chapter 5

Cellular immune responses and skin prick test reactivity to house dust mite allergen in Ghanaian schoolchildren

Abena S. Amoah[1,2], Benedicta B. Obeng[1,2], Yvonne A. Aryeetey[1], Irene A. Larbi[1], Yvonne C. Kruize[2], Franca C. Hartgers[2] , Daniel A. Boakye[1] and Maria Yazdanbakhsh[2]

Affiliations:

[1] Department of Parasitology, Noguchi Memorial Institute for Medical Research, Accra, Ghana
[2] Department of Parasitology, Leiden University Medical Center, Leiden, the Netherlands;

- Manuscript in preparation -

Abstract

Background: In recent years, a marked global rise in the prevalence of allergic disease has been observed. Although a number of factors have been found to account for this increase, few investigations have examined the relationship between cellular immune responses and markers of allergic disease especially in developing countries.

Objective: To study the association between cellular immune responsiveness and house dust mite (HDM) skin prick test (SPT) reactivity among Ghanaian schoolchildren aged 5 to16 years.

Methods: A case-control study was performed in 250 children (95 HDM SPT positive cases 155 controls) selected from a larger cross-sectional investigation. Whole blood samples from study participants were cultured with innate as well as adaptive stimuli for 24 and 72 hours respectively. The innate stimuli used were the TLR 2/1 ligand PAM3CSK4 (Pam3) and the TLR4 ligand lipopolysaccharide (LPS). The adaptive stimuli were purified protein derivative (PPD) and the mitogen phytohaemagglutinin (PHA).

Results: Elevated IL-10 in response to LPS at 24 hours was significantly associated with HDM SPT reactivity (adjusted odds ratio 1.71, 95%CI [1.01 - 2.90], p=0.046). In addition, high IFN-γ to PPD at 72 hours was associated with being a HDM SPT case (adjusted odd ratio 1.77, 95%CI [1.04 - 3.01], p=0.034). No significant associations were observed between cytokine responses to Pam3 or PHA and being a HDM SPT case.

Conclusion: The results of the study suggest that enhanced cellular immune responsiveness to LPS and to PPD are associated with HDM SPT reactivity among Ghanaian children.

Introduction

The prevalence of allergic disorders is on the increase worldwide particularly in low to middle income countries [1] where urbanization and the adoption of a so-called western lifestyle have been linked to the rising incidence of inflammatory diseases [2]. Studies have identified a link between changes in environmental determinants and this increase. Specifically, reduced exposure to pathogens during childhood is thought to be leading to the inadequate maturation of the immune system's regulatory arm thus resulting in uninhibited inflammatory responses toward harmless antigens that include allergens [3, 4]. Key elements of allergic inflammation are mast cells, basophils, eosinophils and immunoglobulin (Ig) E [5]. In addition, during allergic inflammation, T-helper-2 cells regulate type 2 responses through the secretion of cytokines that include IL-4, IL-5, IL-9 and IL-13 [6]. Although type 2 cellular immune responses have been studied extensively in animal models [7] and in a few human studies in western countries [8-10], little is known about these cellular immune responses among allergic children in developing countries.

One commonly used marker of allergic disease in Western countries is atopic sensitization to environmental allergens based on allergen-specific IgE as well as skin prick test (SPT) reactivity. However, a number of studies conducted in helminth-endemic areas have demonstrated the poor diagnostic value of measured allergen-specific IgE due to IgE cross-reactivity [11]. Thus, SPT reactivity to environmental allergens seems more closely related to clinical allergic disease in these countries [12-14] and therefore we have considered this test in our present study.

The aim of our study was to investigate the association between cellular immune responsiveness and house dust mite (HDM) SPT reactivity among schoolchildren living in one region of Ghana.

Materials and methods

Study design and population

We conducted an immunological study among a subset of subjects that had been recruited into a larger cross-sectional study on allergic sensitization and parasitic infections in schoolchildren in Southern Ghana. The cross-sectional survey was conducted between March 2006 and March 2008 and detailed methodology and population description have been reported elsewhere [15]. Briefly, the larger investigation was performed among children aged between 5 and 16 years attending rural and urban schools in the Greater Accra Region. In this cross-sectional study, the percentage of children who were SPT positive to house dust mite allergen was 12.7% (177/1396).

For the current cellular immunological study, an unmatched case-control design was used in which the case definition was house dust mite SPT positivity. Out of 177 HDM SPT positives, 100 randomly selected HDM SPT positives were targeted along with 200 SPT negative controls. Controls were from the same schools as the cases

and were selected in a ratio of one case to two controls. Assessment of immune responsiveness was performed in both groups based on cytokine production following stimulation of whole blood with innate and adaptive stimuli. The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board, Ghana (NMIMR-IRB CPN 012/04-05).

Skin prick testing

For the larger cross-sectional survey, skin prick tests were performed using a panel of food and environmental allergens that included a commercially available extract of house dust mite (*Dermatophagoides* mix - ALK-Abelló, Madrid, Spain). A positive control of histamine hydrochloride (10 mg/ml) and a negative control of saline (ALK-Abelló, Madrid, Spain) were included in the panel. The SPT procedure has been described in detail elsewhere [16]. SPT positivity was defined as a mean wheal diameter ≥3 mm. Controls for the study were SPT negative for all allergens tested.

Whole blood stimulation assay

Four to 6 hours following venipuncture, heparinized blood samples were cultured at a dilution of 1:4 in RPMI 1640 medium (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM glutamate. For the assay, heparinized whole blood was first diluted 1:1 with medium and then 100 µl of the mixture was cultured in each well of a round-bottomed 96-well tissue culture plate (Nunc, VWR International) with 100 µl of either medium alone or stimulus added to medium. The panel of stimuli included innate immune ligands for 24 hour cultures as well as adaptive immune stimuli for 72 hour cultures. The innate stimuli used were the TLR 2/1 ligand PAM3CSK4 (Pam3) and the TLR4 ligand lipopolysaccharide (LPS). The adaptive stimuli were purified protein derivative (PPD) as a marker of adaptive immune response to a vaccine given at birth in Ghana and the mitogen phytohaemagglutinin (PHA) which is a polyclonal T cell stimulus. Both 24 and 72 cultures included responses to culture medium alone as a control. Details of the stimuli used and final concentrations are given in Table 1. A pilot study conducted before the main immunological investigation determined the optimal concentrations to be used. Whole blood cultures were incubated at 37°C in 5% CO_2 for either 24 hours or 72 hours after which supernatants were harvested. Supernatants were frozen at −20°C and later transported on dry ice to a central laboratory for cytokine measurements.

Cytokine measurements

The levels of interleukin IL-10 and tumor necrosis factor (TNF) for 24 hour cultures and IL-10, TNF, interferon (IFN)-γ, IL-13 and IL-17 for 72 hour cultures were determined in harvested supernatants using a Luminex 100 cytometer (Luminex Corporation, Austin Texas, United States) and Luminex cytokine kits (BioSource, Camarillo, California,

Table 1: Panel of stimuli and cytokines measured

STIMULI	ABBR.	CLASSIFICATION	FINAL CONCENTRATION	SOURCE	INCUBATION TIME	CYTOKINES MEASURED
MEDIUM (RPMI)	MED	Negative Control	-	Life technologies	24 hrs. & 72 hrs.	IL-10, TNF (24 hrs.) IL-10, TNF, IFN-γ, IL-13, IL-17 (72 hrs.)
Pam3CSK4	Pam3	TLR2/1 agonist	100 µg /ml	EMC microcollections	24 hrs.	IL-10, TNF
Lipopolysaccharide	LPS	TLR4 agonist	1 ng/ml	Invivogen	24 hrs.	IL-10, TNF
Purified protein derivative	PPD	Tuberculosis antigen	10 µg/ml	Invivogen	72 hrs.	IL-10, TNF, IFN-γ, IL-13
Phytohaemagglutinin	PHA	Mitogen	2 µg/ml	Wellcome Diagnostics, Dartford, UK	72 hrs.	IL-10, TNF, IFN-γ, IL17

United States) according to the manufacturer's instructions. The lower detection limit of the assays was 5 pg/mL for IL-10 and IFN-γ. For TNF, IL-13 and IL-17, the lower detection limit was 10 pg/mL. Samples with concentrations less than the detection limit were assigned one-half the value of this threshold.

Parasitological examinations

For each study participant, one stool sample was collected for the detection of intestinal helminth eggs by the Kato-Katz technique [17] using 25 mg of stool. Intestinal helminths detected were hookworm (*Necator americanus* and *Ancylostoma duodenale*), *Ascaris lumbricoides*, *Trichuris trichiura* and *Schistosoma mansoni*. A urine sample was also collected to determine *Schistosoma haematobium* infection using the standard filtration method [18] in which 10ml of urine is filtered through a nylon nucleopore filter (pore size, 12 μm). A small quantity of blood was used to prepare a Giemsa-stained thick smear slide to detect malaria parasites.

IgE antibody measurements

Immunoglobulin E to house dust mite extract (*Dermatophagoides pteronyssinus*) was assessed using the ImmunoCAP® platform (Thermo Fisher Scientific, Uppsala, Sweden) according to manufacturer's instructions. For our analysis, ≥ 0.35 kU/L was used as the sensitization cut-off.

Anthropometric Measurements

For each study participant, weight and height measurements were determined by weighing scale (BS-8001, capacity: 130 kg) and portable stadiometer respectively. Body Mass Index (BMI) was calculated by dividing weight (kg) by height (m) squared. For each individual, a BMI-for-age z-score based on gender-specific growth chart reference data collected in the World Health Organization Multicentre Growth Reference Study [19] was generated.

Questionnaire

A standard questionnaire was administered to the parents or guardians of study subjects to collect information on demographic and socioeconomic parameters as well as history of asthma within each family. The questionnaire was administered by trained interviewers who were fluent in the local language of each participant. It was pre-tested in a pilot study under field conditions to ensure understanding and acceptability.

Statistical analysis

For our analysis, the exposures of interest were cytokine responses to a panel of innate and adaptive stimuli as assessed by whole blood cultures. Cytokine responses were categorized as either being greater than the median response level (high response)

or below the median response level (low response). Differences between cases and controls according to characteristics of the study population were examined by Pearson's χ^2 tests (with 1 degree of freedom) for categorical variables and Mann-Whitney U test for continuous variables. Crude and adjusted logistic regression models were fitted to determine the associations between immune responses and being a HDM SPT case. In multivariable analysis, models were adjusted for area, age and gender as *a priori* confounders. To examine whether any association between immune response and HDM SPT positivity was different in the urban compared to rural area, for each response, a separate adjusted model was fitted with a product term to examine interaction between immune response and area. A p-value less than 0.05 was taken as the level for statistical significance for multivariable analysis. All analysis was performed using IBM SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of Study Participants

A total of 250 children had complete immunological data and of these, 95 were HDM SPT positive (cases) and 155 SPT negative (controls). Figure 1 shows a flow diagram of study participants from the cross-sectional investigation to the selection for the immunological study. The characteristics of HDM SPT positive cases and controls are presented in Table 2. Overall, a greater proportion of study subjects were from the rural area compared to the urban area as was the case with the larger cross-sectional investigation. In addition, significantly more HDM SPT positives reported wheeze in the last 12 months (p=0.001), doctor-diagnosed asthma (p=0.021) and having an asthmatic father (p=0.038) compared to controls. Specific IgE to house dust mite was also significantly higher among HDM SPT positives compared to controls (p <0.001). With regards to family size, we observed that cases tended to come from families with greater than 6 children while most controls were from families with 1-3 children. However, after adjusting for area, there was no longer an association between family size and being a HDM SPT case. There were no significant differences between cases and controls when it came to parasitic infections or socioeconomic factors.

Innate cytokine responses and HDM SPT positivity

The crude and adjusted associations between cytokine responses to innate stimuli and HDM SPT positivity are shown in Table 3. For IL-10 in response to LPS, a significant association was observed between high response and being an HDM SPT positive case in crude analysis. After adjusting for *a priori* confounders, high IL-10 in response to LPS was still significantly associated with an increased odds of HDM SPT positivity (adjusted odds ratio = 1.71, 95%CI [1.01 - 2.90], p=0.046).

For TNF in response to LPS as well as both cytokine responses to Pam3, high responses were not associated with HDM SPT positivity in crude or multivariable

Table 2: Characteristics of HDM SPT positive cases and controls

FACTORS		Total	HDM SPT +	HDM SPT -	*P-value
Area	Rural	157 (62.8%)	67 (70.5%)	90 (58.1%)	**0.048**
	Urban	93 (37.2%)	28 (29.5%)	65 (41.9%)	
Age	less than 11years	126 (50.4%)	48 (50.5%)	78 (50.3%)	0.975
	11years or greater	124 (49.6%)	47 (49.5%)	77 (49.7%)	
Gender	Male	127 (50.8%)	54 (56.8%)	73 (47.1%)	0.135
	Female	123 (49.2%)	41 (43.2%)	82 (52.9%)	
Family Size	1-3 children	89 (42.8%)	27 (33.8%)	62 (48.4%)	0.075
	4-5 children	48 (23.1%)	19 (23.8%)	29 (22.7%)	
	6+ children	71 (34.1%)	34 (42.5%)	37 (28.9%)	
Birth order	Median (IQR)	2 (1-4)	3 (2-5)	2 (1-4)	0.141
BMI-for-age z-score	Median (IQR)	0.20 (-0.66 – 0.85)	0.12 (-0.64 – 0.85)	0.25 (-0.66 – 0.83)	0.728
Reported Wheeze (12 months)	No	190 (90.9%)	67 (82.7%)	123 (96.1%)	**0.001**
	Yes	19 (9.1%)	14 (17.3%)	5 (3.9%)	
Doctor diagnosed asthma ever	No	195 (93.8%)	72 (88.9%)	123 (96.9%)	**0.021**
	Yes	13 (6.3%)	9 (11.1%)	4 (3.1%)	
Asthma history in child's family	No	118 (57.0%)	42 (52.5%)	76 (59.8%)	0.114
	Yes	72 (34.8%)	34 (42.5%)	38 (29.9%)	
	No Idea	17 (8.2%)	4 (5.0%)	13 (10.2%)	
Asthmatic mother	No	196 (93.8%)	78 (96.3%)	118 (92.2%)	0.231
	Yes	13 (6.2%)	3 (3.7%)	10 (7.8%)	
Asthmatic father	No	199 (95.2%)	74 (91.4%)	125 (97.7%)	**0.038**
	Yes	10 (4.8%)	7 (8.6%)	3 (2.3%)	
Specific IgE to House Dust mite	< 0.35 kU/L	143 (63.3%)	21 (25.9%)	122 (84.1%)	**<0.001**
	≥ 0.35 kU/L	83 (36.7%)	60 (74.1%)	23 (15.9%)	
**Any Helminth	Negative	179 (72.8%)	70 (76.1%)	109 (70.8%)	0.366
	Positive	67 (27.2%)	22 (23.9%)	45 (29.2%)	
***Plasmodium species	Negative	176(72.1%)	64(69.6%)	112(73.7%)	0.487
	Positive	68(27.9%)	28(30.4%)	40(26.3%)	
Primary household fuel	Firewood	75 (36.1%)	34 (42.0%)	41 (32.3%)	0.349
	Charcoal /firewood/ Kerosene	74 (35.6%)	27 (33.3%)	47 (37.0%)	
	LPG/Electricity	59 (28.4%)	20 (24.7%)	39 (30.7%)	
Main toilet option	No toilet	95 (45.7%)	39 (48.1%)	56 (44.1%)	0.189
	Public toilet	33 (15.9%)	17 (21.0%)	16 (12.6%)	
	Shared toilet	46 (22.1%)	13 (16.0%)	33 (26.0%)	
	Indoor /private toilet	34 (16.3%)	12 (14.8%)	22 (17.3%)	
Main water source	River/ Untreated water	100 (47.8%)	44 (54.3%)	56 (43.8%)	0.281
	Well/Borehole	43 (20.6%)	16 (19.8%)	27 (21.1%)	
	Piped /Treated water	66 (31.6%)	21 (25.9%)	45 (35.2%)	

* χ^2 Test or Mann-Whitney U Test p-value.
**Any Helminth = *Schistosoma haematobium, Schistosoma mansoni*, hookworm hookworm (*Ancylostoma duodenale* or *Necator americanus*), *Ascaris lumbricoides or Trichuris trichiura.*
****Plasmodium* species = *Plasmodium falciparum or Plasmodium malariae (the 2 malaria species detected in our population.*
P-values less than 0.05 are shown in bold.

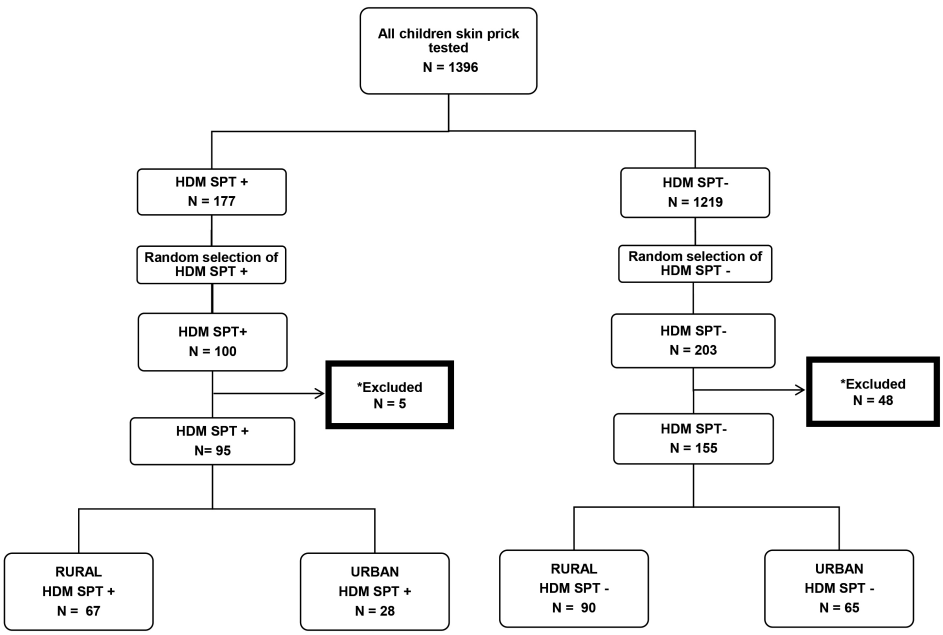

Figure 1: Flowchart of study participants
Flowchart of children selected for the immunological study.
* Subjects were excluded for missing immunological data.

analyses as shown in Table 3. The levels of IL-10 and TNF in response to medium at 24 hours were low and were not related to being a HDM SPT case in crude or multivariable analyses.

The results of adjusted models fitted with product terms to explore interaction between immune response and area are also shown in Table 3. The p-values for the interaction terms ranged from p=0.321 to p=1.000 indicating no evidence that the associations between immune response and HDM SPT reactivity was different in the urban and rural areas for the 24 hour cultures.

Adaptive cytokine responses and HDM SPT positivity

Tables 4A and 4B show the results of the crude and multivariable analyses of HDM SPT positivity and cytokine responses to stimuli that are considered to stimulate the adaptive immune system. For PPD, greater proportions of HDM SPT cases had high responses (i.e. above the median level) for IL-10, TNF and IFN-γ compared to SPT negative controls. In crude logistic regression analysis, high IFN-γ in response to PPD was significantly associated with HDM SPT positivity. This remained significant after adjusting for *a priori* confounders (adjusted OR 1.77 95%CI [1.04 - 3.01], p=0.034). For IL-10 in response to PPD, although the measure of effect from the crude logistic

Table 3: Crude and adjusted associations between immune responses and HDM SPT positivity (24 hour responses)

Stimuli	Cytokine	TOTAL N (%)	HDM SPT + N (%)	HDM SPT - N (%)	Crude Odds Ratio (95% CI)	Wald's Test P-value	Adjusted Odds Ratio (95% CI)	Wald's Test P-value	p-value Interaction Area * Immune Response
MEDIUM	IL-10 ≤2.5 pg/ml	224 (89.6%)	87 (91.6%)	137 (88.4%)	1.00	-	1.00	-	1.000
	IL-10 >2.5 pg/ml	26 (10.4%)	8 (8.4%)	18 (11.6%)	0.70 (0.29 - 1.68)	0.424	0.59 (0.24 - 1.45)	0.247	
	TNF ≤5.0 pg/ml	212 (84.8%)	80 (84.2%)	132 (85.2%)	1.00	-	1.00	-	0.820
	TNF >5.0 pg/ml	38 (15.2%)	15 (15.8%)	23 (14.8%)	1.08 (0.53 - 2.18)	0.839	0.94 (0.45 - 1.95)	0.865	
PAM	IL-10 ≤140.4 pg/ml	125 (50.0%)	44 (46.3%)	81 (52.3%)	1.00	-	1.00	-	0.323
	IL-10 >140.4 pg/ml	125 (50.0%)	51 (53.7%)	74 (47.7%)	1.27 (0.76 - 2.12)	0.362	1.19 (0.70 - 2.02)	0.511	
	TNF ≤267.5 pg/ml	125 (50.0%)	46 (48.4%)	79 (51.0%)	1.00	-	1.00	-	0.696
	TNF >267.5 pg/ml	125 (50.0%)	49 (51.6%)	76 (49.0%)	1.11 (0.66 - 1.85)	0.696	1.03 (0.61 - 1.74)	0.905	
LPS	IL-10 ≤151.6 pg/ml	126 (50.4%)	40 (42.1%)	86 (55.5%)	1.00	-	1.00	-	0.551
	IL-10 >151.6 pg/ml	124 (49.6%)	55 (57.9%)	69 (44.5%)	**1.71 (1.02 - 2.87)**	**0.041**	**1.71 (1.01 - 2.90)**	**0.046**	
	TNF ≤568.2 pg/ml	125 (50.0%)	46 (48.4%)	79 (51.0%)	1.00	-	1.00	-	0.321
	TNF >568.2 pg/ml	125 (50.0%)	49 (51.6%)	76 (49.0%)	1.11 (0.66 - 1.85)	0.696	1.10 (0.66 - 1.85)	0.719	

Models adjusted for age, gender and area.
P-values less than 0.05 are shown in bold.

regression model showed that this response was linked to 1.5 times the odds of a HDM SPT positive case, this was only of borderline significance and in the adjusted model, no longer significant (p=0.132).

Although TNF in response to PPD was not significantly associated with HDM SPT positivity in either crude or multivariable analyses, there was evidence of significant interaction between area and this immune response. Therefore, new models were fitted in which the association between TNF in response to PPD and HDM SPT positivity was examined after stratifying for area. In the rural area, high TNF in response to PPD was associated with HDM SPT positivity after adjusting for confounders (adjusted OR =1.98 95%CI [1.02 – 3.83], p=0.043); this was not seen in the urban area where very fewer cases had elevated TNF in response to PPD compared to controls. For PHA, all cytokine responses were high. However, there were no significant associations between cytokine responses and being an HDM SPT case as shown in Table 4B. From the 72 hour cultures, cytokine responses to medium were generally all low. In addition, no significant associations were observed between cytokine responses to medium and HDM SPT positivity (shown in Table 4A).

Table 4A and 4B also show the results of models fitted with product terms to examine evidence of interaction between cytokine responses and area. For IL-13 and IL-17, the p-values for the interaction term were p> 0.700. For IL-10, TNF, IFN-γ and IL-13 in response to PPD, the significance of the interaction terms ranged from p=0.386 to p=0.845 while for cytokine responses to PHA, the range was from p=0.153 to p=0.915. Therefore, for these specific responses, there was no evidence of significant interaction between area and immune response.

Discussion

We investigated associations between immune responsiveness and allergic sensitization based on house dust mite SPT positivity among urban and rural children in Ghana. A number of factors previously reported to be linked to allergic sensitization based on SPT reactivity were significantly associated with HDM SPT positivity in our study such as wheeze [20], doctor-diagnosed asthma [21], family history of allergic diseases [22] and elevated specific IgE to dust mite [23]. Therefore, HDM SPT positivity was indeed an appropriate marker for the pathogenesis of allergy in our population. Although factors associated with allergic sensitization have been explored in similar settings [20, 24], few studies have examined the effect of cellular immune responsiveness on allergy outcomes.

With regards to innate cytokines, elevated LPS-induced IL-10 was significantly associated with HDM SPT positivity in our study. LPS is a key component of the outer membrane of Gram-negative bacteria that can initiate strong innate immune responses in humans [25]. Our findings seem somewhat unexpected since there are data showing that IL-10 is a suppressory cytokine [26]. Furthermore, a small study conducted among European allergic asthmatic children observed that IL-10 production in whole blood stimulated with LPS was

Table 4: Crude and adjusted associations between immune responses and HDM SPT positivity (72 hour responses)
A. Medium

Stimuli	Cytokine	TOTAL N (%)	HDM SPT + N (%)	HDM SPT - N (%)	Crude Odds Ratio (95% CI)	Wald's Test P-value	Adjusted Odds Ratio (95% CI)	Wald's Test P-value	Area * Immune Response p-value for interaction
MEDIUM	IL-10 ≤2.5 pg/ml	239 (95.6%)	89 (93.7%)	150 (96.8%)	1.00	-	1.00	-	***
	IL-10 >2.5 pg/ml	11 (4.4%)	6 (6.3%)	5 (3.2%)	2.02 (0.60 - 6.82)	0.256	1.87 (0.54 - 6.51)	0.327	
	TNF ≤5.0 pg/ml	230 (92.0%)	89 (93.7%)	141 (91.0%)	1.00	-	1.00	-	****
	TNF >5.0 pg/ml	20 (8.0%)	6 (6.3%)	14 (9.0%)	0.68 (0.25 - 1.83)	0.445	0.54 (0.19 - 1.49)	0.233	
	IFN-γ ≤2.5 pg/ml	245 (98.0%)	93 (97.9%)	152 (98.1%)	1.00	-	1.00	-	****
	IFN-γ >2.5 pg/ml	5 (2.0%)	2 (2.1%)	3 (1.9%)	1.09 (0.18 - 6.64)	0.926	0.83 (0.13 - 5.18)	0.841	
	IL-13 ≤5.0 pg/ml	180 (72.0%)	69 (72.6%)	111 (71.6%)	1.00	-	1.00	-	0.723
	IL-13 >5.0 pg/ml	70 (28.0%)	26 (27.4%)	44 (28.4%)	0.95 (0.54 - 1.68)	0.862	0.95 (0.53 - 1.70)	0.864	
	IL-17 ≤5.0 pg/ml	173 (69.2%)	64 (67.4%)	109 (70.3%)	1.00	-	1.00	-	0.703
	IL-17 >5.0 pg/ml	77 (30.8%)	31 (32.6%)	46 (29.7%)	1.15 (0.66 - 1.99)	0.623	0.93 (0.52 - 1.66)	0.802	

Models adjusted for age, gender and area.
P-values less than 0.05 are shown in bold.
**** Interaction term dropped from model since no urban subjects had cytokine levels greater than the median for this immune response.

B. PPD and PHA

Stimuli	Cytokine	TOTAL N (%)	HDM SPT + N (%)	HDM SPT - N (%)	Crude Odds Ratio (95% CI)	Wald's Test P-value	Adjusted Odds Ratio (95% CI)	Wald's Test P-value	Area * Immune Response p-value Interaction
PPD	IL-10 ≤10.3 pg/ml	125 (50.0%)	41 (43.2%)	84 (54.2%)	1.00	-	1.00	-	0.386
	IL-10 >10.3 pg/ml	125 (50.0%)	54 (56.8%)	71 (45.8%)	1.56 (0.93 - 2.61)	0.091	1.50 (0.89 - 2.54)	0.132	
	TNF ≤5.0 pg/ml	153 (61.2%)	53 (55.8%)	100 (64.5%)	1.00	-	1.00		**0.028**
	TNF >5.0 pg/ml	97 (38.8%)	42 (44.2%)	55 (35.5%)	1.44 (0.86 - 2.43)	0.170	1.31 (0.75 - 2.31)	0.342	
	IFN-γ ≤15.7pg/ml	125 (50.0%)	38 (40.0%)	87 (56.1%)	1.00	-	1.00	-	0.493
	IFN-γ >15.7pg/ml	125 (50.0%)	57 (60.0%)	68 (43.9%)	**1.92 (1.14 - 3.22)**	**0.014**	**1.77 (1.04 - 3.01)**	**0.034**	
	IL-13 ≤5.0 pg/ml	136 (54.4%)	51 (53.7%)	85 (54.8%)	1.00	-	1.00		0.845
	IL-13 >5.0 pg/ml	114 (45.6%)	44 (46.3%)	70 (45.2%)	1.05 (0.63 - 1.75)	0.859	0.99 (0.58 - 1.69)	0.956	
PHA	IL-10 ≤59.4 pg/ml	125 (50.0%)	49 (51.6%)	76 (49.0%)	1.00		1.00	-	0.915
	IL-10 >59.4 pg/ml	125 (50.0%)	46 (48.4%)	79 (51.0%)	0.90 (0.54 - 1.51)	0.696	0.78 (0.46 - 1.32)	0.356	
	TNF ≤ 8.6 pg/ml	126 (50.4%)	45 (47.4%)	81 (52.3%)	1.00		1.00	-	0.153
	TNF > 8.6 pg/ml	124 (49.6%)	50 (52.6%)	74 (47.7%)	1.22 (0.73 - 2.03)	0.453	1.08 (0.63 - 1.84)	0.788	
	IFN-γ ≤23.4 pg/ml	125 (50.0%)	46 (48.4%)	79 (51.0%)	1.00		1.00	-	0.493
	IFN-γ >23.4 pg/ml	125 (50.0%)	49 (51.6%)	76 (49.0%)	1.11 (0.66 - 1.85)	0.696	1.05 (0.62 - 1.77)	0.856	
	IL-17 ≤50.0 pg/ml	125 (50.0%)	50 (52.6%)	75 (48.4%)	1.00		1.00	-	0.803
	IL-17 >50.0 pg/ml	125 (50.0%)	45 (47.4%)	80 (51.6%)	0.84 (0.51 - 1.41)	0.515	0.78 (0.46 - 1.31)	0.342	

Models adjusted for age, gender and area.
P-values less than 0.05 are shown in bold.

significantly lower in the allergic group compared to healthy controls [27]. Although IL-10 will be discussed later, it is important to note that responses to innate immune ligands have been shown to vary greatly across different populations worldwide. For example, an investigation of innate immune responses to TLR ligands measured among infants across four continents (Africa, Europe, North America and South America) using standardized methodology observed that South African infants had lower responsiveness to TLR ligands including LPS compared to infants from the three other sites [28].

An investigation from a helminth-endemic area of Gabon, Central Africa reported that children infected with *S. haematobium* showed lower immune responsiveness when their peripheral blood mononuclear cells were stimulated with LPS compared to uninfected children [29]. It is also known that repeated exposure to TLR ligands such as LPS can lead to a dampening of response in what is known as TLR tolerance [30]. Therefore, it is possible that both the infected children in the study from Gabon and the controls in our study had greater exposure to bacteria and were tolerant to LPS and thus less responsive in general. However, additional studies would be needed to investigate this in our population especially since significant observations were made for LPS only but not for the TLR2/1 ligand Pam3.

PPD, which is a mixture of mycobacterial antigens, was used because 94.8% of our study participants with verifiable records had received BCG vaccine at birth. The magnitude of IFN-γ production in response to PPD by whole blood assay is widely used to assess immunological protection against tuberculosis following BCG vaccination [31]. As this response can be measured years after vaccination [32], PPD provides a suitable antigen to assess adaptive immune responses. With regards to this adaptive immune responsiveness, high IFN-γ in response to PPD was significantly associated with HDM SPT positivity in our study. Moreover, in the rural area, high TNF in response to PPD was significantly associated with being HDM SPT positive.

Although no longer significant after multivariable analysis, IL-10 to PPD was also associated with SPT to HDM. The higher IFN-γ and IL-10 adaptive responses in allergic subjects were not expected. Earlier studies not only in Gabon [33] and Vietnam [34] but also in Australia [35] have shown that the immune suppressory cytokine, IL-10, is negatively associated with SPT. Moreover, in terms of the balance between Th1 and Th2, allergic subjects would be expected to have lower IFN-γ responses [36]. These findings along with the high IL-10 to LPS being associated with SPT positivity, suggest that greater immune responsiveness, rather than a specific cytokine, is associated with HDM positivity.

Similar observations were made among urban children in Brazil where cytokine responses from whole blood cultures stimulated with a mitogen were measured in 1127 children [37]. In this study, the responsive immune phenotype was characterized by generalized production of cytokines above limits of detection [37]. The cytokines measured in the Brazilian study were IFN-γ, IL-5, IL-13 and IL-10, and findings from this investigation indicate that general enhanced responsiveness based on these cytokines was associated with increased odds of SPT reactivity as well as allergen-specific IgE sensitization [37].

Although we observed enhanced cytokine production linked to HDM SPT positivity with PPD, no significant associations were seen in our study when responses to the mitogen PHA and SPT reactivity were considered, which is in contrast to the Brazilian study. However, there were some notable differences between our study and the investigation from Brazil in that they used the mitogen pokeweed which is not as strong a stimulus and assessed cytokines after 120 hours of stimulation instead of 72 hours in our case [37]. Different population dynamics and study methodologies make direct comparisons of cellular immune findings between investigations very problematic.

Our study had a number of limitations such as the relatively small sample size which meant reduced statistical power for some of the associations examined. This reduced power is reflected in some of the borderline significant associations observed and we cannot therefore rule out the possibility of type 2 errors in our study. Moreover, additional studies are needed that measure not only cytokines in supernatants but also at a single cell level in order to identify which cells contribute to the cytokine network in allergic individuals compared to non-allergics.

Acknowledgements

The authors wish to thank Dr. Domingo Barber and Dr. Lucia Jimeno (Alk-Abelló, Madrid, Spain) for providing skin prick testing material as well as Prof. Ronald van Ree for allergen-specific immunoglobulin E measurements. Our appreciation goes to Dr. Dziedzom de Souza for the design of the database, Mr. Richard A. Akuffo for data entry, Miss Linda Tamatey for technical assistance. We would also like to express our sincerest gratitude to the national service personnel involved in the study, community leaders, school authorities and teachers of all participating schools for all their assistance. Finally, we are most indebted to the study participants and their families for their time and commitment. Funding was provided by EuroPrevall (FOOD-CT-2005-514000), GLOFAL (FOOD-CT-2005-517812) and The Wellcome Trust (075791/Z/04/Z).

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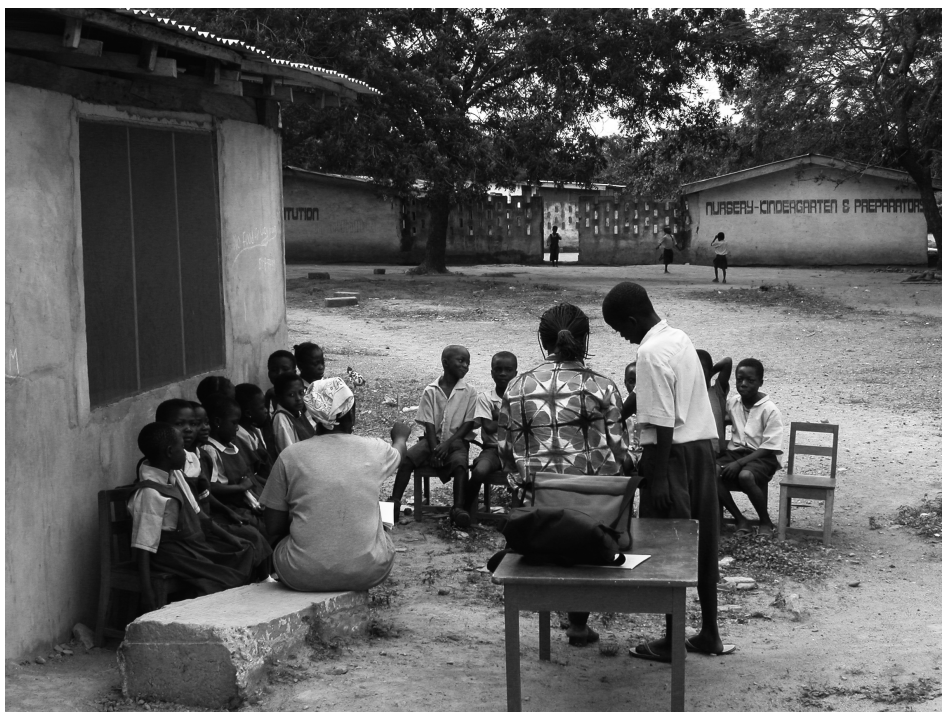
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