

High IFN- γ production by CD8⁺ T cells and early sensitization among infants at high risk of atopy

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Background: High genetic risk (HR) of atopy among unstratified populations of infants is associated with attenuated IFN- γ responses. However, the role of IFN- γ in progression from HR status to active disease is less clear.

Objective: To identify immune function markers in neonates with HR that are associated with positive atopic outcomes at 2 years.

Methods: Cord blood mononuclear cells (CBMCs) were collected from 175 children with HR and cryopreserved. The children were assessed for atopy by skin prick at 0.5 and 2 years. CBMCs were thawed and stimulated with allergens and mitogens PHA and staphylococcal enterotoxin B (SEB), and cytokine responses were determined.

Results: No correlations were observed between allergen-specific CBMC responses and atopic outcomes. In contrast, sensitization was strongly associated with polyclonal IFN- γ responses to both PHA ($P = .002$) and SEB ($P = .005$), and also with SEB-induced IL-5 ($P = .05$), IL-10 ($P = .02$), and IL-13 ($P = .01$). Logistic regression analysis identified elevated PHA-induced IFN- γ and SEB-induced IL-13 responses as the strongest independent predictors of atopy development. Cell separation studies confirmed CD8⁺ T cells as the source of ~90% of IFN- γ production.

Conclusions: IFN- γ produced by CD8⁺ T cells may synergize with T_H2 cytokines in driving atopy development in children with HR. (*J Allergy Clin Immunol* 2004;113:710-6.)

Key words: Atopy, infants, cytokines, CD8⁺ T cells

Our laboratory was the first to identify the inverse relationship between T_H1 functional capacity (as defined by IFN- γ production) in infancy and genetic risk for atopic disease as defined by atopic family history (AFH).¹ Subsequent studies from a range of laboratories have confirmed and extended these observations, including extension of this putative association to include expression of allergic symptoms.²⁻¹⁰

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Abbreviations used

AD: Atopic dermatitis
AFH: Atopic family history
CBMC: Cord blood mononuclear cell
HDM: House dust mite
HR: High genetic risk
SEB: Staphylococcal enterotoxin B
SPT: Skin prick test
TRF: Time-resolved fluorometry

Despite the consistency of these observations across a variety of cross-sectional and longitudinal studies, the precise role of this IFN- γ deficiency in disease pathogenesis in children with high genetic risk (HR) remains to be determined. In particular, it is unclear whether it is primary or secondary in the sensitization process, and whether it is a surrogate marker for other genetic deficiencies that ultimately determine risk for sensitization.

The inherent complexity of this question is further illustrated by the results of a recent prospective study from our group¹¹ suggesting that this T_H1 deficiency may be transient and rebounds by the end of infancy, such that by 18 months of age, T_H1 immune function in AFH⁺ children is equivalent to or greater than that in AFH⁻ children. This finding is consistent with other reports suggesting that older children who eventually develop atopy manifest higher levels of IFN- γ production than their nonatopic counterparts.^{12,13}

In the current study, we have sought to elucidate this complex issue by focusing specifically on the relationship between cytokine (in particular, IFN- γ) production in cord blood mononuclear cells (CBMCs) from AFH⁺ infants and subsequent development of atopy. We reasoned that narrowing the focus of the study to the subpopulation in whom sensitization is most frequent may unmask associations that are obscured within the population at large. We report that despite the inverse relationship between IFN- γ response capacity and susceptibility to atopy within the overall population, the early development of allergic sensitization among the low-IFN- γ -producing HR group is maximal among subjects at the top end of the IFN- γ response spectrum within this subpopulation. This finding suggests a potentially dualistic role for IFN- γ in the pathogenesis of atopy.

METHODS

Subjects

Cord blood was collected from 175 children at birth. All children were enrolled antenatally and were classified as having a positive AFH based on a standard questionnaire⁶ and a positive doctor's diagnosis of asthma, hay fever, or atopic dermatitis (AD) for 1 or both parents (68%, 86%, and 37% of the parents in the study had a doctor's diagnosis of asthma, hay fever, or AD, respectively). The children were assessed by 1 of the authors at 6 months and 2 years for the presence of AD using the criteria of Hanifin and Rajka,¹⁴ and for skin prick test (SPT) reactivity to a standard panel of allergens (Hollister Stier Labs, Spokane, Wash), which included house dust mite (*Dermatophagoides pteronyssinus*; HDM), perennial rye grass, cat pelt, *Alternaria tenuis*, *Aspergillus fumigatus*, fresh cow's milk, and fresh hen's egg. Histamine and distilled water were included as positive and negative controls, respectively. An SPT was regarded as positive if the wheal diameter was ≥ 2 mm.

Cell preparation and culture

As previously described,¹⁵ cord blood was collected into an equal volume of RPMI-1640 (Cytosystems, Castle Hill, Australia) containing preservative-free heparin (20 U/mL). CBMCs were isolated by density gradient centrifugation and cryopreserved in liquid nitrogen as previously described¹⁶ for later batch analysis.

CBMCs were thawed and resuspended in AIM-V serum-free medium (Gibco Life Technologies, New York) supplemented with 2-mercaptoethanol (final concentration, 4×10^{-5} M; Sigma, Castle Hill, Australia). For cultures with HDM and ovalbumin, cells were resuspended at 2×10^6 viable cells/mL, whereas for cultures with PHA and staphylococcal enterotoxin B (SEB), cells were resuspended at 1×10^6 viable cells/mL. Cell aliquots of 0.5 to 1.0 mL were cultured for 48 hours at 37°C with 5% carbon dioxide with medium alone or medium containing HDM (10 μ g/mL, aqueous extract of lyophilized *Dermatophagoides pteronyssinus*; CSL, Parkville, Australia), ovalbumin (100 μ g/mL; Sigma), PHA (1 μ g/mL; Murex Biotech, Kent, United Kingdom), and SEB (200 ng/mL; Sigma). After culture, supernatants were collected and stored at -20°C for cytokine analysis by ELISA or time-resolved fluorometry (TRF).

Detection of cytokine protein

The level of IL-10, IL-13, and IFN- γ in culture supernatants was measured by in-house TRF, whereas IL-5 was measured by in-house ELISA. The PharMingen antibody pairs used for capture and detection were as follows: rat IgG₁ antihuman IL-10 (clone JES3-9D7; PharMingen, San Jose, Calif) and biotinylated rat IgG_{2a} antihuman IL-10 (clone JES3-12G8), rat IgG₁ antihuman IL-13 (clone JES10-5A2) and biotinylated mouse IgG₁ antihuman IL-13 (clone B69-2), mouse IgG₁ antihuman IFN- γ (clone NIB42) and biotinylated mouse IgG₁ antihuman IFN- γ (clone 4SB3), and rat IgG₁ antihuman IL-5 (clone TRFK5) and biotinylated rat IgG_{2a} anti-IL-5 monoclonal antibody (clone JES1-5A10). Standard curves were generated by using serial dilutions of recombinant IL-10, IL-13, IFN- γ , and IL-5 (PharMingen). For detection for TRF, Europium-labelled streptavidin (Delfia Wallac, Turku, Finland) was added, followed by enhancement solution (Delfia Wallac), whereas for ELISA, streptavidin-horseradish peroxidase conjugate (Amersham, Buckinghamshire, United Kingdom) was added, followed by K-blue tetra-methylbenzidine substrate (ELISA systems). The limits of detection for these assays were as follows: 3 pg/mL for IL-10, 3 pg/mL for IL-13, 10 pg/mL for IFN- γ , and 6 pg/mL for IL-5.

TABLE I. Atopic and clinical outcomes at 6 months and 2 years*

Outcome	6 mo		2 y	
	Negative	Positive	Negative	Positive
SPT reactivity				
HDM	168 (96)	7 (4)	137 (78)	38 (22)
<i>Alternaria</i>	172 (98)	3 (2)	171 (98)	4 (2)
Rye grass	174 (99)	1 (1)	154 (88)	21 (12)
Cat	167 (95)	8 (5)	148 (85)	27 (15)
<i>Aspergillus</i>	172 (98)	3 (2)	163 (93)	12 (7)
Cow's milk	158 (90)	17 (10)	152 (87)	23 (13)
Egg white	148 (85)	27 (15)	149 (85)	26 (15)
Any positive SPT	125 (71)	50 (29)	96 (55)	79 (45)
Any SPT to aeroallergen	158 (90)	17 (10)	112 (64)	63 (36)
Any SPT to food allergen	139 (79)	36 (21)	136 (78)	39 (22)
Symptomatology				
AD	96 (55)	79 (45)	65 (37)	110 (63)
Wheeze	164 (94)	11 (6)	139 (79)	36 (21)
Doctor-diagnosed asthma	173 (99)	2 (1)	152 (87)	23 (13)

*A total of 175 children from whom cord blood samples were obtained were skin prick tested to a range of allergens, as shown in Table I. Data shown represent frequency (with percentage in parentheses) of positive responders. Symptoms are also shown for AD, wheeze, and asthma diagnosed by personal physician any time to 2 years of age (percentage in parentheses).

Statistical analysis

Mann-Whitney *U* test for unpaired responses was used for initial univariate analyses of differences in CBMC cytokine responses between groups with positive versus negative atopic outcomes, using SPT as a binary variable. Logistic regression was used to examine relationships between atopic outcomes (as a binary variable) and cytokine responses to the mitogens PHA and SEB, adjusting for the potential confounding effects of responses to the allergens. The statistical package SPSS (SPSS Inc, Chicago, Ill) was used for all analyses.

RESULTS

Clinical assessments

The results of SPT and clinical examinations of the cohort at 6 months and 2 years are shown in Table I and Fig 1. Among these children with HR, the frequency of sensitization to ≥ 1 allergen was 29% at 6 months and increased to 45% by 2 years. Sensitization to dietary allergens dominated at 6 months and was supplanted by inhalant allergens at 2 years, consistent with findings in earlier studies. AD was common in the cohort at 6 months and increased further in frequency by 2 years. A diagnosis of asthma was made in 13% of the children by 2 years.

Allergen-specific cytokine responses

Table II illustrates CBMC cytokine responses to the archetypal inhalant allergen HDM and a corresponding

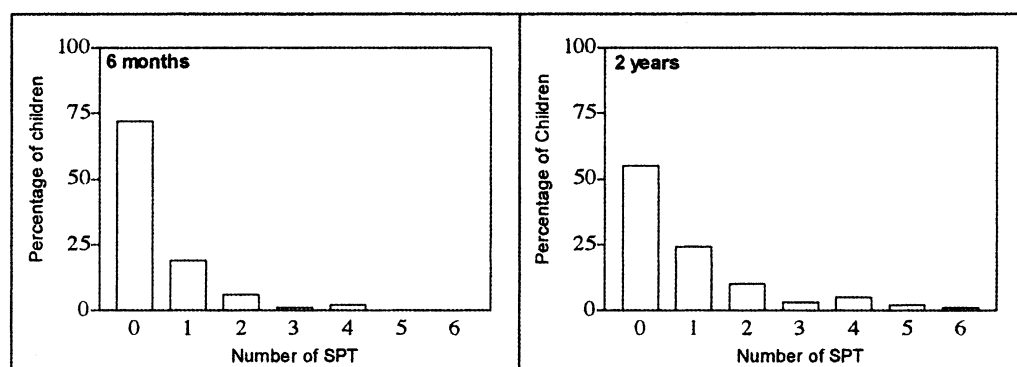


FIG 1. SPT frequencies at 6 months and 2 years. Subjects were skin prick tested to the allergens indicated in Table I. Data are the percentage of children responding to 1 or more allergens at 6 months and 2 years.

TABLE II. Allergen-induced cytokine production by CBMC*

SPT	In vitro stimulus	Cytokine	6 mo	
			Negative	Positive
HDM	HDM	IL-5	0 (0-338) [21]	0 (0-38) [14]
		IL-10	4.6 (0-213) [48]	2.8 (0-28) [43]
		IL-13	4.9 (0-633) [47]	8.6 (0-123) [57]
		IFN- γ	0 (0-2479) [33]	5.2 (0-108) [43]
OVA	OVA	IL-5	0 (0-321) [45]	6.9 (0-474) [56]
		IL-10	32.4 (0-360) [77]	41.8 (0-287) [93]
		IL-13	20.3 (0-328) [76]	22.3 (0-342) [81]
		IFN- γ	18.3 (0-8778) [62]	28.7 (0-2646) [63]
SPT	In vitro stimulus	Cytokine	2 y	
			Negative	Positive
HDM	HDM	IL-5	0 (0-338) [23]	0 (0-255) [19]
		IL-10	4.4 (0-213) [47]	5.6 (0-126) [51]
		IL-13	4.6 (0-633) [47]	4.9 (0-279) [49]
		IFN- γ	0 (0-355) [32]	0 (0-2479) [35]
OVA	OVA	IL-5	0 (0-321) [44]	7.8 (0-474) [57]
		IL-10	36.9 (0-360) [78]	31.7 (0-287) [85]
		IL-13	18.4 (0-328) [74]	25 (0-342) [85]
		IFN- γ	17.1 (0-8778) [60]	36.9 (0-2646) [73]

OVA, Ovalbumin.

*CBMCs were cultured alone or together with HDM or OVA and supernatant cytokine levels determined. The data were then grouped on the basis of SPT status to HDM or OVA at age 6 months or 2 years. Data are expressed as medians (ranges) and [% positive responders]. The significance of the differences between those with a positive or negative SPT at age 6 months or 2 years was determined using a Mann-Whitney *U* test, and none was below the .05 cutoff.

food allergen, ovalbumin, in children with HR respectively negative or positive to these allergens at 6 months or 2 years. Positive cytokine responses were frequent but highly variable in magnitude, and no positive associations (qualitative or quantitative) between allergen-specific CBMC responses and subsequent SPT reactivity to the corresponding allergen, or to clinical symptoms, were detected.

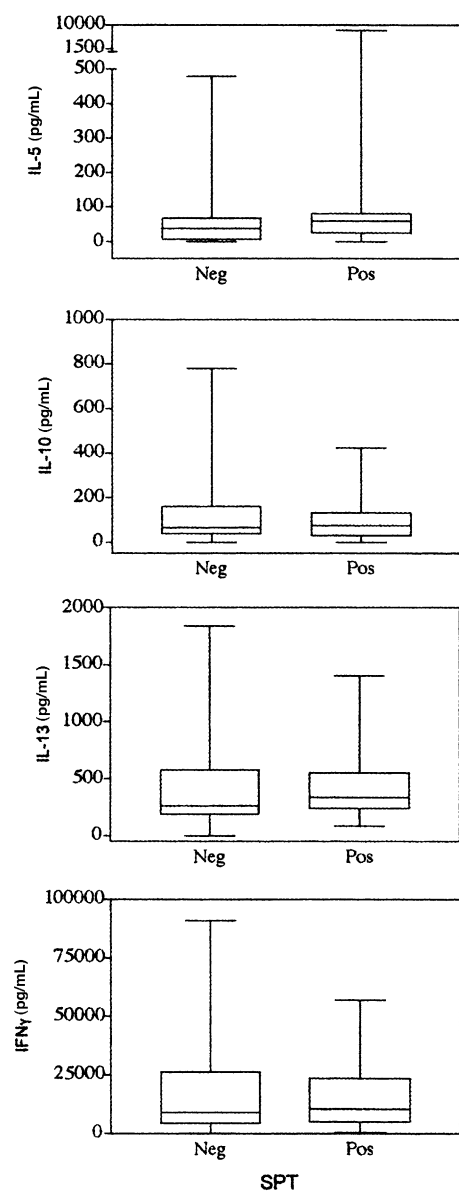
Polyclonal cytokine responses

In addition to stimulation of CBMC with allergen, responses to the polyclonal mitogen PHA and the bacterial superantigen SEB were examined. Fig 2 illustrates findings with SEB; it can be seen that positive atopic outcomes

at 2 years are consistently associated with high-level responsiveness to this agent. The strongest association was seen for IFN- γ production ($P = .005$), and significant associations were also observed for IL-5, IL-10, and IL-13. We also observed a weak association between IL-5 responses to SEB and expression of AD at 2 years ($P = .076$; data not shown) which is consistent with our findings from an earlier study.¹⁵

Fig 3 illustrates parallel findings for the mitogen PHA. In this case, the association was restricted to IFN- γ , production of which in CBMC was strongly correlated with positive atopic outcome ($P = .002$). Further analyses of the associations between SEB and PHA-induced cytokine responses and atopy development were performed by using a logistic regression model, utilizing

SPT at 6 months



SPT at 2 years

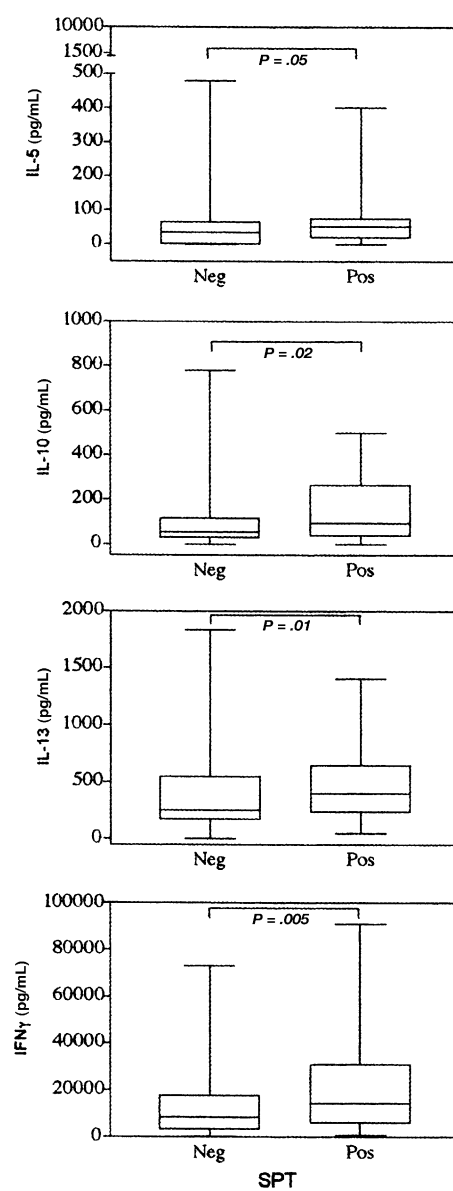


FIG 2. SEB-specific cytokine production by CBMC. SEB-induced cytokine responses were determined, and resulting data were grouped on the basis of SPT outcome at 6 months and 2 years. The data are expressed as box plots. The limits of the boxes represent the 25th and 75th percentiles of the results. The *closed line* represents the median (50th percentile), whereas the *whiskers* represent the 5th and 95th percentiles. Significant differences between those with and without positive SPT outcomes were determined by Mann-Whitney *U* test, and resulting *P* values are shown.

log-transformed data, and including responses to the allergens and parental history of atopy or allergic disease as potential confounding factors. These analyses identified PHA-induced IFN- γ production as the strongest independent predictor of atopy (odds ratio, 2.11 [1.23-3.61]; $P = .007$), in conjunction with SEB-induced IL-13 (odds ratio, 3.03 [1.11-8.30]; $P = .031$). None of the cytokine responses was predictive of symptomatology in the study group, nor was parental history of atopy or symptoms.

In a previous report,¹⁷ we immunomagnetically separated CD4⁺ and CD8⁺ T cells from CBMC before stimulation with the accessory cell-independent mitogens phorbol 12-myristate 13-acetate and Ionomycin, and these experiments suggested that the bulk of IFN- γ production by CBMC is attributable to the CD8⁺ population. We confirmed this in the current study by using the same methodology, with 5 randomly selected CBMC samples. IFN- γ production levels in 48-hour culture supernatants

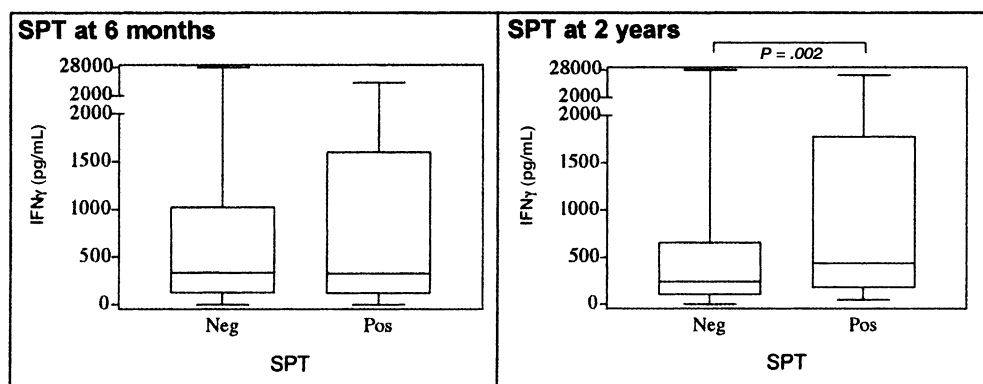


FIG 3. PHA-specific IFN- γ production by CBMC. CBMCs were cultured with PHA and supernatant cytokine content determined. The data are expressed as per Fig 2. Results are shown only for IFN- γ ; other cytokine responses (IL-5, IL-10, and IL-13) did not differ between groups.

from 10^6 CD4⁺ T cells were 2523 ± 937 pg/mL (mean $\pm$ SE) versus 16464 ± 4406 pg/mL for 10^6 CD8⁺ cells; CD8⁺ > CD4⁺, $P < .04$ by Wilcoxon signed-rank test.

DISCUSSION

Earlier findings from a range of independent laboratories indicate that attenuated IFN- γ response capacity during the early postnatal period is a consistent hallmark of children at high risk for subsequent development of allergic sensitization.¹⁻¹⁰ The degree of attenuation in children with HR varies markedly as a function of how IFN- γ production is assessed, ranging from an approximately 2-fold reduction in PBMC cultures^{6,18} to as much as 9-fold to 10-fold in purified CD4⁺ T cells.⁶ The mechanism underlying the association between initial low IFN- γ response capacity in infancy and subsequent allergic sensitization is incompletely understood. However, it is evident from several studies that this life phase represents the period during which allergen-specific T_H memory is most commonly developed, and further, that the cytokine profile in these memory responses in children with HR frequently becomes T_H2-polarized.^{6,19,20} Studies in murine systems have suggested a range of mechanisms by which IFN- γ may directly or indirectly influence T-helper cell differentiation during the early phase of T-cell memory development. In particular, it appears that in the absence of appropriate levels of T_H1-promoting signals, T-cell memory responses preferentially default to the T_H2 pathway, driven in part by spontaneous low-level IL-4 secretion from naive T-helper cells, and IFN- γ has been proposed to play an important role in countermanding this default via both IL-12-dependent and IL-12-independent mechanisms.²¹⁻²⁴

However, once T_H memory is fully developed, the available evidence suggests that cross-regulation either does not occur or is markedly less efficient than during the early phase of the response, because IFN- γ -producing

allergen-specific T_H cells are found in atopic patients concomitantly with T_H2-secreting cells of the same specificity.^{16,25,26} Ongoing studies in our laboratory suggest that T_H1 and T_H2 cytokines are generally not secreted by the same individual cells in atopic patients (Wikström et al, unpublished data), but it is not known whether the relevant cells are from the same clones and manifesting different activation and differentiation states, or alternatively, whether they are from different clones with the same antigen specificity.

Our current results appear at first glance counterintuitive relative to earlier published data (including our own) that have identified low IFN- γ response capacity in neonates as a hallmark of high risk for atopic disease. However, our previous studies in this regard^{1,6} have used unselected study populations containing both AFH⁻ and AFH⁺ subjects from the opposite ends of the atopy risk spectrum. In contrast, this study has deliberately focused on children with HR, and our results suggest that among these low IFN- γ producers, the children whose IFN- γ production levels are at the high end of the response range for this subgroup are most likely to develop sensitization. It is noteworthy that expression of atopy at age 2 years in this cohort was also associated with elevated T_H2 cytokine responses to SEB, in particular IL-13. We^{15,27} and others^{28,29} have previously reported positive associations between cytokine responses to this broad spectrum cellular stimulant and atopy expression; whether this points to an underlying role for SEB produced by epidermal or mucosal flora in driving allergy development, or alternatively reflects its role as a polyclonal mitogen (similar to PHA), remains to be established.

Recent results from other studies in our lab and elsewhere provide a possible explanation for these apparently anomalous findings relating to IFN- γ . In particular, we have recently reported a prospective analysis of vaccine-specific T_H1 and T_H2 responses in an unselected cohort of 130 children. Stratification of tetanus toxoid-specific T_H1/T_H2 responses in these children by atopic family history revealed that responses in AFH⁺ infants were markedly

T_H2-skewed relative to AFH⁺ children at 6 months and 12 months of age.¹¹ However, this T_H1 deficiency was transient, and rebounded by the time of the 18-month bleed, at which the T_H1:T_H2 ratio in vaccine-specific cytokine responses in the AFH⁺ children now exceeded that of their AFH⁺ counterparts.¹¹ We have reported findings consistent with this observation in an earlier study comparing vaccine-specific (and allergen-specific) responses in atopic versus nonatopic 6-year-olds, and T_H1 responses to both these stimuli were significantly higher among the atopic patients.¹² Moreover, a recent report on 12-year-olds revealed a comparable pattern of hyperexpression of T_H1 immunity to HDM, Rye allergen, and SEB among young atopic patients.¹³

Collectively, these findings suggest that transient developmentally related hyporesponsiveness in the T_H1 compartment of the adaptive immune system during infancy, followed by a significant rebound resulting in high IFN- γ production in later childhood, may be an integral part of the immunophenotype of many atopic patients. It is possible that higher (relative to group) IFN- γ production among neonates with HR marks those most likely to manifest this rebound phenomenon; this issue will be addressed in follow-up studies. It is not known whether this state of heightened T_H1 responsiveness is generally short-lived or long-lived during allergy development, but reports (eg,^{25,26}) indicating that allergen-specific responses in a significant number of adult atopic patients commonly contain major T_H1 components and suggest that the state may be persistent in at least a subset of subjects.

It is pertinent to note that IFN- γ is known to be potentially highly toxic if produced at high levels in tissues.³⁰ In the context of allergic diseases, this cytokine has been suggested to play a central role in the inflammatory process at lesional sites in AD³¹ and in airway inflammation in animal models of asthma.^{32,33} Moreover, asthma severity in both children³⁴ and adults³⁵ has been linked with excessive IFN- γ production, in particular by CD8⁺ T cells,^{34,35} and bronchoalveolar lavage studies suggest preferential recruitment of CD8⁺ T cells (relative to CD4⁺ T cells) into the airways during acute asthma exacerbations.³⁶ A recent study using class I MHC tetramer technology has also implicated CD8⁺ T cells responsive to Der p 1 allergen as determinants of the severity of allergic disease in HDM-sensitized adults.³⁷ In a previous study, we demonstrated that although expression of the IFN- γ gene is developmentally constrained in neonatal CD4⁺ T cells via hypermethylation of CpG dinucleotide sites in the proximal promoter, this control mechanism does not appear to operate to the same degree in neonatal CD8⁺ T cells, which are capable of production of IFN- γ at levels close to the adult normal range.¹⁷ We have confirmed this observation, demonstrating that in the order of 90% of the polyclonal IFN- γ response of CBMC is attributable to CD8⁺ cells, which suggests that the association defined here between atopy development in children with HR and elevated IFN- γ production is related principally to variations in CD8⁺ as opposed to CD4⁺ T-cell function.

In summary, our data suggest that IFN- γ may play a dual role in the pathogenesis of atopic disease. On the one hand, as demonstrated in earlier studies, it may antagonize the disease process by limiting T_H2 differentiation during the early stage of priming of allergen-specific T_H memory cells. However, once sensitization is achieved, coexpression of T_H1 immunity at lesional sites may directly or indirectly amplify T_H2-driven tissue inflammation and thus contribute to the persistence and severity of the disease. It is pertinent to note in this context that despite the high frequency of sensitization to inhalant allergens in the community, the expression of severe allergic diseases such as atopic asthma remains comparatively rare.³⁸ This infers that ancillary pathogenic mechanisms additional to those governed by T_H2 cytokines may ultimately determine the nature and degree of symptomatology in atopic patients. Taken together with earlier findings cited here, our current findings suggest that IFN- γ production, particularly by CD8⁺ T cells, is a potential candidate for such an ancillary role.

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