

Reciprocal age-related patterns of allergen-specific T-cell immunity in normal vs. atopic infants

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Summary

By adulthood there is almost universal immunological memory to aeroallergens, and the presence of allergic disease appears to be related to the nature of the underlying T-helper (Th) cell cytokine responses. The hypothesis of this study is that adult patterns of allergen specific Th-cell memory (Th-2 polarized in atopics vs. Th1 in non-atopics) can be determined in early infancy.

Mononuclear cell cytokine responses to house-dust mite were measured at 6-monthly intervals from birth to 2 years of age, using ELISA (IL-10, IL-13, IFN- γ) and sqRT/PCR (IL-4, IL-5, IL-9, IFN- γ) in normal infants ($n = 14$) with no family history or allergic symptoms, and infants with a family history and definite atopy by 2 years ($n = 16$).

Both normals and atopics showed low-level Th2 skewed allergen-specific responses at birth with little accompanying IFN- γ . The Th2 responses to house-dust mite were higher in normal newborns, who then show a rapid downregulation of these responses in the first year of life. Atopic infants instead show a consolidation of their neonatal patterns of Th2 polarized allergen specific immunity.

Earlier studies indicate that neonates at high risk of atopy display diminished capacity for production of the Th1 cytokine IFN- γ . The present study suggests for the first time that neonates who subsequently develop atopy also initially have reduced capacity to mount Th2 responses. However, in contrast to non-atopics who selectively downregulate their fetal Th2 polarized allergen-specific responses, atopic children display age-associated upregulation of Th2 immunity.

Introduction

Adult humans show almost universal T-cell memory to ubiquitous environmental allergens, such as house-dust mite. Differences in the underlying patterns of T-cell cytokine responses influence the clinical outcome. Th2-skewed immunity is the hallmark of the atopic phenotype, and contrasts with the Th1-like pattern which is stably expressed in normal adults throughout life [1,2]. Allergic responses to these airborne allergens are responsible for the bulk of allergic diseases within the community, but little is known about how these disparate forms of immune memory develop in early life, and what determines the expression of the Th2-like phenotype in the genetically at risk individual.

There is extensive epidemiological evidence supporting the concept of a 'sensitization window' during infancy

(reviewed in [3]). This suggests that challenge with allergen during infancy predisposes towards development of long-term Th2-skewed allergen-specific immunological memory. There is also evidence of an allergic 'genotype' [4], given the strong allergic risk of children with atopic parents or siblings. How these environmental and genetic factors interact is also poorly understood.

It has been suggested that delayed postnatal maturation of cellular immune function may be a key determinant of genetic predisposition to atopic disease [5,6]. This is based on the observations of a number of independent groups that neonates with positive atopic family history have diminished capacity for secretion of Th1 cytokines (in particular IFN- γ) relative to their family history negative counterparts [7–11].

In support of this hypothesis, a recent retrospective study of 12-year-old children demonstrated that allergic disease was most common amongst those who failed to develop Th1-dependent delayed tuberculin

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hypersensitivity in response to BCG vaccination during infancy [12]. It is possible that transient deficiencies of Th1 function at the time of primary exposure to allergen may increase the likelihood of allergen-specific Th2 memory consolidation during these early responses.

There is now definitive evidence based on DNA genotyping that allergen reactive T cells in cord blood are of fetal origin [13], suggesting that human T cells are initially primed to allergen before birth, although the mechanisms are still unclear. This paper compares the allergen specific cytokine responses at birth and over the first 2 years of life, in a group of known allergic infants and a similar group of normal infants. This study addresses our hypothesis that the contrasting patterns of immune deviation seen in atopic and normal adults are established within the first years of life, and that atopics have maturational defects in immune function which are more extensive than those previously observed with respect to IFN- γ .

Materials and methods

Study subjects

Subjects were recruited antenatally, and atopic risk determined by maternal IgE, RAST, skin prick testing (SPT) and interview questionnaires. Cord blood was collected at birth by venepuncture of the placental vessels (after wiping with alcohol three times and allowing to dry). Infants were seen at 6-monthly intervals for detailed histories, physical examinations, skin tests and blood collections. The groups were defined on the basis of their clinical allergic at 2 years of age. The 'normal' infants have no family history and no symptoms of allergy at 2 years old, and the 'atopic' group have a positive family history and definite allergic disease at 2 years old. Atopic eczema was diagnosed by a paediatric allergist, and children with 'recurrent wheezing' were only included in the atopic group if they had other evidence of allergic disease (i.e. atopic eczema, positive SPT or RAST). There were 14 normal children and 16 atopic children used for this comparison.

The study was approved by the Princess Margaret Hospital and the King Edward Memorial Hospital Ethics Committees (Perth, Western Australia).

Study design

Mononuclear cells from samples collected at birth and each subsequent visit were cultured with allergen for lymphoproliferation and for Th1 and Th2 cytokine production. These variables were compared in atopic and normal individuals, and for each age.

Methods

For lymphoproliferation, CBMC were cultured in triplicate for 7 days in microwells at 10^6 cells/mL in serum free medium as detailed in [14], either alone or in the presence of house-dust mite allergen extract (10 mg/mL), ovalbumin (100 mg/mL), or tetanus toxoid (0.5 LF units/mL). These concentrations were identified as optimal for *in vitro* T-cell stimulation in preliminary experiments. DNA synthesis was measured as incorporation of ^3H -thymidine at the 7-day time point, after pulse labelling at 6 days. Data were expressed as delta dpm per culture, designating levels of tritium incorporation above background (unstimulated) control cultures. Responses which were > twice backgrounds and > 1000 dpm above backgrounds were considered positive [14].

For cytokine analysis, CBMC were cultured for 24 h in medium alone, or supplemented with optimal stimulating concentrations of house-dust mite allergen (10 mg/mL) or PHA (10 mg/mL). Supernatants were assayed for the presence of IL-10 and IL-13 employing commercial ELISA kits (CLB Laboratories, Amsterdam). For cytokines produced at lower levels (IL-4, IL-5, IL-9 and IFN- γ) together with β -actin, specific mRNA was measured in cell pellets by standardized semiquantitative RT-PCR as described in [15]. 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 IL-4, IL-5, IL-9, IFN- γ and β -actin plus TthPlus DNA polymerase, and were performed in a programmable thermocycler. Individual cycle analyses were performed with each primer set, and cycle numbers chosen that were in the linear phase of the respective amplification reactions. Verification of the expected size of the PCR products was obtained by analysis in 1.5% agarose gels. 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. The intensity of each test sample was expressed as a ratio relative to that of its respective β -actin control.

Analysis

Non-parametric (Mann-Whitney *U*) tests were employed for comparisons of all variables (cytokine protein, mRNA expression, lymphoproliferation and antibody levels) as none of these is normally distributed. The Statistical Package for Social Sciences, and Instat computer programs were used.

Results

Lymphoproliferative responses

As previously shown, newborns show positive proliferative responses to ubiquitous allergens to which mothers have been exposed during pregnancy [13]. As a group, 40% of all neonates responded to house-dust mite extract, and 33% to the food allergen ovalbumin. By comparison only one baby showed any response to tetanus toxoid. There was no difference in the frequency of positive responses in HR and LR samples at birth. However, the median, mean and range of responses to all allergens were higher in the HR group (Fig. 1), although these differences were not statistically significant using nonparametric tests.

Over the age-range tested there was a only a slight increase in the frequency of house-dust mite responses that were already established at birth (data not shown). There were no significant differences in the frequency or magnitude of proliferative responses in normal and atopic infants to any of these stimuli. Responses to ovalbumin are under investigation in a separate study.

Cytokine responses

The neonatal responses of both atopic and normal subjects to house-dust mite allergen is dominated by Th2 cytokines, with little detectable IFN- γ as either protein or mRNA (Fig. 2). However, normals tended to show stronger neonatal Th2 cytokine mRNA expression

compared to atopics, including IL4 mRNA (Fig. 2) IL5 and IL9 (not shown). This pattern was significant for neonatal IL-10 and IL-13 house-dust mite responses which were detectable as protein by ELISA (Fig. 3).

Normal infants show a significant age-dependent decline in Th2 responses to house-dust mite, within the first years of life (shown as IL-13 protein responses in Fig. 4a). In contrast, the atopic infants displayed rapid up-regulation of IL-13 responses over the same period. This trend is also apparent for other Th2 cytokines: IL-5 and IL-9 levels increase with age, but only in atopics (not shown). In parallel with IL-13, normal children also show declining IL-4 levels with age ($P = 0.03$; not shown). IL-10 responses to house-dust mite increase over the first 2 years of life in both normals and atopics, with no differences between these groups (not shown).

At birth IFN- γ responses to house-dust mite were below detection in all atopics, and only two normal babies had detectable responses above background. Mitogen (PHA) was required to generate detectable levels of this Th1 cytokine. We were able to reproduce the observations of other groups [7–11] that neonatal IFN- γ responses in atopics were significantly lower than in normals (Fig. 5).

During the study period only atopic babies showed a showed a significant age-related increase in IFN- γ responses to house-dust mite (Fig. 4b). By 2 years of age the normal children still have quite low levels of

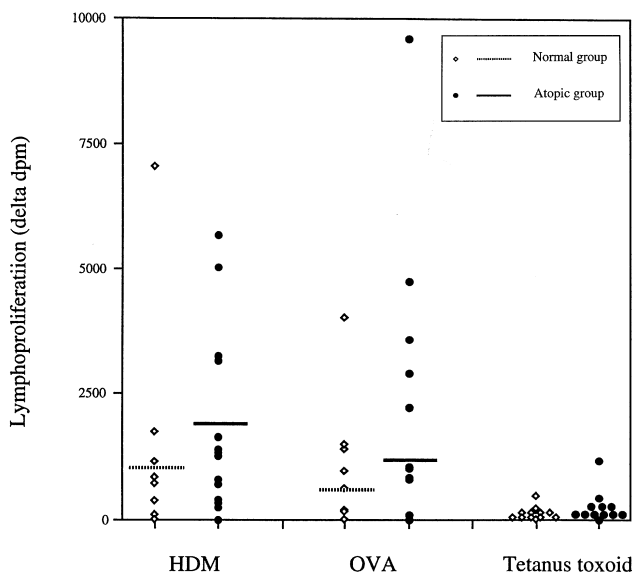


Fig. 1. This shows the lymphoproliferative responses of cord blood MC of individual normal (open squares) and atopic newborns (closed circles) cultured with house-dust mite, ova, and tetanus toxoid for 7 days. Group means are also shown. The levels shown are those above unstimulated background cultures.

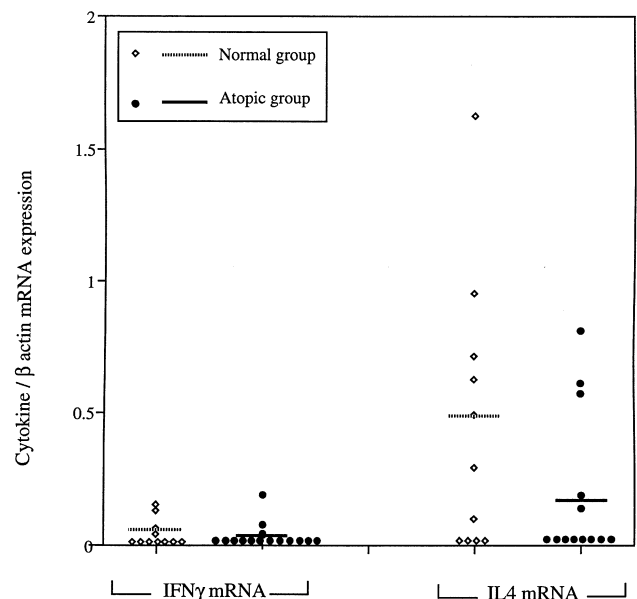


Fig. 2. IL-4 and IFN- γ mRNA responses (as a ratio of bactin mRNA expression) by individual cord blood MC samples cultured with house-dust mite for 24 h are shown. The levels shown are those above unstimulated background controls levels. Mean values for each group are also shown (the normal group as open squares and the atopic group as closed circles).

Fig. 3. This shows neonatal IL-13 and IL-10 protein responses to house-dust mite after 24 h (measured by ELISA). Levels above background unstimulated controls are shown for individual normal babies (open squares) and those who developed atopy by 2 years (closed circles). Group means are also shown.

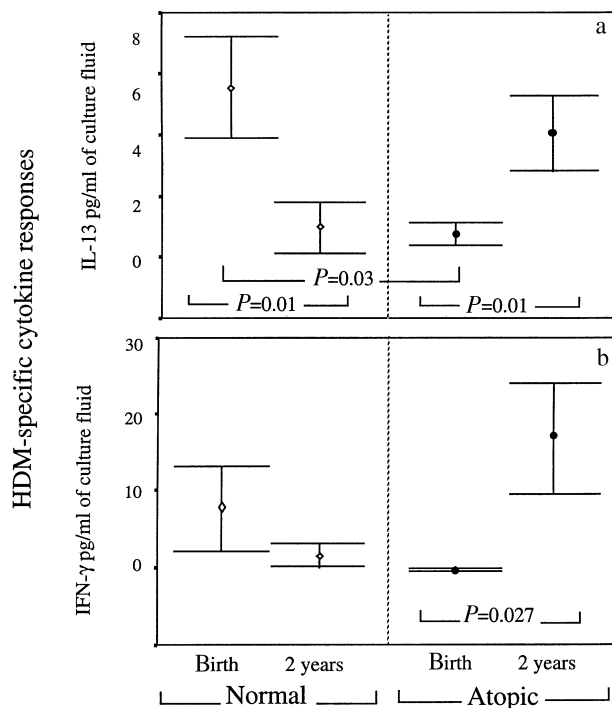
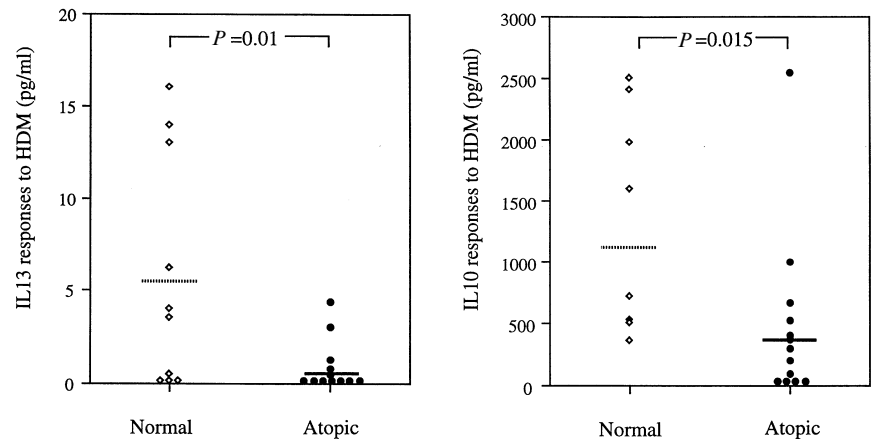


Fig. 4. This compares IL-13 (4a) and IFN- γ (4b) responses to house-dust mite with age (birth and 2 years) for atopic and normal babies (levels in 24 h culture supernatants measured by ELISA). Group means and standard errors are shown.

house-dust mite specific IFN- γ (not significantly different from birth). In contrast, the significant increase in house-dust mite specific IFN- γ responses by atopic children occurs in parallel to their increasing Th2 responses, so that as a groups they are expressing a mixed or Th0 phenotype.

Discussion

In contrast to long-held assumptions, the neonatal cellular immune system is not truly naive with respect

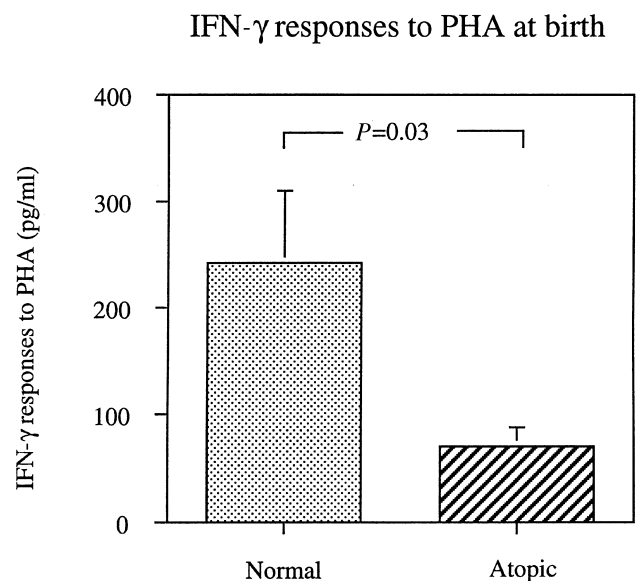


Fig. 5. This compares the cord blood mononuclear cell IFN- γ responses after 24 h cultures with PHA for babies who are normal (specked bar) and atopic (diagonal stripes) at 2 years of age. Group means and standard errors are shown.

to common environmental allergens. Recent work from our group has provided definitive proof of allergen priming *in utero*, and the presence of house-dust mite specific fetal T cells [13]. The present study clearly demonstrates Th2-skewed cytokine production in response to allergen in both normal newborns and those who go on to develop atopic disease by 2 years of age.

Pregnancy itself has been referred to as a 'Th2 state' by Wegmann and colleagues [15]. The healthy placenta produces large amounts of Th2 cytokines which protect the allogeneic fetoplacental unit from the toxic effects of IFN- γ and maternal Th1 cell-mediated immunity. These immunosuppressive effects may also protect the fetus or neonate from fulminant immune responses when they first encounter antigens, which may also be an

important adaptive factor [18,19]. At birth, and clearly for a variable period in postnatal life, there appears to be a persistence of this Th2 skew.

Lymphoproliferative responses to allergens are present in both atopic and normal babies at birth, suggesting that *in utero* allergen priming is a normal rather than pathological process, and is not restricted to babies at high risk of allergy. This may be a normal process by which the fetus is introduced to ubiquitous innocuous proteins in the future environment, in a controlled fashion. If this proves to be the case, the simple allergen avoidance strategies to prevent sensitization may be ineffective. However, the atopic babies in this study tended to have larger responses than the non-atopics (although the differences were not significant in this study), and although similar observations have been made by other groups, the significance of this is not yet clear.

More apparent are differences in the cytokine responses of these allergen reactive mononuclear cells. Although defective cord blood Th1 responses to mitogen in atopics have been previously described [7–11], we show here that this immune defect also includes reduced Th2 responses to allergen. With the exception of Warner *et al.* [8] who showed that children who with eczema at 1 year had reduced IFN- γ responses to food allergens (β LG) at birth, these previous studies only examined the IFN- γ responses induced by polyclonal mitogenic stimulation. The present study focused on allergen specific cytokine responses, and our data suggest that IFN- γ responses airborne allergens (house-dust mite) may also be less marked in atopics (Fig. 4b). In contrast to the normal group, none of the atopic group showed any detectable IFN- γ (protein) in response to house-dust mite at birth, although this needs to be confirmed in a larger group.

The lower levels of mitogen-induced IFN- γ in atopics has lead to speculation that the neonatal Th2 skew may be more marked in atopics, and this may influence the development of later Th2 responses to allergen introduced during this period. A recent small study [18] suggested that babies with atopic parents ($n = 7$) have enhanced ability to generate IL-4 and IL-5 producing house-dust mite-specific T-cell clones, compared to low risk infants ($n = 2$). The current more detailed longitudinal study suggests that aspects of the Th2 component of the response to house-dust mite are also poorly developed in atopic babies, and that the previously observed deficiency in IFN- γ production is thus part of a generalized maturational defect in adaptive immune function. They cannot be simply explained by inhibitory effects of IL-10 which is actually lower in this group (Fig. 3).

It is of interest that these findings contrast the observation of Liao *et al.* [10] that IL-6 responses (to mitogen) are more marked in newborns at risk of atopy. However, they are consistent with earlier studies from our group on mitogen-induced T-cell clones from infants and young children, which demonstrated defective production of both IFN- γ and IL-4 by clones from subjects at risk of atopy [5]. It should be emphasized, however, that the present study measures allergen rather than mitogen-induced IL-6 responses, which were lower in newborns who go onto develop atopy, compared to normals (Fig. 3). Clearly further studies are required to determine the mechanisms of these differences.

Of even greater interest is how the patterns of Th2 cytokine responses of atopics and normals contrast with age, even within the first year of life. IL-13 was used for comparison because it has similar effects to IL-4 in relation to IgE production but is more readily measurable as protein. Normal babies demonstrate a progressive decline in the house-dust mite-specific IL-13 responses with age, while they are continuing to show positive lymphoproliferation, as observed in normal adults. This suggests that maturation of the house-dust mite response occurs during early infancy with progressive dampening of the Th2 responses with age. This appears to occur well before the IFN- γ responses are fully developed, as these are still quite weak at 2 years of age in normal babies. Atopic babies show the opposite pattern with age, with instead a consolidation of their (weak) neonatal Th2 responses. The atopic children in this study show stronger Th1 and Th2 responses against house-dust mite between birth and 2 years of age, during the same period when normal children are showing general suppressed (or possibly tightly regulated) cytokine responses. This suggests that atopic children have defective immune modulation during this crucial period of repeated exposure to new allergens. The stronger IFN- γ responses seen in atopics during this period do not seem to be strong enough to overcome the Th2 responses, which have been shown to dominate by 5 years of age in house-dust mite sensitized (SPT positive) individuals [19].

It would appear that in *all* individuals, T-cell priming to inhalants occurs *in utero*, when there is an initial Th2 skew. This appears to be 'corrected' postnatally by allergen-driven immune deviation towards Th1 selection, in normal individuals. For reasons presently unknown this process fails in atopics with further consolidation of Th2 memory. The mechanisms are still unclear. A number of other potential mechanisms, such as the role of childhood infection (and other Th1 stimuli), and potential defects in APC maturation, are currently being explored in concurrent studies.

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