

Effect of *Mycobacterium vaccae* on cytokine responses in children with atopic dermatitis

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Summary

The increasing prevalence of atopic diseases over the last few decades is thought to be due to reduced exposure to environmental microbes that normally down-regulate allergic responses (hygiene hypothesis). We have shown previously that administration of the environmental microbe *Mycobacterium vaccae* ameliorates atopic dermatitis in school-age children at 3 months post-treatment. The present study tested the hypothesis that *M. vaccae* suppresses Th2-type cytokine activity and increases transforming growth factor (TGF)- β_1 immunomodulatory activity in these children. Interleukin (IL)-4, IL-5, TGF- β_1 and interferon (IFN)- γ activity were assessed in resting and stimulated peripheral blood mononuclear cells (PBMC) isolated from 12 of the children who received *M. vaccae* in our original clinical trial. A cDNA expression array was used to examine a broader range of cytokine pathway transcripts. There were no significant changes in either Th2-type or TGF- β_1 activity. A 5- to 10-fold increase in Th1-type activity was found at 1 month post-*M. vaccae* administration ($P < 0.05$), but it had returned to baseline by 3 months. The results do not support the hypothesis that *M. vaccae* reduces Th2-type or increases TGF- β_1 activity of PBMC isolated from children with atopic dermatitis. The transient surge in IFN- γ at 1 month is unlikely to explain any improvement in eczema score at 3 months.

Keywords: *Mycobacterium vaccae*, atopic dermatitis, cytokines, hygiene hypothesis

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Introduction

An increase in the prevalence of atopic diseases, including atopic dermatitis (AD) [1], in Western societies over the past few decades cannot be explained by genetic factors, but must reflect changes within the environment. The hygiene hypothesis suggests that the rising prevalence is related to a 'cleaner' western lifestyle and reduced exposure to certain environmental microbes [2–4].

Chronic AD is associated with increased Th2-type and to a lesser extent Th1-type cytokine activity. This pattern of cytokine activity is seen characteristically in infants prior to or after low exposure to environmental antigens [5,6]. Exposure to some environmental microbes promotes the selection of a Th1-type rather than the atopy-associated Th2-type phenotype, as well as inducing immunomodulatory

responses, which prevent excessive inflammation [2,7]. Reduced exposure to microbes in early childhood may therefore result in persisting Th2-type predominance as well as a lack of stimulation of immunomodulatory responses leading to the development of atopic disease in genetically susceptible individuals [2].

A study of Japanese school children immunized with Bacille Calmette–Guérin (BCG) showed that responders to tuberculin had a lower prevalence of atopic disease compared to tuberculin non-responders [8]. This implies that, in this population at least, the risk for atopy is associated with lack of exposure or reactivity to mycobacterial antigens. In animal models of asthma, mycobacterial antigens have been shown to directly suppress the allergic response. In addition to promoting strong Th1-type activity [9–11], they may also stimulate immunomodulatory responses. In a mouse model

of asthma, airway hyperactivity was suppressed in association with the stimulation of immunomodulatory cytokines [transforming growth factor (TGF)- β_1 and interleukin (IL)-10] through treatment with a killed *Mycobacterium vaccae* suspension [12].

We have shown previously that administration of a killed *M. vaccae* suspension (SRL172) to children with moderate-to-severe AD was associated with amelioration of their disease [13]. The current study extends these clinical findings using peripheral blood mononuclear cells (PBMC) isolated from these children who were given *M. vaccae*. The aim is to test the hypothesis that administration of *M. vaccae* leads to a reduced Th2-type response and increased expression of the immunomodulatory cytokine TGF- β_1 . Cytokine responses were analysed in both resting and stimulated cells using enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription-polymerase chain reaction (RT-PCR) for specific cytokines on individual samples. Additionally, cDNA expression array was used to illustrate the depth of the effects of *M. vaccae* on Th1-type and Th2-type responses by examining a broader range of transcripts in these pathways using pooled samples. Our PBMC preparation contained not only T lymphocytes, but also other lymphocyte populations and phagocytes. Other cells than T lymphocytes have been shown to be capable of producing interferin (IFN)- γ (e.g. natural killer cells) [14]. Th2-type cytokines (e.g. IL-4 and IL-5) are also produced by basophils and eosinophils [14–16], and TGF- β_1 is produced predominantly by activated monocytes/macrophages [17]. For this reason the terms ‘Th1-type’ (IFN- γ pathway), ‘Th2-type’ (IL-4 and IL-5 pathways) and ‘immunomodulatory’ (TGF- β_1 activity) responses are used to describe the patterns of cytokine expression examined in this study.

Materials and methods

Patients and study design

Details of the children with moderately severe AD from whom blood samples used in this study were taken have been reported previously [13]. Briefly, 41 children aged 5–18 years with moderate-to-severe AD were recruited into a randomized, double-blind, placebo-controlled trial, where they were given either one intradermal injection of killed *M. vaccae* (SRL172) or buffer solution (placebo). All subjects were atopic with median (interquartile range) total serum IgE levels of 2115 (737–2912) ku/l. Permission to conduct the study was obtained from the Medicines Control Agency, Department of Health, London and the Local Research Ethics Committee. Written consent was obtained from parents of all participants.

Blood samples were available from 12 SRL172 treated subjects prior to *M. vaccae* administration. Samples taken 1 month after SRL172 treatment were available from 11 subjects, five of whom also had samples available 3 months post-

treatment. One subject had a sample available at the latter (post-treatment) time-point only. In order to compare like with like, samples obtained 3 months after *M. vaccae* administration (labelled as post-3) were compared only with pre-treatment samples from the same children (labelled as pre-3). Similarly, samples obtained 1 month after *M. vaccae* administration (labelled as post-1) were only compared with pre-treatment samples from the same children (labelled as pre-1).

Isolation and culture of PBMC

PBMC were separated from heparinized blood by Ficoll-Paque gradient centrifugation within 3 h of collection. PBMC, 1×10^5 , were stimulated *in vitro* with AIM-V medium alone (control), *Mycobacterium bovis* (*M. bovis* extract, courtesy of University College Hospital, London, UK, 5 μ g/ml), *Dermatophagoides pteronyssinus* extract (house dust mite allergen (HDM): Bayer, Elkhart, IN, USA, 30 μ g/ml) or phytohaemagglutinin (PHA: Murex Biotech Ltd, Dartford, UK, 1 μ g/ml). Cells and supernatants were harvested at day 3 (PHA) and day 6 (*M. bovis* and HDM). Cell pellets were resuspended in Poly A Tract guanidinium thiocyanate (GTC)-based extraction buffer (Promega Corporation, WI, USA) and samples were stored at -80°C until assayed.

ELISAs

Cytokines were measured in supernatants by ELISA (IFN- γ , IL-5 and IL-2: Diaclone Research, Besançon cedex, France; TGF- β_1 : DRG Instruments GmbH, Marburg, Germany). The sensitivities of the ELISAs were: IFN- γ 12.5 pg/ml, IL-5 7.8 pg/ml, TGF- β_1 19 pg/ml and IL-2 31.2 pg/ml. For all assays, the background absorbance was subtracted from all data points and the amount of cytokine in each sample was determined by extrapolating OD values to cytokine concentrations using the linear range of the standard curve.

Total RNA isolation

Total cellular RNA was extracted from the cells using the acid-guanidinium thiocyanate–phenol–chloroform method as previously described [18]. The RNA pellet was dissolved in 20 μ l RNase-free H_2O , contaminating DNA was removed by DNA-freeTM (Ambion, Inc. TX, USA) and 0.1 volume of Anti-RNase (28 U/ μ l, Ambion, Inc. TX, USA) was added before storage at -80°C .

Real-time RT-PCR

Quantification of IFN- γ , IL-4, TGF- β_1 and IL-2 mRNA was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously [19]. Briefly, 10 μ l aliquots of total RNA were reverse transcribed in 50 μ l reaction volumes using

Table 1. Details of individual probes included in transcription pathway microarray groupings (Atlas human 1·2 array; <http://www.clontech.com/products/cat/HTML/1047.shtml>).

Transcript pathway							
Th1-type		Th2-type	IL-2				
IFN- γ	MIG	IL-4	p56-lck	PIK3RA	JNK1	c-jun	CALNB
IFN- γ R1	STAT1	IL-4R α	ZAP70	PIK3RB	JNK2	junD	NF- κ B
IFN- γ R2	STAT2	IL-5	vav	PKC θ	JNKK1	JAB1	OTF1
IFN- γ R	INOS	IL-5R α	GRB2	p21 ras	MEK1	PKB	IL-2
IL-12p40		IL-13	LAT	raf-1	elk-1	SGK	IL-2R α
IL-12p35		GATA3	PLCG1	rac-1	c-fos	CAMK IV	IL-2R β
IL-12R		STAT6	PIK3CD	ERK1	FRA1	CREB1	IL-2R γ
IP10		EMBP	PIK3CA	ERK2	FRA2	CALNA	PCNA

IP10 = interferon (IFN)- γ -induced protein 10, MIG = IFN- γ -induced monokine, STAT = signal transducer and activator of transcription, INOS = inducible nitric oxide synthase, GATA3 = GATA-binding protein 3, EMBP = eosinophil granule major basic protein, ZAP70 = 70-KDa zeta-associated protein, GRB2 = growth factor receptor-bound protein 2, LAT = linker for activation of T cells, PLCG1 = phospholipase C gamma 1, PIK3CD/CA/RA/RB = phosphatidylinositol 3 kinase catalytic subunit delta isoform/catalytic subunit alpha isoform/regulatory alpha isoform/regulatory beta isoform, PKC θ = protein kinase C theta, ERK1/2 = extracellular signal-related kinase 1/2, JNK1/2 = c-jun N-terminal kinase 1/2, JNKK1 = c-jun N-terminal kinase kinase 1, MEK1 = MAPK/ERK kinase 1, FRA1/2 = fos-related antigen 1/2, JAB1 = jun activation domain binding protein 1, PKB = protein kinase B, SGK = serum and glucocorticoid-regulated serine/threonine protein kinase, CAMK IV = calcium/calmodulin-dependent protein kinase type IV, CREB1 = cyclic AMP response element binding protein 1, CALNA/B = calcineurin A alpha/B isoform 1, NF- κ B = nuclear factor kappa-B DNA binding subunit, OTF1 = octamer-binding transcription factor 1, PCNA = proliferating cyclic nuclear antigen.

Taqman reverse transcription reagents (Applied Biosystems). Pre-developed assay reagent (PDAR) kits of primers and probe for the genes of interest were obtained commercially from Applied Biosystems and were labelled with FAM (6-carboxyfluorescein) at the 5' end and with TAMRA (6-carboxytetramethylrhodamine) at the 3' end. 18S rRNA labelled with VIC was evaluated in the same PCR reactions. Samples were amplified (40 cycles at 95°C for 15 s and 60°C for 1 min) using 12 μ l cDNA, primers, probe and Taqman universal PCR master mix as described in the manufacturer's directions (Applied Biosystems). The fluorescence signals for each amplification product were calculated by determining the point at which the fluorescence intensity was 10 times larger than the standard deviation of the baseline fluorescence (threshold cycle, C_t value). Relative cytokine mRNA levels were determined by normalizing IFN- γ , IL-4, TGF- β_1 and IL-2 specific signals to constitutively expressed 18S rRNA signals using the formula: $2^{-\Delta C_t} = 2^{-(C_t, \text{IFN-}\gamma, \text{IL-4, TGF-}\beta_1 \text{ and IL-2} - C_t, \text{18S rRNA})}$; as described previously [20].

cDNA expression array

Atlas human 1·2 nylon arrays (BD Clontech, Palo Alto, CA, USA) were used for gene expression profiling. The membranes contained the cDNAs [200–600 base pairs (bp)] from 1176 known genes that are involved in a range of important cellular pathways. Prior to data analysis, transcripts were grouped into those involved in Th1-type and Th2-type pathways (Table 1) (immunomodulatory-type transcripts were insufficiently represented on the arrays and therefore were not analysed). Additionally, IL-2 pathway transcripts were grouped and used as a positive control for lymphocyte

stimulation [21,22] (Table 1). A negative control group comprised all transcripts in the array that were not classified in the Th1-type, Th2-type or IL-2 pathways (individual transcripts not shown).

Briefly, equal volumes of individual RNA sample from each of the pre- and post-*M. vaccae* administration time-points were pooled to a total volume of 3·5 μ l. The pooled RNA samples were reverse-transcribed into cDNAs (Atlas SMARTTM PCR cDNA Synthesis kit, BD Clontech), amplified by PCR (Atlas Advantage 2 PCR kit, BD Clontech), subjected to spin column purification, and 500 ng of purified cDNA was then labelled with [α -³²P] dATP (10 μ Ci/ μ l; 3000 Ci/mmol, Amersham Pharmacia AB, Uppsala, Sweden). Hybridization of radiolabelled cDNAs to Atlas human 1·2 nylon arrays and washing were performed as recommended by the manufacturer. mRNA (raw data) was quantified by spot intensity values (arbitrary units) following exposure/data acquisition using an InstantImager®/InstantQuantTM software (Packard Instrument company, CT, USA) for 7 days.

Data analysis

SPSS version 10·1 for windows (SPSS Inc, Chicago IL, USA) was used to test for changes in cytokine protein (ELISA) and mRNA (real-time RT-PCR) data before and after *M. vaccae* administration using the non-parametric Wilcoxon Signed Rank test.

cDNA expression arrays representing the post-*M. vaccae* administration time-points were compared to their corresponding pre-*M. vaccae* administration arrays. The mRNA data for comparable arrays were extracted (raw values) and

then standardized using AtlasImage™ 1.0 software (BD Clontech), in accordance with the manufacturer's instructions [23]. Briefly, this software standardized the signal intensity between two compared arrays (pre- *versus* post-*M. vaccae*) by calculating a normalization coefficient as follows, where *i* = genes on array 1 (pre-*M. vaccae* arrays), *j* = genes on array 2 (post-*M. vaccae* arrays) and *n* = number of detectable genes on the array:

$$\text{Normalization Coefficient} = \frac{\sum_{i=1}^n (\text{intensity} - \text{background})_i}{\sum_{j=1}^n (\text{intensity} - \text{background})_j}$$

Once calculated, this coefficient was applied to the spot intensity values of each gene on array 2 (post-*M. vaccae* arrays), so that it became normalized with respect to array 1 (pre-*M. vaccae* arrays) [23]. Such transformed data were appropriate for non-parametric comparisons, as described further below (log transformation of raw data prior to normalization did not effect the ranking and therefore the outcome of the data). The Wilcoxon Signed Rank test was used to test for differential expression of Th1-type, Th2-type and IL-2 pathway transcripts (Table 1) by comparing spot intensity values between pre- and post-*M. vaccae* administration time-points for each pathway. Additionally, spot intensity differences (post-spot intensity – pre-spot intensity) of each of the three transcript pathways were compared with a negative control transcript group (as described previously), using the Mann–Whitney *U*-test. Comparisons with transcripts in the negative control group were made to allow for the effects of time and the *M. vaccae* injection on overall

levels of gene expression. Significance for all two-tailed tests was considered when $P < 0.05$.

Results

ELISA

IFN- γ , IL-5 and IL-2 proteins were only detected in PBMC stimulated *in vitro* with PHA. For this stimulant, significantly higher levels of IFN- γ protein were found at 1 month but not 3 months post-*M. vaccae* administration compared to baseline ($P = 0.01$) (Table 2). *M. vaccae* had no effect on IL-5, TGF- β_1 (resting or stimulated PBMC) and IL-2 protein levels when administered 1 or 3 months previously (Table 2).

Real-time RT-PCR

IFN- γ mRNA levels were significantly increased at 1 month but not 3 months post-*M. vaccae* administration for all *in vitro* stimulated PBMC conditions ($P < 0.05$) (Table 3). No difference was found in IL-4 (PHA stimulated PBMC), TGF- β_1 (resting or stimulated PBMC) and IL-2 (stimulated PBMC) mRNA levels, 1 and 3 months after *M. vaccae* administration compared with prior to treatment (Table 3). With the exception of TGF- β_1 , cytokine mRNA levels were undetectable at all time-points measured in resting PBMC.

cDNA expression array

Th1-type and Th2-type pathway transcript expression paralleled changes observed with individual cytokines.

Table 2. ELISA quantification of cytokine proteins before, 1 month and 3 months after *M. vaccae* administration. Results are expressed in pg/ml.

Transcript pathway	Cytokine	<i>In vitro</i> PBMC stimulant	Median (interquartile range) pg/ml			Median (interquartile range) pg/ml		
			Pre-1 month†	Post-1 month†	<i>P</i>	Pre-3 months‡	Post-3 months‡	<i>P</i>
Th1-type	IFN- γ	PHA	20 220 (68–66 150)	84 216 (63 923–188 808)	<i>0.01*</i>	13 672 (68–72 371)	13 407 (2515–163 851)	<i>0.2</i>
Th2-type	IL-5	PHA	44 (36–131)	64 (46–162)	<i>0.6</i>	44 (36–66)	44 (36–117)	<i>0.2</i>
Immunomodulatory	TGF- β_1	<i>M. bovis</i>	200 (125–255)	205 (160–280)	<i>0.1</i>	200 (165–250)	195 (170–280)	<i>0.2</i>
		HDM	220 (135–266)	273 (156–321)	<i>0.1</i>	213 (158–279)	193 (148–275)	<i>0.4</i>
		Control (day 6)	185 (155–215)	175 (140–275)	<i>0.8</i>	195 (159–231)	180 (170–295)	<i>1.0</i>
		PHA	173 (133–260)	243 (169–328)	<i>0.1</i>	230 (170–275)	230 (193–273)	<i>0.5</i>
		Control (day 3)	220 (134–263)	213 (139–280)	<i>0.6</i>	220 (143–259)	188 (168–285)	<i>0.8</i>
		PHA	237 (115–773)	480 (193–753)	<i>0.5</i>	128 (115–790)	229 (115–1000)	<i>0.1</i>

Medians (interquartile range) are reported. Data represent 11† or six‡ individuals. *Significance $P < 0.05$. *P* values representing the level of significance are shown in italic; median and interquartile ranges that show a difference (i.e. compared values that are significant) are given in bold; *P* values representing significant values (i.e. $P < 0.05$) are shown in bold and italic.

Table 3. Real-time reverse transcription-polymerase chain reaction (RT-PCR) quantification of cytokine mRNA before, 1 month and 3 months after *M. vaccae* administration. Results are expressed as relative quantities to 18S rRNA ($\times 10^7$).

Transcript pathway	Cytokine	<i>In vitro</i> PBMC stimulant	Median (interquartile range) Relative quantity ($\times 10^7$)			Median (interquartile range) Relative quantity ($\times 10^7$)		
			Pre-1 month†	Post-1 month†	<i>P</i>	Pre-3 months‡	Post-3 months‡	<i>P</i>
Th1-type	IFN- γ	<i>M. bovis</i>	1 (1–3)	6 (3–92)	0.03*	2 (1–4)	3 (2–24)	0.2
		HDM	65 (8–212)	604 (85–2739)	0.01*	56 (4–434)	95 (2–1113)	0.5
		PHA	520 (54–2161)	2895 (792–5160)	0.01*	476 (2–2858)	292 (131–1592)	0.5
Th2-type	IL-4	PHA	3 (1–6)	2 (1–3)	0.6	2 (1–26)	2 (1–2)	0.2
Immunomodulatory	TGF- β_1	<i>M. bovis</i>	1026 (744–3188)	1290 (634–3037)	0.8	1126 (756–4324)	2046 (1238–3798)	0.9
		HDM	1445 (590–2472)	3266 (2245–8601)	0.2	1195 (590–1703)	1478 (97–3000)	0.7
		Control (day 6)	250 (85–1281)	590 (6–828)	0.7	49 (9–268)	565 (180–1478)	0.1
		PHA	5697 (1606–15 186)	3088 (1875–6623)	0.6	7700 (314–28 896)	2208 (402–5323)	0.2
		Control (day 3)	162 (18–468)	15 (3–205)	0.1	162 (20–481)	104 (23–485)	0.3
IL-2	IL-2	<i>M. bovis</i>	7 (1–13)	4 (3–11)	0.8	8 (1–14)	6 (3–14)	0.9
		HDM	21 (3–91)	87 (9–133)	0.2	21 (3–91)	45 (1–155)	1.0
		PHA	39 (3–58)	19 (10–40)	0.8	23 (1–280)	31 (8–84)	0.9

Medians (interquartile range) are reported. Data represent 11† or 6‡ individuals. *Significance $P < 0.05$. *P* values representing the level of significance are shown in italic; median and interquartile ranges that show a difference (i.e. compared values that are significant) are given in bold; *P* values representing significant values (i.e. $P < 0.05$) are shown in bold and italic.

Compared with PBMC isolated from children pre-*M. vaccae* administration, PBMC taken from children 1 month post-*M. vaccae* and stimulated *in vitro* with *M. bovis*, HDM and PHA showed increased expression of transcripts in the Th1-type pathway (Table 4). The increase in Th1-type pathway transcript expression (after *in vitro M. bovis* stimulation of PBMC) was significantly greater than the 293 negative control transcripts detected within the array [median: 20 519 (interquartile range: 9714–34 651) (1 month post-*M. vaccae*) versus 13 848 (3740–47 760) (pre-1 *M. vaccae*)] (Mann–Whitney, $P = 0.002$). Th2-type pathway transcripts were only detected after *in vitro M. bovis* stimulation of PBMC where no significant differences in transcript expression were found (Table 4). *In vitro M. bovis* (but not PHA and HDM) stimulation of PBMC isolated from children given *M. vaccae* 1 month previously was associated with significantly higher levels of IL-2 pathway transcripts when compared to PBMC responses prior to *M. vaccae* administration ($P = 0.0001$) (Table 5). This increase in IL-2 pathway transcript expression at 1 month post-*M. vaccae* administration was greater

than the 293 negative control transcripts detected within the array ($P = 0.0001$).

The pre-3 versus post-3-month *M. vaccae* administration array data were also compatible with the other forms of data, i.e. no difference in Th1-type, Th2-type and IL-2 pathway transcript expression (data not shown). Transcript pathway activity was not detected in resting PBMC at any time-point.

Discussion

This study does not provide any evidence to support the hypothesis that *M. vaccae* reduces Th2-type (IL-4, IL-5) or stimulates TGF- β_1 activity, but shows clearly that administration of *M. vaccae* (SRL172) up-regulates Th1-type (IFN- γ) activity (both at the mRNA and protein level) at 1, but not 3 months post-treatment. It is the first study to document the duration of action of a single dose of killed mycobacterial antigen on Th1-type activity in children with AD. It therefore provides new information as to the time-course of Th1-type responses to mycobacterial antigens, demonstrated

Table 4. Atlas human 1·2 array quantification of Th1-type and Th2-type pathway transcripts before and 1 month after *M. vaccae* administration. Adjusted spot intensity values (after background subtraction and normalization) of individual transcripts detected are shown.

Transcript pathway	<i>In vitro</i> PBMC stimulant	Transcript detected	Adjusted spot intensity (arbitrary units)		<i>P</i>
			Pre-1 month	Post-1 month	
Th1-type	<i>M. bovis</i>	IFN- γ R2	17 536	40 304	
		STAT1	10 144	51 183	
		STAT2	0	13 288	
		IFN- γ R	0	14 016	
		IP10	48	46 464	
		MIG	0	25 024	
		Median	24	32 664	<i>0·03*</i>
		(interquartile range)	(0–11 992)	(13 834–47 644)	
	HDM	IP10	0	4332	
		IFN- γ	0	2586	
Th2-type	PHA	IFN- γ	72	3239	
	<i>M. bovis</i>	IL-5R α	25 948	13 275	
		IL-13	7420	6211	
		IL-4	35 608	33 207	
		IL-5	44 104	32 209	
		Median	30 778	22 742	<i>0·1</i>
		(interquartile range)	(12 052–41 980)	(7977–32 958)	

Medians (interquartile range) are reported (where appropriate). Data represent pooled RNA samples of 11 individuals. *Significance $P < 0·05$. *P* values representing the level of significance are shown in italic; median and interquartile ranges that show a difference (i.e. compared values that are significant) are given in bold; *P* values representing significant values (i.e. $P < 0·05$) are shown in bold and italic.

previously to be crucial to the immunity to this bacteria [24] in non-atopic adults [25–27] and children [28].

Could the augmentation of Th1-type activity by *M. vaccae* have led to the amelioration in clinical eczema scores seen in this cohort of children? This question is prompted by the previous observations of decreased Th1-type (IFN- γ) and increased Th2-type (IL-4, IL-5, IL-13) activity in peripheral blood of atopic individuals compared with healthy controls [29]. The general consensus now is that Th1-type responses do not down-regulate allergic processes, and may indeed exacerbate allergic disorders. Both Th2-type cytokines (IL-4, IL-5 and IL-13) and Th1-type cytokines (IFN- γ) are increased in the skin of patients with chronic AD [30,31]. Furthermore, transferring Th1 lymphocytes into a mouse model of asthma exacerbates rather than ameliorates the disease [32]. Although neither our initial trial of 5–18-year-old children, nor our subsequent study of 2–5-year-olds with AD [33] showed worsening in clinical eczema scores 1 month after *M. vaccae* administration, clinical eczema scores improved significantly only at 3 months post-*M. vaccae* administration [13], at a time when Th1-type activity returned to baseline. We therefore conclude that clinical improvement in AD is unlikely to be mediated by the up-regulation of Th1-type responses to *M. vaccae*.

The increase in IL-2 pathway transcripts post-*M. vaccae* administration in isolated PBMC stimulated with *M. bovis*, but not with non-specific (PHA) or non-cross-reacting (HDM) antigens (as measured by cDNA expression array) shows the selective nature of proinflammatory response to *in*

vivo administration of *M. vaccae*. Our findings are consistent with studies on healthy newborns, which have demonstrated previously that vaccination with BCG results in a Th1-type response that is specific to mycobacterial antigens [34–36]. In our study, the duration of the effect on IL-2 pathway transcripts parallels that observed with the Th1-type pathway and demonstrates that with the dose, route and cohort used, the effects of a single dose of heat-killed *M. vaccae* persist for more than 1 but less than 3 months. These data, as well as the generally high levels of TGF- β_1 , provide positive controls indicating that the low levels of Th2-type cytokines (e.g. post-HDM stimulation) are likely to reflect the true *in vitro* situation and not a technical problem with, for instance, the use of AIM-V serum-free medium [37].

In contrast to the recent published studies showing that *M. vaccae* up-regulates immunomodulatory cytokine expression in a mouse model of asthma [12], our study failed to demonstrate any significant effect on TGF- β_1 expression in children with AD. The differences observed between the two studies may lie with the study design. In the study by Zuany-Amorim *et al.* [12], the up-regulation of immunomodulatory cytokines by *M. vaccae* was found to be directly associated with the expansion/activation of a purified CD4⁺ CD45RB^{lo} regulatory T cell subpopulation. The production of TGF- β_1 from unfractionated PBMC used in the current study may have resulted in the dilution of this cytokine from specific regulatory T cells. Hence, we acknowledge that our data do not exclude the possibility that *M. vaccae* may stimulate regulatory T cell expansion/activation and it may be of

Table 5. Atlas human 1·2 array quantification of IL-2 pathway transcripts before and 1 month after *M. vaccae* administration. Adjusted spot intensity values (after background subtraction and normalization) of individual transcripts detected are shown.

Transcript	Adjusted spot intensity (arbitrary units)					
	<i>M. bovis</i>		HDM		PHA	
	Pre-1 month	Post-1 month	Pre-1 month	Post-1 month	Pre-1 month	Post-1 month
p21 ras	0	20 864	—	—	3448	8233
raf-1	0	13 736	2764	767	—	—
ERK2	12 800	35 123	—	—	—	—
p56-lck	3712	18 244	—	—	—	—
SGK	0	14 072	—	—	—	—
JNK2	14 148	17 352	2484	1492	22 928	15 090
MEK1	0	10 160	—	—	—	—
PIK3CA	2560	13 310	—	—	—	—
CAMK IV	0	10 763	0	3125	—	—
PLCG1	—	—	—	—	14 496	0
rac-1	51 104	62 048	52 268	61 560	36 832	0
GRB2	—	—	55 500	663	19 144	13 111
CREB1	8844	23 716	—	—	21 116	6106
LAT	20 159	25 668	—	—	—	—
PKB	4	16 769	—	—	—	—
PCNA	0	15 029	—	—	—	—
CALNA	0	16 455	—	—	—	—
OTF1	300	8963	—	—	—	—
JAB1	0	31 893	29 184	2052	—	—
IL-2R α	—	—	53 444	65 820	54 096	41 371
IL-2R β	436	16 108	—	—	—	—
NF- κ B	7824	24 217	—	—	—	—
IL-2R γ	31 880	34 462	4176	943	0	9186
Median	368	17 061	16 680	1 772	20 130	8710
(interquartile range)	(0–11 811)	(13 820–25 305)	(2554–53 150)	(811–46 951)	(6210–33 356)	(1527–14 595)
<i>P</i>	<i>0·0001*</i>		<i>0·6</i>		<i>0·1</i>	

Medians (interquartile range) are reported. Data represent pooled RNA samples of 11 individuals. *Significance $P < 0·001$. 0/—: transcript not detected on one/both compared array/s. *P* values representing the level of significance are shown in italic; median and interquartile ranges that show a difference (i.e. compared values that are significant) are given in bold; *P* values representing significant values (i.e. $P < 0·001$) are shown in bold and italic.

more importance to study the expression of immunomodulatory cytokines from these specific cell types.

Although this study focused on TGF- β_1 as a major immunomodulatory cytokine with possible relevance to AD [38], IL-10 also has immunomodulatory properties [39]. Future studies examining the effect of *M. vaccae* on this cytokine may solve some currently unexplained questions as to the possible immunomodulatory mechanisms of *M. vaccae* *in vivo*.

In conclusion, administration of *M. vaccae* to children with AD stimulates a significant increase in Th1-type activity that lasts between 1 and 3 months, but is unlikely to explain the amelioration of these children's disease. *M. vaccae* did not alter the expression of TGF- β_1 , nor did it suppress Th2-type cytokine activity. Further studies examining the direct effects of *M. vaccae* on purified regulatory T cells in patients with atopic disease are required to address more directly the possible immunomodulatory effects of these environmental microbes in atopic diseases.

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