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Immune responses during allergic sensitization and the development of atopy

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In the first years of life, the immune system of children is relatively immature compared to that of adults (1–4). This immaturity of the immune system creates a window period between birth and 2 years of age during which there is an enhanced risk of allergic sensitization and subsequent atopic disease (5). The allergen-specific T-cell responsiveness that distinguishes atopic from nonatopic children is already programmed into the T-helper (Th) cell memory compartment during early childhood (6). In children with “established” atopic disease, a disturbed balance in Th cell subsets (Th1 vs Th2) toward a polarized Th2 phenotype is generally accepted (7–10). The failure of atopic children to develop a long-term, Th1-dependent memory has been proposed to be due to a reduced IFN- γ production (11–13). The shift toward a polarized Th2 phenotype during the process of allergic sensitization in children that develop atopy is not completely understood. Allergen challenge in an atopic patient results in the selective activation, recruitment, and accumulation of specific Th2 cells in the target organs, such as skin and lungs. The importance of Th2 cells in atopic diseases is closely associated with the functional activities of the cytokines they produce. In our studies, we investigated prospectively the possible presence of dysregulations in T-cell responses in children at high risk of developing atopy.

Influence of genetic and environmental risk factors

For our prospective study, 133 neonates at high risk of developing atopy were selected and followed from birth up to 2 years of age. The prevalence of atopy in our cohort increased from 16% at 6 months to 32% at 24 months of age. Atopic dermatitis (AD) was the most frequently diagnosed atopic disease. Of the children with AD at 12 months of age, 22% (5/23) also had a food allergy, confirming previous findings (14, 15). The combination of AD and food allergy in the first 12 months of life proved to be an important risk factor and constituted the most reliable diagnostic parameter associated with the development of asthma-like disease after the first year of life (16).

All of the children included in our study had an atopic mother, and 20% of the children also had an atopic father. The total prevalence of atopy during the first 2 years of life was 27% in the case of an atopic mother only, and 52% in the case of two atopic parents, a finding which is in agreement with the expected genetic predisposition (17). Moreover, atopy in both parents was significantly related (relative risk [RR]=2.13) to the development of atopy in the children at 12 months of age. This clearly confirms the role of genetic predisposition in the development of atopy.

Besides the genetic predisposition, environmental factors influence the development of atopic disease.

Several environmental factors, such as parental smoking, bacterial and/or viral infections, feeding method (bottle or breast-feeding), birth weight, and early exposure to household pets and inhalant allergens, have been implicated as factors increasing the risk of the development of atopic disease (18–21). In our study, we analyzed the relationship between feeding method, exposure to household pets, house-dust-mite (HDM)-avoidance strategies, parental smoking, and the development of atopy before 12 months of age. Of these factors, parental smoking was the only significantly related risk factor ($RR = 2.23$) for the development of atopy in children before 12 months of age.

It was recently reported that parental smoking does not increase the risk of allergic sensitization in children, after excluding asthma and other wheezing disorders (22). However, most published studies show convincing evidence that exposure to tobacco smoke increases the risk of the development of atopic disease (including wheezing bronchitis, asthma, and bronchial hyperreactivity) in unselected children, as well as in children with an atopic predisposition (18–21, 23–26).

As the number of cigarette-smoking young women has steadily increased in the Western industrialized countries over the last 40 years, passive smoking early in life may be one of the factors behind the increasing prevalence of atopy (27). Exposure to maternal smoking appears to have the greatest effect, probably because the mother is in closer contact and spends more time with the infant during the first year of life than anyone else (23). Moreover, maternal smoking during pregnancy could affect the development of the immune system of the fetus (23, 24, 28). It has been documented that maternal smoking causes a significant rise in cord-blood IgE levels and reduces natural killer (NK) cell activity (28). Maternal smoking might also act as an adjuvant factor magnifying the underlying process of allergic sensitization (27). Our data confirm that during the first year of life passive smoking is the best identified and the strongest environmental risk factor for the development of atopic disease. For the development of atopy at later ages, other risk factors, such as exposure to household pets and HDM, may become more important (20). A dose-response relation between exposure to HDM and sensitization to it was recently described for children up to 3 years of age (29). The clinical implications of these markers as risk factors for the development of atopy may become more obvious during longer follow-up.

The immune response during the development of atopic disease

For the process of allergic sensitization, the relative immaturity of the immune system of neonates compared to that of adults could create a window period,

starting *in utero* and lasting up to 2 years of age, of increased susceptibility to the development of atopy. In order to gain more insight into the process of allergic sensitization and the subsequent development of atopic diseases, we examined the development of immunologic responses in high-risk children.

Proliferative responses and cytokine production after polyclonal stimulation

Proliferative responses and cytokine profiles were studied after polyclonal stimulation to investigate whether the intrinsic capacity of T cells to give rise to a Th1 or Th2 cytokine profile was different between atopy-prone children and children who do not develop atopy within their first year of life. Our data showed an increased proliferative capacity of cord-blood mononuclear cells (CBMC) of those children who developed atopy before 12 months of age, a finding which is in agreement with findings of others (30, 31).

The vaginal delivery of a baby usually takes several hours, and during this delivery-related period of stress the fetus' immune system may become nonspecifically activated (32, 33). Such a stress period could explain the higher baseline proliferative response observed in the CBMC of all neonates than in infants at 6 months of age. In addition, the cytokine mRNA expression levels, as determined by a quantitative PCR, were also increased at birth compared to 6 months of age, a finding which provides further evidence for such a nonspecific activation of the immune system of the neonate at birth.

The cytokine profile observed at birth after polyclonal stimulation of CBMC showed a predominance of Th cells of the Th0 phenotype. However, an underlying selective Th2 cytokine profile to specific allergens could still be present although undetected (34). The Th0 cytokine profile observed at birth shifted to a Th2 cytokine profile at 6 months of age in children that developed atopy before 12 months of age. Several groups proposed that the development of a strong Th2-polarized memory response in children that develop atopy was mainly due to reduced IFN- γ production (31, 34–36).

The analysis of IFN- γ is complicated by the rapid degradation of the IFN- γ protein in culture supernatants. Therefore, optimal culture conditions and the combined analysis of mRNA expression levels and protein levels are especially important for IFN- γ . In our study, the mRNA expression levels were determined by a quantitative PCR using the TaqMan technique, which is a sensitive method to detect small differences in expression levels between different patient groups (37).

During the first year of life, no significant differences were observed for mRNA expression or protein levels of IFN- γ between children who did and did not develop atopy. Therefore, we do not consider an intrinsic T-cell defect in the production of IFN- γ to be the cause of the

selective development of the Th2 profile in atopics. If we exclude such an intrinsic T-cell defect for IFN- γ production, possible differences in cytokine production levels between atopics and nonatopics must be the consequence of differential regulation. Infections have been thought to play a negative regulatory role in the development of the Th2 cytokine profile in atopics, in view of the inverse relationship between plasma IgE levels and the number of infections in children (38).

Proliferative responses and cytokine production after allergen-specific stimulation

We and others did not observe differences in allergen-induced proliferative responses of CBMC between neonates who did and did not subsequently develop atopy (11, 39, 40). In addition, neonates who developed food allergy could not be distinguished from the other atopic children by the level of proliferative responses to food stimulation. We therefore conclude that allergen-induced proliferative responses of CBMC do not significantly contribute to the prediction of allergic sensitization during the first year of life.

Remarkably, a Th0, rather than a Th2, cytokine profile was observed after HDM-specific stimulation of CBMC. This is in contrast to the weak Th2 cytokine profile at birth, as originally suggested by Prescott et al. (11). In the latter study, no clear discrimination was made between HDM-induced cytokine profiles in atopic children with or without clinical manifestations of upper-airway allergy. The weak Th2 skewing was proposed to be the result of low levels of environmental allergens crossing the placenta in combination with a local micromilieu (progesterone, PGE₂, IL-4, and IL-10) that is strongly inhibitory to Th1-associated activities (41, 42).

An interesting question concerns the mechanism of processing and presentation of the allergen to the T-cell compartment. At birth, an allergen-specific proliferative response of CBMC is observed, indicating previous interactions with the allergen and the necessary involvement of antigen-presenting cells (APC). The principal APC network responsible for immune surveillance is the dendritic cell (DC) network, which is functionally immature in neonates and infants (43). The capacity of peripheral tissue DC to direct the neonatal T-cell responses to environmental allergens toward the nonatopic Th1 profile may play a role in the development of atopy. In adults, differences in DC functions *in vitro* between atopic and nonatopic individuals have been reported (44, 45). The precise role of APC/DC in the postnatal maturation of Th1-associated functions in infancy has not yet been established. Furthermore, possible differences at the gut and/or bronchial mucosal surfaces between children who do and do not develop atopy could contribute to the nature and the amount of allergens presented to the systemic T-cell compartment.

The process of *allergic sensitization* is currently discussed only in the light of the Th1-Th2 theory. However, the observed cytokine profile of relatively high IL-10 production in combination with reduced IL-4 expression after HDM-specific stimulation in children who develop atopy could also be interpreted as a T-regulatory 1 (Tr1) cytokine profile. Tr1 cells are able to produce high amounts of IL-10 and low amounts of IL-4 in combination with low proliferative responses after repeated antigenic stimulation in the presence of IL-10 (46, 47). Tr1 cells and their high IL-10 production levels in the development of atopy in children may cause a negative feedback in the process of allergic sensitization. High IL-10 levels are principally able to downregulate various components of the allergic reaction; i.e., Th2 development, IgE production, and mast-cell and eosinophil function. This may inhibit and/or delay the development of atopy. In addition, Tr1 cells, by their production of IL-10 and TGF- β , can induce long-lasting anergy in CD4⁺ cells, which may inhibit the antigen-specific responses (46, 47). High IL-10 levels also induce a general inhibition of cytokine release by both Th1 and Th2 cells, but more profoundly inhibit the IFN- γ production (48–50). Therefore, during the development of atopy in children, Tr1 cells might downregulate the Th1 response and thereby indirectly cause a relative increase in the Th2 response.

The analysis of cell-surface markers on CBMC revealed an increased percentage of CD4⁺ and CD4⁺CD45RO⁺ (memory) cells in neonates who later developed atopy, a finding which is in agreement with the data of others (51). In accordance with the data of Miles et al. (52), we did not find a difference in the percentage of CD25⁺ (IL-2R⁺) T cells between neonates who did and did not develop atopy within the first year after birth. The increased percentage CD4⁺CD45RO⁺ cells, in combination with the higher baseline proliferative response of CBMC from children who developed atopy, supports the hypothesis that at the time of birth the Th cells of these children had been previously activated, emphasizing that the initial allergen sensitization could already start *in utero* (32).

CD4⁺CD45RO⁺ cells are considered to be the major T-cell subpopulation which respond to allergens and subsequently produce cytokines (53). However, the mean percentages of CD4⁺CD45RO⁺ cells in cord-blood are very low compared to the percentages found in adults (54, 55), a fact which makes it unlikely that these memory cells are the only source of the allergen-induced cytokines. CD45RO⁺ cells can change into CD45RA⁺ cells. As the CD45RA⁺ T-cell population includes naive T cells, this suggests that the CD45RA⁺ cells are not a homogeneous population. We suggest that subpopulations of CD4⁺CD45RA⁺ cells contribute to the allergen-induced cytokine production by CBMC. This is supported by the finding that CD45RA⁺ T cells from atopic patients produced

IL-4, IL-5, and IFN- γ after stimulation with mitogens (56). Moreover, most of the CD45RO⁺ cells in cord-blood are CD45RO⁺/RA⁺ double-positive cells (57), indicating that CD45RO⁺ cells in cord-blood are not equivalent to the CD45RO⁺ cells in the peripheral blood of older children or adults.

Of the analyzed markers for allergic sensitization, a positive skin test at 12 months of age was significantly related to the clinical expression of AD and food allergy at 24 months of age. Nevertheless, a substantial number of children yielded positive skin prick tests in the absence of clinical symptoms, a finding which makes controversial the practical usefulness of skin prick testing for the diagnosis of food allergy (58, 59).

Food- and HDM-specific IgE antibodies were significantly associated with food and upper-airway allergy, respectively. However, of the children with food-specific IgE antibodies, only 25% had food allergy. Previous studies showed that only 30–40% of patients with food-specific IgE manifested clinical symptoms after ingestion of the involved food (58, 60). This can be the consequence of the nature of the immune response to produce IgE antibodies against food allergens in young children (61). Introduction of solid foods to infants can result in the development of varying plasma levels of IgE antibodies against one or more common food antigens, such as those of cow's milk and egg. The antibody titers usually peak at around 9 months of age and rapidly disappear thereafter. Only in children that develop food allergy do these titers persist beyond the age of 9 months and remain generally higher than the titers measured in nonatopic children. Therefore, the practical usefulness of food-specific IgE for the diagnosis of food sensitization or allergy in children below 2 years of age is disputed (58, 59, 62, 63).

IgG antibodies to food antigens occur in young children before IgE antibodies to food antigens are detectable in the serum. Recently, increased food-specific IgG levels in the serum of 1-year-old children have been proposed as a predictor for increased risk of developing atopy (64).

Immunologic markers predicting atopy at 12 months of age

As stated before, the cord blood of neonates who developed atopy before 12 months of age displayed an increased percentage of CD4⁺ CD45RO⁺ cells and an increased proliferative capacity after polyclonal stimulation of CBMC. Moreover, differences in cytokine production levels were found after allergen-specific stimulation of CBMC of neonates who did and did not develop atopy. Children who developed their first atopic symptoms between 6 and 12 months of age already displayed a Th2 cytokine profile at 6 months. Despite these differences between the subpopulations of children who did and did not develop

atopy before 12 months of age, the ranges of these immunologic markers within the groups were large, resulting in a substantial overlap. Therefore, it is not feasible to use these cellular markers to predict the development of atopy at the level of an individual child in this age range.

Of the investigated markers for allergic sensitization or inflammation, a positive skin test and specific IgE antibodies to food and inhalant allergens were related to, but did not clearly precede, the clinical expression of atopy. This was also suggested in studies which analyzed children older than 2 years of age (21, 65–67). Apparently, analysis at 2 years in our cohort is too early for a definitive conclusion about the predictive value of the investigated markers for atopy development at later ages.

Allergic sensitization and the development of atopy

Intrauterine sensitization

One of the mechanisms underlying the development of atopy concerns intrauterine sensitization. It has been repeatedly documented that cord-blood T cells are capable of proliferating *in vitro* in response to both food and inhalant allergens (11, 39, 40, 68). This suggests that the initial allergen-specific T-cell priming may occur *in utero* as the result of transplacental transfer of small amounts of the relevant allergens to which mothers are exposed during pregnancy. The allergens may cross the placenta as soluble or IgG-complexed peptide fragments already processed by the APC of the mother. Recent data on cytokine production levels at birth suggest that this T-cell priming may be strongest in infants with a positive family history of atopy and in infants who later develop atopy (31, 69).

As stated before, allergen-induced proliferative responses of CBMC from neonates who either did or did not develop atopy before 12 months of age support the hypothesis that the initial allergen sensitization of all neonates could have already started *in utero*. Jones et al. have presented evidence that allergen-specific T-cell priming may already be initiated in the second trimester of pregnancy (70). Such fetal T-cell priming is believed to be dependent on the season and the extent of allergen exposure of the mother (70–72). Very low or very high maternal exposure to allergens seems to be protective against primary fetal sensitization (71). High doses of maternal allergen-specific IgG antibodies are thought to account for the prevention of atopy development in the neonate (73). Moreover, it might well be that differences exist between the intrauterine environments of fetuses of atopic and nonatopic mothers. For example, there is some evidence that the amniotic fluid of atopic mothers contains higher IL-10 levels than that of nonatopic mothers (74). However,

the mechanisms involved, including the role of fetal APC, and the initiation of intrauterine sensitization, are largely unclear and need to be studied further.

Reduced production of IFN- γ during the perinatal period

Another factor proposed to be involved in the mechanism of allergic sensitization is the reduced production of IFN- γ by fetal CBMC and/or neonatal T cells after mitogenic stimulation relative to adult PBMC (75). This reduced IFN- γ production appears to be more marked in neonates at risk of atopy or those who later develop atopic disease (12, 13, 31, 40). After delivery, a rebound Th1-like reaction has been proposed (76), while others have reported a positive effect of pregnancy on both IL-4 and IFN- γ production (77). Increased prolactin levels during breast-feeding may also lead to increased IFN- γ production, inhibiting Th2 development (78). It has been suggested that maternal circulation pregnancy factors stimulate monocyte, but inhibit lymphocyte, activity (79, 80). The resulting maternal (placental) IL-10/IL-12 ratio thus determines fetal antigen-specific T-cell responses and the Th-differentiation pathway. As a consequence, a selective suppression of fetal (and maternal) Th1 responses occurs, preventing possible rejection. Thus, on exposure to antigen, fetal T cells react by a predominantly Th2 response.

We showed that there appears to be no intrinsic defect in the capacity of T cells to produce IFN- γ when children who develop atopy before 12 months of age are compared with children who do not. We cannot exclude, however, a possible difference in the capacity to produce IFN- γ between low- and high-risk neonates, because we only included high-risk children in our study. This indicates that the reduced IFN- γ production is probably the consequence of a difference in regulatory mechanisms between atopic or not yet atopic and nonatopic children. Allergen-specific stimulation apparently reflects more closely the *in vivo* situation.

Therefore, *in vivo*, IFN- γ could still play a role in the mechanism underlying the development and maintenance of allergic sensitization and atopic disease. An explanation for the reduced allergen-induced IFN- γ production by CBMC of neonates who will develop atopy may be the presence of the recently described Tr1 cells (46). After allergen-specific stimulation of CBMC from neonates who developed atopy before 12 months of age, we indeed observed a relatively large IL-10 production in combination with reduced IL-4 expression, suggesting the presence of Tr1 cells. However, in children with established AD, Tr1 cells probably do not explain the reduced allergen-induced IFN- γ production by PBMC, as reduced IL-10 production was observed in these children. This reduced IFN- γ production has been attributed to a "post-transcriptional defect", as these children display a discrepancy between IFN- γ mRNA expression and IFN- γ protein production (81). In

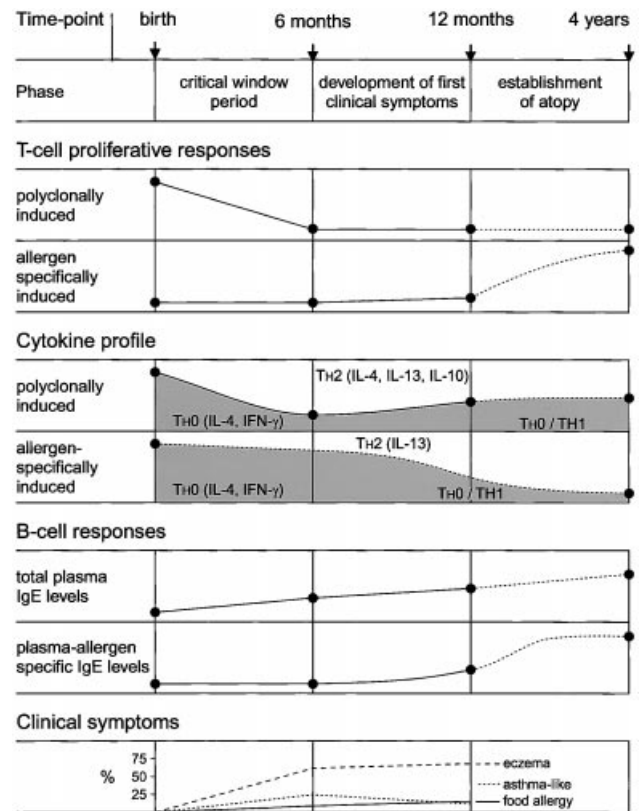


Figure 1. Schematic presentation of sequence of phases implicated in the gradual development and maintenance of atopic disease. Three different phases are distinguished: 1) the critical period between birth and 6 months of age; 2) the development of the first clinical symptoms at 6–12 months of age; and 3) the establishment of atopy between 12 months and 4 years of age. Data for phases 1 and 2 were obtained from samples of children included in a longitudinal study, and data for phase 3 were obtained from samples of children included in a cross-sectional study. The development of detectable T-cell proliferative responses, Th-cell cytokine profiles, B-cell responses, and clinical symptoms is schematically shown during the different phases of the development of allergic sensitization and the establishment of clinically overt atopic disease during childhood. Data represent gradual changes of the immunologic markers with emphasis on the time sequence between the markers in children who develop atopy relative to nonatopic children. Distinction is made between polyclonally induced and allergen-specifically induced T-cell proliferative responses and cytokine profiles. Moreover, the B-cell response is indicated by total IgE as well as allergen-specific IgE plasma levels. ●: value of analyzed data; —: extrapolated course;: expected course.

addition, the IFN- γ production is believed to be primarily transcriptionally regulated with little or no post-transcriptional regulation (82), arguing against a post-transcriptional defect. An explanation for the discrepancy between the comparable mRNA levels and the reduced allergen-induced IFN- γ production in children with established AD could be the rapid degradation of secreted IFN- γ protein in culture supernatants. Alternatively, an increased autocrine receptor-mediated consumption of secreted IFN- γ in children with established AD might account for the disturbed Th1-Th2 cytokine balance.

Proposed sequence of phases implicated in the gradual development and maintenance of atopic disease

Based on the hypothesis of allergic sensitization at the perinatal age, we propose the following sequence of important phases implicated in the allergic sensitization and the maintenance of atopic disease during childhood (Fig. 1). The data represent gradual changes in a critical set of immunologic markers in children who develop atopy relative to nonatopic children. These parameters were analyzed at discrete time points. Eventually, such data might aid in the assessment of selected immunologic markers suitable for the follow-up of high-risk children.

The initial T-cell priming already starts *in utero* and may be stronger in infants that will develop atopy. This results in an increased proliferative capacity of cord-blood T cells and a Th0 cytokine profile after polyclonal and allergen-specific stimulation of CBMC from neonates who will develop atopy. Alternatively, the increased proliferative capacity of CBMC might be a reflection of the period of stress during the delivery and will gradually disappear during the first 6 months of life.

The first 6 months of life appears to be the most critical period for the induction of atopic disease (critical window period). During this period, the inherent capacity to produce a Th0 cytokine profile gradually shifts to a Th2 cytokine profile in children that develop atopy by 12 months of age. Later during infancy, allergen-specific stimulation of PBMC leads to a pronounced Th2 cytokine profile, due to an increased frequency of allergen-specific T cells in the peripheral blood.

At 6–12 months of age, the clinical manifestations of atopic disease usually become more pronounced. During this period, a Th2 cytokine profile is still detectable after polyclonal stimulation of PBMC. In addition, with increasing age, allergen-specific stimulation of PBMC leads to a more pronounced Th2 cytokine profile. In the same period, the nonspecific B-cell response, as determined by total plasma IgE levels, becomes more prominent.

Between 12 months and approximately 4 years of age, the first clinical symptoms of atopy tend to develop into an established atopic disease. By that time, an allergen-specific proliferative response and a pronounced Th2 cytokine profile may be observed after stimulation of PBMC. During this period, allergen-specific IgE appears in the circulation, and plasma total IgE levels also become more typical of atopic children. Thereafter, a more stable phase starts in these children, possibly persisting into adulthood, although variations may well occur (82–84).

Th2 polarization in the first years of life

During the different phases of the development of allergic sensitization and the establishment of the atopic disease in childhood, particular cytokines can be used to distinguish between children who will and will not develop atopy. Around 6 months of age, a brief strong increase in IL-4 protein production after polyclonal stimulation of PBMC is observed in children who will develop atopy. This IL-4 is probably responsible for the initial development of Th2 cells (85). During all phases, small differences in IL-5 mRNA expression and IL-5 protein production are observed after stimulation of PBMC from children who will develop atopy compared to those who will not. Probably, IL-5 is more related to the local tissue inflammation, and therefore the presence of this cytokine in the peripheral blood is less pronounced than that of other Th2 cytokines, such as IL-13.

IL-13 is a key cytokine for atopic diseases that can distinguish between atopic and nonatopic children during all the different phases. Moreover, IL-13 is reported to be related to the presence of specific IgE and to be the most predictive cytokine of the presence of a Th2 pool independent of the age of the child (10, 82).

During the establishment of atopy, the reduced production of IL-10 and IFN- γ protein becomes gradually more prominent. As IL-10 is a strong immunosuppressive cytokine (50), the reduced IL-10 production may result in a lower degree of immunosuppression and subsequently in the maintenance of the allergic inflammation. The imbalance between Th1 and Th2 cells in atopy, with a skewing toward Th2 cells, probably causes the relatively reduced IFN- γ protein levels.

Conclusions

During allergic sensitization and the establishment of atopic disease in childhood, the Th0 cytokine profile as detected at birth gradually shifts to a Th2 cytokine profile. Polyclonally induced responses of circulating cells can reveal atopic sensitization at an earlier age than allergen-induced immune responses. Similarly, allergen-specific T-cell responses precede allergen-specific IgE antibodies in the circulation. The allergen-specific IgE antibody formation correlates with the appearance of clinical symptoms of atopy.

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