

Atopic dermatitis in young children is associated with impaired interleukin-10 and interferon- γ responses to allergens, vaccines and colonizing skin and gut bacteria

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Summary

Background A significant proportion of children with food allergy and more severe forms of atopic dermatitis (AD) go on to develop persistent forms of allergic disease such as asthma. Defining immune dysregulation in these children will be of great value in understanding disease pathogenesis.

Objective In this study we characterized the immune responses of young infants (6–18 months of age) with moderate-to-severe AD (a modified SCORAD ≥ 25) and compared these ($n = 53$) with responses of non-allergic children with no history of dermatitis or sensitization of the same age ($n = 20$).

Methods Mononuclear cell cytokine responses to allergens (egg ovalbumin (OVA), β -lactoglobulin (BLG), house dust mite (HDM)), vaccines (tetanus toxoid (TT), diphtheria toxoid (DT)), intestinal flora (heat-killed *Lactobacillus species* (HKLB)), heat-killed *Staphylococcus aureus* (HKSA), *S. aureus* enterotoxin B (SEB) and mitogen (phytohaemagglutinin (PHA)) were compared in children with AD with unaffected children.

Results Children with AD had significantly lower spontaneous (unstimulated) production of regulatory cytokine IL-10 ($P < 0.001$), as well as IFN- γ ($P < 0.001$) and TNF- α ($P < 0.001$) compared with the unaffected children. After allowing for differences in baseline levels IL-10 responses to virtually all stimuli (food allergens ($P = 0.003$), vaccines $P = 0.01$, intestinal flora (heat-killed *Lactobacillus species* (HKLB), $P = 0.005$) and skin flora (heat-killed *Staphylococcus aureus* (HKSA), $P = 0.003$)) were also significantly attenuated in children with AD. The only exception was HDM, to which responses were stronger in children with AD [$P = 0.05$]. Although there were no significant correlations between HDM IgE and HDM cytokine responses at this age, T-helper type 2 (Th2) IL-5 ($P = 0.014$) and IL-13 ($P = 0.004$) responses to HDM were significantly more frequent in the children with AD. However, while children with AD showed significantly attenuated Th1 IFN- γ responses to food allergens (OVA, $P = 0.007$ and BLG, $P < 0.001$) and vaccines (DT, $P = 0.008$ and TT, $P < 0.001$), these children showed no difference in Th1 IFN- γ responses to HDM or microbial agents (HKSA and HKLB).

Conclusion A increase in propensity for Th2 responses to aeroallergens in children with AD is associated with early impaired production of IL-10 regulatory cytokine to a broad range of environmental stimuli including foods, intestinal flora, *S. aureus*, and vaccines.

Keywords atopic dermatitis, food allergy, IL-10, infants, intestinal flora staphylococcus, Th2 cytokines

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Introduction

Atopic dermatitis (AD) is frequently the first manifestation of atopic disease in infancy [1]. Although the pathogenesis of this condition is still not clear, a number of observations suggest that this is the initial cutaneous manifestation of a systemic disorder that also leads to other atopic diseases including food allergy, asthma and allergic rhinitis (AR) [2,3].

There is a strong association between AD and allergic sensitization, particularly in children with more severe cutaneous disease. These children are also at increased risk (as much as 80%) of developing sensitization to inhalant allergens (such as house dust mite (HDM)) and persistent respiratory tract disease (AR and asthma) [1]. Logically, this early period could provide an early window for prevention of respiratory allergic disease [4]. Thus, early events associated with aeroallergen sensitization are of prime interest in this very high-risk population.

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Even before significant cutaneous and gastrointestinal exposures, cord blood studies show that neonates who go on to develop AD, have increased T-helper type 2 (Th2) IL-5 responses to staphylococcus enterotoxin B (SEB) (and other microbial antigens), as well as increased IL-10 responses to HDM [5]. These observations suggest early pre-symptomatic differences in responses to both microbes and allergens, although the significance is not yet clear. More recently, our collaborators performed preliminary studies [6] on a small group of children (aged 5–25 months) with AD ($n = 11$) and noted that affected children produced higher pro-allergic Th2 cytokine responses (IL-5 and IL-13) to SEB but not heat killed *Staphylococcus aureus* (HKSA), compared to unaffected children ($n = 7$). It is not clear if these children also had early differences in HDM responses as this was not examined. This larger study now examines for differences in early systemic immune responses to inhalant allergens, Staphylococcal antigens, intestinal flora and other stimuli (including food allergens, vaccines and other microbial stimuli) in very young children (6–18 months of age) with AD.

There is growing interest in the role of percutaneous sensitization to inhalants such as HDM in AD. Animal models suggest that percutaneous sensitization is more likely to result in allergic Th2 sensitization when the skin barrier is disrupted [7], and bacterial colonization (particularly with *S. aureus*) appears to play a role in amplifying skin inflammation and barrier disruption through a number of different pathways. These include superantigen effects [8] and direct Th2 promoting effects of lipotechoic acid from *S. aureus* [9]. More recent studies have shown that SEB can inhibit the suppressive properties of CD4⁺CD25⁺ regulatory T cells [10], which suggests a novel mechanism by which staphylococcal superantigens could augment T cell activation in patients with AD. It is not yet clear how these early postnatal interactions influence evolving responses to HDM in young children.

Intestinal colonization is an important source of microbial-driven immune regulation in early life, and changing intestinal flora is thought to be important in the pathogenesis of allergic disease. Infants who develop allergic disease have notable differences in very early colonization patterns [11, 12]. This suggests that intestinal flora influence maturation of the large pool of immature precursor cells that circulate through the gut and subsequently home to tissues throughout the body [13, 14], particularly to the skin and mucosal surfaces where they develop their mature functional attributes. Alteration in microflora or events that lead to mucosal inflammation could logically modify the local milieu and the rate and pattern of precursor maturation and effect local immune development in remote tissues.

We therefore examined the early patterns of cytokine responses to skin (*S. aureus*) and intestinal flora (*Lactobacillus species*), in addition to food and inhalant allergens in young children with moderate-to-severe AD.

Methods

In this cross-sectional study, allergen-specific peripheral blood mononuclear cell (PBMC) responses to allergens, mitogens and microbial antigens were compared in children with ($n = 53$) and without AD ($n = 20$) at 6–18 months of age.

The number of subjects in this study was based on previous studies by the host laboratory comparing young children with symptomatic allergic disease [15]. Power calculations determined that our sample size was sufficient for the detection of a difference of 15% in cytokine levels between affected and unaffected groups at a significance level of $P = 0.05$.

Participants

Children with moderate or severe AD ($n = 53$) were recruited between April and November 2003 from the general community or outpatient clinics. All children diagnosed with AD met the Hanifin and Rajka criteria for AD and had a modified SCORAD ≥ 25 [16, 17]. These were compared with 'unaffected' children of the same age (6–18 months of age) who had no evidence of AD or sensitization (on either skin prick testing (SPT) or RAST for allergen-specific IgE antibodies). These non-allergic children in this study were recruited from the general community. These children were already being followed prospectively in longitudinal studies examining immune development. Eligible children were randomly selected from the database if they (a) no diagnosis of any allergic disease, (b) had no history of any possible allergic symptoms, (c) no history of sensitization to common allergens, and (d) had not received any specific intervention for allergy prevention. Children were ineligible for the study if they had any other major medical problems. The trial protocol was approved by the Ethics Committee at Princess Margaret Hospital and all parents gave informed consent.

Clinical assessment

A detailed history was obtained by interview questionnaire. A SCORAD assessment was also performed by a clinician. This tool [18] was used to assess the severity of AD by combining evaluation of extent, intensity of lesions and subjective symptoms (pruritus and sleep loss). A modified SCORAD was obtained by using only the assessment of extent and intensity, omitting subjective criteria [16]. To ensure consistency, a single investigator performed all SCORAD assessments. A diagnosis of asthma was made in children with recurrent wheezing (three or more episodes, at least one confirmed by paediatrician or general practitioner). All children diagnosed with asthma were responsive to bronchodilator therapy. Chronic cough (in the absence of apparent infection) and recurrent wheeze (on more than two occasions) were also assessed in the clinical history. A diagnosis of AR was considered where there was a history or recurrent sneezing, itching (eyes and nose), and nasal congestion in the absence of a cold. All children had either a RAST (using commercial Pharmacia CAPSystem, Uppsala, Sweden) or a SPT (Hollister-Stier Laboratories, Spokane, WA, USA) to assess for evidence of sensitization (specific IgE antibodies) to common allergens (hen's egg, cows milk, peanut, HDM, cat and grass pollens). A SPT weal diameter of >2 mm was considered positive.

Lymphocyte responses to allergen, microbial and mitogen stimulation

All samples were processed together under the same conditions within the same 6-week time period. Peripheral

blood samples were collected into heparinized RPMI (Life Technology Ltd, Paisley, Scotland) tissue culture medium. Mononuclear cells (PBMC) were isolated from blood using Lymphoprep (Nycomed Pharma, Asker, Norway) gradient centrifugation and cryopreserved for subsequent batch analysis of allergen-specific cytokine responses. For cytokine analysis 2×10^9 PBMC/L were cultured in AIM V (Gibco, Life Technology Ltd, Paisley, Scotland) serum-free medium [19] for 48 h without stimuli (for spontaneous production of cytokines) or with a) allergens, including HDM extract 20 µg/mL (CSL, Melbourne, Australia), ovalbumin (OVA) 200 µg/mL (Sigma, Castle Hill, Australia), β -lactoglobulin (BLG) 100 µg/mL (Sigma, Australia), b) vaccine antigens, including tetanus toxoid (TT) 0.5 fL and diphtheria toxoid (DT) 0.5 fL (CSL, Australia), c) *Staphylococcus* antigens, including SEB (Sigma) 200 ng/mL and heat-killed *S. aureus* 10^7 colony-forming units (CFU)/mL, (d) heat-killed *Lactobacillus fermentum* 10^7 CFU/mL or, (e) phytohemagglutinin (PHA) mitogen 1 µg/mL (HA16, Murex, Biotech Ltd, Dartford, UK). For PHA stimulation (1 µg/mL) 10^9 CBMC/L were used.

The *S. aureus* strain was originally isolated from an infant with AD, and had been previously identified using three tests; latex agglutination assay (Staphatech[®], Oxoid, Basingstoke, UK), DNase assay (Oxoid) and coagulase activity assay (Oxoid). *S. aureus* was cultured overnight in LB medium (Oxoid), washed twice with sterile PBS and CFU per millilitre quantified by spread plating 10-fold serial dilutions on LB agar (Oxoid) and incubating for 24 h at 37 °C. Bacteria were heat killed at 60 °C for 60 min and stored in aliquots at –80 °C until use. An aliquot was checked for sterility by plating on LB agar for 48 h.

PCC[®] *Lactobacillus fermentum* VRI 003 (Probiomix Ltd, NSW, Sydney, Australia) was cultured overnight in De Man, Rogosa, Sharpe (MRS) broth (Oxoid) and the cells washed twice in PBS prior to enumeration of the viable cells by 10-fold serial dilution, and spread plating on MRS agar. Colony-forming units per millilitre of suspension were enumerated after anaerobic incubation at 37 °C for 24 h. The bacterial suspension was heat treated at 75 °C for 45 min and aliquots stored at –80 °C until use. An aliquot was checked for sterility by plating on MRS agar.

Supernatants were analysed for IL-5 using an in-house ELISA technique as previously described [20]. The limit of detection was 3 pg/mL. TGF- β was determined using an ELISA kit (R&D Systems Europe, Abingdon, UK) for the detection of latent TGF- β 1. The limit of detection was 7.8 pg/mL. The concentration in many stimulated cell culture supernatants was reduced compared with the controls and this was reported as percentage suppression determined by the formula $([\text{control-stimulated}/\text{control}]) \times 100$. IL-10, IL-13 and IFN- γ were quantified by an in-house ELISA using a time resolved fluorometry detection system (DELPHIA, PerkinElmer, Life Sciences, MA, USA) as previously described [21]. 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-10, IL-13 or IFN- γ (Pharmingen), were linear between 5 and 30 000 pg/mL. Cytokine data was expressed as

the difference between the stimulated culture and the control (pg/mL) and as detected (> 5 pg/mL) or not detected.

Cytokine data are shown as spontaneous or stimulated production as indicated. The responses to various stimuli are shown as the level above the parallel background (unstimulated) control cultures. As TGF β 1 is spontaneously produced in high levels in PBMC cultures and is generally down-regulated with activation, the data for this cytokine were defined in terms of the percentage suppression. This is the level of down-regulation (background minus stimulated) as a percentage of the spontaneous production [22].

Analysis

Cytokine data were analysed as (a) continuous data, and (b) dichotomous data (detected or non-detected). The cytokine data were generally not normally distributed and were unable to be normalized with logarithmic transformation. These data are therefore displayed as median and interquartile range and a non-parametric test (Mann–Whitney *U*-test) was used to determine differences between the groups. Differences between the groups for dichotomous data were determined by χ^2 . Correlations between disease severity (SCORAD index) and PBMC cytokine response to stimuli were determined only on infants with AD ($n = 53$). Correlations were assessed using Spearman's or Kendall's τ_b (where a proportion of the variables of interest shared 'zero' values) in order to avoid the problems associated with 'ties' within the data. All statistical analyses were performed using SPSS software (Version 11 for Macintosh). A significant level of 0.05 was considered statistically significant for all analyses. Because of the exploratory nature of this study, we did not wish to exclude any important relationships by using stringent correction factors for multiple analyses. However, we recognize the potential for type 1 error, and data have been interpreted conservatively in this context. Accordingly, our discussion has focussed on differences that are likely to be of greatest biological significance (namely differences of $P < 0.01$).

Results

Characteristics of the populations

The characteristics of the study population are detailed in Table 1. There was no statistically significant difference in the ages of the groups although there was a larger age range in the AD group compared with the control group. All children with AD had a modified SCORAD of ≥ 25 , with a mean SCORAD of 42.2 (and a mean modified SCORAD of 33.2). None of the control group had any previous history or current evidence of AD or other allergic disease. Seventy five percent (75%) of the infants with AD were sensitized to allergens (mostly food allergens) at the time of testing (raised serum IgE or positive SPT) compared with none in the control group.

There were no differences in childcare attendance or the number of children in the household between the groups. Most children (95.9%) were breastfed (for a mean of 7.5 months). The duration of breastfeeding was not associated with either the development of AD (odds ratio (OR) = 1.08,

Table 1. Characteristics of the study population with atopic dermatitis ($n = 53$) and without atopic dermatitis ($n = 20$)

	No atopic dermatitis ($n = 20$)	Atopic dermatitis ($n = 53$)	Significance P
Age (months) (mean $\pm$ SD)	12.65 $\pm$ 0.52	10.96 $\pm$ 3.87	0.056
Maternal allergy (no. pos (%))	7 (35)	38 (71)	0.004
SCORAD index (mean $\pm$ SD)	0	42.19 $\pm$ 9.06	<0.001
Modified SCORAD (mean $\pm$ SD)	0	33.23 $\pm$ 7.28	<0.001
Symptomatic IgE mediated food allergy (no. pos (%))	0	16 (30.2)	0.005
Symptoms suggestive of allergic rhinitis (no. pos (%))	0	19 (35.8)	0.008
Doctor diagnosed asthma (no. pos (%))	0	3 (5.7)	0.557
Allergen-specific IgE positive (no. pos (%))	0	40 (75.5)	<0.001
Food-specific IgE positive (no. pos (%))	0	37 (69.8)	<0.001
HDM-specific IgE positive (no. pos (%))	0	12 (22.6)	0.029
Other inhalant-specific IgE positive (no. pos (%))	0	0	N/A

$P < 0.05$ was considered statistically significant.

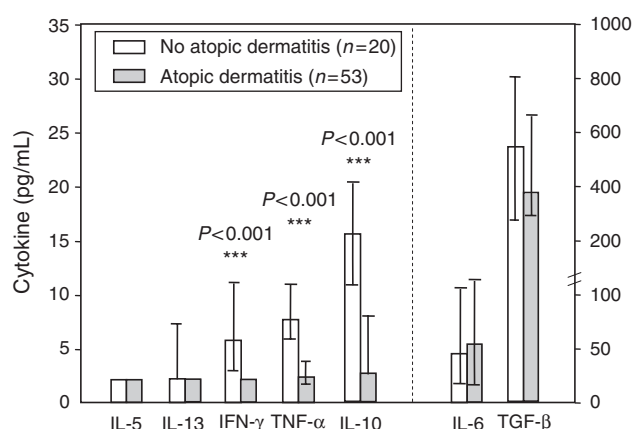


Fig. 1. Comparison of constitutive cytokine production by peripheral blood mononuclear cells (PBMC) from children and without atopic dermatitis. This shows cytokine (IL-5, IL-6, IL-13, IL-10, IFN- γ , TNF- α) production by PBMC incubated for 48 h in tissue culture medium. The responses of children with atopic dermatitis (AD) ($n = 53$) are compared with those of children without AD ($n = 20$). Cytokine data are expressed as median (and interquartile ranges). Differences between the groups were determined by Mann-Whitney U -test and $P < 0.05$ was considered statistically significant. Significance levels are indicated and denoted * if $P < 0.05$, ** if $P < 0.01$, or *** if $P < 0.001$.

95% confidence interval (CI) 0.97–1.2, $P = 0.170$) or PBMC immune responses (data not shown). None of these children was exposed to cigarette smoking in pregnancy, and very few parents were current smokers. Smoking in the home did not contribute to the differences in cytokine patterns with AD described below.

Children with atopic dermatitis have reduced spontaneous cytokine production

Infants with AD demonstrated reduced constitutive IL-10 ($P < 0.001$), TNF- α ($P < 0.001$), and IFN- γ ($P < 0.001$) production compared with infants without eczema (as shown in Fig. 1).

Children with atopic dermatitis have reduced interferon- γ and tumour necrosis factor- α responses to food allergens and vaccines

Stimulated cytokine responses were assessed after subtracting the constitutive production during the same period (48 h).

Children with AD had reduced IFN- γ response to food allergens (both OVA, $P = 0.007$ and BLG, $P < 0.001$) as well as to vaccine antigens (DT, $P = 0.008$ and TT, $P < 0.001$). These impaired IFN- γ responses in children with AD were seen independently of the sensitization status. Children with AD also tended to have lower IFN- γ responses to PHA ($P = 0.053$) compared with children with no AD. The notable exception was HDM, with no difference in IFN- γ response to HDM between children with moderate-severe AD compared to those with no disease ($P = 0.373$). TNF- α responses were also significantly attenuated in children with AD in response to all stimuli (food allergens, bacteria and vaccine antigens) (data not shown). The only exception was with responses to HDM, which were at similar levels as the children without AD. Again, this was seen equally in AD children with ($n = 40$) and without ($n = 13$) sensitization.

Children with atopic dermatitis have reduced regulatory cytokine (interleukin-10) responses to most stimuli

After allowing for differences in constitutive production, children with AD had reduced IL-10 responses to virtually all specific stimuli including allergens (OVA, $P = 0.003$ and BLG, $P = 0.052$) vaccine antigens (TT, $P = 0.010$ and DT, $P = 0.012$) and colonizing skin (HKSA, $P = 0.003$) and intestinal (HKLB, $P = 0.005$) bacteria compared with infants without AD (Fig. 2a). The notable exceptions were IL-10 responses to HDM, which were low level and tended to be slightly stronger in children with AD. There were no differences in IL-10 responses to polyclonal stimulation with PHA or SEB superantigen.

Of note, the impaired IL-10 responses appeared to be more strongly related to the presence of AD rather than the presence of sensitization, in fact the attenuation of IL-10 was more significant in children with AD but no sensitization ($n = 13$) (data not shown). For the combined groups of AD patients, the severity of disease (SCORAD index) in AD patients was directly proportional to the IL-10 responses to both allergen (OVA, $\tau = 0.22$, $P = 0.022$) and staphylococcus (HKSA, $\tau = 0.21$, $P = 0.029$).

IL-10 production was strongly correlated with the production of all other cytokines for each stimulus (Table 2), including both Th2 (IL-5 and IL-13), Th1 (IFN- γ) and pro-inflammatory cytokine responses (TNF- α and IL-6).

The other main regulatory cytokine of interest was TGF- β , which is produced constitutively in large amounts (see Fig. 1). The detection of TGF- β , is complex and can be difficult to interpret [22]. As reported by others, we noted that stimulation generally resulted in significant suppression of TGF- β

production relative to constitutive production (notably after stimulation with PHA and SEB; $P < 0.001$). The percentage suppression of TGF- β relative to background production was no different between the groups except for polyclonal stimulation where children with AD showed significant less suppression in response to both PHA ($P = 0.008$) and SEB ($P = 0.003$) compared with non-AD children.

However, the severity of AD (SCORAD index within the AD group) was negatively associated with gross TGF- β response to virtually all stimuli including HDM ($r = -0.32$, $P = 0.032$), OVA ($r = -0.37$, $P = 0.012$), BLG ($r = -0.37$, $P = 0.015$), TT ($r = -0.34$, $P = 0.026$), DT ($r = -0.30$, $P = 0.052$), HKSA ($r = -0.38$, $P = 0.011$) and PHA ($r = -0.35$, $P = 0.020$).

Children with atopic dermatitis have enhanced T-helper type 2 responses to house dust mite and food allergens

Children with AD had significantly higher Th2 IL-13 (Fig. 2c) and IL-5 production in response to HDM allergen ($P = < 0.001$ and 0.015, respectively). Disease severity (SCORAD index) was directly proportional to the level of HDM-induced IL-13 production ($r = 0.28$, $P = 0.042$). No non-allergic children had detectable HDM-specific IL-5, whereas 25% of children with AD showed IL-5 responses to HDM ($P = 0.014$). In this study the production of IL-13 and IL-5 were associated more strongly with atopy (sensitization) than with AD *per se*. Specifically, the elevated IL-15 and IL-13 responses described above were only seen in AD children who also had evidence of sensitization, but not in the subgroup without sensitization ($n = 13$) (data not shown).

As noted above, in contrast to the reduced IL-10 responses to most other stimuli (Fig. 2a), IL-10 responses to HDM tended to be higher ($P = 0.05$) compared with the unaffected children. There were no significant correlations between HDM IgE levels/sensitization and HDM-specific cytokine responses.

Despite the high frequency of food sensitization in the children with AD, the production of food-specific IL-5 and IL-13 was not elevated relative to the control group. As this may have been due effects of the age range, the AD children were divided into two groups: those 6–12 months ($n = 34$) and those 12–18 months of age ($n = 19$). Infants with AD (6–12 months of age) had significantly higher IL-5 and IL-13 Th2 responses to food allergens than 12–18-month-old children with AD (as shown in Fig. 3), but there were no significant

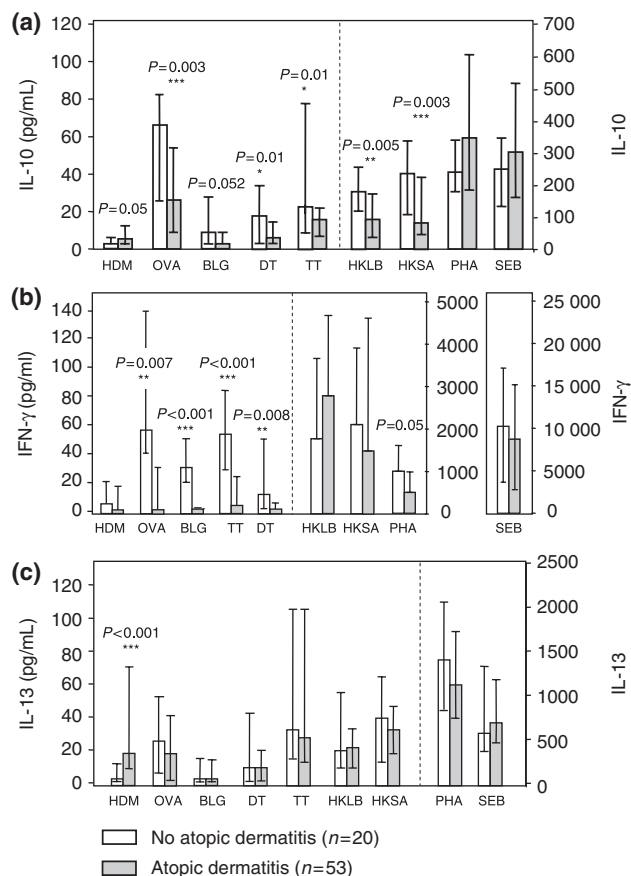


Fig. 2. Comparison of stimulated cytokine production by peripheral blood mononuclear cells (PBMC) from children with and without atopic dermatitis (AD). Cytokine responses after 48 h culture with various stimuli are shown for children with ($n = 53$) and without AD ($n = 20$). Values shown are for (a) IL-10, (b) IFN- γ , and (c) IL-13 cytokine production over and above parallel unstimulated control cultures (i.e. the level after subtracting spontaneous production). Cytokine data are expressed as median (and interquartile ranges). Differences between the groups were determined by Mann–Whitney U -test and $P < 0.05$ was considered statistically significant. Significance levels are indicated and denoted * if $P < 0.05$, ** if $P < 0.01$, or *** if $P < 0.001$.

Table 2. Relationship (Kendall's τ b) between IL-10 responses and other cytokine responses to the same stimuli ($n = 73$)

Cytokine response to same stimuli	IL-10 responses to various stimuli				
	HDM	OVA	TT	HKSA	SEB
IL-13	0.30 ($P = 0.001$)***	0.41 ($P < 0.001$)***	0.33 ($P < 0.001$)***	0.38 ($P < 0.001$)***	0.29 ($P < .001$)***
IL-5	0.39 ($P < 0.001$)***	0.16 ($P = 0.1$)	0.29 ($P = 0.002$)**	0.07 ($P = 0.427$)	0.30 ($P < 0.001$)***
IFN- γ	0.30 ($P = 0.002$)**	0.44 ($P < 0.001$)***	0.35 ($P < 0.001$)***	0.12 ($P = 0.123$)	0.35 ($P < 0.001$)***
TNF- α	0.21 ($P = 0.045$)*	0.42 ($P < 0.001$)***	0.271 ($P = 0.002$)*	0.56 ($P < 0.001$)***	0.36 ($P < 0.001$)***

The relationships between IL-10 responses and other cytokine responses to the same stimuli (HDM, house dust mite; OVA, ovalbumin; TT, tetanus toxoid; HKSA, heat killed *Staphylococcus aureus*; SEB staphylococcal enterotoxin B) are shown for the total study population ($n = 73$) including infants with ($n = 53$) and without ($n = 20$) atopic dermatitis. Correlations are expressed as Kendall's τ b. $P < 0.05$ was considered statistically significant and significance levels are indicated and denoted * if $P < 0.05$, ** if $P < 0.01$, or *** if $P < 0.001$.

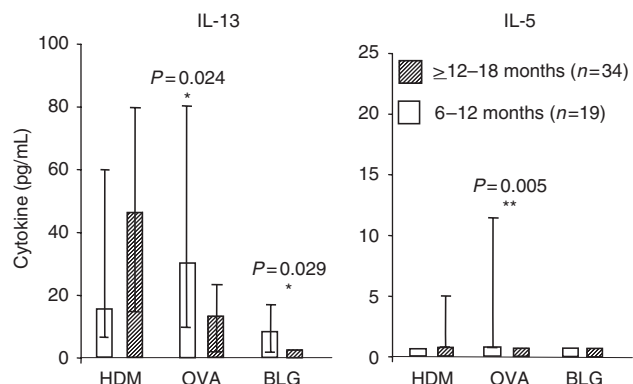


Fig. 3. Comparison of allergen-specific T-helper type 2 (Th2) cytokine responses by children aged less or greater than 12 months of age with atopic dermatitis (AD). a) IL-5 and b) IL-13 cytokine responses by children with AD were compared for children aged 6–12 months ($n = 34$) relative to those who were of aged 12–18 months ($n = 19$). PBMC from these children were stimulated with allergens (HDM, house dust mite; OVA, ovalbumin; or BLG, β -lactoglobulin). Cytokine data are expressed as median (and interquartile ranges). Differences between the groups were determined by Mann–Whitney U -test and $P < 0.05$ was considered statistically significant. Significance levels are indicated and denoted * if $P < 0.05$, or ** if $P < 0.01$.

differences in other cytokine responses to foods, vaccines or any other microbial stimuli between these age groups. There was a non-significant trend for higher Th2 responses to HDM in 12–18-month-old children (Fig. 3).

Responses to skin heat-killed *Staphylococcus aureus* and *Staphylococcus aureus enterotoxin B* and enteric heat-killed *Lactobacillus* species microflora

Cytokine responses to whole killed bacteria and super-antigens (SEB) were much higher than antigen-specific responses. IFN- γ responses to HKSA and HKLB were 100-fold higher (Fig. 2b), and 1000-fold higher to SEB compared with allergen and vaccine responses with no differences in children with AD compared with controls. Consistent with a Th1-dominant response to bacteria, Th2 responses to these stimuli were generally low level with no differences between groups (Fig. 2c). However, these bacteria-induced significant IL-10 responses (at levels 10-fold higher than antigen-specific responses) with detectable differences between groups:

- Reduced IL-10 responses to *Staphylococcus* in AD:** Children with AD had significantly attenuated IL-10 responses to *S. aureus* (HKSA; $P = 0.003$), however there were no differences in any other cytokine responses to HKSA. In contrast to previous studies [6] this group of children with moderate and severe AD did not have significant differences in any of the measured cytokine responses to SEB (including Th2, Th1 and regulatory cytokines).
- Reduced IL-10 responses to *Lactobacilli* in AD:** The only significant difference in responses to HKLB in children with AD was for lower IL-10 responses ($P = 0.005$), compared with children with no AD. All other cytokine responses were comparable between the groups. Although there were no significant differences in TGF- β responses, there were significant correlations between the production of these regulatory cytokines (TGF- β and IL-10) in response to HKLB ($r = 0.29$, $P = 0.022$).

- Relationships between microbial and allergen responses:** Although IL-10 production is induced by different pathways in response to these stimuli, there were consistent strong positive correlations between IL-10 responses to bacteria (both skin and enteric) and IL-10 responses to other antigens, namely food and vaccines (as shown in Table 3). Although this could reflect the general state of IL-10 maturation, there were no relationships between microbial responses and the capacity for PHA mitogen-induced IL-10 secretion (Table 3).

The magnitude of IL-13 responses to microflora (both HKLB and HKSA) were also significantly associated with the production of IL-13 in response to allergens, vaccines and mitogens (Table 3). At this age, the production of IFN- γ to microflora was strongly associated with the maturation status of IFN- γ function (as assessed by polyclonal PHA responses), and while there were some relationships with allergen responses there were no relationships with vaccine responses (Table 3).

Discussion

Our findings confirm that very young children with AD have significant differences in both resting and stimulated cytokine responses to multiple stimuli. Specifically, PBMC from affected children have significantly lower spontaneous production of IL-10, IFN- γ and TNF- α (Fig. 1). Furthermore, these children also had reduced capacity to produce these same cytokines (IL-10, IFN- γ and TNF- α) in response to food (but not HDM) allergens and vaccines compared with unaffected children (Figs 2a and b) after allowing for differences in baseline production. In this population the associations between AD and reduced IL-10, IFN- γ and TNF- α production was independent of sensitization status, suggesting independent pathways. Because of the cross-sectional nature of this study it is not possible to determine if these differences in cytokine production pre-dated the onset of clinical disease (and if they are therefore causally implicated) although we have previously noted impaired cytokine responses (including IL-10 and IFN- γ responses) in neonates at high risk of allergic disease [23].

It is of interest that the attenuated IL-10 responses in AD were seen for all specific stimuli except HDM. IL-10 responses to HDM were generally lower than food (OVA) responses at this age and it is possible that these observations reflect known differences in the rate maturation of pathways that regulate food and inhalant allergen responsiveness and different mechanisms of tolerance.

Although initially classified as a Th2 cytokine, there has been growing recognition of the immuno-regulatory properties of IL-10. There has been growing interest in the role of potential IL-10 in regulating or inhibiting allergic responses and the expression of allergic disease states ([24] and many others). The findings in our study are consistent with this notion, and strongly suggest that very young children with moderate-to-severe AD have impaired capacity for IL-10 responses to many different types of stimuli. This also supports observations that children with evidence of allergic disease in the first years of life have impaired PBMC IL-10

Table 3. The relationship (Kendall's τ b) between cytokine responses to microflora (HKSA and HKLB) and responses to other antigens ($n = 73$)

	IL-10			IL-13			IFN- γ		
	OVA	BLG	HDM	OVA	BLG	HDM	OVA	BLG	HDM
(a) Relationship to allergen (OVA, BLG and HDM) responses									
HKLB	0.47 (<0.001)***	0.23 (0.007)**	0.09 (0.32)	0.49 (<0.001)***	0.45 (0.001)**	0.14 (0.105)	0.22 (0.01)*	− 0.08 (0.384)	0.18 (0.044)*
HKSA	0.45 (<0.001)***	0.25 (0.004)**	0.10 (0.235)	0.55 (0.001)**	0.37 (<0.001)***	0.17 (0.037)*	0.29 (0.001)**	− 0.03 (0.776)	0.13 (0.128)
	IL-10			IL-13			IFN- γ		
	TT	DT	PHA	TT	DT	PHA	TT	DT	PHA
(b) Relationship to vaccine (DT and TT) responses (and polyclonal stimulation)									
HKLB	0.21 (0.008)**	0.22 (0.012)*	− 0.04 (0.665)	0.20 (0.017)*	0.15 (0.091)	0.21 (0.011)*	0.12 (0.146)	0.06 (0.50)	0.22 (0.005)**
HKSA	0.25 (0.002)**	0.26 (0.003)**	0.002 (0.977)	0.23 (0.004)**	0.22 (0.011)*	0.22 (0.006)**	0.12 (0.147)	0.10 (0.258)	0.24 (0.003)**

The relationships between cytokine responses to microflora (HKSA, heat-killed *Staphylococcus aureus*; HKLB, heat-killed *Lactobacillus fermentum*) and responses to (a) allergens (HDM, house dust mite; BLG betalactoglobulin; OVA, ovalbumin) and (b) vaccine (TT, tetanus toxoid; DT, diphtheria toxoid) and (c) polyclonal stimulation (PHA, phytohemagglutinin) are shown for the total study population ($n = 73$) including infants with ($n = 53$) and without ($n = 20$) atopic dermatitis. Correlations are expressed as Kendall's τ b. $P < 0.05$ was considered statistically significant and significance levels are indicated and denoted *if $P < 0.05$, **if $P < 0.01$, or ***if $P < 0.001$.

responses to allergens [25] and polyclonal stimuli [26]. IL-10 is likely to be produced by a number of cells in these mixed culture systems, including T cells, B cells and monocytes. It has previously been noted that purified T cells from children (less than 4 years of age) with AD express significantly lower levels of IL-10 mRNA compared with unaffected children [27], although this does not exclude defects in other cells. Our finding that high-level IL-10 responses to bacteria were also attenuated in AD suggest that cells (such as monocytes) which are strongly stimulated through these innate pathways are also affected although this needs to be investigated further. These findings lend further support to observations that the relationship between IL-10 and AD appears to be very different in early childhood than in adulthood when disease is more established. Although IL-10 responses appear attenuated in most [25–27] but not all [28] studies of childhood AD, studies in adults show that AD is associated with higher numbers of IL-10-producing cells in circulation [29], higher spontaneous IL-10 mRNA expression [30], and stronger IL-10 responses HDM to allergens (HDM) [31] and LPS [32].

We did not see inverse relationships between this presumed inhibitory cytokine and the production of effector cytokines. Rather, we noted consistently strong *positive* correlations between IL-10 responses and all other cytokines including Th1, Th2 and pro-inflammatory cytokines. This was also seen for most stimuli and further suggests that this pattern of attenuated *in vitro* responses in AD is not limited to allergen-induced cytokine responses.

Clearly, immunologic events in the skin are more relevant to AD than those in peripheral blood, which provide a useful but indirect measure of immune reactivity. Allergen-reactive T cell clones derived from skin (in adults) produce more IL-4, IL-5 and IL-10 compared with clones derived from peripheral blood of same individual [33], suggesting that local factors play an important role in programming the patterns responsiveness of skin homing immune cells. Well recognized 'barrier disruption' in AD skin is likely to result in increased contact between the local immune system and environmental exposures including *S. aureus* which colonizes AD skin more

heavily, and contact allergens. In addition to the well-recognized polyclonal T cell activation through the 'super-antigen' effects of *S. aureus* toxins such as SEB, recent studies show that SEB may also inhibit the immunosuppressive activity of CD4⁺CD25⁺ regulatory T cells [10], suggesting another mechanism by which *S. aureus* could amplify local T cell activation in patients with AD. As another important aspect of local immune regulation, *S. aureus*-induced IL-10 responses may be also important for dampening excessive response on encounter with *S. aureus*, including non-toxicogenic forms. Our finding that children with AD also have significantly attenuated IL-10 responses to *S. aureus* (HKSA; $P = 0.003$) (Fig. 2a) suggest a further mechanism by which *S. aureus* species may promote poorly controlled T cell activation.

There is also speculation that early barrier disruption results in increased allergen encounter and that this may be an important factor driving local immune reactivity as well as patterns of systemic sensitization in patients with AD. Animal studies confirm that barrier disruption favours Th2-dominant response after percutaneous application of inhalant allergen (HDM) [7]. In our study, children with AD have significantly higher Th2 (IL-13 and IL-5) responses, and the magnitude of the IL-13 response was proportional to the SCORAD index ($r = 0.28$, $P = 0.042$). In general, however, these higher Th2 IL-5 and IL-13 responses (to HDM) were a function of atopy (sensitization) rather than AD *per se*, as they were not seen in AD children who were not sensitized (to this or other allergens). Although most (70%) of the children with AD also had food allergen sensitization, the production of Th2 cytokines to food allergens was not a defining feature in this population (Fig. 2c). This potential relationship may have been masked by the age range in the study (6–18 months), which could include some children who have Th2-dominant response and others who are beginning to develop tolerance [15]. In support of this we observed (Fig. 3) that younger children with AD (less than 12 months) did have stronger food allergen-specific Th2 responses compared with 12–18 month olds with AD.

In this study, there was a consistent relationship between AD and deficient IFN- γ responses. Impaired Th1 responses in early life have been associated with future risk [34] and subsequent development of allergic disease [35–37], including AD in children [28, 38] and adults [39, 40]. However, our findings are at odds with the observations by Smart and Kemp [41] (and others) who have previously noted that allergic children have increased IFN- γ responses to allergens. However, this population is much younger (11.4 months) than the population in the aforementioned study by Smart and Kemp [41] (age range 2–16 years), and it is possible that the weaker IFN- γ responses in affected children in this study may be a residual reflection of a impaired neonatal IFN- γ responses frequently seen in future atopics [35–37]. It is still not clear how this early deficiency is implicated in the subsequent excessive (poorly regulated) responses at older ages, although it could indicate early failure of regulatory systems. Collectively these observations support the concept that maturation of systemic IFN- γ responses may play a role in the inhibition of allergic sensitization and associated cutaneous disease. Although this may be because of differences in intrinsic T cell function [38], differences in APC maturation are also likely but not well documented in this context and we have not examined differences in separate cell types in this study. The microbial environment plays a major role in immune maturation, the gut is the largest source of microbial stimulation. Differences in exposure and other intestinal events appear to influence the function of peripheral blood lymphocytes [42], which circulate through the gut in their immature forms before seeding to peripheral tissues including the skin. Although they do not transit the gut, tissue APC appear equally dependent on environmental microbial exposure as the major stimulus for normal maturation [43]. Delays in systemic maturation of the APC compartment have been implicated as one of the most likely mechanisms for the increasing propensity for allergic disease [44]; as a presumed result of reduced microbial burden in infancy [44]. In support of this, circulating monocytes in infant animals mature at significantly different rates depending on enteric microflora exposure [45]. In this study we examined for potential differences in responses to a common intestinal species, namely *Lactobacillus* and observed that children with AD had deficient IL-10 responses compared with normal children. Although other cytokine responses were similar a relatively IL-10 depleted milieu in the gut mucosa in response to these organisms could have significant effects on immune development.

This group of young children with AD is at highest risk of developing persistent aeroallergen sensitization and associated respiratory diseases (asthma and AR) particularly those with more severe disease [1] or food allergen sensitization [46]. As the earliest manifestation of allergic disease in many children, AD may offer future opportunities for more definitive targeted interventions to prevent the progression to other more persistent forms of disease. As shown in this study, immune dysregulation is a very early event in affected children, and this is associated with impaired systemic IL-10, IFN- γ and TNF- α responses to multiple stimuli, as well as very early Th2 responses to HDM. A better understanding of how these events lead to aeroallergen disease may lead to novel prevention strategies, including strategies to improve skin barrier integrity and factors which may enhance normal immune development in the gut.

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