

Neonatal interleukin-12 capacity is associated with variations in allergen-specific immune responses in the neonatal and postnatal periods

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Summary

Background and objectives A reduced capacity of antigen presenting cells (APC) to provide pro-T helper 1 (Th1) signals, such as IL-12, to T cells during early life may be implicated in the development of T helper 2 (Th2)-mediated allergic disease. In this study we examined the relationships between the capacity for IL-12 responses in the neonatal period and atopic risk (family allergy), *in vitro* T cell responses to allergens, and the subsequent development of allergic disease at 6 years

Methods The capacity of circulating neonatal (and maternal) APC to produce IL-12 p70 in response to LPS (and IFN- γ) stimulation was assessed in a group of 60 children with previously well-characterized immune responses to allergens and atopic outcomes. The IL-12 responses were compared with allergen-induced lymphoproliferation (to house dust mite (HDM) ovalbumin (OVA), cat and β -lactoglobulin (BLG)) and IL-13 and IFN- γ cytokine responses (to OVA, HDM and phytohaemagglutinin (PHA)) in the neonatal and postnatal periods. IL-12 responses were also compared according to atopic risk and atopic outcomes (doctor-diagnosed asthma, eczema, food allergies and sensitization as evidenced by skin prick testing) at 6 years clinical follow-up.

Results Maternal peripheral blood mononuclear cells (PBMC) synthesized significantly greater amounts of IL-12 than neonatal PBMC, though within maternal-infant pairs IL-12 responses were significantly correlated ($r = 0.4$, $P = 0.019$). Moreover, neonatal IL-12 responses were positively correlated with neonatal allergen proliferation for HDM ($r = 0.6$, $P < 0.0001$), OVA ($r = 0.55$, $P < 0.0001$), cat ($r = 0.5$, $P = 0.003$) and BLG ($r = 0.55$, $P = 0.001$), but negatively correlated with neonatal IL-13 responses to both allergens tested (HDM: $r = -0.4$, $P = 0.03$ and OVA: $r = -0.5$, $P = 0.001$). Both neonatal and maternal IL-12 responses were positively correlated with postnatal IFN- γ responses to HDM at 12, 18 and 24 months of age (responses after age of 2 years were not assessed). There was no relationship between atopic risk and IL-12 capacity in the neonatal period, but there was a (non-significant) trend for neonatal IL-12 responses to be lower in the high-risk children who developed clinical allergy at 6 years (compared with the low risk group) although the number in this analysis was small.

Conclusions Reduced APC IL-12 production in the perinatal period was associated with reduced T cell activation (lymphoproliferation), stronger neonatal Th2 responses, and weaker Th1 responses to allergen in the postnatal period. This supports the notion that variations in APC function in early life may contribute to altered allergen-specific cytokine responses associated with later allergy.

Keywords allergens, allergy, APC, cord blood, cytokines, IL-12, infants

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Introduction

The early immunological events that predispose to the development of allergic immune responses have become a research priority. Although the mechanisms are still unclear, the development of allergen-specific Th cell memory that is dominated by T helper Type 2 (Th2) cytokines appears to be key.

The immunological events that drive T helper memory development are initiated early in infancy [1], possibly even before birth [2, 3]. Already during the perinatal period, there are immunological differences in neonates at high risk of allergy, namely relatively reduced capacity for Type 1 (Th1) interferon gamma (IFN- γ) responses, compared with low-risk neonates with no family history of allergy [4–6]. These findings have focused attention on the role of antigen presenting cells (APC) during early life, given the key influence that APC have on T cell activation and the pattern of Th1/Th2 polarization. One major objective is now to determine if variations in the capacity of APC to activate T cells and provide Th1 maturation signals are key factors in the development of allergy.

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APC provide a number of critical signals to developing T cells. After presentation of antigen, T cell activation appears dependent on obligatory costimulatory second signals through surface molecules, such as CD80/86, on APC, which are up-regulated in the presence of local activating factors (such as inflammation). In the absence of these costimulatory signals, T-cell activation does not occur, leading to the development of antigen-specific tolerance [7]. APC also produce pro-Th1 cytokines in the form of IL-12 (and other cytokines IFN- α , IL-18, IL-23) that influence the cytokine profile of T cells once activated. It has been suggested that a relative absence of IL-12 favours the development of a default Th2 cytokine profile, although this remains controversial [8, 9]. In the neonatal period, when APC function is known to be less mature [10], less efficient pro-Th1 cytokine production and costimulation is likely to contribute to the observed increased susceptibility to both tolerance [11] and Th2 responses [12].

Little is known currently concerning the mechanisms by which allergen-specific T cell responses are regulated in the neonatal period. Cytokines released by APCs, especially IL-12, are thought to be important in Th1 polarization in mature adult humans/animals. However, the capacity to synthesize bioactive IL-12 is impaired in neonates, as compared with adults, so it is not clear how Th1 polarization is induced in early life, and whether there is any relationship between the capacity to produce IL-12 and synthesis of Th1/Th2 cytokines in allergen-stimulated cord blood cultures. We hypothesized that variations in APC function (IL-12 production) in the neonatal period might affect the magnitude of perinatal T cell responses to allergen and the patterns of cytokines produced. Furthermore, we reasoned that the neonatal capacity for IL-12 responses might predict the postnatal pattern of cytokine responses to allergen. Thus, the main aims of this study were to assess the relationships between capacity for neonatal IL-12 responses to LPS and (a) cord blood mononuclear responses to allergens (lymphoproliferation and cytokine responses), (b) postnatal immune development, and (c) atopic risk (family history) and the subsequent development of allergic disease (at 6 years).

Materials and methods

Overview

A group of 60 children were recruited previously for longitudinal assessment of allergen-specific immune responses [1, 3]. The current study utilized cryopreserved mononuclear cells (MC) to assess IL-12 responses in this well-characterized population. This included 49 neonatal and 59 maternal samples. The resulting IL-12 responses were then analysed in relation to previously measured allergen-specific responses (from birth to 2 years), family history and atopic outcomes at 6 years.

Study population

Of the 60 neonates, 16 had no family history of allergy, and 44 had one or more first-degree relatives with allergic disease. Fifty-three were followed for clinical assessment at 6 years of age, which included 28 allergic children and 25 children with no evidence of disease or sensitization. Children were defined as allergic if they had doctor-diagnosed asthma, atopic eczema/

dermatitis syndrome (AEDS) [13] or hayfever and a current or past positive skin prick test (SPT) to house dust mite (HDM), grass pollen, cat, dog, milk, egg or peanut. Further details of this population have been published previously [1].

Samples

The original cord blood (CB) samples were collected from the placental vessels by venepuncture immediately after Caesarean delivery. Maternal peripheral blood was collected after delivery by routine venesection. Follow-up infant blood samples were collected at 6, 12, 18 and 24 months of age. As described previously [1, 3], MC were isolated using Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and cryopreserved for subsequent allergen-specific responses [1, 3], but IL-12 assays were not readily available at the time of the original studies. Additional aliquots of MC (which were only available from maternal and neonatal samples) were previously kept in liquid nitrogen storage. These were thawed for IL-12 assays in the present study. All samples were checked for viability on thawing. We have previously demonstrated (unpublished) that long-term cryopreservation does not significantly alter the pattern of cellular function.

IL-12 methods

The capacity for IL-12 responses was assessed using paired maternal and cord blood samples (as discussed above), in order to determine if there was a relationship between maternal and neonatal IL-12 responses and infant *in vitro* response to allergens. Lipopolysaccharide (LPS) stimulation (after IFN- γ priming) was used to evoke IL-12 responses. This method of stimulation allows assessment of APC capacity to respond under intense pro-inflammatory conditions (also see Results and discussion). Thawed maternal and fetal MC were primed with 10 ng/mL IFN- γ (Pharmingen, Becton Dickinson, Perth, Australia) for 2 to 3 h before stimulation with 10 ng/mL lipopolysaccharide (*E. coli* LPS, Sigma, Castle Hill, Australia) for a further 24 h in 10% fetal calf serum and Roswell Park Memorial Institute 1640 (RPMI, Gibco BRL, Glen Waverly, Australia) culture medium. Supernatants were harvested at 24 h and frozen (at -80°C) for subsequent IL-12 (p70) measurement by commercial ELISA (OptEIA, Pharmingen, Becton Dickinson).

Allergen-specific immune responses

Lymphoproliferation and cytokine responses to allergens were previously assessed in this infant population [1, 3, 14]. Briefly, lymphoproliferation was assessed using fetal and infant MC in round bottom plates. Mononuclear cells (2×10^5 in 200 μL of AIM V (Gibco, Life Technology) serum free medium) were cultured alone or with optimal stimulating doses of HDM (CSL, Castle Hill, Australia, 10 $\mu\text{g/mL}$), ovalbumin (OVA, Sigma chemicals, 10 $\mu\text{g/mL}$), Fel d 1 (ALK, Horsholm, Denmark, 30 $\mu\text{g/mL}$) or β -lactoglobulin (β -LG, CSL, Australia, 100 $\mu\text{g/mL}$) for 6 days (neonates) or 7 days (infants). DNA synthesis was measured as incorporation of 3H-thymidine, after pulse labelling 16 to 18 h prior to harvest. Data were expressed as delta dpm per culture, designating levels of incorporation above background (unstimulated) control cultures.

To assess cytokine responses, MC from the same samples were cultured for 24 h in medium alone, or supplemented with optimal stimulating concentrations (as above) of HDM, OVA

or 1 µg/mL phytohaemagglutinin (PHA) mitogen (HA16, Murex, Biotech Ltd, UK). Cytokine protein (IL-13, IFN-γ and IL-10) was measured in supernatants employing commercial ELISA kits (Pharmingen, Becktin Dickenson). Because IFN-γ responses to allergens were often below detection, IFN-γ mRNA was measured in the cell pellets from the same cultures, using semiquantitative reverse transcriptase PCR as described previously [3]. Briefly, RNA was extracted from cell pellets using RNeasy B, and cDNA was transcribed using oligo (dT) 15 and avian myeloblastosis virus reverse transcriptase in the presence of ribonuclease inhibitor. The PCR reactions employed primers specific for IFN-γ and β-actin plus TthPlus DNA polymerase (Biotech, Western Australia), and were performed in a programmable thermocycler. Samples of the PCR products were manually dot-blotted onto nylon membranes, and hybridized overnight with biotinylated double stranded probes, and binding visualized by chemiluminescence using a commercial kit. Probe binding was quantified by computer assisted densitometric scanning and the intensity of each test sample was expressed as a ratio relative to that of its respective β-actin control.

IgE measurements

IgE measurements were previously performed on serum from both infants (at 2 years) and mothers by the Diagnostic Immunology Department at Princess Margaret Hospital using a standardized commercial fluoroimmunoassays (Pharmacia CAPSystem for specific IgE, and the Pharmacia ImmunoCAP for total serum IgE).

Statistical analysis

The relationship between IL-12 responses and allergen-specific responses in the pre- and postnatal period were assessed using non-parametric (Spearman) correlation coefficients. The Mann-Whitney *U*-test was employed when making comparisons between groups. All analyses were performed using SPSS version 10 (for Macintosh).

Ethical approval

Ethical approval for follow-up and repeat analysis of this population was granted by the Princess Margaret Hospital Research and Ethics Committee.

Results

Relationship between IL-12 responses and maternal factors (excluding allergy)

We first examined the capacity of PBMC from maternal infant pairs to synthesize IL-12 p70 in response to LPS stimulation following IFN-γ priming. (Due to limitations on numbers of available cells, it was not possible to repeat these experiments in older babies.) IL-12 p70 is predominantly synthesized by APC in peripheral blood [15], so this allowed us to assess the functional capacity of APC *in vitro* under strong proinflammatory conditions. In keeping with the recently documented pattern of IL-12 ontogeny [15], maternal IL-12 responses were an order of magnitude greater than fetal IL-12 responses (not shown). Of note, IL-12 responses to LPS/IFN-γ within maternal-infant pairs were significantly correlated ($r = 0.4$, $P = 0.019$).

However, there were no relationships between maternal (or fetal) IL-12 responses and parity, gravidity, or maternal smoking in pregnancy.

Relationship between capacity for neonatal IL-12 responses and CBMC lymphoproliferative responses to allergens

There were consistent and highly significant relationships between cord blood IL-12 responses and the magnitude of lymphoproliferative responses to allergens (see Fig. 1 and Table 1). This was significant for all allergen responses assessed, specifically HDM ($r = 0.6$, $P < 0.0001$), OVA ($r = 0.55$, $P < 0.0001$), cat ($r = 0.5$, $P = 0.003$) and BLG ($r = 0.55$, $P = 0.001$).

Relationship between capacity for neonatal IL-12 responses and CBMC cytokine responses to allergens and mitogens

There were significant inverse relationships between the magnitude of LPS/IFN-γ-induced neonatal IL-12 responses and IL-13 responses to both OVA ($r = -0.5$, $P = 0.001$) and HDM ($r = -0.4$, $P = 0.03$) as shown in Table 1. IL-12 responses were significantly stronger in the subgroup of neonates with undetectable IL-13 responses to HDM ($P = 0.001$) and OVA ($P = 0.001$) as seen in Fig. 2. In contrast, there was no obvious relationship between neonatal IL-12 responses and IFN-γ responses to either allergens or PHA, though this may be partially attributable to the generally low level IFN-γ responses typically seen in the neonatal period. Because IFN-γ protein responses to allergens were largely below detection in the neonatal samples, we also examined CBMC IFN-γ mRNA responses to allergens, but again there was no clear relationship to IL-12 synthesis. HDM and OVA were the only allergens used to stimulate cytokine responses in this study due to the numbers of available cells. There were no significant relationships between IL-12 and IL-10 responses to LPS in the neonatal period.

Relationship between capacity for neonatal IL-12 responses and postnatal immune responses

There was a significant positive association between both maternal and fetal IL-12 responses at birth and Th1 (IFN-γ) responses to HDM in the postnatal period (Table 2). This was true at 12, 18 and 24 months of age (but not 6 months when even IFN-γ mRNA responses were still very weak). The comparisons at 12 and 18 months are made using IFN-γ mRNA levels because IFN-γ protein responses were also largely below detection at these ages. There were no consistent relationships between neonatal IL-12 responses and postnatal IL-13 responses to HDM. There were also no significant relationships between neonatal IL-12 responses and postnatal lymphoproliferation or cytokine responses to OVA. The exception was IL-10 responses to OVA at 12 months, which were positively correlated to neonatal IL-12 responses ($r = 0.5$, $P = 0.02$). IL-10 responses were not measured at 6 or 18 months, and this relationship was no longer evident at 2 years of age. There were no significant relationships between perinatal IL-12 responses and postnatal lymphoproliferation.

Relationship between capacity for neonatal IL-12 responses and atopic risk and outcome measures

There were no clear relationships between fetal IL-12 responses and maternal atopy and family atopy. Similarly, there were no significant relationships between perinatal IL-12 responses and

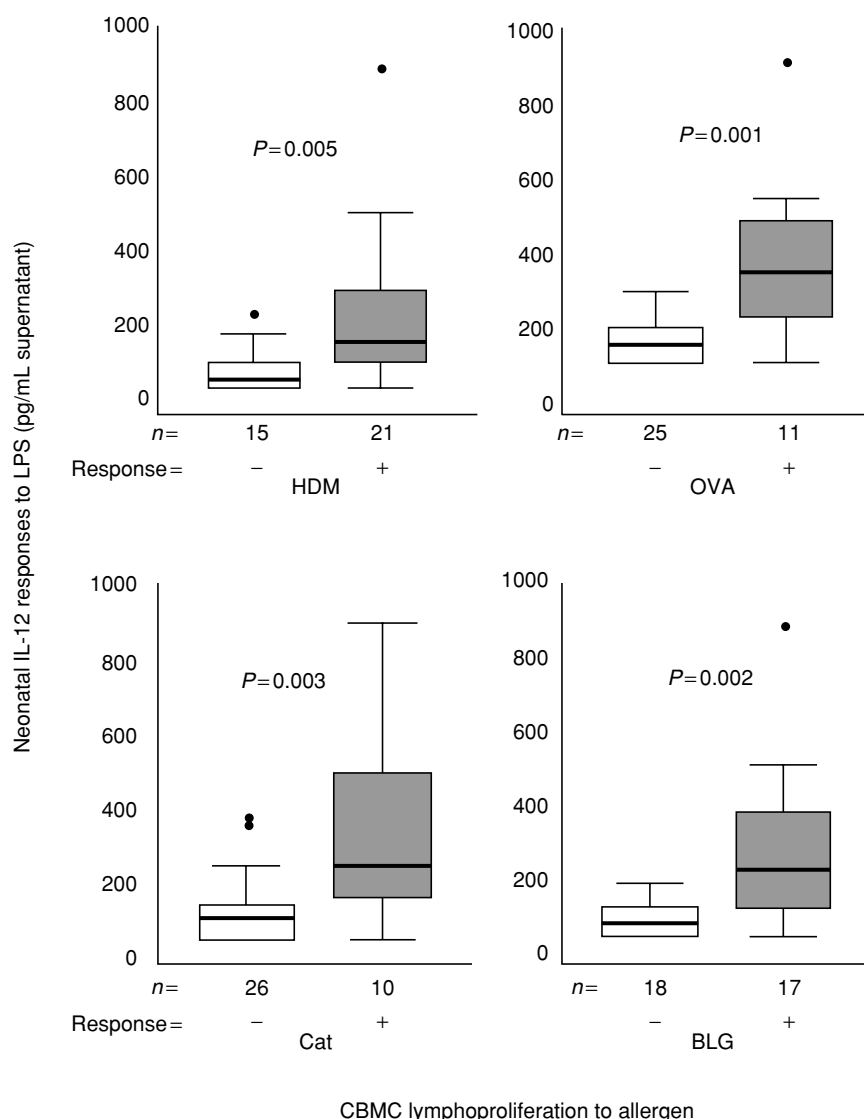


Fig. 1. The relationship between neonatal IL-12 responses and neonatal allergen specific lymphoproliferation. The IL-12 responses in neonates who showed positive (shaded boxes) and negative (empty boxes) responses to allergens (HDM, OVA, cat and BLG) were compared. Responses were defined as 'positive' if they were greater than twice the background and greater than 1000 dpm above background unstimulated cultures. The IL-12 responses (pg/mL) are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. Groups were compared using nonparametric Mann-Whitney *U*-test (significance values are as shown).

Table 1. Correlation between neonatal IL-12 responses and allergen-induced responses at birth. This illustrates the relationship between LPS-induced neonatal IL-12 responses and neonatal Th1 (IFN- γ mRNA) responses, Th2 (IL-13 protein) responses and lymphoproliferative responses to allergens. Non-parametric (Spearman) correlation coefficients (and significance values) are shown

Response	HDM	OVA	CAT	BLG
Lymphoproliferation	0.6 $P < 0.0001$	0.55 $P < 0.0001$	0.5 $P = 0.003$	0.55 $P = 0.001$
IFN- γ (mRNA)	NS	NS	Not done	Not done
IL-13 (protein)	-0.4 $P = 0.03$	-0.5 $P = 0.001$	Not done	Not done

specific infants outcomes at 6 years of age, including a history of childhood wheezing, asthma or eczema, or positive allergen SPTs or IgE levels. However, there was a clear trend for neonatal IL-12 responses to be lower in children who went on to develop atopic disease within the first 6 years of life. The

numbers in this analysis are small and the observation is not statistically significant (Fig. 3), but further analysis appears warranted in future studies.

Discussion

Signals from APC have a key influence on T cell development and the pattern of subsequent cytokine response. Immature or defective APC function is associated with reduced costimulation and pro-Th1 cytokine signals involved in T cell activation. This is most evident in the neonatal period, when immature APC function may contribute to both the apparent susceptibility to tolerance induction and the tendency for Th2 responses by activated cells [10, 11].

While APC immaturity is characteristic in the neonatal period, it has been postulated that some infants may have more significant and prolonged functional impairment in APC function, which predisposes them to developing Th2-mediated allergic disease [16]. The present study aimed to investigate how

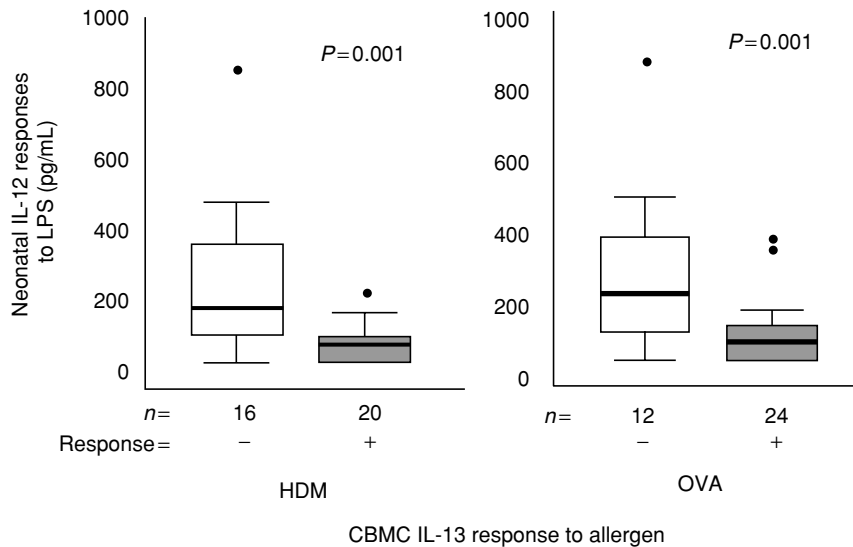


Fig. 2. The relationship between neonatal IL-12 responses and neonatal allergen-specific IL-13 responses. The IL-12 responses in neonates who showed positive (shaded boxes) and negative (empty boxes) IL-13 responses to allergens (HDM and OVA) were compared. Responses were defined as 'positive' if they were above 2 pg/mL (the limit of detection of the ELISA). The IL-12 responses (pg/mL) are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. Groups were compared using non-parametric Mann-Whitney *U*-test (significance values are as shown).

Table 2. Correlation between neonatal IL-12 capacity and postnatal IFN- γ responses to HDM. This illustrates the relationship between LPS-induced neonatal (and maternal) IL-12 responses and postnatal Th1 (IFN- γ) responses to HDM. IFN- γ responses are given as IFN- γ mRNA at < 18 months of age (as HDM-specific IFN- γ protein responses were below detection in most infants below this age), IFN- γ protein at 2 years. Non-parametric (Spearman) correlation coefficients (and significance values) are shown

Postnatal IFN- γ responses to HDM	Maternal IL-12 responses (LPS/IFN- γ)	Neonatal IL-12 responses (LPS/IFN- γ)
12 months (IFN- γ mRNA)	0.5 $P=0.05$	0.52 $P=0.03$
18 months (IFN- γ mRNA)	0.47 $P=0.03$	0.52 $P=0.02$
24 months (IFN- γ protein)	0.7 $P=0.003$	0.45 $P=0.059$

variations in APC function, in particular capacity to secrete the key regulatory cytokine IL-12, were related to T cell responses to allergens in infancy. The method used to stimulate APC in this study was chosen to provide an insight into the capacity of these cells to synthesize IL-12 under intense pro-inflammatory conditions, and therefore provide a measure of APC maturation.

In this study we have demonstrated consistent relationships between the capacity to secrete IL-12 in the neonatal period and both neonatal and postnatal immune responses to allergens. IL-12 responses correlated with neonatal lymphoproliferative responses for all the allergens tested. An obvious interpretation is that IL-12 responses are a marker of the efficiency of APC to provide activating signals to neonatal T cells. This also appears consistent with the inverse relationship between IL-12 production and neonatal IL-13 response to allergen, as weaker pro-Th1 signals from APC are likely to favour a Th2 default response. Recently, Gabriellsson et al. [17] demonstrated that a stronger Th2 bias in CBMC cultures from children with atopic heredity, was associated with reduced numbers of IL-12 producing cells after allergen stimulation. These findings are also consistent with the current observations that APC IL-12

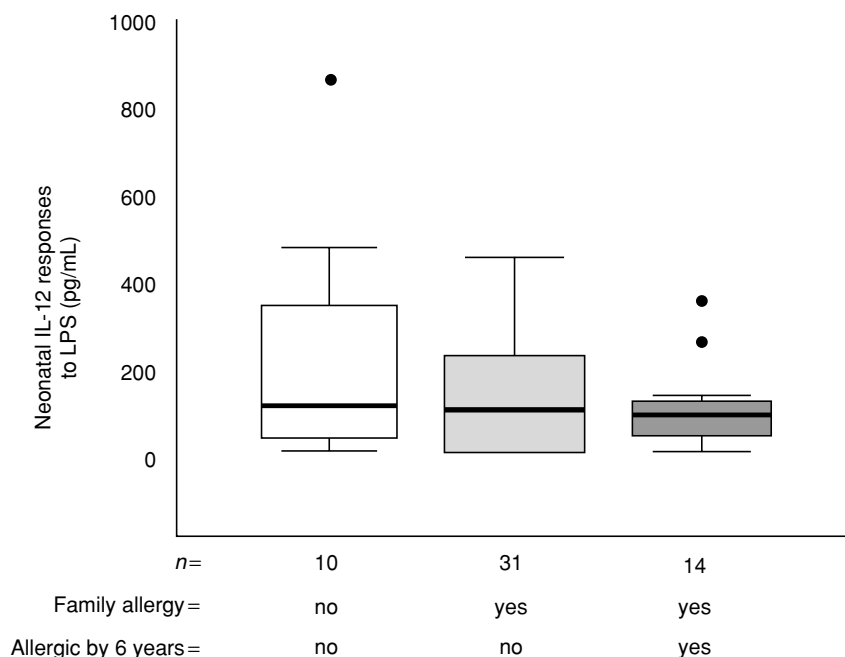
production in the neonatal period is inversely related to Th2 responses, and suggest that this is also inversely related to the risk of atopy.

Although other previous studies have not examined IL-12 capacity at birth, there are conflicting reports about the relationship between atopic risk and neonatal Th2 responses. As with Gabriellsson et al. [17], some groups have observed stronger neonatal Th2 skewing [18, 19] in high-risk babies while others have observed the opposite [3, 20]. The significance of previously reported trends for stronger allergen lymphoproliferation in high-risk infants [3, 6, 21] is also unclear here. There is clearly still considerable uncertainty surrounding the origin and significance of allergen-specific responses in early life, particularly in the perinatal period. There is mounting evidence that these early responses may not represent priming of conventional naïve T cells. The exact role of APC in previously observed T priming and Th2 immune deviation [3] is therefore still unclear, and needs to be further investigated in light of our observations here.

As one of the key factors defining atopic risk is maternal atopy, the positive correlation between maternal and fetal IL-12 production found here is also of key interest. There is growing speculation that the mother may exert 'direct' influences on the fetus (in addition to genetic factors) explaining the stronger effect of maternal atopy than paternal atopy in conferring atopic risk. The nature of maternal influence is unclear and could include both maternal immune interactions with the fetus and maternal environmental exposures. Both could influence APC function (and IL-12 production) as suggested by our findings here. This further highlights the need to consider the gestational environment in studies of allergic disease pathogenesis.

This study also provides new evidence of a relationship between neonatal APC function and postnatal Th1 immune development. The consistent positive correlations between neonatal IL-12 responses and postnatal IFN- γ responses to inhalant allergen, suggest that variations in capacity for IL-12 production during the perinatal period could influence subsequent Th1 function. This may provide preliminary evidence to support a role for APC immaturity in impaired postnatal Th1

Fig. 3. The relationship between neonatal IL-12 responses and atopic risk (allergy in immediate family) and subsequent atopic disease at age 6 years. The neonatal IL-12 responses in children are defined according to their clinical allergy status at 6 years. This shows comparisons between atopy negative/family history negative children (empty boxes), atopy negative/family history positive (light shaded boxes) and atopy positive/family history positive children (dark shaded boxes). These clinical definitions are described in the methods. The IL-12 responses (pg/mL) are displayed as median, 5, 25, 75, 95 percentile ranges and outlying values. Groups were compared using non-parametric Mann-Whitney *U*-test (significance values are as shown).



development. This has previously been proposed as a potential mechanism for increased propensity for Th2 responses and allergic disease. Although the trend for lower neonatal IL-12 responses in the high-risk children who developed allergy was not significant, the numbers in this part of the analysis were small, warranting larger studies to examine this further. Similarly, although we saw a positive relationship between neonatal pro-Th1 (IL-12) activity responses and anti-inflammatory (IL-10) responses to OVA at 1 year of age, it is difficult to draw conclusions about this without more information about responses in the intervening period.

Events in the first years of life appear critical in the development of 'tolerance' to environmental allergens, which evolves very differently for aeroallergens and food allergens. During this period tolerance to aeroallergens in non-atopics appears to develop by positive selection of Th1-like allergen-responsive T cells [22], and here we provide indirect evidence that IL-12 may have a role in this process. In contrast, food allergen tolerance appears to result from negative selection of responsive cells, presumably by clonal deletion or anergy induction [23]. Consistent with this premise, during infancy OVA responses (both lymphoproliferation and cytokine production) fell to almost undetectable levels in most of the children in this cohort, whereas HDM responses increased [1]. Thus, although neonatal HDM and OVA responses were similar and showed the same relationships with IL-12 production, a positive relationship between IL-12 and postnatal Th1 function was only observed for aeroallergen (HDM), and not for food allergen (OVA). This is further evidence that different regulatory processes are involved in tolerance induction to these two classes of allergen.

In conclusion, the consistent associations between IL-12 production and neonatal allergen-specific responses clearly justify further investigation. Our findings also suggest that APC have an important role in postnatal development of immune competence, particularly in maturation of Th1 responses to aeroallergens.

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