Prior *Bordetella pertussis* infection modulates allergen priming and the severity of airway pathology in a murine model of allergic asthma

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Summary

Background It has been proposed that T helper (Th)2-driven immune deviation in early life can be countered by Th1 inducing childhood infections and that such counter-regulation can protect against allergic asthma.

Objective To test whether Th1-inducing infection with *Bordetella pertussis* protects against allergic asthma using well-characterized murine models.

Methods Groups of mice were sensitized to ovalbumin (OVA) in the presence or absence of *B. pertussis,* a well-characterized Th1 inducing respiratory infection. Immunological, pathological and physiological parameters were measured to assess the impact of infection on immune deviation and airway function.

Results We demonstrate that OVA sensitization does not affect the development of *B. pertussis*-specific immune responses dominated by IgG2a and IFN-γ and does not impair Th1-mediated clearance of airway infection. In contrast, *B. pertussis* infection at the time of sensitization modulated the response to OVA and significantly reduced total serum and OVA-specific IgE. The pattern of cytokine responses, in particular OVA-specific IL-5 responses in the spleen was also modulated. However, *B. pertussis* did not cause global suppression as IL-10 and IL-13 levels were enhanced in OVA-stimulated spleen cell cultures and in lavage fluid from infected co-sensitized mice. Histopathological examination revealed that *B. pertussis* infection prior to OVA sensitization resulted in increased inflammation of bronchiolar walls with accompanying hyperplasia and mucous metaplasia of lining epithelia. These pathological changes were accompanied by increased bronchial hyper-reactivity to methacholine exposure.

Conclusion Contrary to the above premise, a Th1 response induced by a common childhood infection does not protect against bronchial hyper-reactivity, but rather exacerbates the allergic asthmatic response, despite modulation of immune mediators.

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Introduction

Asthma is a chronic disease of the respiratory tract that has increased dramatically in prevalence in western society [1]. The inflammatory response in asthma is tightly associated with airway hyper-responsiveness (AHR), increased mucus production and an infiltration of the bronchial mucosa with CD4$^+$ T cells [2]. There is evidence of an altered local T cell response in favour of T helper (Th)2 cytokine release (IL-4, IL-5 and IL-13) resulting in B cell isotype switching to IgE, mast cell, eosinophil and basophil recruitment and production of a wide range of inflammatory mediators [3]. The resulting pulmonary inflammation leads to bronchoconstriction and ultimately to airway remodelling [4].

The current understanding of the pathophysiology of allergic asthma is that it results from a breakdown in the normal tolerance to inhaled antigens, associated with Th2 cytokine production [5, 6]. The murine ovalbumin (OVA) model of AHR exhibits many of the features of human asthma, including airway hyper-reactivity, airway inflammation and increased serum IgE levels [7, 8]. This model has been used extensively to probe mechanisms of asthma [7, 9].

The increased incidence of asthma has been linked to improved sanitation in industrialized societies, which in turn has reduced the incidence of childhood infections [10]. One current attempt to explain these observations, loosely termed the hygiene hypothesis, states that childhood asthma develops as a result of decreased exposure to infectious agents during infancy and early childhood, which results in the persistence of the neonatal Th1 deficit, thereby predisposing the child to atopic disease [11]. While Th2 cells promote airway inflammation in asthma, it has been proposed that because Th1 cells antagonize Th2 cell function, immune deviation towards Th1

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may be protective in asthma [12, 13]. One prediction arising from the above hypothesis is that a powerful Th1-inducing infection during or prior to airway sensitization should diminish or protect against Th2-mediated allergic asthma.

Bordetella pertussis is a Gram-negative bacterium that causes the severe infant disease whooping cough. *B. pertussis* respiratory challenge of mice is a well-characterized model of airway Th1-induced immunity, which correlates well to immunity in humans [14]. Recovery from infection is associated with the development of *B. pertussis*-specific Th1 cells in both humans and mice [15]. Th1 cells producing IFN-γ play an essential, non-redundant role in the clearance of the bacteria from the respiratory tract [16]. Murine respiratory challenge by aerosol administration of the bacteria has been used extensively for studies of *B. pertussis* immunity and pathogenesis and although mice lack the characteristic cough, in other respects the course of infection and many of the systemic effects are similar to those observed in infants [17]. In order to test the above hypothesis, we used the Th1 driving *B. pertussis* model, in combination with the murine OVA model of allergic asthma. Based on the prediction above, prior infection with *B. pertussis* might be expected to reduce OVA-induced Th2-mediated AHR and immunopathology.

Our findings demonstrate that although dampening of the Th2 response was seen at the local and systemic level, OVA sensitization during *B. pertussis* infection led to enhanced production of airway IL-10 and IL-13, coupled with a subsequent increase in AHR and pathology. This suggests that although IL-10 may be regarded as a regulatory cytokine, it has broader functions that may not always protect against inflammatory disease. These data have implications with regard to the validity of the hygiene hypothesis and raise concerns regarding therapies based on the conversion of Th2-dominated allergic inflammatory responses into Th1-dominated responses based on protective effects of Th1 cells in allergy and asthma.

Materials and methods

Animals

Six- to eight-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.

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 two groups of mice on 0 day (Bp and BpOVA 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.

Immunization and airway delivery of OVA

Two groups of 6–8-week-old female BALB/c mice (OVA and BpOVA) were sensitized by i.p. injection of 100 μg OVA (Grade V; Sigma, Dorset, UK) emulsified in 2% Alhydrogel® adjuvant (Superfos Biosector, Sweden) at 10 and 24 days after bacterial or saline challenge. Control groups (Ctrl and Bp) received saline alone (i.p.). On 35, 36 and 37 days, OVA and BpOVA-sensitized mice received 50 μg OVA intra–nasally, whereas Ctrl and Bp groups received saline only.

Enumeration of viable bacteria in the lungs

Lungs were removed aseptically into 1 mL of sterile physiological saline with 1% casein. One hundred microliotres 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 for individual lungs from four or more mice.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) fluids were obtained by repeat administration and aspiration of 0.5 mL volumes (total 5 mL) of phospate-buffered saline (PBS) via cannulation of the trachea of mice from three experiments ($n = 5$). Cells from the lavage fluid were recovered by centrifugation at 300 g for 6 min and resuspended in PBS; total leucocytes were counted and cytospin preparations were stained with a combined Alcian blue/Discombe's stain to determine the differential cell count. Supernatants were collected for cytokine analysis and stored at − 80 °C.

Measurement of ovalbumin- and Bordetella pertussis-specific antibody

OVA- and *B. pertussis*-specific-IgG1, IgG2a, IgG2b and IgG3 present in collected sera were measured by ELISA as previously described [16, 18]. Briefly, plates were coated with OVA protein (5 μg/mL) or sonicated *B. pertussis* antigen (1 μg/mL) overnight at 4 °C. After blocking and the addition of serum samples, alkaline phosphatase-labelled rat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Pharmingen) were used to detect OVA- and *B. pertussis*-specific antibody as previously described [19]. Total and OVA-specific IgE was measured using a rat anti-mouse IgE monoclonal antibody (Pharmingen). The IgE concentration was expressed as micrograms per millilitre after comparison with murine IgE standards.

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 ± SE. At the 72-h time-point, culture supernatants were sampled for cytokine analysis, although the kinetics of cytokine produc-

tion varies this time-point has previously proved acceptable for detection of most cytokines [14].

Cytokine measurement

Concentrations of IL-4, IL-5, IL-10, IL-13 and IFN-γ from spleen and broncho-alveolar lavage fluid (BALF) were assessed by ELISA (Pharmingen). Cytokine concentrations were calculated by comparison with known cytokine standards as previously described [16].

Whole-body plethysmography

Airway responsiveness was assessed by methacholine (MCh)-induced airflow obstruction from conscious mice using whole-body plethysmography (Buxco Electronics, USA) as previously described [20]. 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 (3.3–50 mg/mL) of MCh delivered by aerosol [21].

Respiratory tract histology

Animals ($n = 5$ per group per experiment) were killed 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), Discombe's (identification of eosinophils)/Alcian blue (identification of mucus), Periodic Acid-Schiff (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 (Table 2). All experiments were performed at least twice ($n = 5$) on each occasion.

Results

Ovalbumin sensitization does not impair T helper 1-mediated mechanisms of bacterial clearance

In order to examine the effects of immune cross-regulation we tested the effect of OVA sensitization upon the development of a protective Th1 response to infection. Groups of mice were sensitized to OVA in the presence or absence of a prior *B. pertussis* infection (Table 1). The kinetics of bacterial clearance from the lungs of experimental animals were monitored by performing colony counts on whole-lung homogenates at different times post-bacterial challenge. Mice received either saline (Ctrl and OVA) or viable *B. pertussis* (Bp and BpOVA) by aerosol. This was followed by OVA (OVA and BpOVA) or sham sensitization (Table 1). Groups infected with *B. pertussis* (Bp and combined BpOVA groups) showed similar kinetics of bacterial clearance. No bacteria were recovered from the OVA or Ctrl group (Fig. 1), which were uninfected but received saline by aerosol. Bacterial burden in the Bp and BpOVA groups peaked at 10 days and declined thereafter. By 35 days-post-challenge, both the Bp and the BpOVA groups showed complete bacterial clearance (Fig. 1). Therefore, sensitization with OVA does not impair the effector function associated with the Th1-mediated clearance of a bacterial disease.

Table 1. Experimental design

Description*	Time (days)			
	0	10	24	35/36/37
Control	Saline aerosol	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
Bp infection	Bp aerosol infection	Saline (i.p.)	Saline (i.p. and i.n.)	Saline (i.n.)
OVA sensitization	Saline aerosol	OVA (i.p.)	OVA (i.p and i.n.)	OVA (i.n.)
Bp infection and OVA sensitization (BpOVA)	Bp aerosol infection	OVA (i.p.)	OVA (i.p and i.n.)	OVA (i.n.)

*Groups of 6–8-week-old female BALB/c mice ($n = 4$ or more per replicate per time-point) were treated as follows: Control (Ctrl) mice were sham infected on 0 day and sham sensitized at 10 days and 24 days. A second group (Bp) were infected with *Bordetella pertussis* at 0 day and sham sensitized at 10 day and 24 days. The third group (OVA) were sham infected but sensitized with OVA (100 μg, i.p.) at 10 and 24.days and then again (50 μg i.n.) at 24, 35, 36, and 37 days. The final group (BpOVA) were infected with *B. pertussis* on 0 day, and sensitized as above.

Table 2. Histological assessment of airway pathology

Treatment group	Epithelial mucous metaplasia	Epithelial hyperplasia	Smooth muscle hypertrophy	Peri-airway inflammation*					
				Overall	E	N	L	M	F
Control	−	−	−	−	−	−	−	−	−
Bp	−	+	+	+	−	+	++	−	−
OVA	++	++	++	++	+	++	++	−	−
BpOVA	+++	+++	++	+++	++	+++	+++	−	−

A semi-quantitative score (−, absent; +, mild; ++, moderate; +++, severe) was assigned to features of airway pathology observed.
*Peri-airway inflammation 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). Observations are representative of at least two experiments where $n = 5$ or more in each case.
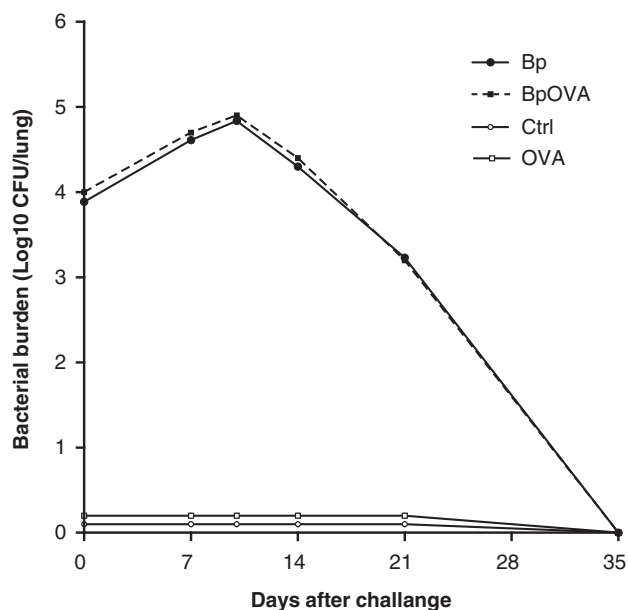
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Fig. 1. Course of *Bordetella pertussis* infection in experimental and control (Ctrl) mice. Groups of mice were killed at intervals after challenge and the number of viable bacteria estimated by performing colony counts on individual lung homogenates. Results are representative from two experiments and are presented as mean CFU in the lungs determined individually per group from four mice at each time point. Data for Ctrl and ovalbumin (OVA) groups have been offset from zero for clarity.

Bordetella pertussis *suppresses ovalbumin-specific humoral immune responses*

Although OVA-induced sensitization does not impair Th1 clearance of *B. pertussis,* we wanted to examine the influence of *B. pertussis* infection on responses associated with OVA Th2 sensitization. OVA-specific IgG was not detected from mice infected with *B. pertussis* only; similarly *B. pertussis*-specific IgG could not be detected in OVA-sensitized animals, suggesting no significant cross-reaction between the two immunogens occurred (Figs 2a and b). An analysis of antibody subclasses revealed that infection induced greater serum titres of *B. pertussis*-specific IgG2a than IgG1 (Figs 2a and b), consistent with our previous findings [19]. Sensitization of infected mice with OVA (BpOVA) did not significantly alter this profile. OVA sensitization in the absence of infection resulted in strong antibody responses almost exclusively of the IgG1 subclass, consistent with a Th2 model of priming. In contrast to the minimal influence of OVA on the response to infection (Fig. 2a), bacterial *B. pertussis* infection suppressed or modulated immunity to OVA. For example, OVA-specific IgG1 titres were significantly lower ($P<0.001$) in the combined group (Fig. 2b). Suppression was not confined to IgG subclasses. Total serum and OVA-specific IgE was also significantly reduced ($P<0.001$) between the OVA and the Bp/OVA groups (Figs 2c and d), supporting a role for *B. pertussis* in suppressing or regulating the immune response to antigen exposure during infection.

Ovalbumin sensitization during Bordetella pertussis *infection enhances airway Interleukin-10 and Interleukin-13*

In order to dissect the nature of *B. pertussis* suppression, cell-mediated immune responses were examined from spleen

cultures. As previously reported [22], *B. pertussis* infection induced strong splenic proliferative and IFN-γ responses but very little IL-5 (Figs 3a–c). This was consistent with the observed protection (Fig. 1) and the antibody subclass data (Fig. 2). Again, IFN-γ was not significantly reduced by OVA sensitization (Fig. 3c). As expected, OVA sensitization alone induced significant IL-5 but no IFN-γ (Figs 3a and c). However *B. pertussis* did influence cytokine responses to OVA. Reduced levels of IL-5 were detected in the combination group suggesting that *B. pertussis* suppressed Th2 responses to OVA, mirroring the reduction in titre of specific antibody detected. Interestingly *B. pertussis* infection did induce specific IL-10 as well as IL-13 responses (Figs 3b and d). While OVA sensitization had little other effect on the immune response induced by infection, it significantly enhanced the levels of IL-10 and IL-13 ($P<0.01$ and <0.01, respectively) produced in response to the bacterium (Figs 3b and d).

To extend these findings, we examined the levels of cytokines present in BALF. OVA sensitization, but not infection, induced IL-4 (Fig. 4a); however, prior infection with *B. pertussis* suppressed this. *B. pertussis* infection induced local IFN-γ, which was not reduced by OVA sensitization (Fig. 4d). Interestingly, while OVA sensitization induced IL-10 and IL-13 detectable in BALF, this was significantly ($P<0.01$ and <0.01, respectively) enhanced if sensitization followed infection (Figs 4b and c).

A T-helper 1 infection in the respiratory tract does not protect, but exacerbates the allergic asthmatic response

It has been proposed that prior Th1 responses to bacterial infections protect against allergic disease by dampening the activity of Th2 effector cells. It might also be predicted that the suppressive or modulatory effects of *B. pertussis* outlined above would protect against Th2-driven pathology. We used whole-body plethysmography in order to measure airway reactivity in mice infected with *B. pertussis* prior to OVA sensitization in comparison with controls (Fig. 5). We found that contrary to the above premise, a Th1 response induced by infection in the respiratory tract did not protect against bronchial hyper-reactivity but rather exacerbated the allergic asthmatic response. Statistical analysis using two-way ANOVA showed that mice sensitized to OVA following *B. pertussis* infection displayed significantly greater bronchial hyper-reactivity compared with OVA sensitized alone ($P<0.001$) (Fig. 5). This demonstrates that infection with *B. pertussis* of the respiratory tract prior to sensitization results in increased airway reactivity and exacerbates the allergic response.

B. pertussis infection also modulated the quality of the inflammatory influx to the respiratory tract. There was a marked reduction in eosinophil numbers observed in BpOVA compared with OVA-sensitized airways (Table 3). Histological examination of lung tissue showed that pathological changes were largely focussed on bronchioles and adjacent peribronchiolar blood vessels with varying degrees of airway wall inflammation and smooth muscle hypertrophy accompanied by varying degrees of epithelial hyperplasia and mucous metaplasia (Fig. 6). A semi-quantitative histopathological scoring system was used to facilitate comparisons between groups (Table 2). Minimal changes were observed in
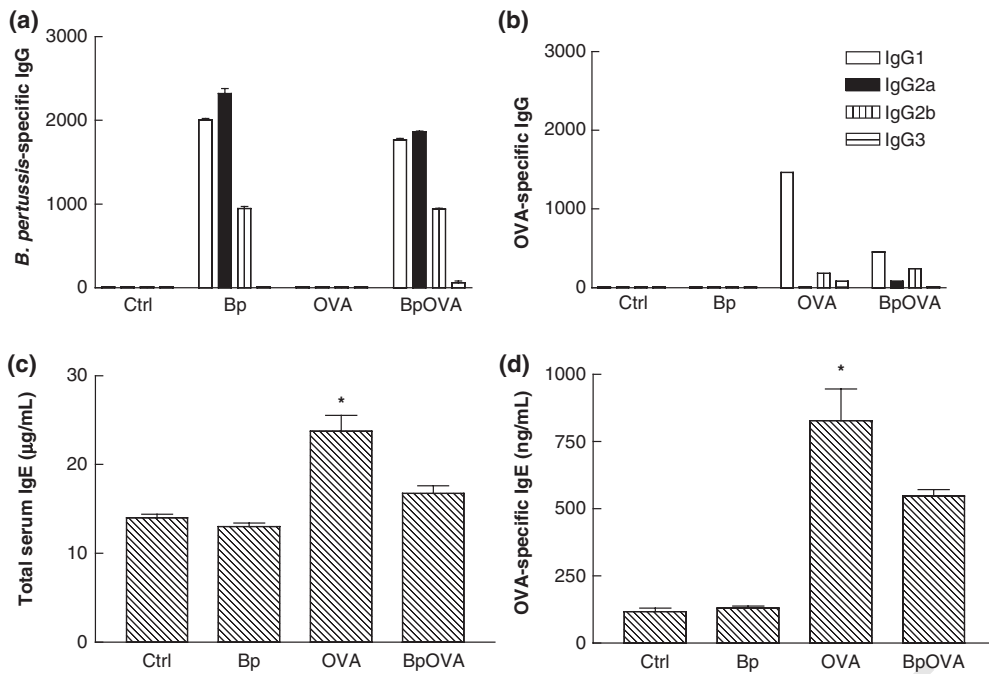
Fig. 2. Serum and ovalbumin (OVA)-specific IgE and IgG subclasses elicited by bacterial infection and allergic sensitization. (a) *Bordetella pertussis* and (b) OVA-specific serum antibody responses by IgG subclass elicited in control (Ctrl), infected (Bp), sensitized (OVA) mice, or in mice infected with *B. pertussis* prior to sensitization (BpOVA); expressed as geometric mean titre of antibody ($\pm$ SE). (c) Total IgE and (d) OVA-specific IgE present in sera from each experimental group expressed as μg/mL or ng/mL, respectively. OVA-specific IgE (ng/mL) is a relative measure determined by adaptation of a standard IgE ELISA. Results are representative of three experiments from four animals performed independently in triplicate. *Statistical significance, $P < 0.001$ compared with BpOVA-treated group.
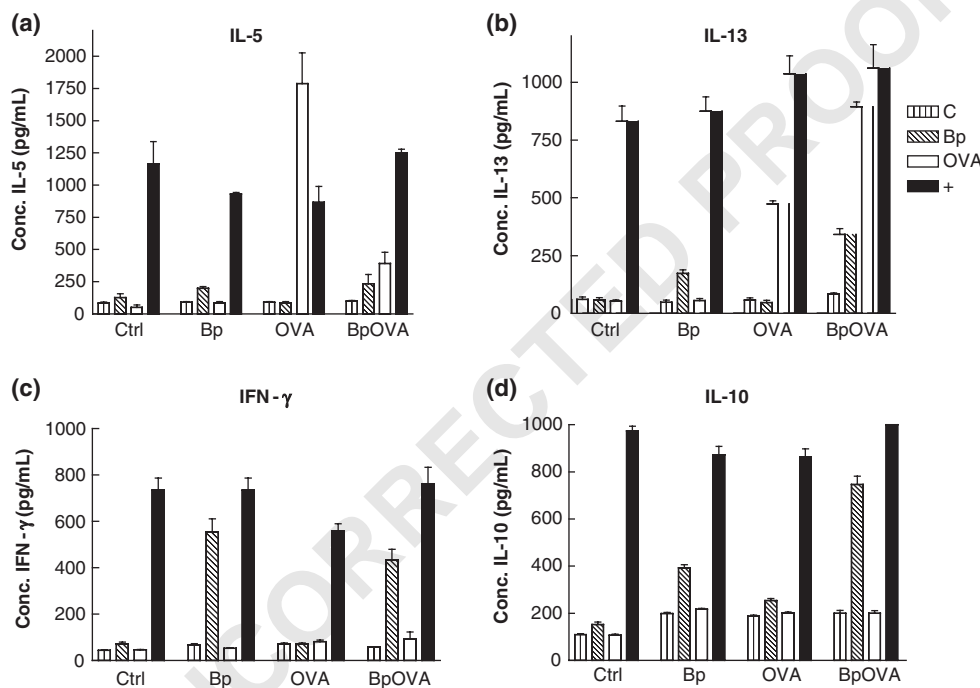

Fig. 3. Cell-mediated immune responses from spleen, elicited by bacterial infection and allergic sensitization. IL-5 (a), IL-13 (b), IFN-γ (c), and IL-10 (d) responses from spleen cell cultures stimulated with medium alone (negative control, vertical shading), heat inactivated *Bordetella pertussis* sonicate at 1×10^4 CFU/mL (hatched bar), ovalbumin (OVA) (20 mg/mL) (open bar) or Con A (positive control, black bar). Responses are representative of triplicate experiments each assay was performed in triplicate on individual samples from four mice per group and results are expressed as mean ($\pm$ SE).

mice challenged with *B. pertussis* only, as infection had resolved by the 37 days time-point. OVA sensitization resulted in typical inflammation of airway walls with infiltration of eosinophils, neutrophils and lymphocytes (Fig. 6c). However, BpOVA mice displayed more severe airway wall inflammation with a greater degree of both epithelial hyperplasia and mucous metaplasia than Bp, OVA or control mice (Fig. 6d). Given that airway resistance (R) is inversely proportional to the fourth power of the airway luminal radius (r^4) even minimal narrowing caused by processes such as transmural inflammation, epithelial hyper-

plasia or mucus exudation subsequent to epithelial mucous metaplasia can profoundly increase pulmonary resistance as indicated by plethysmography (Fig. 5).

Discussion

The present study demonstrates that prior infection with viable *B. pertussis* modulates the immune response induced by allergen sensitization. Infection suppresses antibody and cell-
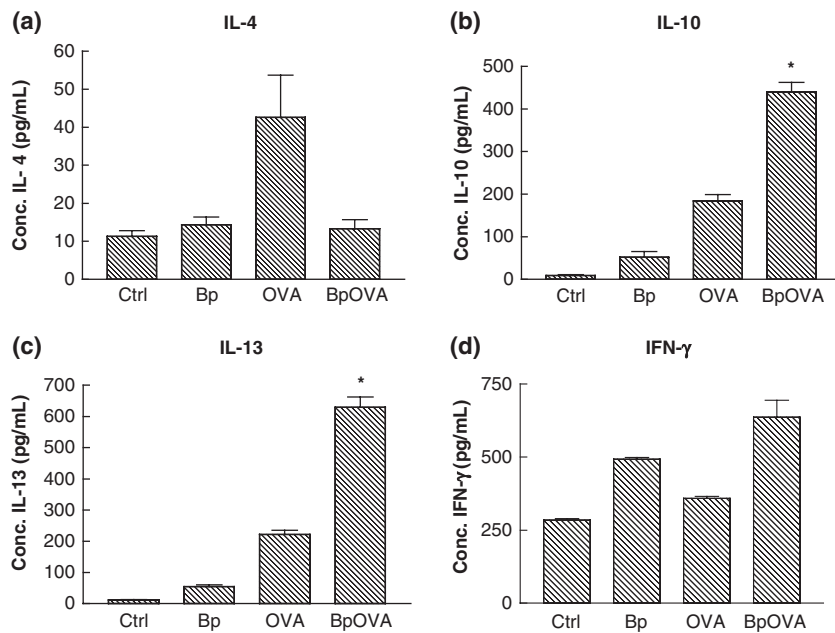
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(a)

IL-4

(b)

IL-10

(c)

IL-13

(d)

IFN-γ

Fig. 4. *Bordetella pertussis* infection modulates the local cytokine response to allergen. Groups of mice were treated as described in the legend to Fig. 1. At 37 days, diluted bronchoalveolar lavage was pooled from five mice per group and concentrations of IL-4 (a), IL-10 (b), IL-13 (c) and IFN-γ (d) responses determined by enzyme immunoassay. Results are representative of triplicate experiments, assays were performed in triplicate, values are expressed as mean ± SE between experimental and control groups. $*P < 0.01$
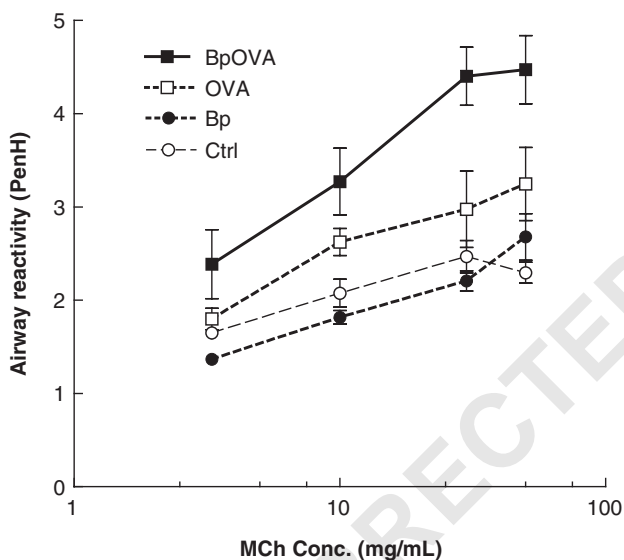

Fig. 5. *Bordetella pertussis* exacerbates bronchial hyper-responsiveness to sensitizing antigen. Groups of mice were treated as described in the legend to Fig. 1. At 37 days, airway hyper-reactivity in response to increasing concentrations of inhaled methacholine (MCh) was measured by whole-body plethysmography. Results are representative of three experiments (*n* = 4 per group) and values are expressed as mean enhanced pause (PenH) ± SE.

mediated responses against OVA locally and systemically, while enhancing the levels of the regulatory cytokine IL-10 and IL-13. Despite this modulation, *B. pertussis* exacerbates OVA-induced airway pathology, leading to the development of more pronounced allergen-induced airway inflammation as well as the induction of enhanced AHR.

Asthma is a chronic inflammatory disease of the airways, the prevalence of which has increased substantially in recent decades [24, 25]. The explanation that has attracted most attention is the hygiene hypothesis, which suggests that the increase in allergic disease is caused by a cleaner environment

and fewer childhood infections [26]. The goal of this study was to test the hypothesis that a powerful Th1-mediated infection such as *B. pertussis* would diminish or protect in a murine model of allergic asthma. Zuany-Amorim et al. [27] used a similar model, but employed heat-killed *Mycobacterium vaccae* that was effective in blocking allergic inflammation but by a mechanism independent of IFN-γ. The same authors went on to find that mycobacteria induce IL-10-producing regulatory T cells. Intriguing recent data from McGuirk et al. [15] have shown that the filamentous haemagluttinin component of *B. pertussis* behaves in a similar way. Our observation of IL-10 in lavage fluid and following *in vitro* stimulation of spleen cells supports the latter finding. In the case of the protection generated by mycobacterial exposure, it is proposed that IL-10 has an essential role in modulating the immune system by inducing a shift from an allergen-specific Th2 response [28]. In the present study, we observe a similar modulation but this does not result in protection against airway hyper-reactivity, implying that although the restoration of a putative balance between Th1 and Th2 is an attractive theory, it is unlikely to provide a universal explanation of the pathogenesis of asthma.

Studies with Th1 and Th2 cells in diabetes mellitus and autoimmune encephalomyelitis indicate that cross-regulation does not always operate and in some instances can be unexpectedly harmful [29]. For example, Genain et al. [30] showed in a model of multiple sclerosis that a shift in cytokine production from a Th1 to a Th2 pattern increased concentrations of pathogenic autoantibodies and in some instances exacerbated autoimmune disease. Pakala et al. [31] also showed that immune deviation towards a Th2 response did not reduce, but rather exacerbated pathology and disease. In the present study, the Th2-associated effect of OVA sensitization had little influence on the Th1-mediated clearance of a bacterial infection of the airways. In contrast, *B. pertussis* infection suppresses IgG and IgE responses associated with OVA sensitization. This provides compelling

Table 3. Leucocytes present in BAL fluid

Group	Total leucocytes ($\times 10^4$)	Eosinophils ($\times 10^4$)	Macrophages ($\times 10^4$)	Lymphocytes ($\times 10^4$)	Neutrophils ($\times 10^4$)
Control	5.0 ± 0.5	<0.005	3.9 ± 2.0	0.2 ± 0.1	<0.005
Bp	5.0 ± 0.4	<0.005	4.6 ± 0.9	1.1 ± 0.7	0.2 ± 0.1
OVA	8.0 ± 1.7	4.1 ± 0.9	2.9 ± 1.2	0.6 ± 0.1	0.5 ± 0.2
BpOVA	5.0 ± 0.8	2.4 ± 0.8*	2.1 ± 0.8	0.4 ± 0.1	0.5 ± 0.3

Groups of mice were killed (37 days), bronchoalveolar lavage (BAL) cells were collected, counted, and cytospin preparations stained to obtain the differential leucocyte count. Data represent mean ($\pm$ SE) values; $n = 5$–8 mice per experiment.
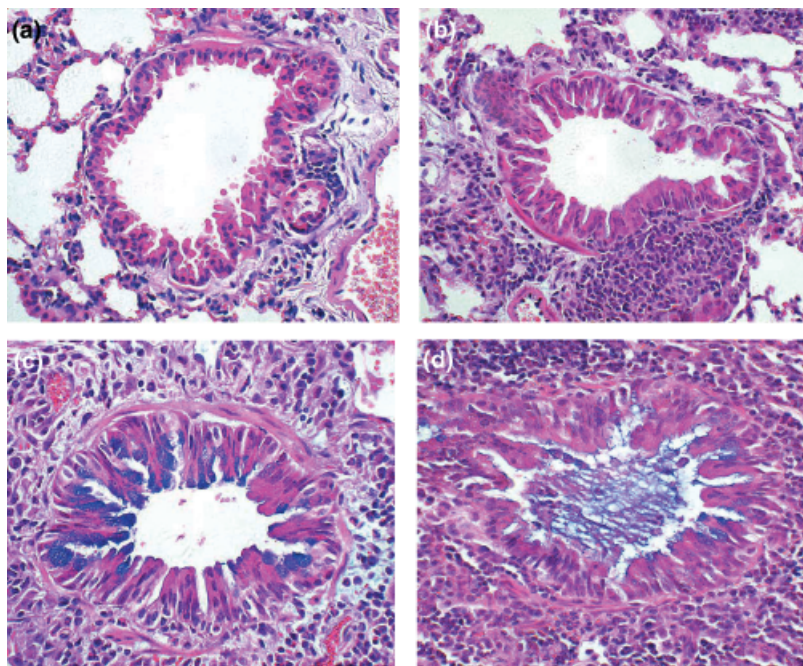*$P < 0.05$ vs. OVA group.

Fig. 6. *Bordetella pertussis* increases the severity of airway pathology to sensitizing antigen. Photomicrographs a–d illustrate representative morphological changes in transverse sections of bronchioles at 37 days ($n = 5$ per group). (a) Control group; (b) *B. pertussis* infected group (mild mural and peri-airway inflammation evident); (c) ovalbumin (OVA)-sensitized group illustrating moderate mural and peri-airway inflammation with accompanying moderate mucus metaplasia (blue staining goblet cells) and hyperplasia of epithelium; (d) 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 (P). All sections stained with a combined Discombes/Alcian blue stain. Original magnification $\times 400$.

evidence that *B. pertussis* powerfully modulates the response to the third party antigens. These data are again consistent with studies in which *M. vaccae* injection to OVA-immunized mice significantly suppressed serum IgE [32] but are in direct contrast to Zuany-Amorim et al. [27] in which they failed to see any effect on IgG2a levels or serum IgE. Although *M. vaccae* and *B. pertussis* provoke similar immune responses, radically different pathologies are induced. The resolution of this paradox may lie in the different aetiologies of both *M. vaccae* and *B. pertussis*. Respiratory challenge by *M. vaccae* causes minimal epithelial damage and limited airway pathology. In stark contrast *B. pertussis* causes significant damage to the epithelial lining of the airways [33]. This is mediated through a bacterial virulence factor called tracheal cytotoxin, which induces IL-1β and reactive nitrogen intermediates that bring about ciliostasis, followed by airway remodelling [34, 35]. Thus, while *M. vaccae* and *B. pertussis* induce very similar immune responses, it may be that during *B. pertussis* infection there is a combination of epithelial damage, IFN-γ, IL-10 and IL-13 production that has profound influences on the epithelium and its local environment, which serves to exacerbate rather than protect against asthma.

In asthma, the bronchial epithelium is highly abnormal with structural changes involving separation of columnar cells from their basal attachments and functional changes resulting in increased expression and release of pro-inflammatory cytokines and growth factors [36]. Beneath the damaged and dysfunctional epithelium lie increased numbers of subepithelial myofibroblasts that deposit interstitial collagens causing thickening of the basement membrane [4]. These effects are seen in the present study in the OVA group (Fig. 6c) but are more prominent in the combination group that received *B. pertussis* prior to OVA sensitization (Fig. 6d). Evidence suggests that the epithelium should not be viewed in isolation, as airway smooth muscle cells contribute to the perpetuation of airway inflammation and airway remodelling [37]. Grunstein et al. [38] has suggested that IL-10 may play an important role in allergic asthma by acting directly on the sensitized airway smooth muscle itself. The present study suggests that although IL-10 may be a regulatory cytokine, it has broader functions that may not always protect against inflammatory disease, particularly if there has been damage to the superficial epithelium. Lee et al. [39] have also demonstrated that IL-10 induces IL-13 production *in vivo* and that this induction was responsible for the mucus, but not the inflammatory and fibrotic effects of IL-10. This would appear to be consistent with our own data (Figs 3b and d, 4b and c) where we see an increase in both IL-10 and IL-13 at the systemic and local level. The decrease in eosinophilia (Table

2) observed in the BpOVA group is most likely because of the modulation of IL-5 responses in these mice (Fig. 3a). IL-5 is a cytokine necessary for the regulation of eosinophil growth, differentiation, activation and survival and plays a critical role in the recruitment of eosinophils to the lung [40]. Similar responses were observed by Wu et al. [41] using murine cytomegalovirus infection in conjunction with the murine model of OVA-induced allergic airway disease. Previous studies in humans have demonstrated that IL-13 mRNA and protein levels are elevated in the lungs of atopic and non-atopic asthmatics [42, 43], suggesting that overproduction of IL-13 may predispose toward the development of both types of asthma [43]. Walter et al. [44] showed that an OVA-specific Th2 line generated from IL-13$^{-/-}$ mice, which produced high levels of IL-4 and IL-5, but not IL-13, failed to induce AHR, demonstrating the essential role of IL-13 in the development of AHR. IL-13 may also increase AHR directly. IL-13 induces smooth muscle proliferation *in vitro* [45] and can aid contractions of tracheal smooth muscle [46]. Airway smooth muscle cells have also been shown to express IL-13 receptors, including both components of the IL-13R complex [47]. Amrani et al. [48] have suggested that increased levels of IFN-γ in asthmatic individuals may promote AHR and exacerbate asthma by directly modulating contractile responses. OVA sensitization of infected mice in the combination group induced significant levels of IFN-γ detectable in the BALF (Fig. 4d), suggesting that IFN-γ may contribute to the observed exacerbation of pathology (Fig. 6d). This correlates well with studies in humans where increased IFN-γ was seen in asthmatic patients compared with normal subjects [49, 50]. It has also been shown that AHR can manifest independently of pulmonary inflammation [51] although this was not seen in this study. Respiratory syncytial virus (RSV), commonly associated with lower lung infections in infancy is also known to exacerbate asthma [52, 53]. Matsuse et al. [54] found that the effect of RSV infection varies depending upon the inflammatory context of the lung. Both primary and recurrent RSV infections augment ongoing allergic inflammation, however, in the absence of allergic sensitization, the effects of RSV were transient. Comparable with our own study with *B. pertussis,* Lukacs et al. [55] found that an initial RSV infection can initiate a pro-asthmatic environment that promotes a more severe asthmatic response, even when the allergic response is initiated at a time after clearance of the RSV-induced reactions. Likewise, OVA sensitization of mice infected intravenously with *Listeria monocytogenes,* converts a non-lethal infection to a lethal disease. In that model IL-10 plays a critical role in the suppression of anti-listerial resistance in OVA-immunized mice [56]. Other groups have shown that heat-killed *Mycobacterium bovis*-BCG suppressed the development of OVA-induced airway eosinophila [57, 58]. In many of these studies the timing of sensitization and infection influence the outcome, however, the exacerbation mediated by *B. pertussis* appears to be persistent and long lived (data not shown). Taken together, these studies suggest that pathogens may induce an altered cytokine environment in the context of airway remodelling that ultimately provides for an exacerbated asthmatic-type response [55].

Immune counter-regulation based on Th1/Th2 mechanisms or even regulatory T cells secreting IL-10 have been suggested as mechanisms that could protect against asthma. We show here that this response must be viewed in the broader context of the host–pathogen interaction. *B. pertussis* fulfils many of the criteria for a potent immunomodulator that should protect against asthma. However, the potent influence on airway remodelling during infection means that this bacterium has the opposite effect. This study clearly shows that while the hygiene hypothesis is an attractive theory, it is of limited validity as currently stated. Furthermore, our results raise concerns regarding immunomodulatory therapies aimed at the conversion of Th2-dominated allergic inflammatory responses into Th1-dominated responses based on counter-regulation that may be of limited efficacy, or even harmful.

Acknowledgements

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