pathology in a murine model of allergic asthma [17] and we have previously shown that Pw

immunization induces a similar immune response to infection [34] and that although variables such as route, dose and timing influence T-cell responses in animal models, Pw is a consistent inducer of Th1 responses [20]. In order to test whether immunization with Pw exacerbated asthma, we employed a well-characterized murine model of whole-cell pertussis vaccination and *B. pertussis* infection in combination with the murine OVA model of airway hyperresponsiveness. We show that although Pw induces a Th1 type immune response to *B. pertussis* infection, it does not exacerbate pathology in a model of allergic asthma. Our findings demonstrate that Pw immunization prevents *B. pertussis* enhancement of OVA-induced IL-10 and IL-13, which results in a subsequent decrease in airway hyperresponsiveness and pathology. This study finds no evidence of a mechanism to support speculation linking Pw immunization and asthma.

2. Materials and methods

2.1. Animals and experimental approach

6- to 8-week-old female BALB/c (Harlan, UK) mice were used under the guidelines of the Irish Department of Health and the research ethics committee of the National University of Ireland Maynooth. The experimental approach is outlined in Table 1, briefly groups of mice were immunized with whole-cell pertussis vaccine (Pw), infected with *B. pertussis*, and then sensitised to ovalbumin (OVA) at the peak of infection as detailed below. Control mice received similar treatment in which 0.9% (w/v) (aq) NaCl (hereafter termed Saline) replaced experimental treatment.

2.2. Immunization, sensitization and airway delivery of OVA

Four groups of at least thirty-five 6–8-week-old female BALB/c mice (Pw, PwBp, PwOVA and PwBpOVA) were immunized i.p. with 0.16 I.U. of whole-cell pertussis vaccine (Pw) (Third International Standard, 1998, pertussis whole-cell vaccine, NIBSC, UK), equivalent to 1/25th of the human dose according to the schedule outlined in Table 1. At 0 day mice were infected with *B. pertussis*, selected groups were then sensitized with ovalbumin (OVA). Sensitization involved 100 μ g OVA (Grade V; Sigma, Dorset, UK) emulsified in Alhydrogel[®] adjuvant (Superfos Biosector, Sweden) (1 mg/mouse aluminium hydroxide) administered as 0.2 ml i.p. at 10 and 24 days. Control group (Ctrl) received saline alone (i.p.). On 35, 36, and 37 days, PwOVA and PwBpOVA sensitized mice received 10 μ l containing 50 μ g OVA intra-nasally (i.n.) whereas remaining groups received saline only (Table 1). All experiments were repeated at least twice.

Table 1
Experimental design

	Time (days)					
	–42	–14	0	10	24	35/36/37
Group^a						
Pw	Pw	Pw	Saline aerosol	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
Pw and <i>B. pertussis</i> infection (PwBp)	Pw	Pw	<i>B. pertussis</i> aerosol infection	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
Pw and OVA sensitization (PwOVA)	Pw	Pw	Saline aerosol	OVA (i.p.)	OVA (i.p. and i.n.)	OVA (i.n.)
Pw, <i>B. pertussis</i> infection and OVA sensitization (PwBpOVA)	Pw	Pw	<i>B. pertussis</i> aerosol infection	OVA (i.p.)	OVA (i.p. and i.n.)	OVA
Group^b						
Control (Ctrl)	Saline (i.p.)	Saline (i.p.)	Saline aerosol	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
<i>B. pertussis</i> infection (Bp)	Saline (i.p.)	Saline (i.p.)	<i>B. pertussis</i> aerosol infection	Saline(i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
OVA sensitization (OVA)	Saline (i.p.)	Saline (i.p.)	Saline aerosol	OVA (i.p.)	OVA (i.p. and i.n.)	OVA (i.n.)
<i>B. pertussis</i> infection and OVA sensitization (BpOVA)	Saline (i.p.)	Saline (i.p.)	<i>B. pertussis</i> aerosol infection	OVA (i.p.)	OVA (i.p. and i.n.)	OVA (i.n.)

^a Groups of 6–8-week-old female BALB/c mice were immunized (i.p.) and boosted with whole-cell pertussis vaccine (Pw) at –42 and –14 days. On 0 day, mice were either sham infected or infected with *B. pertussis* (Bp) by aerosol. At 10 and 24 days selected groups were sensitised to OVA by i.p. alone or i.p. and i.n. routes, respectively. On 35, 36 and 37 days mice were exposed to either saline or OVA by the i.n. route (i.e. 25, 26, and 27 days post-OVA priming and after bacterial clearance). Bacterial burdens in the airways were measured between 0 and 37 days. All other readouts, including plethysmography were performed at 37 days.

^b For comparison, further groups of unimmunized (sham immunized) mice were treated as follows: control (Ctrl) mice were sham infected on 0 day and sham sensitised with saline. A second group (Bp) were infected with *B. pertussis* at 0 day and sham sensitized. The third group (OVA) were sham infected but sensitized with OVA (100 µg, i.p.) at 10 and 24 days and again (50 µg i.n.) at 24, 35, 36, and 37 days. A separate group (BpOVA) were infected with *B. pertussis* on 0 day, and sensitized as above. Each experiment was repeated at least twice, on each occasion $n > 35$ mice per group, bacterial burdens were measured between 0 and 37 days, all other readouts were at 37 days.

2.3. *B. pertussis* aerosol infection

Respiratory infection was initiated by aerosol challenge with *B. pertussis* strain W28, following growth under agitation conditions at 37 °C in Stainer-Scholte liquid medium. Bacteria from a log-phase culture were resuspended at a concentration of 2×10^{10} CFU/ml in 1% (w/v) casein in 0.9% (w/v) saline. The challenge inoculum was administered to groups of mice on 0 day (Bp, PwBp and PwBpOVA groups). Administration was by aerosol over a period of 15 min using a nebulizer. Groups of four or more mice were killed at various time points after aerosol challenge to assess the number of viable *B. pertussis* in the lungs. Remaining mice received a similar aerosol of sterile saline alone.

2.4. Enumeration of viable bacteria in the lungs

Lungs were removed aseptically into 1 ml of sterile physiological saline with 1% casein. One hundred microlitres of serially diluted homogenate from individual lungs were placed onto triplicate Bordet-Gengou agar plates and the number of CFU determined after incubation at 37 °C for 4 days. Results are reported as the mean number of *B. pertussis* CFU (\pm S.E.M.) for individual lungs, each determined in triplicate, from four or more mice per time point. All experiments were repeated twice.

2.5. Bronchoalveolar lavage

Bronchoalveolar lavage fluids (BALF) were obtained by cannulation of the trachea followed by repeat administration

and aspiration of 0.5 ml PBS per mouse. This was pooled from five mice (total 2.5 ml) per experimental group. All experiments were performed at least twice. Diluted BALF was assessed for the presence of cytokines.

2.6. Measurement of OVA and *B. pertussis*-specific antibody

OVA and *B. pertussis*-specific IgG1, 2a, 2b, and 3 present in collected sera were measured on day 37 by ELISA as previously described [35,36]. Total and OVA-specific IgE was measured using a rat anti-mouse IgE monoclonal antibody (BD, Pharmingen, San Diego, CA, USA). The IgE concentration was expressed as µg/ml after comparison to murine IgE standards.

2.7. T-cell proliferation assays

Spleen cells (2×10^6 /ml) from infected, sensitized and control mice ($n=4$ or more per group) were tested for in vitro proliferation against heat-inactivated *B. pertussis* (1×10^4 CFU/ml), OVA (20 µg/ml), Concanavalin A (Con A) (5 µg/ml, positive control), or medium alone (negative control). After 72 h, cell proliferation was assessed by liquid scintillation counting of [³H]-thymidine incorporation and results were expressed as mean CPM of triplicate wells \pm S.E. At the 72 h time point, culture supernatants were sampled for cytokine analysis, although the kinetics of cytokine production varies this time point has previously proved acceptable for detection of most cytokines [22].

2.8. Cytokine measurement

Concentrations of IL-5, IL-10, IL-13 and IFN- γ from spleen, and BALF were assessed by ELISA (BD, Pharmingen, San Diego, CA, USA). Cytokine concentrations were calculated by comparison with known cytokine standards as previously described [35], all determinations were made in triplicate, results are presented as mean cytokine concentration (\pm S.E.M.).

2.9. Whole body plethysmography

Airway responsiveness on day 37 was assessed by methacholine (MCh) induced airflow obstruction from conscious mice using whole-body plethysmography (Buxco Electronics, Sharon, CT, USA) as previously described [37]. Pulmonary airflow obstruction was measured by enhanced pause (PenH), a value determined from the ratio of expiratory time and relaxation time to peak expiratory flow and peak inspiratory flow and thought to correlate with airway responsiveness. Measurements were obtained after exposure of mice for 3 min to PBS (baseline) followed by incremental doses (3.3 mg–50 mg/ml) of MCh delivered by aerosol [38].

2.10. Respiratory tract histology

Animals ($n = 5$ per group per experiment) were sacrificed at 37 days. Lungs were removed, fixed in a paraformaldehyde/lysine/periodate fixative, paraffin embedded, sectioned and stained using the haematoxylin and eosin (H&E), Discombes (identification of eosinophils), alcian blue (identification of mucus), PAS (assessment of basement membrane thickness), azure-A (identification of mast cells) and Van Gieson (identification of fibrosis) methods. Histopathological changes evident were graded according to a semi-quantitative scoring system as mild, moderate or severe by two researchers without prior knowledge of the treatment group using a previously established scoring system [17]. All experiments were performed at least twice.

2.11. Statistical methods

Results are expressed as the mean \pm S.E.M. of the indicated number of animals. A Student's t -test was used to determine significance among the groups. A value of $P < 0.05$ was considered significant. Analyses were performed using the Graph-Pad PrismTM software (GraphPad, San Diego, CA).

3. Results

3.1. Ovalbumin sensitization does not impair vaccine-mediated clearance of *B. pertussis*

The murine OVA model of airway hyperresponsiveness induces a powerful Th2 response [7] whereas both *B. pertus-*

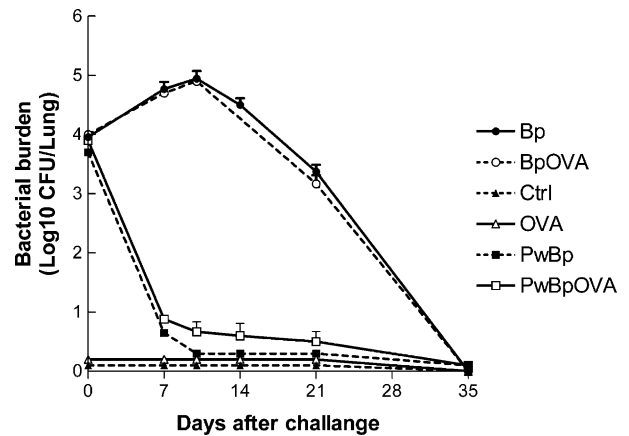


Fig. 1. Course of *B. pertussis* infection in experimental and control mice. Groups of mice were sacrificed at intervals after challenge and the number of viable bacteria estimated by performing colony counts on individual lung homogenates. Results are representative from two experiments and are presented as mean (\pm S.E.M.) CFU in the lungs, determined individually from four mice at each time point, and for each experimental group. Data for Ctrl and OVA groups have been offset from zero for clarity.

sis infection and Pw immunization induce a powerful Th1 response [29]. In order to examine immune cross-regulation and potential interference with immunization, we tested the effect of OVA sensitization upon the development of a protective response to infection in Pw immunized and non-immunized mice. Mice received combinations of OVA sensitization, Pw immunization and aerosol challenge with virulent *B. pertussis* (Table 1). Groups of mice infected with *B. pertussis* (Bp and BpOVA) showed similar kinetics of bacterial clearance (Fig. 1), indicating that OVA sensitization does not influence bacterial clearance rates. Likewise, OVA sensitized and non-sensitized mice that had been immunized prior to bacterial challenge (PwBpOVA and PwBp, respectively) showed identical kinetics of clearance. No bacteria were recovered from the OVA sensitized or control (Ctrl) groups, which were uninfected but received saline by aerosol (Fig. 1). The bacterial burden in the Bp and BpOVA groups peaked at 10 days and declined thereafter. Pw immunized mice cleared subsequent infection by *B. pertussis* by 7 days. In contrast unimmunized mice (Bp and the BpOVA groups) only showed complete bacterial clearance by 35 days (Fig. 1). Therefore sensitization with OVA did not impair vaccine-mediated clearance of *B. pertussis* in this model.

3.2. OVA-specific IgE production is modulated by Pw immunization

The goal of this study was to examine the influence of Pw immunization on responses associated with allergic sensitization. Although OVA-induced sensitization does not impair vaccine-mediated clearance of *B. pertussis*, it was possible that Pw influenced allergic sensitization. OVA-specific IgG was not detected from mice infected with *B. pertussis*

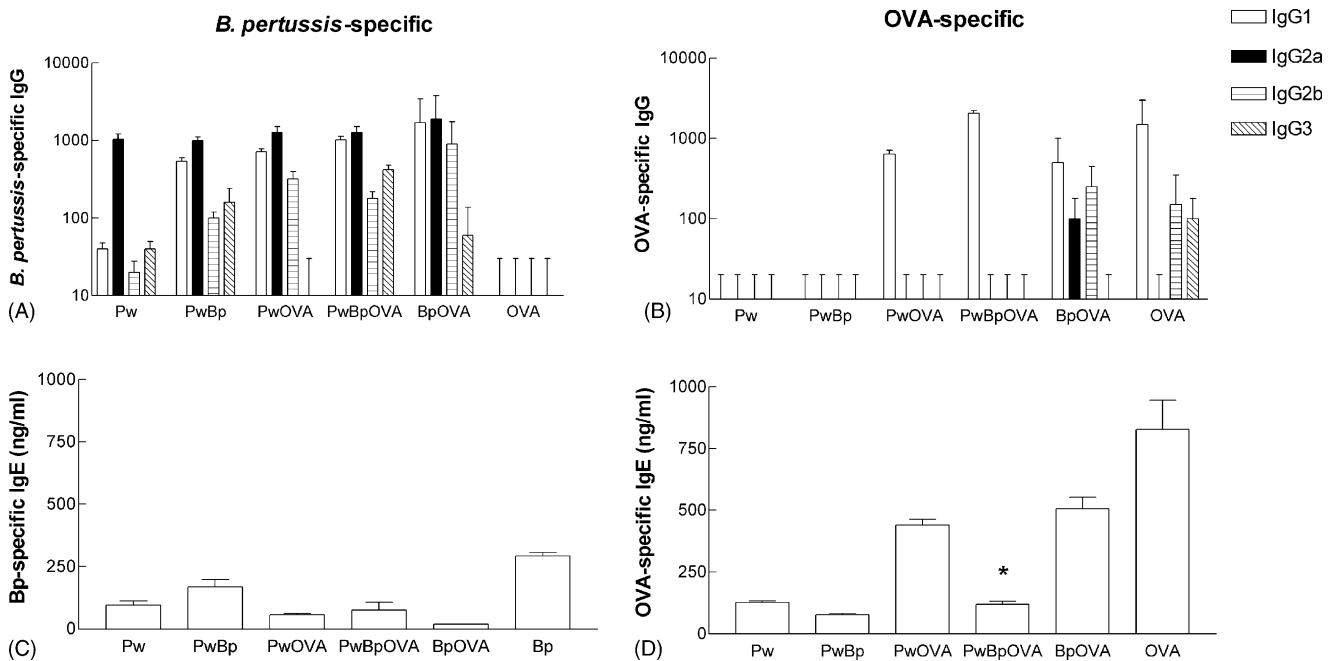


Fig. 2. Serum IgE and IgG subclasses elicited by Pw vaccination, bacterial infection and allergic sensitization. (A) *B. pertussis* or (B) OVA-specific serum antibody responses by IgG subclasses elicited in whole-cell *B. pertussis* (Pw), vaccinated plus *Bp* infection (PwBp), vaccinated plus sensitized (PwOVA), or in mice vaccinated then infected with *B. pertussis* prior to sensitization (PwBpOVA), response from mice that received OVA sensitization alone (OVA) or combined with infection (BpOVA) are shown for comparison. (C) *B. pertussis*-specific serum IgE and (D) OVA-specific serum IgE present from each experimental group. Results are representative of two experiments expressed as geometric mean titre or ng/ml of antibody (\pm S.E.M.) from four animals each determined independently in triplicate. * $P < 0.05$ compared to the PwOVA treated group.

only; similarly *B. pertussis*-specific IgG could not be detected in OVA sensitized animals, suggesting no significant cross-reaction between the two immunogens (Fig. 2A and B). An analysis of the serum antibody subclasses evoked revealed that Pw predominantly induced pertussis-specific IgG2a (Fig. 2A) as previously reported [28,34]. IgG2a remained the dominant subclass of pertussis-specific antibody when Pw immunization was combined with OVA sensitization (PwOVA) or *B. pertussis* infection (PwBp) although IgG1 and IgG3 was detected in the latter groups (Fig. 2A). Pw immunization prior to OVA sensitization (PwOVA) did not significantly alter the dominant OVA-specific IgG1 response. Infection of these mice with *B. pertussis* (PwBpOVA) did not broaden the subclasses of OVA-specific antibody detected although the titer was increased (Fig. 2B). Taken together, these data indicate that Pw immunization does not modulate serum IgG subclasses induced by allergic sensitization. In contrast to the conservative effects on serum IgG subclasses, significant differences were observed in the induction of IgE (Fig. 2C and D). Pw induces little *B. pertussis*-specific IgE, and this is not altered by OVA sensitization or infection (Fig. 2C). OVA sensitization induces high levels of OVA-specific IgE but this is significantly reduced by prior Pw immunization (Fig. 2D). However, a combination of immunization and infection prior to OVA sensitization resulted in a significant reduction of IgE ($P < 0.05$) (Fig. 2D) compared to the PwOVA group.

3.3. Pw immunization prevents *B. pertussis* enhancement of OVA-induced IL-10 and IL-13

B. pertussis infection enhances OVA-induced IL-10 and IL-13 [17]. Pw immunization has hitherto been regarded as inducing essentially similar immune responses to those induced by infection [22]. In order to dissect the influence of immunization on airway hyperresponsiveness, we examined cell-mediated immune responses in the various study groups. Pw immunization alone or in combination with *B. pertussis* infection (Pw or PwBp) induced very little IL-5 but strong IFN- γ responses (Fig. 3A and B). This was consistent with the protection observed earlier (Fig. 1) and previous data [34]. Pw immunization reduced levels of IL-5, IL-13 and IFN- γ (Fig. 3A–C) in all immunized groups suggesting that Pw immunization prevents live *B. pertussis* enhancement of these indices; mirroring the reduction in OVA-specific IgE (Fig. 2D). Interestingly, previous results have shown that *B. pertussis* infection induced specific IL-10 as well as IL-13 responses [17]. Here we demonstrate that in contrast to infection, Pw-immunization resulted in significantly reduced levels of IL-10, and IL-13 (Fig. 3A–D).

To extend these findings, we examined the levels of cytokines present in bronchoalveolar lavage fluid (BALF) from each group of mice. Pw immunization alone induced little or no detectable cytokines in BALF. As expected, OVA sensitization induced high levels of IL-5, -10 and -13 but the levels of IL-10 and -13 in particular, known to rise in infected mice,

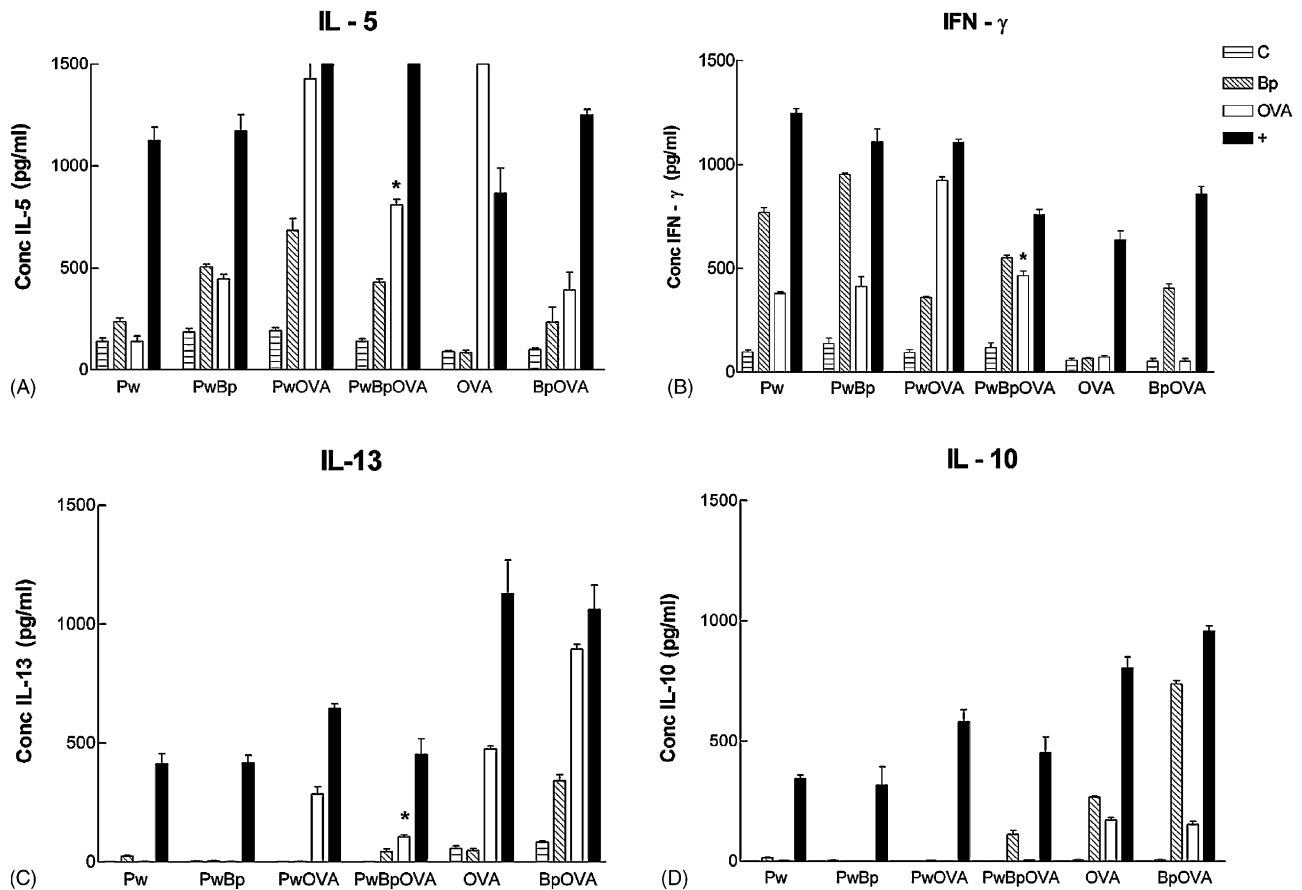


Fig. 3. Cell mediated immune responses from spleen, elicited by Pw vaccination, bacterial infection and allergic sensitization. IL-5 (A), IFN- γ (B), IL-13 (C) and IL-10 (D) responses from spleen cell cultures stimulated with medium alone (–ve control, horizontal shading), heat inactivated *B. pertussis* at 1×10^4 CFU/ml (hatched bar), OVA (open bar) or Con A (+ve control, black bar). Responses are representative of duplicate experiments each performed in triplicate on individual samples from four mice per group and are expressed as mean (\pm S.E.M.). * $P < 0.05$ compared to the PwOVA treated group.

were reduced in immunized mice that had been infected with *B. pertussis* (PwBpOVA) (Fig. 4A–D).

3.4. Pw immunization prior to *B. pertussis* infection decreases bronchial hyperresponsiveness to sensitizing antigen

It has been proposed that prior Th1 responses to bacterial infections protect against allergic disease however, Th1-inducing *B. pertussis* infection exacerbates airway hyperresponsiveness in OVA sensitized mice. It might be predicted that Pw, which induces a very similar immune response to *B. pertussis* would have a similar exacerbating influence. In fact, this is not the case. We used whole body plethysmography as a surrogate measure of airway reactivity in mice immunized with Pw and infected with *B. pertussis* prior to OVA sensitization (Fig. 5). Prior immunization with Pw does not enhance but protects against *B. pertussis* exacerbated airway hyperresponsiveness in comparison to controls. Statistical analysis using two-way analysis of variance (ANOVA) showed that mice vaccinated with Pw, and sensitized to OVA following *B. pertussis* infection (PwBpOVA) displayed significantly reduced bronchial hyperreactivity

compared to BpOVA sensitized animals ($P < 0.05$) (Fig. 5D). Thus demonstrating that vaccination with Pw protects against *B. pertussis* exacerbation of allergic asthma.

B. pertussis infection is known to modulate the quality of the inflammatory influx of the respiratory tract, with a marked reduction in eosinophil numbers accompanied by varying degrees of epithelial hyperplasia, mucous metaplasia, and airway pathology [17]. Lung tissue was assessed histologically (Table 2). Minimal pathology was observed in mice immunized with Pw or those immunized and infected with *B. pertussis* (PwBp) (Fig. 6A and B). Pw and OVA sensitized (PwOVA) mice illustrated moderate mural and peri-airway inflammation with accompanying mild mucous metaplasia and moderate hyperplasia of the epithelium (Fig. 6C). The combination of Pw immunization, *B. pertussis* infection and OVA sensitization did not show enhanced pathology but only moderate mucous metaplasia and moderate hyperplasia of the epithelium (Fig. 6D). Given that previous work has shown that *B. pertussis* infection in combination with OVA sensitization (BpOVA) displayed more severe airway inflammation with a greater degree of both epithelial hyperplasia and mucous metaplasia, it can be clearly seen here that Pw immunization reduces the severity of airway pathology (Fig. 6D)

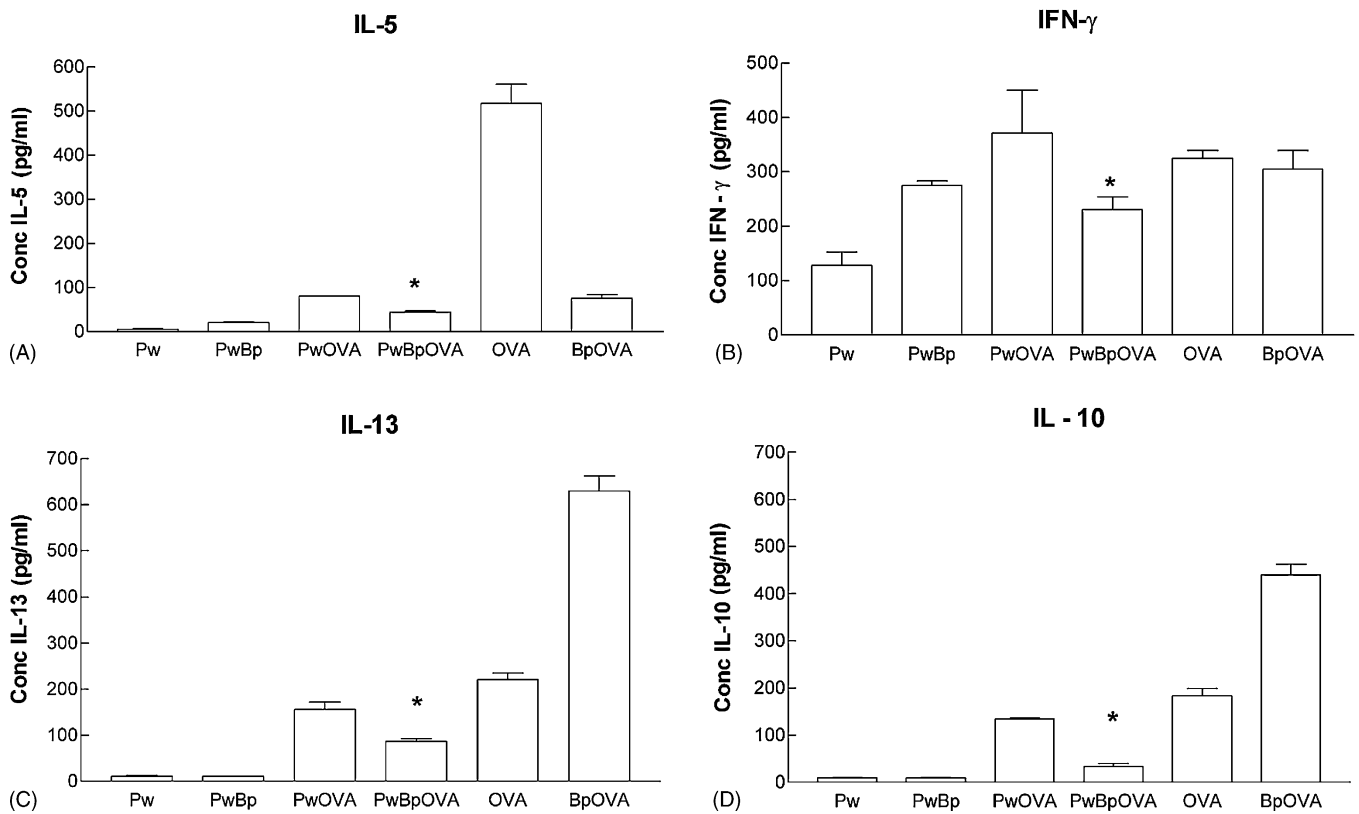


Fig. 4. Pw immunization modulates the local cytokine response to *B. pertussis* infection and OVA sensitization. Diluted BALF (0.5 ml per mouse) was pooled from five mice per group and concentrations of IL-5 (A), IFN- γ (B), IL-13 (C) and IL-10 (D) were determined by EIA. Results are representative of duplicate experiments. Cytokine concentrations were assayed in triplicate with values expressed as mean cytokine concentration in diluted BALF (\pm S.E.M.). * $P < 0.05$ compared to the PwOVA treated group.

and reduces pulmonary resistance as indicated by plethysmography (Fig. 5D).

4. Discussion

The present study demonstrates that Pw immunization protects against *B. pertussis* exacerbation of OVA-induced

airway hyperresponsiveness in a murine model. Pw immunization suppresses antibody and cell mediated responses against OVA sensitization in combination with *B. pertussis* infection at both the local and systemic level, coupled with a subsequent reduction in airway reactivity and pathology. It has been previously shown that *B. pertussis* exacerbates OVA-induced airway pathology leading to the development of more pronounced allergen-induced airway inflammation

Table 2
Histological assessment of airway pathology

Treatment group	Mucous metaplasia of airway epithelium	Hyper plasia of airway epithelium	Smooth muscle hypertrophy of airway wall	Peri-airway/vascular inflammation ^a					
				Overall degree	E	N	L	M	F
Ctrl	–	–	–	–	–	–	–	–	–
Bp	–	+	+	+	–	+	++	–	–
Ova	++	++	++	++ ^b	+	++	++ ^c	–	–
BpOVA	+++	+++	++	+++ ^b	++	+++	+++ ^c	–	–
Pw	–	–	–	–	–	–	–	–	–
PwBp	–	+	+	–	–	–	–	–	–
PwOVA	+	++	+	++ ^b	++	++	++ ^c	–	–
PwBpOVA	++	++	++	++ ^b	++	++	++ ^c	–	–

A semi-quantitative score (– absent, + mild, ++ moderate, +++ severe) was assigned to features of airway pathology observed according to previously described criteria [17].

^a Peri-airway/vascular inflammation was assessed in terms of overall degree and of numbers of infiltrating eosinophils (E), neutrophils (N), lymphocytes, plasma cells and macrophages (L), mast cells (M) and in terms of circumscribing fibrosis (F).

^b Inflammation extending into surrounding pulmonary interstitium and alveolar spaces.

^c Macrophage giant cells form part of inflammatory exudates within surrounding alveolar spaces.

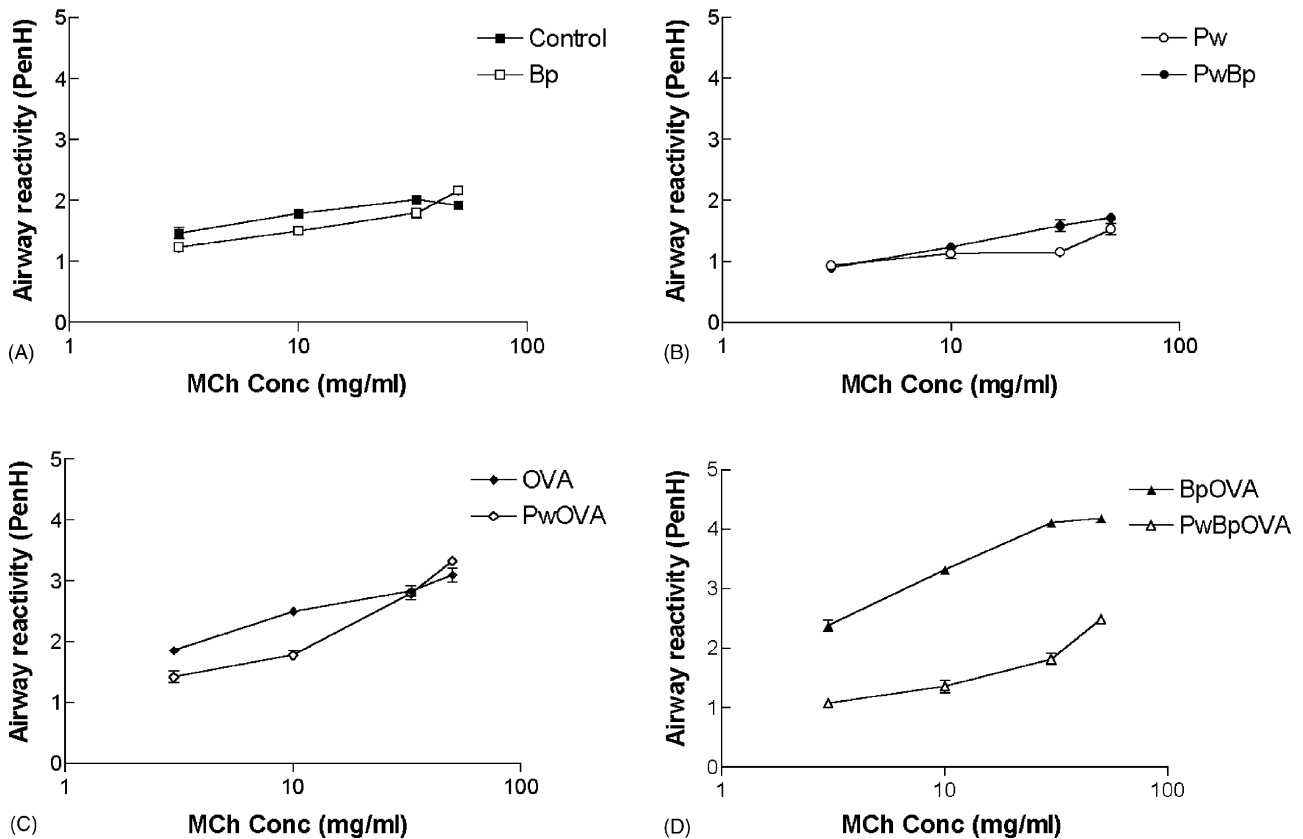


Fig. 5. Pw immunization prior to *B. pertussis* infection decreases bronchial hyperresponsiveness to sensitizing antigen. Airway hyperreactivity in response to increasing concentrations of inhaled methacholine (MCh) was measured by whole-body plethysmography. (A) Control and Bp infected mice (B), Pw and PwBp (C), OVA and PwOVA (D), BpOVA and PwBpOVA groups, respectively. Results are representative of two experiments ($n=4$) and values are expressed as mean enhanced pause (PenH) \pm S.E.M., in groups where no errors are visible, error bars are shorter than the size of the data point symbol.

[17]. We demonstrate that Pw immunization protects against this as well as reducing airway hyperresponsiveness.

The prevalence of asthma and allergic disease has increased in many countries [39,40] and there has been speculation as to possible causes [41,42], including the possible role of immunization in promoting allergic sensitization [43]. For example, pertussis vaccination acts as an adjuvant for antigen-specific responses in laboratory animals [30,33]; a specific IgE response to pertussis toxin itself has been identified in children receiving pertussis immunization [44]; and vaccination with some other organisms such as *Haemophilus influenzae* enhances histamine release in laboratory animals [45]. Active pertussis toxin has a similar effect [46]. In addition, two studies have suggested that pertussis infection increased the risk of atopy [47,48]. It is therefore theoretically possible that Pw immunization might contribute to the development of allergic disease.

The goal of this study was to test whether immunization with whole-cell pertussis (Pw) vaccine would protect against *B. pertussis* exacerbation of allergic asthma. *B. pertussis* infection modulates allergen priming and the severity of airway pathology in a murine model [17]. It has been proposed that IL-10 plays an essential role in modulating the immune responses by inducing towards regulatory T-cell responses [49]

however Lee et al have demonstrated that IL-10 induces IL-13 production in vivo and that this is responsible for the mucus, but not the inflammatory/fibrotic effects of IL-10 [50]. In the present study, we observe that Pw immunization prevents induction of IL-10 and IL-13 and protects against airway hyperreactivity. Although IL-10 is known to act in an immune regulatory manner, we and others have suggested that it has broader functions that may not always protect against inflammatory disease [17,51]. For example, Grunstein et al. have suggested that IL-10 may play an important role in allergic asthma by acting directly on the airway smooth muscle itself [51]. Previous studies in humans have demonstrated that IL-13 mRNA and protein levels are elevated in the lungs of atopic and non-atopic asthmatics [52] suggesting that over expression of IL-13 may predispose toward the development of both types of asthma [53]. The reduction in both IL-10 and IL-13 at the systemic and local levels suggests that by removing the damaging effects of pertussis infection and consequently reducing IL-10 and IL-13, Pw exerts a protective effect. Interestingly, very recent work by Kim et al has shown that components of *B. pertussis* can inhibit airway hyperresponsiveness [54]. That study demonstrated that unmethylated CpG sequences from *B. pertussis* DNA inhibited Th2 cytokines in the airways via a TLR9 interaction [54]. Our

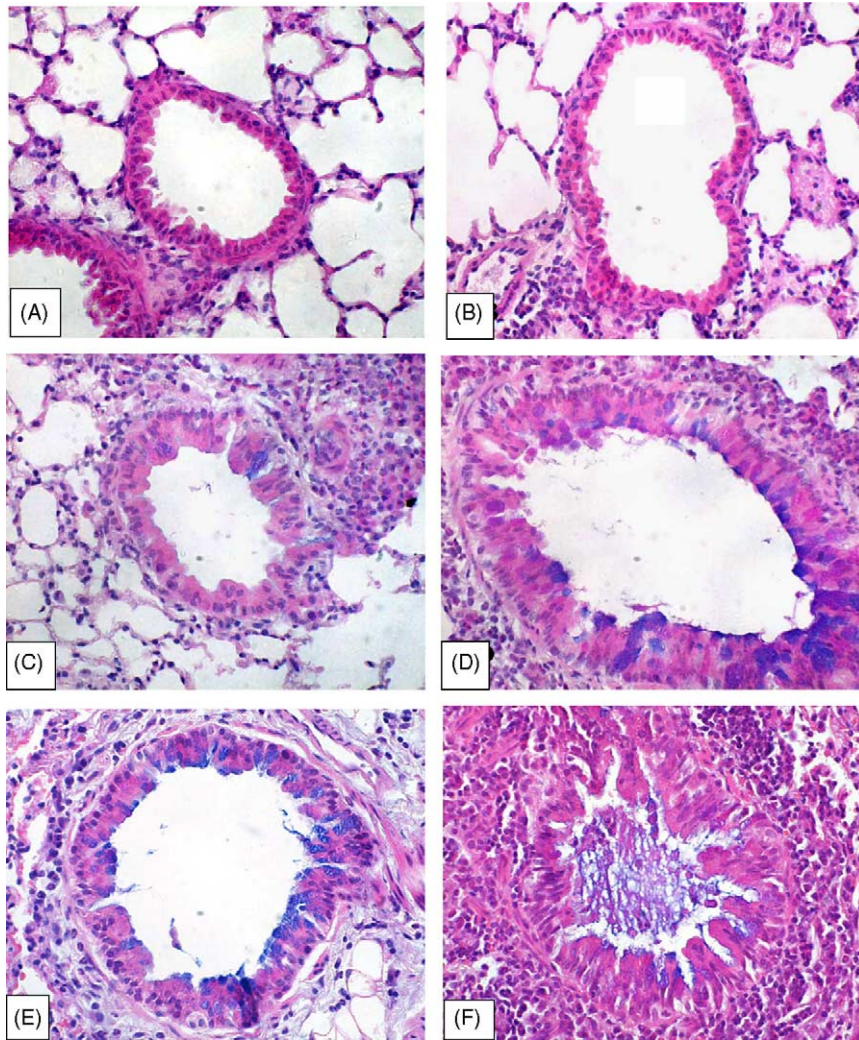


Fig. 6. Whole-cell *B. pertussis* vaccine reduces the severity of airway pathology to sensitizing antigen both in the presence and absence of *B. pertussis* infection. Representative morphological changes at 37 days in transverse sections of bronchioles from (A) Pw immunized mice showing no changes evident; (B) Pw/Bp mice, minimal changes evident; (C) Pw/OVA treated mice, illustrating moderate mural and peri-airway inflammation with accompanying mild mucous metaplasia (blue staining goblet cells) and moderate hyperplasia of epithelium; (D) Combined Pw/Bp/OVA treated group, illustrating a moderate mural and peri-airway inflammation, moderate mucous metaplasia and moderate epithelial hyperplasia; (E) OVA sensitized group illustrating moderate mural and peri-airway inflammation with accompanying moderate mucous metaplasia (blue staining goblet cells) and hyperplasia of epithelium; (F) combined *B. pertussis*/OVA treated group illustrating severe mural and peri-airway inflammation, moderate epithelial hyperplasia and severe mucous metaplasia with accompanying mucus plugging of the lumen. All sections are representative of groups of five mice per experiment, performed at least twice. Sections stained with a combined Discombe's/Alcian blue stain, original magnification 400 \times .

observation of reduced inflammation and OVA-specific IgE (Figs. 2 and 6) in PwBpOVA mice is consistent with that finding. TLR9 mediated reduction in Th2 cytokines can be invoked in this situation, because these mice will be exposed to bacterial DNA in a context where prior immunization will limit tissue damage or the other immunomodulatory effects of viable *B. pertussis* [17,54].

The most common formulation of the hygiene hypothesis is based upon a lack of immunological stimuli in early infancy that results in aberrant Th2 responses [10,12]. Clearly infant pertussis immunization or infection could potentially influence this process. Studies in neonatal mice support our findings that Pw is a strong Th1 inducer, but it is less

effective in inducing antibody responses during this period [28,55,56]. A study by Gruber et al. revealed no evidence for an allergy promoting effect of common childhood vaccines in a prospectively followed atopy risk-enhanced birth cohort [26]. Moreover, they found that children with a better vaccination coverage seemed to be better protected against the development of atopy in their second and third years of life. In particular, measles/mumps, pertussis, and diphtheria/tetanus immunization were associated with a transient reduction of atopy, whereas immunization against polio and *H. influenzae* had no effect. Furthermore, immunization of children with Pw down regulated the IgE response to co-administered diphtheria and tetanus toxoids [57]. In contrast, a study

involving the effects of diphtheria–tetanus–pertussis (DTP) or tetanus vaccination on allergies among children in the U.S. reported that DTP or tetanus vaccination appeared to increase the risk of allergies and related respiratory symptoms, one contentious interpretation of this study is that vaccine components may be responsible for a portion of the increased prevalence of asthma and allergies in U.S. children [24].

We have established models that allow examination of the mechanisms of interaction between protective immunization and allergic sensitization. Pw immunization reduces IL-13 and IL-10 in BALF (Fig. 4C and D) and also protects against airway hyperresponsiveness (Fig. 5D). These data indicate that although Pw induces a similar immune response to *B. pertussis* infection, these responses are not identical. Pw does not enhance airway IL-13 and consequently does not enhance but protects against the exacerbation of allergic responses. We find no evidence of Pw contributing to allergic asthma, but rather provide evidence of a mechanism whereby whole-cell pertussis vaccination has a protective role.

Acknowledgements

We thank Mrs. Sheila Worrell, Mr. Joseph Brady and Ms. Bernadette Ruane for their expert technical assistance. This work was supported by grants from the Irish HEA PRTL programme (Darren Ennis). Bernard Mahon is a Wellcome Trust/HRB new blood fellow (GR 054236).

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