

Original article

The value of perinatal immune responses in predicting allergic disease at 6 years of age

Background: Characterizing early abnormalities in immune development of allergic individuals provides an important basis for defining disease pathogenesis and future prevention strategies. This study compares patterns of early immune responses in an established cohort based on allergic outcomes and allergen skin prick test (SPT) reactions at 6 years of age.

Methods: Children from an original birth cohort ($n = 60$) consisting of 44 high risk (HR) (family history of allergy) and 16 low risk (LR) (no family history) were reassessed at 6 years of age. Detailed clinical information about allergic disease was obtained ($n = 53$) and a subgroup ($n = 31$) consented to have allergen SPT to common food and inhalant allergens. Data from previous immunological assessments performed at birth, 1 and 2 years of age, including lymphoproliferation and cytokine [interleukin (IL)-4, IL-5, IL-6, IL-10, IL-13 and interferon (IFN)- γ] responses to ovalbumin (OVA), house dust mite (HDM), cat allergen (Fel d 1), phytohaemagglutinin (PHA) and tetanus toxoid, were re-analysed based on the 6-year clinical outcomes.

Results: Twenty-eight HR and three LR children had a clinical history of allergic disease at 6 years of age including doctor diagnosed asthma ($n = 17$) and/or eczema ($n = 24$). Most children (78%) with atopy at 6 years had positive SPT to the allergens tested, and 70% had symptoms within the last year. Children at genetic risk (family history) of allergy had weaker ($P = 0.017$) polyclonal T helper 1 (Th1) IFN- γ responses in the neonatal period compared with LR children. Although children with allergic disease at 6 years also tended to have weaker neonatal IFN- γ responses compared to those with no symptoms, but this was not quite significant ($P = 0.05$). A positive SPT to HDM at 6 years was associated with higher IL-13 responses to HDM at 1 year ($P = 0.02$), whereas allergic disease at 6 years was associated with higher IL-5 messenger RNA (mRNA) responses to HDM at 1 year ($P = 0.01$). Despite these associations, regression analysis demonstrated that the only significant early predictors of allergic sensitization at 6 years of age were a family history of allergic disease, and atopic symptoms at 2 years. Importantly, none of the early immunological parameters measured were significantly predictive of allergic disease or allergic sensitization in these 6-year-olds.

Conclusions: Although our observations suggest that subtle differential alterations in cytokine responses during early immune development are associated with different aspects of subsequent atopy, there are still no early predictive biomarkers of disease. A positive family history of allergy remains the dominant predictive factor.

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Although allergic diseases now affect almost half the population in some 'western' countries at enormous personal and societal costs, the disease pathogenesis and aetiology are still poorly understood. Numerous studies suggest that individuals with characteristic immunoglobulin (Ig)E-mediated inflammation have altered underlying cellular immune responses to allergens compared with nonallergic individuals. However, this is likely to be more

complex than a simple shift from the T helper cell type 1 (Th1)-like responses typical of nonallergic responses adults, to the T helper cell type 2 (Th2) cytokine (IL-4, IL-5, IL-13) patterns which have been associated with the allergic response.

Early life events appear to have a critical influence on the ultimate pattern of immune maturation (1), and there has been a pressing need for longitudinal studies to better

define how immune responses evolve in atopic and nonatopic children. In a prospective cohort study (2), we previously compared the early postnatal immune responses in children considered at high risk (HR) of allergy because of affected first-degree relatives, with low risk (LR) children with no family history of allergy. This demonstrated that HR children who developed symptoms suggestive of allergy by 2 years of age showed presymptomatic allergen-specific Th2 responses, often within the first year of life. This was in contrast to asymptomatic LR children, who demonstrated down-regulation of the apparently normal neonatal pattern of Th2 responses within the first year of life. We speculated previously that relative immaturity of Th1 function in the perinatal period might be a potential mechanism for the failed suppression of Th2 responses in atopics. However, despite similar observations of impaired neonatal Th1 responses in either HR or symptomatic infants by a number of other groups (3–8), the significance and predictive value of altered IFN- γ responses in the neonatal period has remained unclear.

One reason for this controversy has been the difficulty relying on either atopic 'risk' or often-ambiguous clinical symptoms in infancy to define atopic status. This was a recognized shortcoming of our original publication in the *Lancet* (2). Here, we demonstrate that the previously observed relationships remain consistent after clinical re-evaluation of the same cohort at 6 years of age. We also explore the relationship between early immune responses and allergic sensitization at 6 years of age, and the predictive value of these measures. To this end, we have examined clinical outcomes (and not assessed the immune responses) at 6 years of age, as this has already been addressed in other studies by our group (9) and others. We have focused the predictive value of early immune responses because this is a more novel area and remains of great interest.

Methods

The original cohort

This cohort of 60 children was originally recruited prior to birth in 1995 (10). All women with uncomplicated pregnancies booked for elective caesarean sections (at King Edward Memorial Hospital and St John of God Hospital Subiaco, Western Australia) between April and June 1995 were approached. As the initial focus of the study was the T-cell immune responses of the unstressed term fetus, uncomplicated elective caesarean deliveries seemed ideal for this purpose, as far less inflammatory mediators are released during caesarean section than during vaginal delivery (11). The most common indication for caesarean section was a previous elective or nonelective caesarean section (46.7%). Other indications included breech presentation or unstable lie (18.3%), cephalo-pelvic disproportion (6.7%), previous maternal abdominal surgery (3.3%), previous large for gestational age baby or birth trauma (3.4%), and miscellaneous causes (8.2%). In 13.3% of cases there were no clear medical indications for the operative delivery.

Initial assessments of atopic risk

The allergic risk of each infant was based on maternal and family allergy. This was determined by detailed interview-questionnaires and maternal allergen skin prick testing (SPT). All mothers with atopic symptoms had positive SPT. Neonates were considered to be at high atopic risk if there was a history of doctor diagnosed allergies [asthma, atopic eczema/dermatitis syndrome (AEDS), or seasonal allergic rhinitis] in one or more first-degree relatives. Those with no family history were considered to be at LR. Based on these criteria, there were 44 HR and 16 LR babies in the initial study population.

Atopic status and follow-up assessments

The children were initially seen at 6 monthly intervals (until 2 years of age) for clinical history (from mother), physical examination, and blood collection. SPT were performed at 1 and 2 years of age. They were then seen for a further clinical assessment and SPT at 6 years of age. Clinical information was available on most children ($n = 53$) at 6 years, but only a subgroup ($n = 31$) agreed to have SPT performed at this age.

A diagnosis of AEDS (12) was made in infants with typical skin lesions (13), or doctor diagnosed eczema responsive to topical steroids. The diagnosis of asthma was made in children with recurrent wheezing (three or more episodes, at least one confirmed by pediatrician or general practitioner). All study children diagnosed with asthma were responsive to bronchodilator therapy. Most (all except one) of these children had other evidence of atopic sensitization [positive radioallergosorbent (RAST), SPT and/or other atopic disease]. Children with possible rhinitis, mild eczema, or urticaria were only included as 'atopic' if other features atopic disease (including positive SPT) were present.

Allergen skin prick testing

Standardized allergens for SPT were all manufactured by Hollister Stier, Miles Inc., Pharmaceutical Division (Elkhart, IN). These included house dust mite (HDM) (standardized mite *Dermatophagoides pteronyssinus*) 30 000 AU/ml, southern grass mix (1 : 20 w/v), rye grass (perennial *Lolium perenne*) (1 : 20 w/v), mold mix o. 4 (1 : 10 w/v), standardized cat pelt (10 000 AU/ml), whole egg (1 : 20 w/v), peanut mix (Runner, Virginia, Spanish) 1 : 10 w/v, whole cows milk (1 : 20 w/v), negative control (glycerine) and positive control (histamine phosphate 10 mg/ml). Skin prick tests were performed according to standardized techniques (14). The wheal size was measured as the mean diameter (of the longest and the midpoint orthogonal diameters). The test was regarded as positive if the wheal diameter was > 3 mm. Positive results were all clearly positive with wheals of > 5 mm in most cases.

Sample collection

Cord blood was collected as previously described (10). Peripheral blood collection was performed by routine venepuncture from the cubital fossa at 6, 12, 18 and 24 months of age.

Immunological measurements

Mononuclear cells from samples collected at birth (CBMC) and each subsequent visit (PBMC) were cultured with allergen (and mitogen) for lymphoproliferation and cytokine assays. These

methods have been documented in previous publications from this cohort (2, 10, 15).

Briefly, for lymphoproliferation assays CBMC were cultured in triplicate for 7 days in microwells at 10^6 cells/ml in serum free medium, either alone or in the presence of aeroallergens (HDM extract 10 µg/ml, rye grass 10 µg/ml, Fel d 1 30 µg/ml), food allergens [ovalbumin (OVA) 100 µg/ml and β LG 100 µg/ml] or tetanus toxoid (0.5 Lf), at 37°C in 5% CO₂. The PBMC collected during subsequent visits were cultured in the same conditions, but for 6 days. Data were expressed as delta dpm per culture, as previously, and responses were considered positive if twice background levels and more than 1000 dpm above background levels (16).

For cytokine analysis mononuclear cells from birth and each postnatal visit were cultured for 24 h in medium alone, or supplemented with optimal stimulating concentrations of HDM (10 µg/ml), OVA (10 µg/ml), Fel d 1 (30 µg/ml) or tetanus toxoid (0.5 Lf) (2, 9). Responses to phytohaemagglutinin (PHA, 1 µg/ml) were measured under the same conditions, but at birth only.

Supernatants (from cultures at birth, 1 and 2 years) were assayed for the presence of IL-6, IL-10, IL-13 and IFN-γ employing commercial enzyme-linked immunosorbent assay (ELISA) kits (CLB Laboratories, Amsterdam, The Netherlands). The sensitivities of the ELISAs were 4 pg/ml (IL-5), 30 pg/ml (IL-10), 2 pg/ml (IL-13) and 5 pg/ml (IFN-γ). Cell pellets from each visit were processed for cytokine messenger RNA (mRNA) expression as in Ref. (10). IL-4, IL-5, IL-9 and IFN-γ were detected together with β-actin, as specific mRNA measured in cell pellets (or the same 24-h cultures) by standardized semi-quantitative RT-PCR as described in Ref. (10). The intensity of each test sample was expressed as a ratio to its respective β-actin mRNA expression. In all cases the results were expressed as the magnitude of mRNA ratio, or protein level in the antigen-stimulated cultures, above the unstimulated cultures (i.e. the level of up-regulation after stimulation).

Statistical analysis

All comparative statistical tests used nonparametric models, unless otherwise stated. InStat (version 2.0 for Macintosh) was used for Fisher's exact tests. For larger data sets, SPSS (version 10 for Macintosh) was used for unpaired Mann-Whitney *U*-tests, logistic regression and Spearman (nonparametric) correlation coefficients, under the guidance of a biostatistician.

Results

Clinical outcomes

Clinical information was available on 53 of the 60 children at 6 years of age (and seven were lost to follow-up or declined further involvement). Twenty-eight children had a clinical history of allergic disease at 6 years of age, including 25 of 40 (62%) HR infants and only three of 13 (23%) LR infants reviewed at 6 years. These children had a history of asthma or recurrent (bronchodilator responsive) wheeze ($n = 17$), and/or doctor diagnosed AEDS ($n = 24$). Nineteen (19) had active disease (in the last 6 months). Thirteen ($n = 13$) had additional symptoms of allergic rhinitis or other symptoms ($n = 10$) including previous food allergy or urticaria. Children with rhinitis or urticaria were only classified as atopic if they had positive SPT (≥ 3 mm wheal) or other features to suggest atopy (most had AEDS and asthma). There were

no differences in cigarette smoke exposure when comparing children on the basis of either SPT reactivity or atopic symptoms.

There was a strong relationship between the diagnosis and classification of probable allergy at 2 years (2) with confirmed history of clinical disease at the 6-year visit ($P < 0.001$). Ninety-four percent of those with symptoms at 2 years children also had atopic features at 6 years. Of the initial HR infants 62% (25 of 40) developed atopic disease by 6 years of age, compared with only 23% (three of 13) of the LR infants ($P = 0.02$) seen at follow-up (see Table 1). Only two of 13 (15%) of the LR children developed positive SPT by 6 years of age, compared with 18 of 26 (69%) of the HR children ($P = 0.002$). While most children (72%) with no symptoms at 2 years remained asymptomatic at 6 years (21 of 29), some of these (28%) did go on to develop later allergic symptoms (eight of 29).

Most children (78%) with atopy at 6 years had positive SPT to at least one of the allergens tested (14 of 18). However, 38% of the children with no symptoms of allergy (at 6 years) also had positive SPT to one or more allergens tested (Table 1). All SPT positive children were reactive to aeroallergens (although two also had responses to foods).

Table 1. Association between atopic disease at 6 years, family history, skin prick test (SPT) reactivity and previous symptoms (at 2 years). Children with allergic disease at 6 years are compared on the basis of atopic risk (family history of allergy), probably symptoms of allergy at 2 years, and SPT reactivity

	Atopic at 6 years ($n = 28$)	Nonatopic at 6 years ($n = 25$)
Atopic risk (family history)		
High risk ($n = 40$)	25	15
Low risk ($n = 13$)	3	10
	$(P = 0.02)$	
Atopic symptoms at 2 years of age		
Symptoms ($n = 18$)	16	1
No symptoms ($n = 32$)	8	21
Missing data	4	3
	$(P < 0.0001)$	
+SPT at 6 years (to any allergen)		
Positive	14	5
Negative	4	8
(Not known)	10	12
	$(P = 0.06)$	
+SPT at 6 years (HDM only)		
Positive	11	4
Negative	6	9
(Not known)	11	12
	$(P = 0.1)$	
+SPT at any age (1, 2, 6 years)		
Positive	15	5
Negative	5	9
(Not known)	8	11
	$(P = 0.03)$	

Statistical comparisons were made using Fisher's exact test. Significance (P) values are shown.

Relationship between atopic symptoms at 6 years and immune responses at birth

Neonatal PHA-induced lymphoproliferative responses were significantly higher ($P = 0.019$) in babies who developed atopic disease by 6 years of age compared with those who did not (not shown). However, there were no significant differences in the magnitude of allergen-induced lymphoproliferation between these groups.

Although the atopic children had stronger neonatal CBMC proliferative responses to PHA, the neonatal Th1 cytokine responses tended to follow an inverse pattern. Cord blood IFN- γ responses (to PHA) were previously noted to be lower in the HR children in this cohort (14), although children with allergic disease at 6 years also tended to have lower neonatal IFN- γ responses (Fig. 1), this was not quite significant ($P = 0.05$). IFN- γ responses to HDM followed the same pattern, but were also not statistically significant ($P = 0.06$).

There were no significant differences in neonatal Th2 responses to HDM in children with diagnosed allergic disease at 6 years of age, compared with nonallergic children (Fig. 2). Neonatal IL-6 responses to Fel d 1 ($P = 0.008$) were lower in children with allergic disease at 6 years (not shown). However, the subgroups of children with a diagnosis of asthma at 6 years had weaker IL-13 responses to OVA ($P = 0.03$) and Fel d 1 ($P = 0.008$) at birth. IL-4 mRNA responses to OVA also tended to be lower ($P = 0.05$). There were no other significant differences in the neonatal immune responses.

Relationship between SPT reactivity at 6 years and immune responses at birth

Neonatal IFN- γ responses (to PHA) also tended to be lower in children with positive SPT at 6 years but this was not statistically significant (Fig. 1).

There was a consistent trend for lower Th2 like cytokine responses at birth in the SPT positive group (Fig. 2). Children with positive SPT to HDM at 6 years had lower IL-13 responses to HDM at birth ($P = 0.01$), although this relationship reversed early in the postnatal period (see below). There were similar trends for IL-10 and IL-6 responses (to HDM) but these were not quite significant ($P = 0.07$ and 0.06 , respectively). Children with positive SPT (to one or more allergens) also had lower IL-6 and IL-13 responses to tetanus toxoid ($P = 0.007$ and 0.05 , respectively) and Fel d 1 ($P = 0.05$ and 0.008 , respectively). There were no other significant differences in neonatal responses of SPT positive and negative children.

Relationship between atopic symptoms at 6 years and early postnatal immune responses (at 1–2 years of age)

Children with atopic disease at 6 years showed an increase in Th2 cytokine responses after birth. This was most evident for IL-5 mRNA responses to HDM, which increased significantly between birth and 2 years of age in the atopic group ($P = 0.01$) but did not change significant in the nonatopic group (not shown). The same pattern

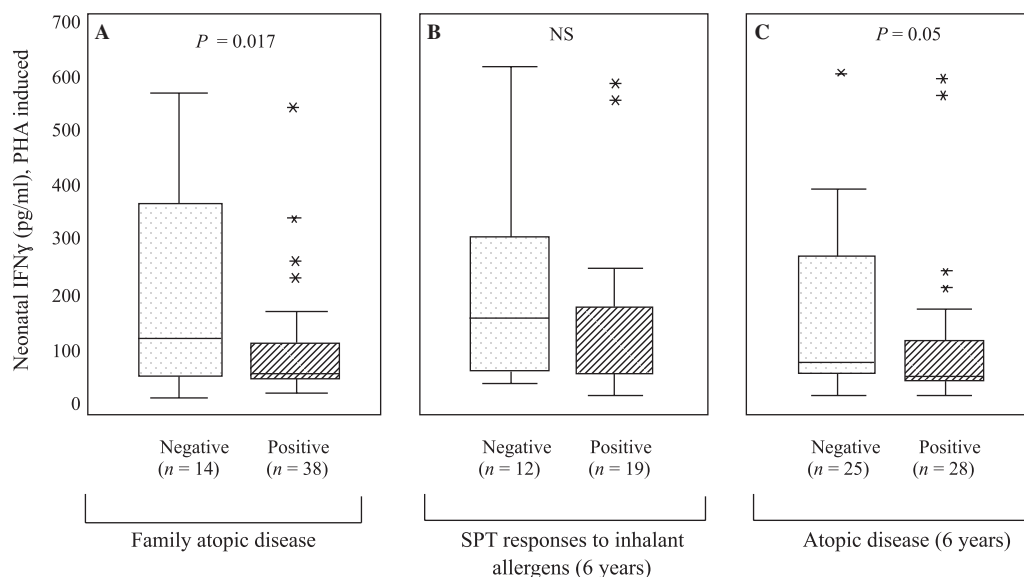


Figure 1. Comparison of neonatal phytohaemagglutinin (PHA)-induced interferon (IFN)- γ responses according to atopic heredity and atopic outcomes at 6 years of age. Neonatal IFN- γ responses to PHA are compared on the basis of: (A) family history of allergy, (B) SPT responses to inhalant allergens at 6 years, and (C) atopic disease at the age of 6 years. The IFN- γ levels in culture supernatants (pg/ml) are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. All responses are shown as the levels in stimulated cultures after subtracting levels in the unstimulated control cultures. Groups were compared using nonparametric Mann-Whitney U -test (significance values are as shown).

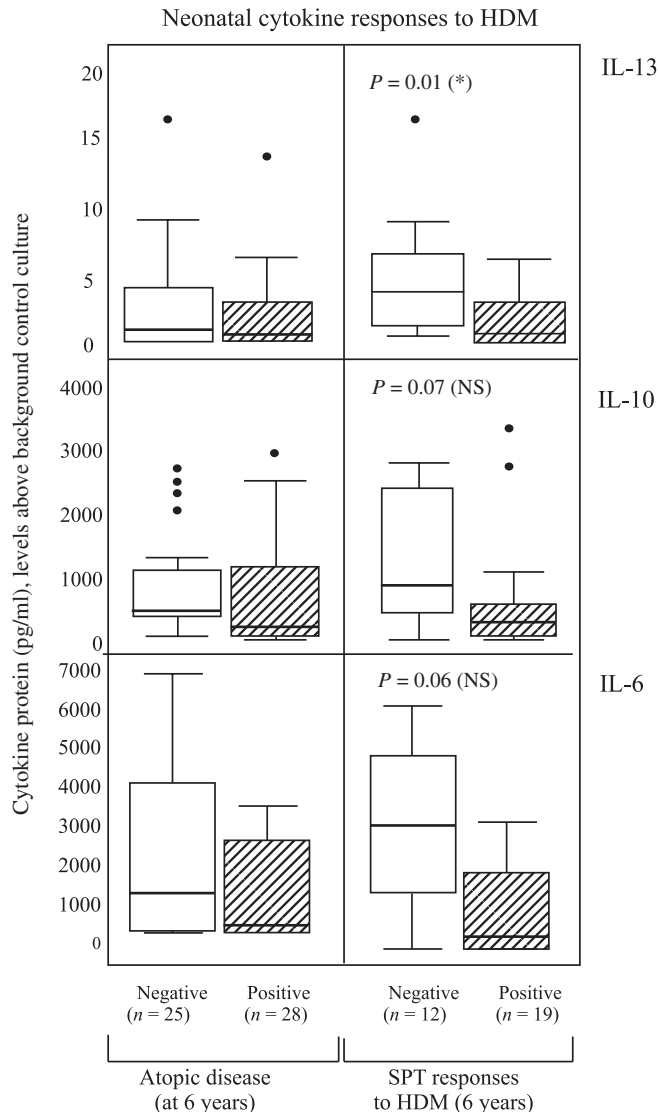


Figure 2. Comparison of neonatal HDM-cytokine responses by atopic outcomes at 6 years of age. Neonatal Th2-like cytokine (IL-13, IL-10, IL-6) responses to HDM are compared on the basis of atopic disease at the age of 6 years (left panels), or SPT responses to inhalant allergens at 6 years (right panels). The cytokine levels in culture supernatants (pg/ml) are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. All responses are shown as the levels in stimulated cultures after subtracting levels in the unstimulated control cultures. Groups were compared using nonparametric Mann-Whitney *U*-test (with significance values as shown).

was seen for IL-13 but this was not statistically significant (not shown).

Interleukin-5 mRNA responses were significantly higher ($P = 0.01$) in the atopic children at 1 year of age (Fig. 3). Although IL-13 and tumor necrosis factor (TNF)- α responses to HDM also tended to be higher in atopic infants during infancy (at 1 and 2 years) these

differences were not statistically significant (Fig. 3). There were no significant differences in responses to OVA or other allergens.

Relationship between SPT reactivity at 6 years and early postnatal immune responses

Interleukin-13 responses to HDM increased significantly ($P = 0.04$) from birth to 2 years in the children who were SPT positive at 6 years. There were parallel changes in IL-4 although this was not statistically significant. These patterns were not seen in the SPT negative children (not shown).

Compared with SPT negative children (Fig. 4), the SPT positive group had significantly higher HDM-specific IL-13 ($P = 0.02$) and TNF- α ($P = 0.03$) responses at 1 year, and a similar but nonsignificant trend for higher IL-5 mRNA ($P = 0.08$). IL-5 mRNA responses to OVA at 1 year of age were also higher ($P = 0.01$) in the children who had positive (aeroallergen) SPT responses at 6 years of age (not shown). Only one of these children had a history of symptomatic egg allergy.

Conversely, IFN- γ Th1 responses to HDM at 1 year of age tended to have weaker ($P = 0.06$) in these SPT positive children (Fig. 4E).

There were no differences in IL-10 and IL-6 responses based on family history, SPT reactions, or allergic disease at 6 years of age. There were no additional relationships found when comparing children on the basis of 'current' symptoms as opposed to 'previous' symptoms.

Value of perinatal immune response in predicting allergic disease/sensitization at 6 years

A key objective of this follow-up study was to determine whether early immunological markers can be used to 'predict' allergic disease and SPT reactivity in later childhood. We also examined 'family history' and 'allergic symptoms at 2 years as predictors of allergic outcomes in 6-year-olds. Using univariate logistic regression with neonatal IFN- γ responses to PHA as the sole predictor variable, we demonstrated that this factor did not significantly predict either SPT reactivity (weight coefficient = -0.001 ; SE = 0.002 ; $\chi^2 = 0.36$; $P = 0.5$; OR = 0.999 ; 95% CI = 0.994 – 1.003) or allergic disease (weight coefficient = -0.001 ; SE = 0.415 ; $\chi^2 = 0.07$; $P = 0.8$; OR = 0.99 ; 95% CI = 0.995 – 1.003) at 6 years of age. In separate analyses, none of the other immunological variables discussed above was significantly predictive of allergic disease or SPT reactivity in 6-year-olds. Only family history (weight coefficient = 2.1 ; SE = 0.94 ; $\chi^2 = 5.9$; $P = 0.014$; OR = 8.59 ; 95% CI = 1.3 – 54.1) and allergic symptoms at 2 years (weight coefficient = 3.1 ; SE = 1.1 ; $\chi^2 = 7.1$; $P = 0.008$; OR = 21.9 ; 95% CI = 2.2 – 212) had any significant predictive effects on allergic sensitization at 6 years.

Discussion

The major aim of the study was to prospectively assess whether there was a *predictive relationship* between *immune responses in early infancy* and later *clinical* (rather than immunological) *outcomes* at 6 years of age. Immune responses at 6 years (not measured) were not relevant to this aim. Furthermore, many other studies have already assessed immune responses in established disease *vs* no

disease at this age, including those by our own group (9), and would add new information here. On the other hand, the predictive value of early immune responses focused on here, is a more novel area and remains of great interest.

Genetic risk (family history) is an established risk factor for allergy, and has been associated with weaker neonatal IFN- γ responses (3–8). This pattern was also seen in the current study, and supports the notion that the development of atopy is linked with an exaggerated neonatal immaturity of Th1 function. Weaker neonatal IFN- γ responses to PHA were not associated with reduced capacity for mononuclear cell activation, as cell division (measured by lymphoproliferation) was actually increased in the atopic group.

In this population, variations in neonatal IFN- γ responses were related more strongly to family history (Fig. 1A) than atopic outcomes (Fig. 1B,C). Although atopic children (with allergic disease or SPT reactivity at 6 years of age) also tended to have lower IFN- γ responses to PHA in the neonatal period compared with nonatopic children, this was quite not significant (Fig. 1B,C), possibly because of the smaller numbers in this analysis. However, even the association with family history was not strong enough for IFN- γ responses to have any value in predicting allergy outcomes at 6 years.

Beyond the neonatal period the relationship between allergy and IFN- γ responses is less clear. Mitogen-induced responses were not measured after birth, and allergen-specific responses were generally low level. Children with allergic disease or family allergic disease did not have significantly lower IFN- γ responses to HDM or OVA. Studies in other populations show high IFN- γ responses to both food (17) and inhalant (18) allergens in allergic children, and question the presence of 'IFN- γ deficiency' in allergy. Further studies are necessary to clarify this, and there is growing evidence that atopic disease is associated with stronger mixed Th0 (Th1 and Th2) cytokine responses rather than Th2 skewed (Th1 deficient) responses. Vaccine studies indicate that this 'mixed Th0 hyper-responsiveness' is not limited to allergens. Infants at HR of atopy also show transiently stronger Th0 (Th1 and Th2) responses to both vaccine

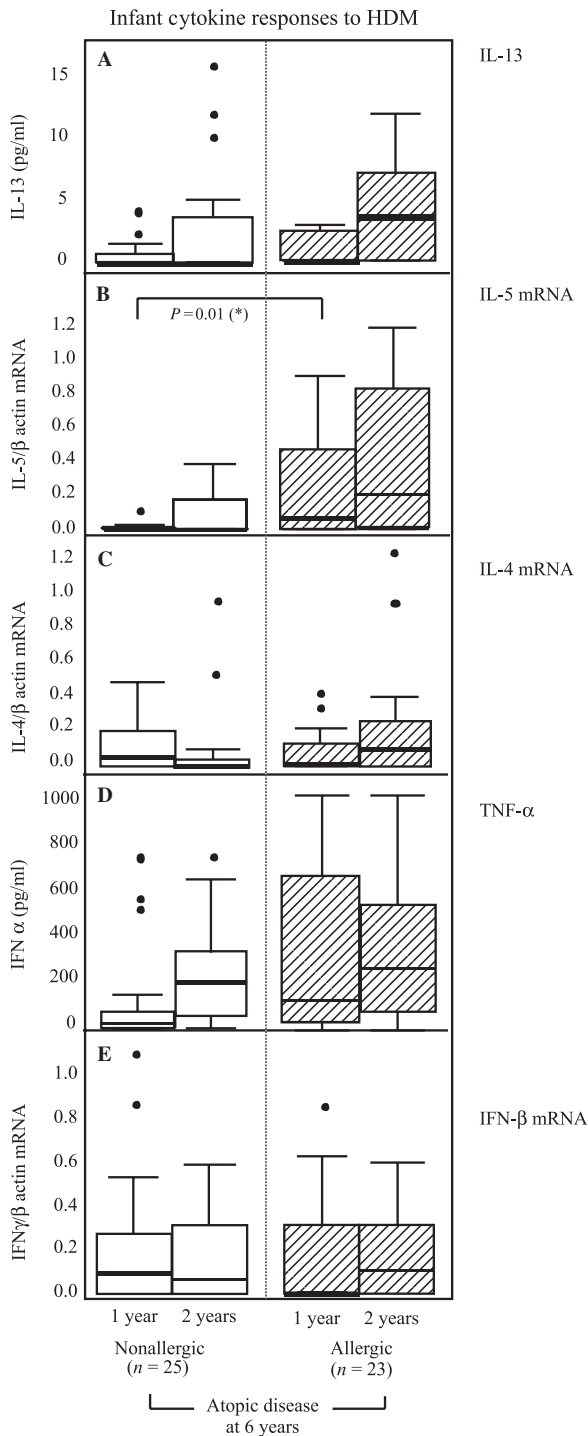
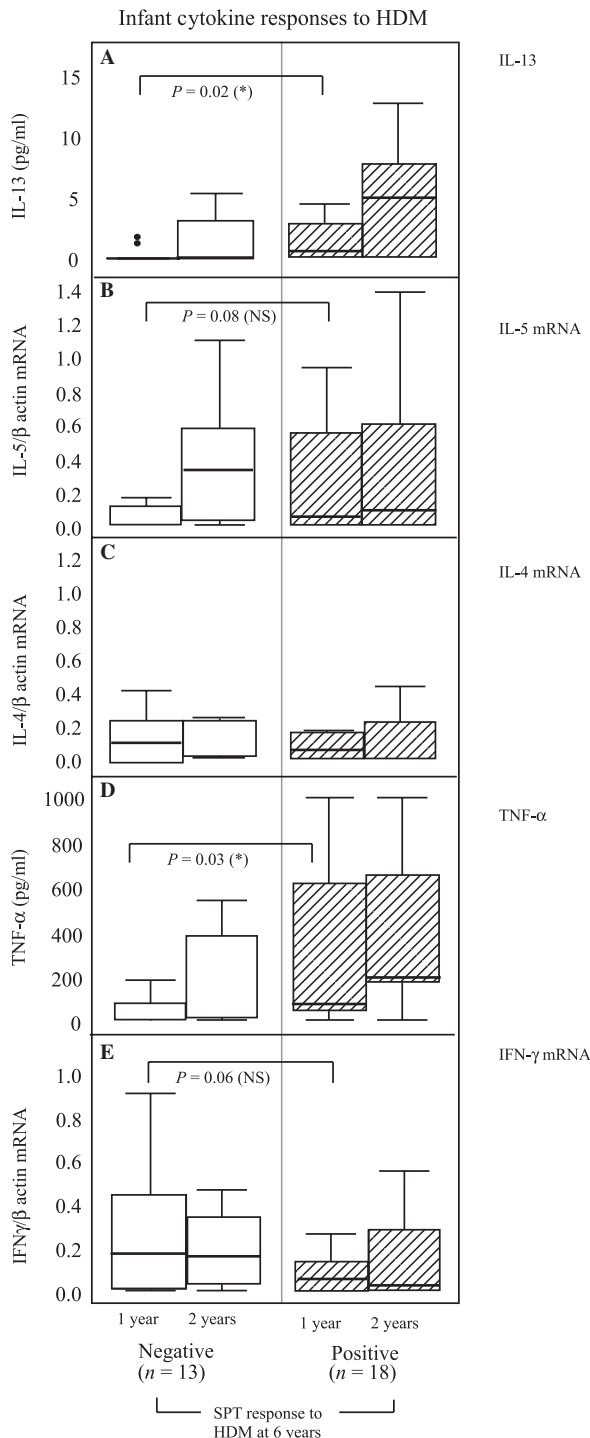


Figure 3. Comparison of early postnatal HDM-cytokine responses in allergic and nonallergic children (based on outcomes at 6 years). Postnatal cytokine (IL-13, IL-5 mRNA, IL-4 mRNA, TNF- α , IFN- γ mRNA) responses to HDM at 1 and 2 years of age are compared on the basis of atopic disease at the age of 6 years. The cytokine levels in culture supernatants (pg/ml) are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. All responses are shown as the levels in stimulated cultures after subtracting levels in the the unstimulated control cultures. Data from allergic children (shaded bars) were compared with the nonallergic group (unshaded bars) using nonparametric Mann–Whitney *U*-test (with significance values as shown).

antigens (tetanus toxoid) and allergens compared with LR infants (18).

This study also demonstrates that our clinical assessments at 2 years (2, 15) were reliable and predictive of later allergic disease and sensitization (by SPT). There was a strong relationship between the diagnosis and classification of allergy at 2 years with confirmed history of clinical disease at the 6-year visit ($P < 0.001$).



Although the diagnosis of allergic diseases is difficult in very young children, the presence of 'probable' atopic symptoms at 2 years in the context of atopic heredity strongly predicted later atopic disease and/or positive SPT at 6 years.

The main limitations of this study are the relatively small population size, and the heterogeneous nature of allergic diseases. Despite these factors, some clear patterns in early allergen-specific immune responses emerge in this population. We previously noted (2) that nonallergic children (particularly those with no family history of allergy) progressively down-regulate Th2 (IL-4, IL-5 and IL-13) responses to HDM in the postnatal period. In contrast, the Th2 cytokine responses of HR children (who develop symptoms suspicious of allergic disease at 2 years) persisted or continued to increase with age. With this additional follow-up period at 6 years of age, these patterns are confirmed and are based on more certain clinical definitions.

The significance of weaker neonatal allergen-specific Th2 responses (IL-13), which were only associated with later SPT reactivity (not later allergic disease), is not clear. This has been noted by some (19) but not all (20) groups and could suggest a more global immaturity of immune responses. This trend in Th2 responses was reversed early in the postnatal period. At 1 year of age, children who were later sensitized to HDM (at 6 years of age) had stronger cytokine responses to HDM, even in the absence of any clinical disease (Fig. 4). It is of note that elevated HDM-specific IL-13 at 1 year was associated more with HDM 'SPT reactivity' (specific IgE) at 6 years rather than 'allergic disease' *per se*. Conversely, elevated HDM-specific IL-5 responses were associated more with 'allergic disease' rather than specific IgE at 6 years. This supports a role of specific IL-13 responses in IgE regulation in children, and suggests that IL-5 (and eosinophil regulation) may have a role in disease expression. It also supports the notion that 'Th2' cytokines have independent actions, differential regulation and vary differently with the clinical phenotype. Despite this, the associations were not strong enough to be of value in predicting allergic outcomes. The significance of elevated TNF- α responses to HDM in SPT positive children is not

Figure 4. Comparison of early postnatal HDM-cytokine responses in HDM SPT positive and SPT negative children (based on outcomes at 6 years). Postnatal cytokine (IL-13, IL-5 mRNA, IL-4 mRNA, TNF- α , IFN- γ mRNA) responses to HDM at 1 and 2 years of age are compared on the basis of SPT responses to inhalant allergens at 6 years. The cytokine levels in culture supernatants (pg/ml) are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. All responses are shown as the levels in stimulated cultures after subtracting levels in the unstimulated control cultures. Data from SPT positive children (shaded bars) were compared with the SPT negative group (unshaded bars) using nonparametric Mann-Whitney *U*-test (with significance values as shown).

clear, but suggests that allergen-responsiveness in these children is not limited to 'Th2-like' cytokines.

In conclusion, our observations clearly indicate that a positive family history of allergic disease remains is the best predictor of subsequent allergic sensitization and disease. As this is still fairly imprecise, there is an ongoing search for more accurate early predictive biomarkers of disease. Although the findings of this study suggest that very early alterations in immune responses during a 'critical period' of immune development may be associated with persistent Th2 disease, these early biomarkers did not clearly predict disease. Further studies are required to define the early processes which lead to

allergic disease, in order to more accurately at risk individuals and to develop prevention strategies.

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