

The effects of *Mycobacteria vaccae* derivative on allergen-specific responses in children with atopic dermatitis

J. A. Dunstan,* S. Brothers,[†] J. Bauer,*
M. Hodder,* M. M. Jaksic,[†]
M. I. Asher[†] and S. L. Prescott*

*University of Western Australia, School of
Paediatrics and Child Health Research, Perth,
WA, Australia, and [†]Department of Paediatrics:
Child and Youth Health, Faculty of Medical and
Health Sciences, The University of Auckland,
Auckland, New Zealand

Summary

The capacity of microbial products to inhibit allergic inflammation make them logical candidates for novel therapies in allergic diseases such as atopic dermatitis. To assess the effects of intradermal *Mycobacterium vaccae* derivative on allergen-specific immune responses in children with moderate to severe atopic dermatitis. Peripheral blood mononuclear cells were isolated from children aged 5–16 years who received intradermal injections of *M. vaccae* derivative AVACTM ($n = 26$) or placebo ($n = 34$) three times at 2-weekly intervals, weeks 0, 2 and 4. Cytokine [interleukin (IL)-13, interferon (IFN)- γ and IL-10] responses to allergen [house dust mite (HDM)], mitogen [phytohaemagglutinin (PHA)], Staphylococcal enterotoxin B (SEB) and Toll-like receptor (TLR) ligands were assessed. At week 8 (1 month after all injections given) children in the AVAC group showed a significant increase in IL-10 ($P = 0.009$), T helper type 1 (Th1) IFN- γ ($P = 0.017$) and Th2 IL-13 ($P = 0.004$) responses to HDM compared with baseline (week 0). There were no significant changes in any cytokine production in the placebo. HDM-specific IL-10 responses remained significantly higher ($P = 0.014$) than at baseline in the AVAC group by week 12; however, the HDM-specific IL-13 and IFN- γ responses were no longer significantly different from baseline. IL-13 ($r = 0.46$, $P < 0.001$) and IL-10 ($r = 0.27$, $P = 0.044$) responses to HDM were correlated with total immunoglobulin E but not with disease severity. There were no effects of AVAC on mitogen, SEB, TLR-2- or TLR-4-mediated responses. This *M. vaccae* derivative appeared to modulate responses to HDM selectively, suggesting the capacity for *in vivo* effects on allergen-specific immune responses.

Keywords: atopic dermatitis, BCG, children, cytokines, mycobacteria

Accepted for publication 27 January 2011

Correspondence: S. Prescott, Princess Margaret
Hospital, University of Western Australia,
School of Pediatrics and Child Health Research,
PO Box D184, Perth, WA 6001, Australia.
E-mail: sprescott@meddent.uwa.edu.au

Introduction

The powerful immunomodulatory effects of microbial products have generated intense interest in their role in inhibiting the inappropriate T helper type 2 (Th2) responses that characterize allergic disease. Although bacterial 'vaccines' were examined for the treatment of conditions such as asthma more than 40 years ago [1], there was renewed interest in this approach with the advent of the hygiene hypothesis [2,3]. Population studies have shown a strong inverse association between delayed hypersensitivity to *Mycobacterium tuberculosis* vaccine [bacillus Calmette–Guérin (BCG)] and atopy, leading to speculation that exposure to *M. tuberculosis* may inhibit allergic disease by immune modification [4]. Although this has not been seen in other populations [5,6], a

number of animal models have shown consistently that mycobacterial antigens inhibit allergic immune responses [7–10]. At least some of this effect appears to be due to the induction of strong Th1 responses [7–9]. This is in keeping with the early models of the hygiene hypothesis, which proposed that inadequate microbial-driven Th1 maturation leads to Th1/Th2 imbalance. As this paradigm expanded to recognize the central role of regulatory pathways in the suppression of inappropriate responses, it became clear that microbial exposure is also critical for inducing regulatory cytokines [interleukin (IL)-10 and transforming growth factor (TGF)- β] in groups of specialized regulatory cells [10]. It has been shown since that the allergy suppressive effects of *Mycobacterium vaccae* in mice are due to the induction of IL-10 and TGF- β producing regulatory T cells [11].

In one of the first human studies to examine the effects of heat-killed *M. vaccae*, Arkwright and David showed a significant improvement in the disease of children with atopic dermatitis [12]. Subsequent assessment of the underlying immune mechanisms in the same population was limited by the small number of children with blood samples ($n = 12$) and the inability to detect allergen-specific Th1 or Th2 responses [13]. Although the authors saw no effect of the *M. vaccae* on TGF- β production, IL-10 responses were not examined [13]. The purpose of the current study was to examine more fully the effects of *M. vaccae* on allergen-specific immune responses, with a specific focus on IL-10.

Since the first study by Arkwright and David [12] there have been several larger randomized controlled trials to examine the effects of *M. vaccae* derivatives on atopic dermatitis [14,15], including our own study [14]. Although the clinical effects in both studies have been disappointing, with no difference in the improvement in the severity of eczema in the active compared with the placebo group [14,15], this does not exclude immunomodulatory effects on allergen-specific responses. We have taken the opportunity to investigate further in our study population [14].

Methods

Details of the original randomized controlled trial have been published previously [14]. The study was designed to evaluate the safety, efficacy and tolerability of a delipidated, deglycolipidated and arabinogalactan-depleted derivative of heat-killed *M. vaccae* (AVACTM, Genesis Research and Development Corporation Limited, Auckland, New Zealand) in children with moderate to severe atopic dermatitis (AD).

Summary of parent study population

Briefly, 129 children aged 5–16 years with moderate to severe atopic dermatitis were recruited between October 2003 and June 2004. Atopic dermatitis was defined by the UK Working Party's diagnostic criteria for atopic dermatitis [16] and severity was defined by the Rajka and Langeland criteria [17].

A total of 125 children completed the study. Children were required to be in good general health and were ineligible if they had a history of anaphylaxis or had used non-standard atopic dermatitis medications [14]. Retention of participants in the study was high: only four participants withdrew from the study. However, of the 125 who completed the study, collection for peripheral blood mononuclear cells (PBMC) isolation was complete at all three time-points in only 60 patients. Contributory factors included: laboratory logistical issues, difficulty with venepunctures in some children, and some participants did not consent to this aspect of the study. None of these factors are likely to have introduced bias in the participants from whom blood was analysed in this study.

Parents of all participants gave written informed consent prior to the study and the approval to conduct the study was obtained from the Auckland Ethics Committee.

The intervention

Participants were randomized using a computerized number generator to receive a total of three 0.05 ml intradermal injections of either a delipidated, deglycolipidated and arabinogalactan-depleted derivative of heat-killed *M. vaccae* (12.5 μ g AVACTM; Genesis Research and Development Corporation Limited, Auckland, New Zealand) in phosphate-buffered saline (PBS) or placebo (PBS alone) at 2-weekly intervals at weeks 0, 2 and 4.

Clinical assessments and previously reported clinical findings

Clinical evaluation of disease severity and extent were performed at baseline (week 0), 2 and 4 weeks and at 2, 3 and 6 months. Severity was assessed using a six-area six-sign atopic dermatitis [(SASSAD) score [18]. Blood for immunoglobulin (Ig)E analysis was collected at the screening visit at baseline (within 2 weeks before the first injection of *M. vaccae* or placebo at week 0), 8 and 12 weeks. Serum IgE levels were analysed by fluoroenzyme immunoassay [Phadia ImmunoCAP 250 for house dust mite (HDM)-specific IgE and UniCAP 100 system for total IgE; Phadia, Uppsala, Sweden]. The core study findings have been reported elsewhere [14], and in summary, *M. vaccae* did not improve the severity or extent of atopic dermatitis at 3 or 6 months. However, IgE levels at 2 months had decreased in the AVAC group compared to placebo, although this did not reach statistical significance. By 3 months the IgE levels had returned to baseline.

Samples collected

The purpose of the present study was to assess effects of AVAC on mononuclear cell responses, particularly allergen-specific responses. Peripheral blood samples for cell culture were collected into heparinized tubes at baseline (within 2 weeks before the first injection of *M. vaccae* or placebo at week 0), 8 and 12 weeks. Samples were available at all three time-points. PBMC were isolated using lymphoprep (Nycomed Pharma, Oslo, Norway) gradient centrifugation and were then cryopreserved for subsequent batch analysis of cytokine responses to various stimuli.

Lymphocyte responses to allergen, microbial and mitogen stimulation

For cytokine analysis 2×10^6 PBMC/l were cultured in AIM V (Gibco, Life Technology, Paisley, UK) serum-free medium [19] for 48 h without stimuli (for spontaneous production of cytokines) or with whole HDM extract 20 μ g/ml (CSL, Melbourne, Australia), *Staphylococcus enterotoxin B* (SEB)

200 ng/ml and phytohaemagglutinin (PHA) mitogen 1 µg/ml (HA16; Murex, Biotech Ltd, Warminster, UK). For PHA and SEB stimulation 10⁹ PBMC /l were used.

Assessment of Toll-like receptor (TLR) function

As mycobacteria are known to activate a number of innate immune pathways, including Toll-like receptor 2 (TLR-2) and TLR-4 [20,21], we assessed the function of these pathways. PBMC were cultured alone or with optimized doses of lipoteichoic acid (20 µg, 1 µg/ml; Invivogen, San Diego, CA, USA) as a TLR-2 ligand and lipopolysaccharide (LPS) (*Escherichia coli* LPS; Sigma, Castle Hill, Australia; 10 ng/ml) as a TLR-4 ligand, as described previously [22].

Supernatants were analysed for IL-12 using an in-house enzyme-linked immunosorbent assay (ELISA) technique as described previously [23]. The limit of detection was 3 pg/ml. IL-6, IL-10, IL-13, TNF-α and IFN-γ were quantified by an in-house ELISA using a time-resolved fluorometry (TRF) detection system (DELPHIA; PerkinElmer, Life Sciences, Waltham, MA, USA), as described previously [24]. Briefly, the ELISA method was followed using paired antibodies (Pharmingen, Sydney, NSW, Australia) and the biotinylated antibody was detected using Europium-labelled streptavidin and fluorescence was quantified using a fluorometer (WALLAC VICTOR², PerkinElmer, Life Sciences). Standard curves, generated using serial dilutions of recombinant human IL-6, IL-10, IL-13, TNF-α or IFN-γ (Pharmingen), were linear between 5 and 30 000 pg/ml. Cytokine data were expressed as the difference between the stimulated and the unstimulated (control) cultures (pg/ml) and described as 'detected' (>5 pg/ml) or 'not detected'.

Statistical analysis

Where the data could be normalized with logarithmic transformation they are displayed as geometric mean and 95% confidence intervals (CI), and groups compared by Student's *t*-tests. For within-group changes the paired Student's *t*-test was used. Data that did not achieve log-normal distribution

were compared using non-parametric tests (Mann–Whitney *U*-test for between-group comparisons and Wilcoxon's test for within-group changes). For measures of correlation, parametric tests (Pearson's correlation) were used where both variables were distributed normally. Non-parametric correlations were calculated (Spearman's Rho) where one or both variables were not distributed normally. All statistical analyses were performed using SPSS software (version 16.0 for Macintosh). A *P*-value < 0.05 was considered statistically significant for all analyses.

Results

Baseline characteristics

Sixty children with blood samples available at all three time-points were included in this analysis, including 26 in the AVAC group and 34 in the placebo group. The characteristics of these groups are shown in Table 1. For the main population, there were no significant differences in age, gender, initial SASSAD score for severity of disease or the extent of disease in the AVAC compared with the placebo group. However, IgE levels were significantly higher in the AVAC group compared to placebo (*P* = 0.04) (Table 1). Forty-six of the 60 children were sensitized to HDM antigen and this proportion was not different between the two groups.

Effects of AVAC on allergen-specific cytokine responses

The most notable effects of AVAC were on HDM allergen-specific immune responses, as shown in Fig. 1. At week 8, 4 weeks after the final injection, there was significant increase in IL-10 (*P* = 0.009), Th1 IFN-γ (*P* = 0.017) and Th2 IL-13 (*P* = 0.004) responses to HDM in the AVAC group (compared to levels prior to receiving AVAC). In contrast, there were no changes in the placebo group. Cytokine responses also tended to be higher in the AVAC group compared with the placebo group, although this only approached statistical significance for IL-13 (*P* = 0.06). To allow for any differences

Table 1. Characteristics of the population at baseline.

Characteristic	AVAC	Placebo	<i>P</i> -value
Total number of children	26	34	
Age; mean years (+ s.d.)	10.31 ± 3.16	10.53 ± 3.31	0.794
Male (%)	11 (42.3%)	16 (47.1%)	0.714
Female (%)	15 (57.7%)	18 (52.9%)	0.714
Asthma (%)	15 (57.7%)	14 (41.2%)	0.205
Rhinoconjunctivitis (%)	15 (57.7%)	12 (44.4%)	0.084
BSA GM (95% CI)	26.1 (21.1–32.2)	27.6 (22.72–33.57)	0.698
SASSAD GM (95% CI)	27.2 (23–32.2)	29.3 (25.1–34.2)	0.516
Total IgE (GM) (95% CI)	729.5 (381–1397)	287.5 (155–532)	0.04*
House dust mite sIgE-positive	21 (80.8%)	25 (73.5%)	0.511

BSA: body surface area; CI: confidence interval; GM: geometric mean; sIgE serum: immunoglobulin E; SASSAD: six-area, six-sign atopic dermatitis; s.d.: standard deviation.

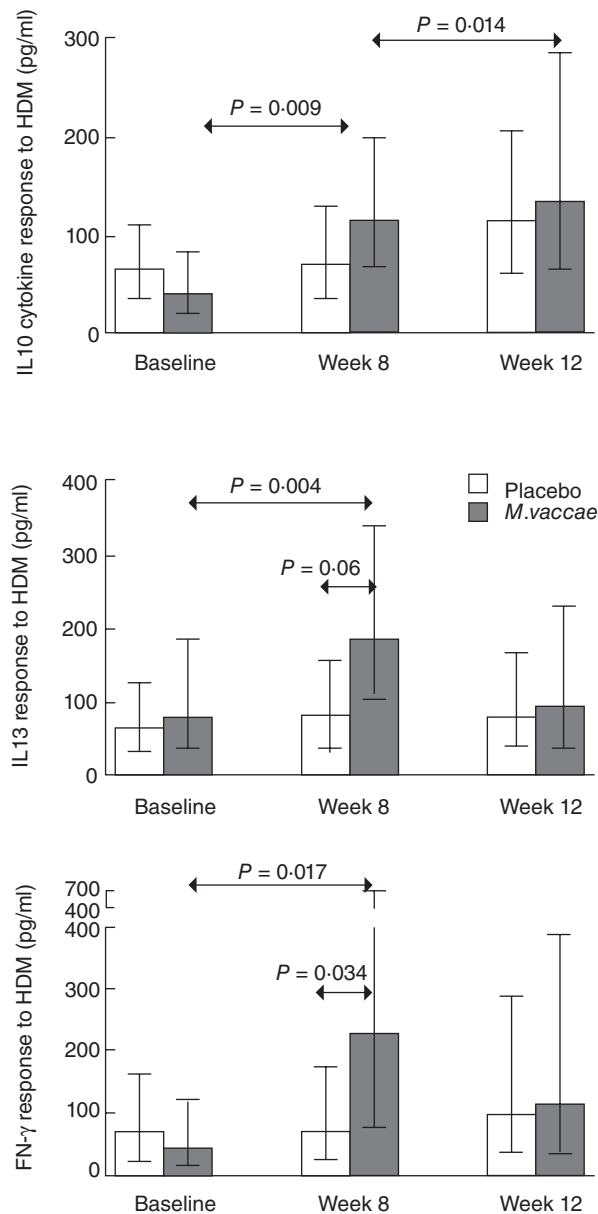


Fig. 1. Effects of AVAC on house dust mite (HDM)-specific cytokine responses: peripheral blood mononuclear cell cytokine responses [interleukin (IL)-10, IL-13 and interferon (IFN)- γ] to HDM stimulation are shown for the AVAC group ($n = 22$; dark-shaded) compared to the placebo group ($n = 32$; light-shaded). Cytokines are expressed as geometric mean $\pm$ 95% confidence interval (pg/ml). Differences between the two groups were determined by independent t -test. Significance levels are shown and $*P < 0.05$ is considered to be a significant difference between the two groups.

in baseline responses we also compared the change in cytokine responses between baseline and week 8 (Fig. 2). The AVAC group showed a statistically significant increase in IL-13 ($P = 0.031$) and IFN- γ ($P = 0.032$) responses to HDM compared with the placebo group, with a similar but non-significant trend for IL-10 responses. These differences

remained significant after adjusting for baseline IgE and HDM sensitization status. Notably, the AVAC-induced increase in IL-13 response to HDM was seen largely in the subgroup of children who were sensitized to HDM ($n = 40$; $P = 0.03$), with no significant changes in the AVAC children who were not HDM-sensitized ($n = 13$) (data not shown). Conversely, the increase in IFN- γ HDM responses following AVAC tended to be in the non-HDM-sensitized subgroup, although this was not statistically significant ($P = 0.08$). There were no differences in the IL-10 HDM responses or in clinical effects according to HDM sensitization status.

By week 12, the increases in Th1 IFN- γ and Th2 IL-13 responses in the AVAC group were no longer evident suggesting that, if anything, this effect was transient. However, the IL-10 responses in the AVAC group remained significantly higher than at baseline ($P = 0.014$) (Fig. 1). However, there were no differences in the cytokine levels between the groups.

Effects of AVAC on polyclonal cytokine responses

Cytokine responses to other stimuli (PHA and SEB) did not change significantly in the AVAC group and there were no significant differences between the groups (Table 2).

Effects of AVAC on innate TLR-mediated cytokine responses

As Mycobacteria are known to activate a number of innate immune pathways, including TLR-2 and TLR-4 [20,21], we

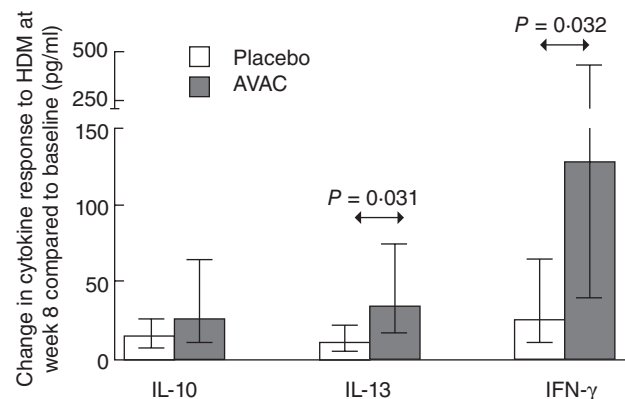


Fig. 2. Changes in cytokine responses to house dust mite (HDM) 8 weeks post-vaccination with *Mycobacterium vaccae*: the change in peripheral blood mononuclear cell cytokine responses [interleukin (IL)-10, IL-13 and interferon (IFN)- γ] to HDM stimulation are shown after vaccination with *M. vaccae* ($n = 22$; dark-shaded) compared to the placebo group ($n = 32$; light-shaded). Change in cytokine responses (week 8 minus week 0 baseline) are expressed as geometric mean $\pm$ 95% confidence interval (pg/ml). Differences between the two groups were determined by independent t -test. Significance levels are shown and $P < 0.05$ is considered to be a significant difference between the two groups.

Table 2. Cytokine responses to phytohaemagglutinin (PHA) and *Staphylococcus enterotoxin B* (SEB) in the *Mycobacterium vaccae*-vaccinated group ($n = 23$) compared to placebo ($n = 32$).

	Geometric mean (95% CI) pg/ml		
	Baseline	Week 8	Week 12
IL-10			
PHA			
Placebo	158.9 (95.9; 263.2)	248.3 (162.2; 367.8)	309.8 (210.7; 455.6)
<i>M. vaccae</i>	250.1 (144.8; 431.9)	312.8 (209.6; 466.8)	303.5 (172.7; 533.6)
SEB			
Placebo	245.2 (156; 385.2)	400.1 (296.7; 539.6)	464.3 (353.9; 609.4)
<i>M. vaccae</i>	331.7 (214; 514)	538.6 (389.9; 744)	399.8 (249.8; 640)
IL-13			
PHA			
Placebo	497.8 (274.5; 902.6)	551.1 (314.8; 964.7)	777.7 (491.7; 1230.3)
<i>M. vaccae</i>	646.8 (330.8; 1264.7)	595.2 (296.5; 1195)	456.5 (194.4; 1071.7)
SEB			
Placebo	477.7 (363.8; 627.2)	504.3 (384.4; 661.6)	575.5 (437.2; 757.7)
<i>M. vaccae</i>	506.3 (364; 704.3)	487 (285.1; 831.9)	391.4 (218.1; 702.6)
IFN-γ			
PHA			
Placebo	1714.4 (884.4; 3323.6)	2003.0 (1239.5; 3236.7)	2200.0 (1150.2; 4208.2)
<i>M. vaccae</i>	2401.9 (1358.3; 4247.1)	3114.1 (1775.4; 5461.6)	2768.9 (1453; 5276.4)
SEB			
Placebo	11322.0 (6051.1; 21186.1)	13207.0 (6651.5; 26221.6)	12928.6 (5626.3; 29708.8)
<i>M. vaccae</i>	15867.1 (10339.2; 24352.7)	24824.8 (17790.1; 34644.7)	17123.4 (10626.4; 27589.8)

There were no significant differences in these responses [interleukin (IL)-10, IL-13 and interferon (IFN)- γ] between the groups. CI: confidence interval.

examined for effects on the function of these pathways. There were no significant changes and no differences between the groups for any in cytokine responses (including TNF- α , IL-6, IL-10 and IL-12) to TLR ligands (Table 3).

Effects of AVAC on IgE levels

As reported previously in the parent study ($n = 129$), the children receiving AVAC showed a transient fall in serum IgE levels at 8 weeks ($P = 0.09$), which was no longer evident at week 12 [14]. Similar changes were seen in the subpopulation examined in this study (Fig. 3). There was no change in the placebo group ($P = 0.381$). The decline in IgE levels in the AVAC group was proportional to the increase in IL-10 ($r = -0.546$, $P = 0.009$), IL-13 ($r = -0.382$, $P = 0.079$) and IFN- γ ($r = -0.662$, $P = 0.001$) responses seen during the same period.

The relationship between cytokine responses, IgE, HDM sensitization and allergic phenotype

Prior to the intervention, IL-13 response to HDM was associated strongly with HDM-specific IgE ($r = 0.484$, $P = 0.002$) as well as the level of total serum IgE ($r = 0.466$, $P < 0.001$). The correlation between IL-13 response to HDM allergen and IgE levels was also evident at weeks 8 and 12 and was not influenced by the treatment.

HDM-stimulated IL-10 responses correlated with HDM-specific IgE ($r = 0.275$, $P = 0.094$) and total IgE levels ($r = 0.265$, $P = 0.051$), although these relationships were not statistically significant. There were no relationships between HDM-stimulated Th1 IFN- γ responses, HDM-specific IgE or total IgE levels.

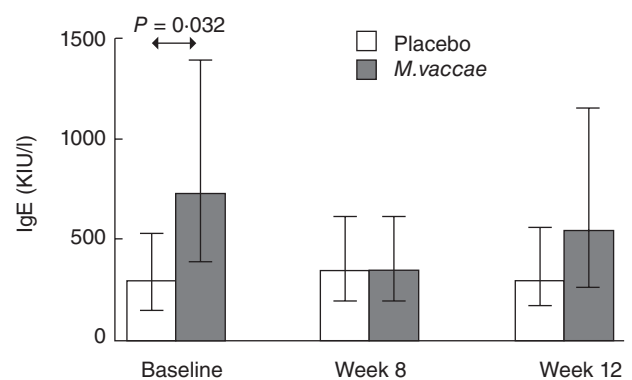


Fig. 3. Immunoglobulin (Ig)E levels: peripheral blood IgE levels at weeks 0, 8 and 12 are shown for the AVAC group ($n = 26$; dark-shaded) compared to the placebo group ($n = 34$; light-shaded). IgE expressed as geometric mean $\pm$ 95% confidence interval (pg/ml). Differences between the two groups were determined by independent t -test. Significance levels are shown and $P < 0.05$ is considered to be a significant difference between the two groups.

Table 3. Cytokine responses to lipopolysaccharide (LPS) and lipotechoic acid (LTA) in the *Mycobacterium vaccae*-vaccinated group ($n = 21$) compared to placebo ($n = 29$).

	Geometric mean (95% CI) pg/ml		
	Baseline	Week 8	Week 12
IL-6			
LPS			
Placebo	16118.1 (11533.4; 22525.4)	17783.0 (8995.7; 35154.2)	24173.2 (17236.8; 33901.0)
<i>M. vaccae</i>	15953.0 (11999.3; 21209.4)	21631.4 (17415.2; 26868.2)	18610.8 (11403.8; 30372.7)
LTA			
Placebo	8275.5 (3432.3; 20008.3)	8881.3 (2914.3; 27065.1)	12158.7 (5110.7; 28296.1)
<i>M. vaccae</i>	14764.8 (11007.1; 19805.2)	20547.6 (15432.0; 27356.2)	16108.5 (10281.5; 25357.8)
IL-10			
LPS			
Placebo	1196.1 (776.6; 1842.2)	1713.5 (1269.5; 2312.5)	1639.1 (1126.5; 2385.1)
<i>M. vaccae</i>	1251.9 (876.3; 1788.3)	1212.0 (591.9; 2481.2)	892.6 (397.1; 2006.4)
LTA			
Placebo	933.5 (438.7; 1986.0)	1389.4 (660.1; 2924.0)	1191.3 (534.8; 2653.9)
<i>M. vaccae</i>	1459.4 (953.4; 2234.1)	1388.4 (641.4; 3005.5)	1540.9 (933.6; 2542.7)
IL-12			
LPS			
Placebo	230.4 (131.0; 405.2)	262.9 (149.7; 461.9)	330.5 (198.6; 549.8)
<i>M. vaccae</i>	227.1 (118.0; 436.9)	232.4 (108.7; 497.0)	269.4 (134.2; 540.7)
LTA			
Placebo	4.8 (0; 8.0)	5.7 (3.4; 9.4)	3.5 (0; 4.4)
<i>M. vaccae</i>	4.7 (3.1; 7.2)	5.6 (3.0; 10.7)	4.5 (3.2; 6.3)
TNF- α			
LPS			
Placebo	2992.0 (2071.4; 4321.7)	3974.3 (2999.8; 5265.3)	3903.0 (2334.8; 6524.4)
<i>M. vaccae</i>	22816.6 (2053.1; 3863.8)	2726.6 (1278.3; 5815.0)	2902.1 (1478.4; 5697.0)
LTA			
Placebo	311.8 (151.2; 643.2)	511.6 (247.6; 1057.1)	377.8 (161.0; 886.2)
<i>M. vaccae</i>	423.5 (205.8; 871.6)	553.6 (271.7; 1128.1)	468.2 (209.0; 1048.6)

There were no significant differences in these responses [interleukin (IL)-6, IL-10, IL-13 and tumour necrosis factor- α] between the groups at any of the time-points. CI: confidence interval.

There were no relationships between HDM-specific cytokine responses and the extent assessed by body surface area (BSA) or severity (SASSAD) of the atopic dermatitis (either at baseline or after the intervention) or with a history of asthma, rhinoconjunctivitis or food allergy. In the HDM-sensitized subgroup there was also no relationship between HDM-specific cytokine responses and BSA or SASSAD. Polyclonal cytokine responses (to PHA and SEB) were generally associated inversely with disease severity, i.e. those with more severe disease had lower IFN- γ ($r = -0.292$, $P = 0.03$) and IL-13 ($r = -0.257$, $P = 0.059$) response to PHA and IL-13 responses to SEB ($r = -0.311$, $P = 0.021$). These relationships were also evident after treatment.

Total IgE levels correlated with extent of disease BSA ($r = 0.344$, $P = 0.007$) but not with severity of disease SASSAD ($r = 0.214$, $P = 0.1$). Total IgE ($r = 0.334$, $P = 0.009$) and HDM-specific IgE ($r = 0.368$, $P = 0.017$) was associated with a history of rhinoconjunctivitis but not with asthma or food allergy.

Discussion

Microbial products offer a major avenue for inhibiting both the development and the expression of allergic disease, by modulating underlying immune responses to allergens. This study explored whether mycobacterial antigens can modify specific immune responses to the ubiquitous HDM allergen in children with atopic dermatitis. We observed transient increases in both Th1 and Th2 responses, but a potentially more sustained increase in allergen-specific IL-10 responses in the AVAC group. This was increased significantly at both 8 and 12 weeks after commencing the three doses of *M. vaccae*, and was not seen in the placebo group. However, despite this significant within-group change, the lack of difference between the groups casts uncertainty over the biological significance of this finding. The mechanism of such an effect is also unclear, as there were no effects of AVAC on the antigen-presenting cell (APC)-driven innate immune responses (to TLR ligands).

It is notable that Hadley *et al.* also saw a transient but marked increase in IFN- γ responses after administration of

M. vaccae in children of a similar age [13], although these responses were to PHA and not to allergen, as in the present study. In that study, levels increased five- to 10-fold 4 weeks after treatments but returned to pretreatment levels by week 12, much the same as in our study. This suggests that the changes we have observed in the *M. vaccae* group are real effects. The significance of the transient effects on both Th1 and Th2 responses to HDM is not clear, but is consistent with 'bystander' activation of T cell clones. Although mycobacteria (BCG) induce a potent Th1-type response to mycobacterial antigens, it has been observed previously to induce both Th1- and Th2-type cytokines in response to unrelated antigens, [25], although some of this effect may be age-dependent. While we could speculate that the parallel increase in IL-10 production may contribute to the subsequent suppression of these responses after 8 weeks, this is not known. The lack of sustained effects on Th1 or Th2 responses does not exclude lasting immunomodulation, as many of the disease suppressive effects of mycobacteria in animals have been independent of effector T cell responses [11,26]. The persistently elevated IL-10 responses also support a more lasting effect on allergen-specific responses, although we do not have data beyond 3 months.

The literature, mainly in animal models, supports an effect of *M. vaccae* on IL-10 pathways, with suppression of allergic airway inflammation by IL-10-producing regulatory T cells [11]. A number of other animal studies have also shown that allergy suppressive effects of mycobacteria (BCG) are associated with increased IL-10 production when given either by injection [27] or orally [28]. Similarly, neonatal BCG has been shown to prevent development of allergic airways disease in mice, with associated increases in allergen-specific IL-10 and TGF- β production and CD4⁺CD25⁺forkhead box P3 (FoxP3)⁺ regulatory cells [29]. *In vitro* studies of human dendritic cells (DC) derived from HDM-sensitized asthmatics also demonstrate that DC incubated with BCG produce more IL-10 and modify T cell responses to HDM, apparently without suppression of Th2 responses [30]. In the present study we cannot confirm the source of IL-10, but it is notable that while the AVAC group showed an increased in allergen-specific IL-10 production, there was no change in IL-10 following innate (APC) stimulation (with either TLR-2 or TLR-4 ligands).

Cutaneous HDM exposure can also exacerbate atopic dermatitis in HDM-sensitized children. However, while HDM-specific cytokine responses (particularly IL-13) were associated with atopy and IgE levels, none of the T cell responses detected (IL-13, IFN- γ or IL-10) was associated with the severity of the atopic dermatitis lesions. Thus, although AVAC has no effect on the severity of atopic dermatitis in the present study, it is possible that any modulation of HDM-specific immune responses following AVAC had other effects, particularly on respiratory symptoms that may be triggered by HDM reactivity. However, there is no evidence that children with inhalant disease (asthma and

allergic rhinitis) responded differently to AVAC (clinically or immunologically) compared with unaffected children. Previous studies of *M. vaccae* in allergen-sensitized asthmatic adults have shown conflicting results, with fewer symptoms, less salbutamol requirement and increased *in vitro* IFN- γ production in one study [31], but no effects in another [32].

Finally, it is likely that the effects of BCG may depend on both genetic factors and the allergic phenotype. In a previous population study Marks *et al.* examined the relationship between neonatal BCG vaccination and subsequent allergic disease and allergen-specific immune responses in 7–14-year-olds [33]. They found that the beneficial effect of BCG vaccination was limited to a subgroup of children with an inherited predisposition to allergic disease, with significantly lower rates of asthma (wheezing with airway hyper-responsiveness) in this subgroup (relative risk, 0.46; 95% CI, 0.22–0.95) [33]. In the present study, as with most studies, there is a degree of heterogeneity in the clinical phenotypes of the children studied, but adjustment for allergic status and IgE levels did not affect the findings significantly.

One of the limitations of our study is that we only had consecutive blood samples on a subgroup of 60 subjects. However, we have no reason to suspect any bias in this subgroup and the generalizability of our results is unlikely to be affected. Furthermore, we have previously detected biologically relevant differences in immune function in similar study sizes [34], and the sample size of the present study is larger than a previous study examining the underlying immune mechanisms of the effect of heat-killed *M. vaccae* in children with atopic dermatitis [13].

The capacity for microbial elements to modulate immune development provides many opportunities to prevent or treat allergic disease. The effects are likely to vary with the kind of microbial product, the route, the dose and the schedule, as well as the age of the host, allergic phenotype and other environmental interactions. Thus, although the specific effects of *M. vaccae* in the amelioration of both atopic dermatitis and asthma have been conflicting, further research remains important. At this stage most of these factors are not well understood, and optimizing conditions for these immunomodulatory microbial products remains a logical approach in advancing the treatment and prevention of allergic disease. This is increasingly imperative, with new evidence of burgeoning rates of disease in populous developing countries in Asia, Africa and South America [35–38], highlighting that allergic disease is fast becoming a global health issue of major importance.

Acknowledgements

We wish to thank Genesis Research and Development Corporation Limited, who developed AVAC, for their support and funding for this research, Dr J. Sinclair for her advice, Dr W. Walker (Middlemore Hospital site lead investigator),

The Centre for Clinical Research and Effective Practice for assistance in conducting the study, and most importantly the participants and their families.

Disclosures

This study was funded by Genesis Research and Development Corporation Limited and AVAC™ is the property of this company. The authors were employed by the University of Western Australia or by The University of Auckland or its subsidiary UniServices. None of the researchers had any financial interest in the outcome of this research.

References

- 1 Barr SE, Brown H, Fuchs M *et al.* A double-blind study of the effects of bacterial vaccine on infective asthma. *J Allergy Clin Immunol* 1965; **36**:47–61.
- 2 Gerrard JW, Geddes CA, Reggin PL, Gerrard CD, Horne S. Serum IgE levels in white and metis communities in Saskatchewan. *Ann Allergy* 1976; **37**:91–100.
- 3 Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989; **299**:1259–60.
- 4 Shirakawa T, Enomoto T, Shimazu S, Hopkin J. Inverse association between tuberculin responses and atopic disorder. *Science* 1997; **272**:77–9.
- 5 Strannegård IL, Larsson LO, Wennergren G, Strannegård Ö. Prevalence of allergy in children in relation to prior BCG vaccination and infection with atypical mycobacteria. *Allergy* 1998; **53**:249–54.
- 6 Alm JS, Lilja G, Pershagen G, Scheynius A. Early BCG vaccination and development of atopy. *Lancet* 1997; **350**:400–03.
- 7 Erb KJ, Holloway JW, Sobeck A, Moll H, Le Gros G. Infection of mice with *Mycobacterium bovis*-bacillus Calmette–Guerin (BCG) suppresses allergen-induced airway eosinophilia. *J Exp Med* 1998; **187**:561–69.
- 8 Herz U, Gerhold K, Gruber C *et al.* BCG infection suppresses allergic sensitization and development of increased airway reactivity in an animal model. *J Allergy Clin Immunol* 1998; **102**:867–74.
- 9 Wang CC, Rook GA. Inhibition of an established allergic response to ovalbumin in BALB/c mice by killed *Mycobacterium vaccae*. *Immunology* 1998; **93**:307–13.
- 10 Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. *Nat Rev Immunol* 2001; **1**:69–75.
- 11 Zuany-Amorim C, Sawicka E, Manlius C *et al.* Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. *Nat Med* 2002; **8**:625–29.
- 12 Arkwright PD, David TJ. Intradermal administration of a killed *Mycobacterium vaccae* suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderate-to-severe disease. *J Allergy Clin Immunol* 2001; **107**:531–34.
- 13 Hadley EA, Smillie FI, Turner MA, Custovic A, Woodcock A, Arkwright PD. Effect of *Mycobacterium vaccae* on cytokine responses in children with atopic dermatitis. *Clin Exp Immunol* 2005; **140**:101–08.
- 14 Brothers S, Asher MI, Jaksic M, Stewart AW. Effect of a *Mycobacterium vaccae* derivative on paediatric atopic dermatitis: a randomized, controlled trial. *Clin Exp Dermatol* 2009; **34**:770–75.
- 15 Berth-Jones J, Arkwright PD, Marasovic D *et al.* Killed *Mycobacterium vaccae* suspension in children with moderate-to-severe atopic dermatitis: a randomized, double-blind, placebo-controlled trial. *Clin Exp Allergy* 2006; **36**:1115–21.
- 16 Williams HC, Burney PG, Pembroke AC, Hay RJ. Validation of the U.K. diagnostic criteria for atopic dermatitis in a population setting. U.K. Diagnostic Criteria for Atopic Dermatitis Working Party. *Br J Dermatol* 1996; **135**:12–7.
- 17 Rajka G, Langeland T. Grading of the severity of atopic dermatitis. *Acta Derm Venereol Suppl (Stockh)* 1989; **144**:13–4.
- 18 Berth-Jones J. Six area, six sign atopic dermatitis (SASSAD) severity score: a simple system for monitoring disease activity in atopic dermatitis. *Br J Dermatol* 1996; **135**:25–30.
- 19 Upham JW, Holt BJ, Baron-Hay MJ *et al.* Inhalant allergen-specific T-cell reactivity is detectable in close to 100% of atopic and normal individuals: covert responses are unmasked by serum-free medium. *Clin Exp Allergy* 1995; **25**:634–42.
- 20 Jang S, Uematsu S, Akira S, Salgame P. IL-6 and IL-10 induction from dendritic cells in response to *Mycobacterium tuberculosis* is predominantly dependent on TLR2-mediated recognition. *J Immunol* 2004; **173**:3392–97.
- 21 Pompei L, Jang S, Zamlynny B *et al.* Disparity in IL-12 release in dendritic cells and macrophages in response to *Mycobacterium tuberculosis* is due to use of distinct TLRs. *J Immunol* 2007; **178**:5192–99.
- 22 Prescott SL, Noakes P, Chow BW *et al.* Presymptomatic differences in Toll-like receptor function in infants who have allergy. *J Allergy Clin Immunol* 2008; **122**:391–99.
- 23 Rowe J, Macaubas C, Monger T *et al.* Heterogeneity in diphtheria–tetanus–acellular pertussis vaccine-specific cellular immunity during infancy: relationship to variations in the kinetics of postnatal maturation of systemic Th1 function. *J Infect Dis* 2001; **184**:80–8.
- 24 Dunstan J, Mori TA, Barden A *et al.* Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomised controlled trial. *J Allergy Clin Immunol* 2003; **112**:1178–84.
- 25 Ota MO, Vekemans J, Schlegel-Haueter SE *et al.* Influence of *Mycobacterium bovis* bacillus Calmette–Guerin on antibody and cytokine responses to human neonatal vaccination. *J Immunol* 2002; **168**:919–25.
- 26 Shen H, Huang H, Wang J *et al.* Neonatal vaccination with bacillus Calmette–Guerin elicits long-term protection in mouse-allergic responses. *Allergy* 2008; **63**:555–63.
- 27 Lagranderie M, Abolhassani M, Vanoirbeek J *et al.* *Mycobacterium bovis* BCG killed by extended freeze-drying reduces airway hyperresponsiveness in 2 animal models. *J Allergy Clin Immunol* 2008; **121**:471–78.
- 28 Hunt JR, Martinelli R, Adams VC, Rook GA, Brunet LR. Intragastric administration of *Mycobacterium vaccae* inhibits severe pulmonary allergic inflammation in a mouse model. *Clin Exp Allergy* 2005; **35**:685–90.
- 29 Li Q, Shen HH. Neonatal bacillus Calmette–Guerin vaccination inhibits *de novo* allergic inflammatory response in mice via alteration of CD4+CD25+ T-regulatory cells. *Acta Pharmacol Sin* 2009; **30**:125–33.
- 30 Koh YI, Choi IS, Lee JJ. Effects of cytokine milieu secreted by BCG-treated dendritic cells on allergen-specific Th immune response. *J Korean Med Sci* 2004; **19**:640–46.
- 31 Hopkin JM, Shaldon S, Ferry B. Mycobacterial immunisation in grass pollen asthma and rhinitis. *Thorax* 1998; **53** (Suppl. 4):S63.

- 32 Shirtcliffe PM, Easthope SE, Cheng S *et al.* The effect of delipidated deglycolipidated (DDMV) and heat-killed *Mycobacterium vaccae* in asthma. *Am J Respir Crit Care Med* 2001; **163**:1410–14.
- 33 Marks GB, Ng K, Zhou J *et al.* The effect of neonatal BCG vaccination on atopy and asthma at age 7 to 14 years: an historical cohort study in a community with a very low prevalence of tuberculosis infection and a high prevalence of atopic disease. *J Allergy Clin Immunol* 2003; **111**:541–49.
- 34 Prescott S, Macaubas C, Smallacombe T *et al.* Development of allergen-specific T-cell memory in atopic and normal children. *Lancet* 1999; **353**:196–00.
- 35 Asher MI, Montefort S, Bjorksten B *et al.* Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* 2006; **368**:733–43.
- 36 Lai CK, Beasley R, Crane J, Foliaki S, Shah J, Weiland S. Global variation in the prevalence and severity of asthma symptoms: phase three of the International Study of Asthma and Allergies in Childhood (ISAAC). *Thorax* 2009; **64**:476–83.
- 37 Ait-Khaled N, Pearce N, Anderson HR, Ellwood P, Montefort S, Shah J. Global map of the prevalence of symptoms of rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC) Phase Three. *Allergy* 2009; **64**:123–48.
- 38 Odhiambo JA, Williams HC, Clayton TO, Robertson CF, Asher MI, and the ISAAC Phase Three Study Group. Global variations in prevalence of eczema symptoms in children from the ISAAC Phase Three. *J Allergy Clin Immunol* 2009; **124**:1251–8.