

Selective development of a strong Th2 cytokine profile in high-risk children who develop atopy: risk factors and regulatory role of IFN- γ , IL-4 and IL-10

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Summary

Background The immunological processes in early life and their relation to allergic sensitization leading to a Th2 cytokine profile are still not well understood.

Objective To analyse the environmental and genetic risk factors and immunological responses at birth in relation to the development of atopic disease at 12 months of age in a longitudinal study of high-risk children.

Methods High-risk children were followed from birth till 12 months of age. Mononuclear cells obtained at birth and 6 and 12 months thereafter were analysed for their proliferative and cytokine responses after polyclonal and allergen-specific stimulation.

Results At 12 months of age 25% children had developed an atopic disease. Two atopic parents, parental smoking and atopic dermatitis of at least one of the parents were significant risk factors. In cord blood of newborns who developed atopy, an increased percentage of CD4⁺CD45RO⁺ cells and an increased polyclonal-stimulated proliferation were observed. Furthermore, an impaired allergen-induced, but not polyclonal-stimulated IFN- γ production was found, suggesting a regulatory defect. At 6 and 12 months of age, a strong Th2 profile (characterized by increased levels of IL-4, IL-5, and IL-13) after both polyclonal and, to a lesser extent, allergen-specific stimulation was found in the children developing atopy. Allergen-induced IL-10 production at 12 months of age was only observed in the non-atopic children.

Conclusion Our data indicate that the first 6 months of life represent a critical time window for the initiation of immunological changes resulting in the development of atopy. The selective development of a Th2 cytokine profile in high-risk children who develop atopy is due to increased production of Th2 cytokines, possibly caused by impaired allergen-induced IFN- γ production in the neonatal period. Furthermore, the decreased allergen-induced IL-10 levels observed in the atopic children at 12 months of age may result in a lack of down-regulation of the inflammatory process.

Keywords: atopy, high-risk children, Th1/Th2, cytokines, cord blood, allergen, risk factors

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Introduction

The shift in the balance of T helper cell subsets towards a polarized Th2 population is generally accepted to occur in atopic disease. Indeed, in adults and children with

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established atopic disease, increased levels of IL-4, IL-5 and IL-13 in combination with a decreased IFN- γ production have been described both after polyclonal and allergen-specific stimulation of peripheral blood mononuclear cells (PBMC) or purified T cells [1–4]. It is, however, still largely unknown when and how these Th2 responses develop.

Recent studies have focused on the analysis of Th1 and Th2 responses in the immediate postnatal period. Data have become available suggesting that the initial sensitization to allergens could already start *in utero* [5,6]. The T cell memory that develops against allergens perinatally is currently believed to be the result of complex interactions between genetic susceptibility and environmental factors. Factors such as parental smoking, infections, feeding methods, birth weight, elevated total IgE levels in cord blood, early exposure to household pets and inhalant allergens are assumed to increase the risk for developing atopic disease.

In newborns many immune functions are less mature: the capacity of cord blood T cells to produce cytokines, such as IL-4 and IFN- γ is diminished [7–9], the secretion of immunoglobulins by cord blood B cells is strongly decreased [10], and cord blood antigen-presenting cells are functionally immature [11]. Moreover, the percentage of cord blood CD45RO⁺ T cells, a marker for memory T cells, is lower than in peripheral blood of adults [10,11]. This relative immaturity of the immune system of newborns could create a time window, starting *in utero* and lasting up to 12 months of age, in which an increased susceptibility to develop atopy exists. Holt *et al.* proposed that a weak Th2 skewing of stimulated cord blood mononuclear cells (CBMC) is present in all children at time of birth, irrespective of the development of atopic disease later in life [12]. This Th2 phenotype could lead to failure of tolerance induction or lack of immune deviation towards a Th1 response. As a result, such children may develop a long-term Th2-skewed allergen-specific immunological memory and atopic disease upon allergen exposure. In contrast, other studies have suggested that newborns that develop atopy have a delayed postnatal maturation of cellular immune functions, especially a reduction in the production of IFN- γ [5,7,13,14]. In agreement with this, a decreased IFN- γ production was found after polyclonal stimulation of CBMC in those children who developed atopy compared to children who did not develop atopy before 2 years of age [5,15].

Clearly, it is still largely unknown when and how the shift towards a polarized Th2 population in children developing an atopic disease occurs. Therefore, we aimed to analyse differences in cytokine profiles between atopy-prone children that did or did not develop atopy within their

first year of life, in order to obtain more insight in the immunological processes leading to the establishment of a Th2 profile in the atopic children. We therefore started a prospective study on a group of 133 high-risk newborns and followed them from birth onwards for the development of clinical signs of atopic disease together with a detailed analysis of their allergen-specific and polyclonally stimulated cytokine profiles and proliferative responses. High-risk children were chosen as in this group of children approximately 30% should develop an atopic disease in the first years of life [16], which allows analysis of both atopic and non-atopic children in a relatively small but homogeneous population.

Methods

Study population

Cord blood samples were obtained from 133 newborns at high risk of developing atopy. At least the mother of the newborns was atopic, as established by clinical history and a positive skin prick test (SPT) and/or specific IgE to common inhalant and/or food allergens. After permission from the pregnant women, informed consent was obtained from the parents of all children prior to their participation in the study. The Medical Ethical Committees of the participating hospitals approved this study.

All children were followed for at least one year in order to monitor the development of atopic diseases such as atopic dermatitis (AD), asthma-like disease and food or upper-airway allergy. At the ages 6 and 12 months, physical examinations were performed, questionnaires on environmental factors and histories of symptoms of atopy were filled out and venous blood samples were obtained. The environmental factors were the presence of pets, strategies for house dust mite (HDM) avoidance, parental smoking and feeding method. Between 9 and 12 months of age, two different skin tests were performed for cow's milk, egg and peanut [17].

Definition of atopy

At the age of 12 months, a clinical judgement was made without knowledge of the immunological results. Atopic children were defined as children with AD, asthma-like disease, food allergy, or a combination of these diseases.

AD was based on Sampson's criteria [18]. Asthma or asthma-like disease was diagnosed if children either had more than two separate wheezing episodes each lasting three days or more, or had more than two separate episodes of paroxysmal nocturnal cough with sleep disturbance for three consecutive nights, or required anti-asthma therapy [19]. Food allergy was defined as a clinical manifestation

of erythematous, papular, macrovesicular skin eruptions, diarrhoea, vomiting, or respiratory problems in relation to food ingestion. For the definitive diagnosis a positive prick-prick test or a positive skin application food test (SAFT) [20] or specific IgE was necessary. Specific IgE was measured using Phadebas CAP RAST (Pharmacia, Uppsala, Sweden) [21].

Isolation of CBMC and PBMC

CBMC and PBMC were isolated from heparinized umbilical cord blood samples or peripheral blood by density gradient centrifugation over Ficoll-Hypaque (Pharmacia). In order to control for maternal blood contamination in the cord blood, IgA content was determined in cord blood samples of all newborns whose IgE values were detectable. Two cord blood samples with an IgA content higher than 10 mg/L were therefore excluded from this study.

Determination of IgE and IgA

The total IgE concentrations in cord blood were determined using a modified radioimmunoassay which resulted in a lower detection limit of 0.2 kU/L. At the age of 12 months a sample of venous blood was obtained to determine specific IgE levels directed towards the most common inhalation allergens (HDM, grass and birch pollen, cat, dog and moulds) and food allergens (peanut, chicken egg white, cow's milk, codfish, wheat and soy). Measurements were performed using the Pharmacia CAP system Phadiatop® RAST and the Pharmacia CAP RAST (Kabi Pharmacia, Uppsala, Sweden). IgA was determined by a nephelometric method with a detection limit of 10 mg/L.

Phenotypic analyses of CBMC

Flow cytometry was performed by staining CBMC with combinations of the following antibodies: CD19(Leu12)-PE, CD3(Leu4)-FITC, CD14(My4)-FITC, CD16(Leu11)-PE and CD56(Leu19)-PE (Becton Dickinson, Franklin Lakes, NJ, USA), CD4(Leu3)-PE, CD8(Leu2)-PE, CD45RA(2H4)-PE (Coulter Cytometry, Hialeah, FL, USA), and CD45RO(UCHL1)-PE (DAKO, Glostrup, Denmark). After staining (10 min at 18–20°C) and lysing normoblasts with NH₄Cl, the CBMC were washed twice, resuspended in FACSflow (Becton Dickinson) and analysed on a FACScan (Becton Dickinson).

Proliferative responses

Lymphocyte proliferation assays were performed as described previously [22]. Cells were stimulated with

phorbol-12-myristate-13-acetate (PMA) (2 ng/mL; Sigma, St Louis, MO, USA) and Ca-ionophore (1 µg/mL; A23187, Sigma), *D. pteronyssinus* extract (HDM, 50 µg/mL, ALK-Benelux, Groningen, the Netherlands), egg-white extract (egg, 50 µg/mL, ALK), or cow's milk extract (CM, 25 µg/mL, ALK) for 3 (polyclonal) or 7 days (allergen), after which proliferation was measured using [³H]-thymidine incorporation. The results are expressed as mean cpm or as stimulation index (SI), equal to the ratio of mean cpm in stimulated cultures over mean cpm in unstimulated cultures. A SI > 3 is considered to reflect significant and specific proliferation.

Detection of cytokine protein production in CBMC cultures

The levels of IFN-γ, IL-4, IL-10 and IL-13 in the culture supernatants and in plasma were determined using a Pelikan Compact™ human cytokine kit (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands) according to the instructions of the manufacturer. IL-5 was determined using an ELISA based on TRFK5 (a kind gift of Dr R. L. Coffman (DNAX Research Institute, Palo Alto, CA, USA)) as a capture mAb and biotin-labelled rat anti-human IL-5 (JES1-5A10, 0.25 µg/mL, Pharmingen, San Diego, CA, USA) as detection mAb. The lower detection limits were 2 pg/mL for IFN-γ, 1 pg/mL for IL-4, 2.5 pg/mL for IL-5, 2 pg/mL for IL-10 and 0.5 pg/mL for IL-13.

Statistical analyses

Statistical analyses were performed using SSPS for Windows (version 8.0) and Microsoft Excel 97. Environmental and genetic factors were evaluated by computing the relative risk (RR) with Cornfield's 95% confidence intervals (CI) as a measure of association or by the Chi-square linear association test. Immunological parameters, which generally showed a normal distribution, were tested using an independent sample *t*-test. Correlation was assessed using Spearman's rank correlation coefficient. *P*-values < 0.05 were considered significant.

Results

Prevalence of atopic disease at 12 months

At the age of 12 months, 110 of the 133 included children (83%) could be assessed for the presence of clinical symptoms of atopic disease defined as AD, asthma-like disease and food allergy. Twenty-three children dropped out of the study, the main reasons being difficulties to visit the hospital and non-acceptance by the parents of repeated venapunctures of their child. At this time point 27 of these

Table 1. Characteristics and prevalence of atopic diseases in children who did and did not develop atopy at 12 months of age

	Atopy status at 12 months		Clinical signs of atopy		
	Non-atopic	Atopic	AD	AD and food allergy	Asthma-like disease
<i>n</i>	83	27	23	5	4
%	75.5	24.5	85.2‡	18.5‡	14.8‡
sex M/F	36/47	13/14	11/12	2/3	2/2
total IgE*	27/63	8/22	8/20	1/4	0/2
birth weight (g)†	3190 (1670–4750)	3355 (2350–4580)	3360 (2710–4210)	3150 (2350–3440)	3785 (3070–4580)
SCORAD†			7 (2–35)	2 (2–11)	

*Data represent number of newborns with detectable IgE levels in cord blood. Total IgE levels were determined by IgE CAP RAST with a lower detection limit of 0.2 kU/L.

†Data represents the median value (range).

‡Percentage of children relative to the total group of atopic children.

children (25%, Table 1) had developed an atopic disease, almost exclusively AD. The median severity of the AD, determined by the objective severity score of AD (SCORAD), was classified as mild AD. Of the atopic children, 5 children had a food allergy (one against cow's milk and four children against egg). Four children had developed asthma-like disease, but no HDM-specific IgE could be detected in these patients at 12 months of age. The prevalence of atopy was similar in boys and girls. Cord blood IgE levels above the detection limit of 0.2 kU/L were detected in 35 children (32%), of which 8 (23%) had developed atopy at 12 months of age. No relationship between the development of atopy and birth weight was found. A detailed analysis of early markers for sensitization in relation to clinical manifestations of atopic disease in this study cohort has been published recently [17].

Risk factors for the development of atopy

Several genetic and environmental risk factors were studied for the development of atopy within the first months of life (Table 2). Three risk factors were significantly related to the presence of atopy in the children at 12 months of age: smoking by at least one of the parents (present in 31% of the parents), AD of at least one of the parents (present in 26%), and atopy of both parents (present in 18%) (Table 2).

Cord blood IgE levels, using cut-off values of either 0.5 or 0.9 kU/L [23,24], were not a risk factor for the development of atopy. The analysis of the feeding method revealed that 5 children received a restricted diet of casein hydrolysate. Formula feeding was received by 32 children and 73 children were breast-fed for various periods of time.

No significant association between the feeding method and the presence of atopy at 12 months of age was found (data not shown).

Phenotypic analyses of CBMC

Flow cytometric analyses revealed that the percentage of CD4⁺ T cells was significantly increased in the CBMC of those children who had developed atopy at 12 months of age (Fig. 1). Furthermore, a significantly increased percentage of CD4⁺CD45RO⁺ (activated or memory) cells was found in those children who later developed atopy (Fig. 1). No significant differences were found for CD25⁺ T cells

Table 2. Risk factors for the development of atopy in high-risk children

Risk factor	Relative risk	95% coincidence interval
Atopy both parents	2.13	1.14–3.99*
Asthma of parents	1.51	0.75–3.02
AD of parents	2.04	1.10–3.78*
Male sex	1.17	0.62–2.21
Cord IgE > 0.5 kU/l	1.64	0.26–10.16
Cord IgE > 0.9 kU/l	1.35	0.23–8.07
Parental smoking†	2.23	1.20–4.14*
Pets	1.06	0.55–2.03
HDM avoidance	0.84	0.40–1.80

**P* < 0.05.

†Smoking of either one of the parents.

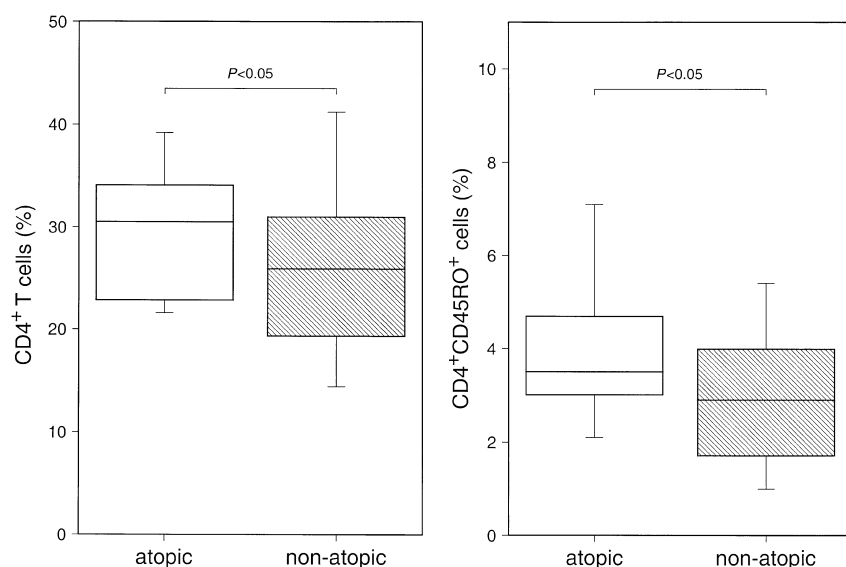


Fig. 1. T cell subpopulations in CBMC of children who did (white boxes) and did not (shaded boxes) develop atopy within the first year of life. Boxplots of mean percentages of CD4⁺ T cells (left) and CD4⁺CD45RO⁺ T cells as determined by flow cytometry.

and other leucocyte subsets analysed (NK cells, B cells, CD8⁺ T cells, and monocytes; data not shown). These data suggest that in cord blood of children developing atopy within the first 12 months of life, increased numbers of previously activated T cells are present.

Proliferative capacity after polyclonal stimulation

The baseline proliferation of CBMC of children who had developed atopy at 12 months (median = 2750 cpm, range 646–13249) was significantly increased compared to those who did not (median = 1674 cpm, range 152–8386). Also proliferative responses after polyclonal stimulation of the CBMC were significantly increased in the group of children who had developed atopy at 12 months (median = 47589 cpm, range 744–107374) compared to those who did not (median = 37631 cpm, range 3599–103391). These results, together with the increased percentage of CD4⁺CD45RO⁺ cells but not CD25⁺ cells, indicate that newborns who had developed atopy at 12 months of age had a pool of apparently previously activated T cells at birth, which had the capacity to respond more vigorously to non-specific stimuli. At 6 and 12 months, no significant differences in polyclonally stimulated proliferative responses were observed (data not shown).

Proliferative capacity after allergen-specific stimulation

In response to allergen-specific stimulation (CM, egg, HDM), proliferative responses were significantly increased as compared to unstimulated cells (Table 3). However, no differences were observed in the proliferative responses between CBMC of children who had and had not developed

atopy at the age of 12 months (Table 3). Furthermore, children who had developed a food allergy at the age of 12 months did not distinguish themselves at birth with regard to their proliferative responses, compared to atopic children without a food allergy. Analysis of allergen-specific proliferative responses over time showed that these responses decreased from birth to 12 months of age, both in children being atopic at that time and those who were not. Significant proliferation (SI > 3) after HDM stimulation of CBMC was observed in 41% of the children, whereas it was 30% at 12 months. Specific proliferation against CM (24% at birth, 13% at 12 months) and egg (14% at birth, 8% at 12 months) showed comparable changes. These results suggest that CBMC show relatively strong allergen-specific proliferative responses to allergens and that this capacity is gradually lost by 12 months of age. This feature

Table 3. Proliferative responses of CBMC of children who did and did not develop atopy at 12 months of age*

	Atopic at 12 months		Non-atopic at 12 months	
Medium	5.7	(0.1–28.4)†	4.4	(0.2–25.5)
CM	10.2	(0.1–31.1)	9.6	(0.2–36.7)
Egg	7.5	(0.1–24.1)	6.5	(0.2–27.7)
HDM	13.1	(0.1–32.8)	14.3	(0.2–47.1)

*Proliferation of CBMC was determined by measuring [³H]-thymidine incorporation on day 7 of culture after stimulation with various allergens or medium only.

†Data are expressed as mean total cpm × 10³. Between parenthesis the range is shown.

however, is similar in children that develop atopy within 12 months and those who don't.

Cytokine profiles after allergen-specific stimulation

Cytokine profiles of CBMC after stimulation with the food allergen egg and the inhalant allergen HDM were studied for the occurrence of allergen-specific memory cells at birth. The levels of IL-4, IL-5 and IL-13 proteins were generally below the detection limit of the sensitive ELISA method employed. Stimulation with HDM significantly increased IL-10 levels (Fig. 2a), both in children who had developed atopy at 12 months of age and those who do not. IFN- γ was significantly increased in both groups of children, but HDM-stimulated IFN- γ levels were significantly higher in the non-atopic children (Fig. 2a). After egg stimulation, no significant increase in IFN- γ levels was observed (Fig. 2a). IL-10 levels were significantly increased in both groups of children, but this

increase was significantly higher in the children developing atopy (Fig. 2a). These data show that children developing atopy show a decreased capacity to produce IFN- γ upon HDM stimulation. Furthermore, these data indicate that the cytokine pattern is dependent upon the allergen used for stimulation. HDM stimulation generally results in a stronger cytokine response than egg stimulation does, which is in accordance with the significantly higher effect of HDM on the proliferative response (Table 3).

At 12 months of age, stimulation of PBMC with HDM resulted in significantly increased IFN- γ levels as compared to unstimulated cells, both in atopic and non-atopic children (Fig. 2b). In contrast, HDM stimulation increased IL-10 production in the non-atopic children only (Fig. 2b). IL-5 and IL-13 levels were generally below or near the detection limit of the assay (< 5 pg/mL). After egg stimulation, IL-10 and IFN- γ levels were only significantly increased in the non-atopic children (Fig. 2b). In contrast,

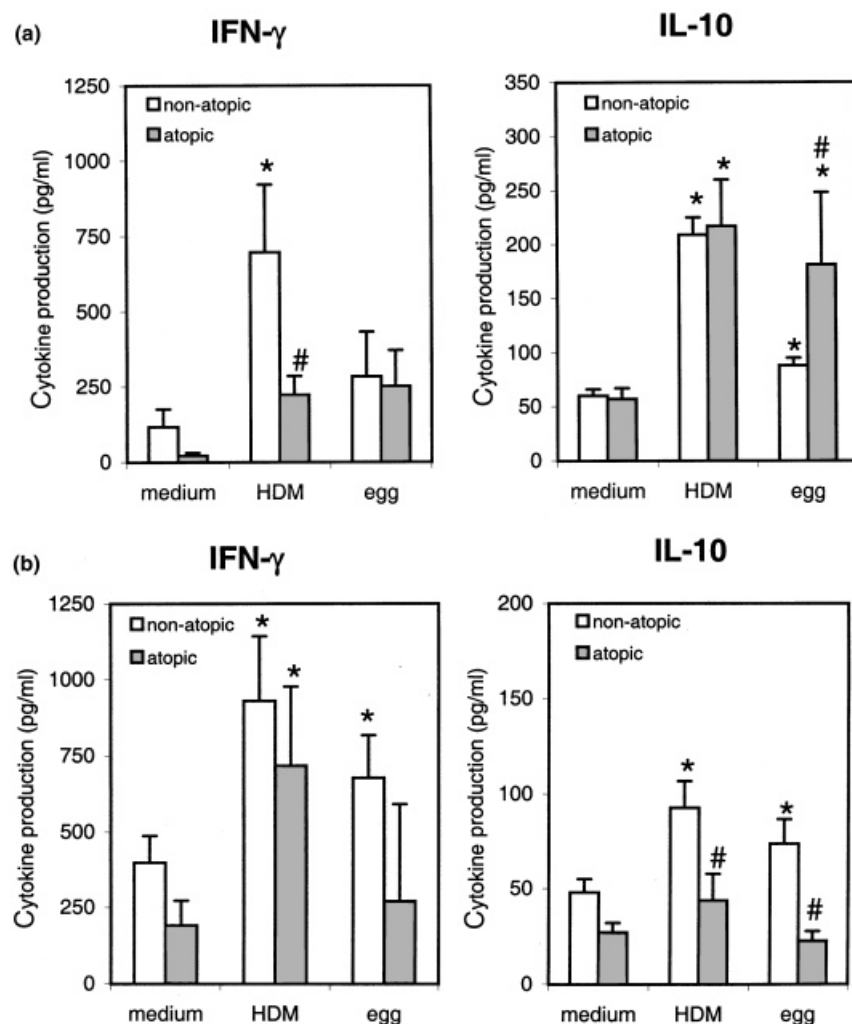


Fig. 2. (a) Allergen-induced cytokine production by CBMC of children who did (shaded boxes) or did not (white boxes) develop atopy at 12 months of age. (b) Allergen-induced cytokine production by PBMC of atopic (shaded boxes) and non-atopic (white boxes) children at 12 months of age. CBMC or PBMC were stimulated allergen-specifically for 4 days after which cytokine production in the culture supernatants was measured by ELISA. Group means and standard deviations are shown. * $P < 0.05$ between unstimulated and stimulated cells; # $P < 0.05$ between children who had developed atopy at 12 months of age and those who had not.

egg stimulation significantly increased IL-13 levels in the atopic children (medium: 2.8 ± 0.5 pg/mL vs. egg: 7.2 ± 2.0 pg/mL), but not in the non-atopic children (medium: 2.8 ± 0.4 pg/mL vs. egg: 3.9 ± 0.8 pg/mL). These data suggest that in atopic children a Th2 cytokine profile had developed at 12 months of age and that this was accompanied by an impaired allergen-induced IL-10 production.

Cytokine profiles after polyclonal stimulation

We then aimed to analyse whether the changes observed after allergen-induced stimulation were due to an intrinsic defect of the cells or rather due to divergent regulatory mechanisms. Therefore, we studied the capacity to produce a certain cytokine profile after polyclonal stimulation. Without stimulation, only IL-5, IL-10 and IFN- γ were detectable (data not shown), while after polyclonal stimulation all analysed cytokines were detectable. No significant differences were observed in the level of IL-5, IL-10, IL-13 and IFN- γ between CBMC of both groups of new-borns (Fig. 3 and data not shown), whereas a trend of increased IL-4 production levels occurred in those children

who later developed atopy. These data suggest that CBMC from newborns developing atopy are able to produce normal IFN- γ levels.

Time-course analysis showed that in the non-atopic children, the levels of IL-10 and all Th2 cytokines analysed (IL-4, IL-5, and IL-13) did not significantly differ from birth to 12 months of age (Fig. 3 and data not shown). In contrast, a significant decrease in IFN- γ protein production was observed during the first 6 months of life, resulting in significant lower IFN- γ protein levels at 6 and 12 months of age (Fig. 3d). In children developing atopy in the first 12 months of life, IFN- γ protein levels also significantly decreased during the first 6 months (Fig. 3d), with no significant difference between the atopic and non-atopic children. In contrast, at 6 months of age IL-4, IL-5 and IL-13 levels were significantly increased in the atopic children compared to the non-atopic children (Figs 3a, b and c). IL-10 levels were not different between both groups of children (data not shown). These changes resulted in the development of a profound Th2 cytokine profile in the majority of the atopic children, whereas all of the non-atopic children showed a concomitant production of IL-4 and IFN- γ (data not shown).

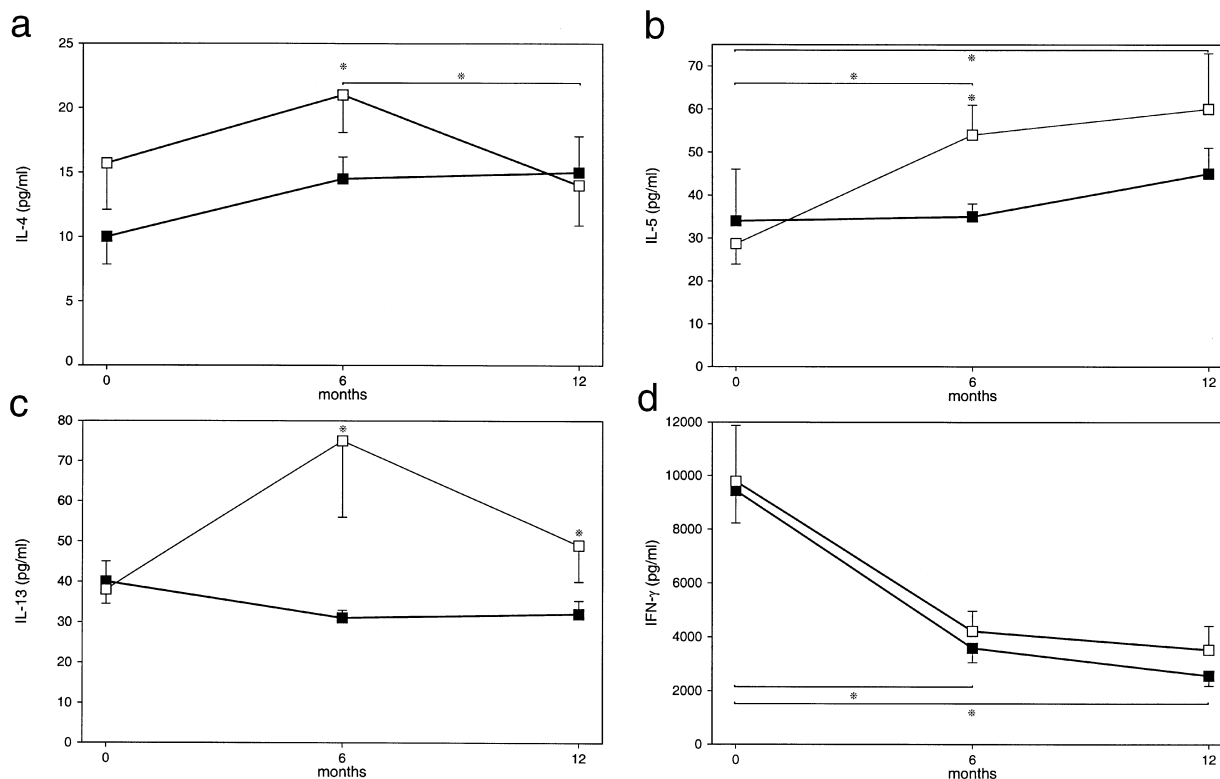


Fig. 3. Time course of cytokine protein production by PBMC of children who had (atopic, $n = 27$; open square) and had not (non-atopic, $n = 83$; closed square) developed atopy at the age of 12 months. CBMC and PBMC were polyclonally stimulated with PMA and Ca-ionophore for 16–18 h. IL-4, IL-5, IL-13 and IFN- γ levels were measured in the culture supernatants by ELISA. Data are expressed as mean $\pm$ SD. * $P < 0.05$ between atopic children and non-atopic children or $P < 0.05$ between two time-points.

At 12 months of age, IL-13 protein levels in supernatants of polyclonally-stimulated PBMC were significantly increased in the atopic children (Fig. 3). IL-5 protein levels were also increased in the atopic children, although this did not reach statistical significance. No significant differences were observed in IL-4, IL-10 and IFN- γ protein levels between atopic and non-atopic children (Fig. 3 and data not shown). Remarkably, IL-4 protein levels in the atopic children significantly decreased between 6 and 12 months of age (Fig. 3). This kinetic analysis shows that in both atopic and non-atopic children, polyclonally-stimulated IFN- γ production decreased during the first 6 months of life. In children developing atopy, Th2 cytokine levels were increased at 6 months (IL-4, IL-5, and IL-13) and 12 months (IL-13). This indicates a transition from a Th0-like cytokine profile at birth to a Th2 cytokine profile at 6 months of age.

Discussion

In the present prospective study, 110 newborns were followed in their first year of life for the development of clinical signs of atopic disease together with a detailed analysis of their cytokine profiles. Our results provide evidence that, in those high-risk children developing atopic disease in their first year of life, a strong Th2 cytokine profile develops within the first 6 months. This skewing to a strong Th2 cytokine profile, characterized by high levels of IL-4, IL-5, and IL-13, may be related to impaired allergen-induced IFN- γ and/or IL-10 production.

In our study, 27/110 children developed an atopic disease within the first 12 months of life, which is in agreement with the expected genetic predisposition of 30% in the case of an atopic mother [16]. Nevertheless, it should be noted that the clinical outcome used to divide infants into 'atopic' and 'non-atopic' groups has some limitations. Firstly, the non-atopic group will be heterogeneous, as a limited number of children will develop an atopic disease after the first year of life [17]. Secondly, the diagnosis of atopy, especially asthma-like disease, can be difficult at 12 months of age. Clearly, it will be of interest to follow this study population to clarify clinical outcome.

The most consistently documented environmental risk factors for the development of atopic disease in children are passive smoking [16] and the presence of atopy in the family [1–4], and this is confirmed by our study. Furthermore, the present study confirms that the cord blood IgE level is a poor predictor of atopic disease [3,24]. Preliminary data indicate that atopic children who had two atopic parents showed an increased proliferative capacity and an increased IL-13 production by CBMC compared to

atopic children with one atopic parent, indicating genetic influences on the development of atopy (unpublished data).

Cord blood of children being atopic at 12 months of age showed a significantly increased percentage of CD4⁺CD45RO⁺ T cells, whereas the percentage of CD25⁺ (activated) T cells was not different. Together with the increased base line proliferative response in CBMC of children developing atopy, these data support the presence of a previously activated pool of memory T cells [5,25,26]. Nevertheless, the presence of these previously activated T cells did not result in a higher proliferative response or production of cytokines after allergen-stimulation as compared to newborns that did not develop atopy. Interestingly, after HDM stimulation of CBMC obtained from non-atopic children, IFN- γ production was significantly increased, whereas much lower IFN- γ production was observed in the children developing atopy within the first 12 months of life. Such a decrease in allergen-stimulated IFN- γ production has previously been considered as evidence for a weak Th2 profile [6,26,27]. We now show that after polyclonal stimulation, IFN- γ levels are comparable between atopic and non-atopic children. This indicates that the impaired allergen-stimulated IFN- γ production in the atopy-developing children was not due to an intrinsic defect but rather due to divergent regulatory mechanisms, possibly related to the immaturity of the antigen-presenting cell compartment [9,28,29].

At 6 months of age, a significantly increased production of IL-4 was observed in the children developing atopy compared to the non-atopic children, whereas this difference was resolved at 12 months of age. This transient increase in IL-4 production, pointing to an active development of Th2 cells (as shown by a similarly increased production of IL-5 and IL-13), correlated in time with the first signs of clinical symptoms characteristic of an atopic disease. Therefore, the first 6 months of life may represent a critical time window for the initiation of immunological changes reflecting the allergic sensitization that becomes clinically detectable at 12 months. The transient increase of IL-4 at 6 months of age is accompanied by a long-lasting increase in IL-13 levels in the atopic children. Therefore, we hypothesize that IL-4 may be essential in the skewing of the immune system towards a Th2 cytokine profile, whereas IL-13 may be the effector cytokine in established atopic diseases.

Our data are consistent with a transition from a weak Th2 cytokine profile at birth to a strong Th2 cytokine profile at 6 months of age in atopic children. This transition is characterized by an increased capacity to produce the Th2 cytokines IL-4, IL-5 and IL-13 in atopic children relative to non-atopic children after polyclonal stimulation of PBMC. These data are supported by TaqMan analysis of mRNA levels, showing increased mRNA levels of IL-4 (at birth),

IL-5 (at birth, 6 and 12 months), and IL-13 (at birth, 6 and 12 months) in children developing atopy (unpublished results). Other reports have described that decreased IFN- γ production is responsible for the profound Th2 skewing present in early life [5,6,14,15,27,30,31]. However, in our study polyclonal-stimulated IFN- γ levels were similarly decreased in atopic and non-atopic children and impaired IFN- γ production was only observed after allergen-specific stimulation of CBMC from children developing atopy. Several explanations for this apparent discrepancy can be put forward. First, this may be due to differences in the patient groups studied. We included only high-risk newborns divided into the presence or absence of an atopic disease at 12 months of age. Other studies compared high-risk with low-risk newborns and did not follow them with respect to the development of atopy [7,23] or compared high-risk children who developed atopy with low-risk children who did not develop atopy [27,31]. Therefore, the decreased production of IFN- γ may only be present between high-risk and low-risk children, but not between atopic and non-atopic high-risk children. Secondly, the discrepancy may be dependent on the different definitions for atopy and the different follow-up period of the newborns up to two or more years [5,23,31]. The third explanation may be the delivery process itself. In our cohort, most children (> 90%) were born by vaginal delivery taking hours. Delivery-related stress may non-specifically activate the immune system of the foetus *in vivo* [32,33]. Most other studies have used children born by caesarean operation, which may result in much lower non-specific activation of the immune system. Fourthly, differences in protein or mRNA detection methods, culture conditions, and stimulation protocols may contribute to the discrepant results [5–7,12–14,22,23,27,31].

At 12 months of age, allergen-specific and polyclonal cytokine responses showed an increased production of IL-13 and, to a lesser extent, IL-5, in the atopic children. These data indicate that at 12 months of age the shift to the Th2 phenotype is already established. In contrast to the Th2 cytokines, allergen-stimulated IFN- γ levels did not differ, whereas IL-10 levels were significantly decreased in the allergic children. It has been shown that IL-10 may have a general inhibitory function on the immune system [34–37]. Therefore, decreased IL-10 levels in established atopic diseases, as also found in a previous study [38], may result in a diminished immunosuppressive function of T cells [34–36,39], leading to a lack of down-regulation of the inflammatory process.

In conclusion, our data demonstrate that in high-risk children the first 6 months of life create a time period in which decreased IFN- γ levels and/or increased IL-4 levels skew the immune system towards a Th2 cytokine profile. Furthermore, the observed decreased allergen-induced

IL-10 production in atopic children may facilitate the development of atopic disease by a lack of down-regulation of the inflammatory process.

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