

Letter to the Editor

In vivo maturation of T_H cells in relation to atopy

To the Editor:

The immune system at birth has a default setting toward T_H2-type responses. In children with allergic diseases, the T_H2 predominance persists.¹ Although the role of regulatory T cells² in the development of peripheral tolerance and the prevention of allergic sensitization has become more clear, the relation of the more recently identified T_H9, T_H17, and T follicular helper cell subsets² to atopy is not unraveled.

We studied CD4 T_H cell maturation in a Belgian birth cohort (Follow-up of Newborn's Immune Development in Relation to Allergic Outcome cohort, n = 107). We measured a series of representative cytokines (IL-4, IFN- γ , IL-9, IL-17A, and IL-21) in culture supernatants of anti-CD3/CD80-stimulated cord blood mononuclear cells (CBMCs) and PBMCs obtained at the age of 12 and 24 months (see the [Methods](#) and [Results](#) sections in this article's Online Repository at www.jacionline.org). Allergic sensitization and symptoms were assessed at 12 and 24 months and 6 years of age (n = 84, see the [Methods](#) section in this article's Online Repository). Those children who were already allergic before or at the age of 24 months were labeled as "early allergic" (n = 17). Those who had allergy between the age of 2 and 6 years were labeled as "late allergic" (n = 10; see [Tables E1, E2, and E3](#) in this article's Online Repository at www.jacionline.org for patients' characteristics and sensitization profiles).

In accordance with our previous findings,³ IL-4 was detectable in almost all CBMC cultures (see [Table E4](#) in this article's Online Repository at www.jacionline.org). High neonatal IL-4 production (>50 pg/mL) predicted development of early allergy (relative risk [RR], 3.03; 95% CI, 1.1-8.3) but not late allergy (RR, 0.5; [Table I](#)). This is in agreement with the findings of Herberth et al.,⁴ showing an increased risk of atopic dermatitis within the 2 first years of life for children with a high percentage of cord blood IL-4-producing cells.⁴ IL-17A was detectable in only 4

of the CBMC cultures, even though IL-4 bioactivity in the cultures was blocked ([Fig 1](#)). Notably, the 4 children with measurable IL-17A production by CBMCs remained healthy. IL-9 production was completely absent at birth (<5 pg/mL).

IFN- γ (see [Table E4](#)) and, surprisingly, IL-21 ([Fig 1](#)) were measurable in almost all CBMC cultures, but levels were not related to later allergy development. Low cord blood IFN- γ production has been reported by several authors to be associated with allergy development.^{4,5} This discrepancy with our results might be explained by the fact that we measured IFN- γ production by total CBMCs instead of by isolated CD4⁺ T cells. IFN- γ was present intracellularly in 10% (mean of 3 experiments) of lymphocytes and $\pm 0.6\%$ (mean of 3 experiments) of isolated CD4⁺ T cells, indicating that other cell fractions beside CD4⁺ T cells (eg, CD8⁺ T cells) were predominantly responsible for its accumulation in the supernatants. Moreover, IFN- γ production by cord blood CD8⁺ T cells was reported not to be defective in atopic children.^{4,5}

We also confirmed IL-21 production by CD4⁺ CBMCs using intracellular staining of 3 additional cord blood samples. IL-21 production by cord blood CD4⁺ T cells was strongly upregulated in the presence of IL-23 and IL-1 β ($\pm 8.5\%$) compared with 0.35% in its absence (data not shown). Only a minor fraction of IL-21-producing CD4⁺ T cells were also IFN- γ producers (see [Fig E1](#) in this article's Online Repository at www.jacionline.org). The presence of IL-21-producing cells in cord blood was surprising. Whether they represent naive T_H0 cells, circulating T follicular helper-like cells, or cells in the early differentiation pathway toward T_H17 cells requires further investigation.

At the age of 12 and 24 months, we again studied cytokine production in PBMC cultures. IL-4 production increased significantly over time (from birth to age 24 months) in both allergic and nonallergic children (see [Table E4](#)). At age 24 months, IL-4 production correlated with the allergic state at the moment of evaluation, and this confirms earlier observations that the T_H2 predominance persists in atopic children.¹ However, high IL-4

TABLE I. RR assessment for early or late allergy by means of evaluation of CBMC and PBMC cytokine production

	Cutoff value for cytokine production (pg/mL)*	RR	95% CI	P value, Fisher exact test
IL-4				
CBMCs	50	Early: 3.03	1.1-8.3	.03
		Late: 0.5	0.06-3.6	.4
12-mo PBMCs	150	Late: 6.3	0.8-47	.03
24-mo PBMCs	200	Late: 2	0.5-7.2	.2
IL-9				
CBMCs	NA			
12-mo PBMCs	400	Late: 1.9	0.6-9	.2
24-mo PBMCs	600	Late: 4.48	1.01-19.72	.03
IFN-γ				
CBMCs	20,000	Early: 0.7	0.2-2.3	.4
		Late: 0.3	0.04-2.4	.2
12-mo PBMCs	70,000	Late: 0.4	0.08-1.6	.1
24-mo PBMCs	70,000	Late: 0.3	0.04-2.4	.2

*CBMCs (5×10^5) or PBMCs (5×10^5) obtained at 12 and 24 months were stimulated with anti-CD3/CD80 in the absence (IFN- γ or IL-9) or presence (IL-4) of anti-IL-4 receptor mAb. Supernatants were collected after 3 days; cytokine production was measured by means of ELISA. The table represents RRs for early or late allergy (see the [Methods](#) section in this article's Online Repository) and 95% CIs for cytokine production expressed as concentrations in supernatants in picograms per milliliter; the cutoff value was based on the 75th percentile in nonallergic children. Because only 3 children allergic at age 2 years were not allergic at age 1 year, no RR for early allergy was studied at age 1 year.

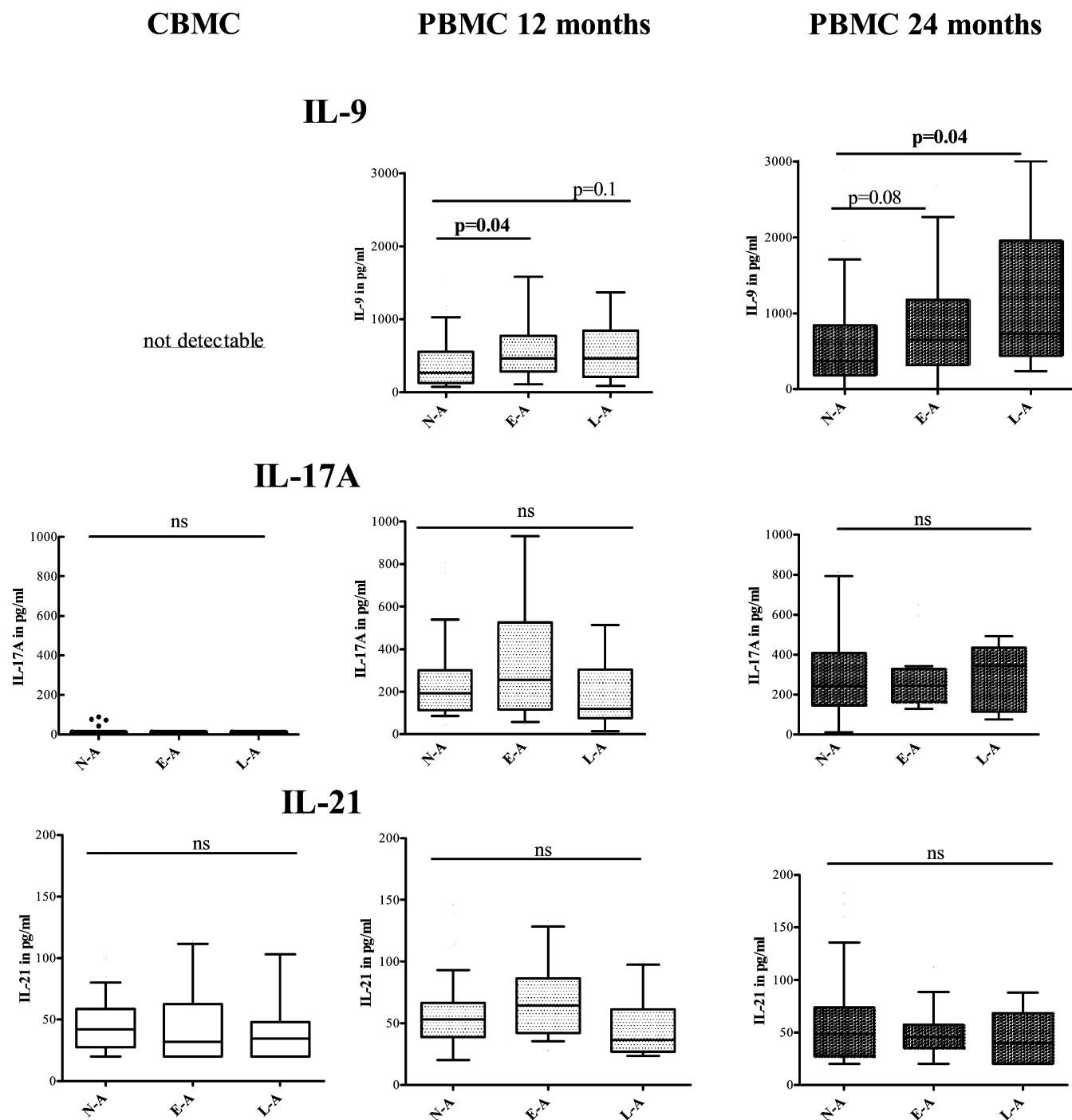


FIG 1. Cytokine production by polyclonally activated CBMCs and PBMCs at the age of 12 and 24 months. CBMCs (5×10^5) obtained within 4 hours after birth or PBMCs (5×10^5) obtained at age 12 and 24 months were cultured with soluble anti-CD3 mAb (UCHT-1, $2 \mu\text{g/mL}$) captured by murine P815/CD80 cells (1:1 ratio) in the absence (IL-9) or presence (IL-17 and IL-21) of the anti-IL-4 receptor mAb ($2.5 \mu\text{g/mL}$). Supernatants were collected after 3 days, and cytokine production was measured by means of ELISA. Box plots represent p10, median, and p90 values, and whiskers extend to minimum and maximum values. Allergic outcome was assessed at 2 and 6 years: E-A, early allergic; L-A, late allergic; N-A, not allergic.

production at age 12 or 24 months (Table I) did not predict late allergy development.

IFN- γ production increased significantly during the first 12 months (see Table E4). Interestingly, in the subpopulation of children with “late allergy” but not in healthy children or “early

allergic” children, IFN- γ production then again decreased significantly during the second year of life ($P = .004$). Decreasing IFN- γ production thus precedes the development of allergy later in life and apparently reflects the predisposition for allergic sensitization.

Importantly, IL-9 production was significantly higher at the age of 12 months, as well as at the age of 24 months, in both children with early and those with late allergy when compared with that seen in children without allergy (Fig 1). Moreover, the RR for late allergy in children with high *in vitro* polyclonal IL-9 production at 24 months is 4.48 (95% CI, 1.01-19.72; $P = .03$, Fisher exact test; Table I). Thus polyclonal IL-9 responses at young age are predictive for allergy development. The difference in the predictive value of IL-4 and IL-9 production supports the concept that IL-4 and IL-9 are produced by separate T-cell lineages.² Earlier observations demonstrated that allergen-induced IL-9 production is associated with asthmatic symptoms in 12-year-old allergic children, also pointing toward the important role for IL-9-producing cells in allergy.⁶ Furthermore, allergen-induced IL-9 production in allergic adults has been shown to be an excellent marker for atopy because its production is virtually absent in nonallergic control subjects and correlates significantly with allergen-specific IgE levels in allergic patients.⁷

IL-17A production could be induced in the PBMC cultures at the age of 12 months. IL-17A production was not different between healthy and allergic children at any age. At the age of 24 months, no further increase in IL-17A production was noted (Fig 1). The appearance of IL-9 and IL-17 production during the first year of life might represent the differentiation of T_H9 and T_H17 cells on immune maturation. Because of its early production at birth, IL-21 might contribute to T_H17 differentiation (as it does *in vitro* if associated with TGF- β) and to T_H9 differentiation (in association with type I interferons).⁸

Especially those children allergic before the age of 2 years might have a high risk for the atopic march. Interestingly, our findings on differences in IL-4, IL-9, and IFN- γ production at birth and at young age between children who will become allergic early and those who will become allergic at a later time point suggest that both groups of children are immunologically different; their sensitization pattern is also different (see Tables E2 and E3). The recent findings by Matricardi et al⁹ indicating that total and specific IgE profiles, as well as clinical characteristics, differ between children sensitized early and those sensitized late also support this hypothesis. Furthermore, recent observations by Simpson et al¹⁰ revealed differences in asthmatic outcomes between infants sensitized early and those sensitized late, pointing to different phenotypes in IgE-mediated allergy.

In conclusion, our results show that differences in T-cell maturation between atopic and nonatopic children can be detected in early life. In particular, the data suggest that evaluation of IL-4 and IL-9 production at birth and in early life, respectively, are useful for estimation of allergy risks. Although the source of the cells responsible for this cytokine production still has to be defined, our results strongly point toward a role for T_H9 cells in allergy development. Moreover, the detection of

IL-21-producing CD4⁺ T cells in cord blood warrants further study on the subset source and physiological role of this early IL-21.

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METHODS

Study design

The Follow-up of Newborn's Immune Development in Relation to Allergic Outcome study is a prospective study of newborns from 139 pregnant women in the province of Flemish-Brabant, Belgium. Pregnant women between the 12th and 38th weeks of pregnancy in 2001 to 2003 answered interview questionnaires in view of potential inclusion. Allergic parents, siblings, or both were defined based on a clinical history of doctor-diagnosed allergic rhinoconjunctivitis, asthma, anaphylaxis, or food allergy. In case of at least 1 allergic first-degree relative of the expected newborn, mothers who consented were all included in the study. When there was no familial risk factor, mothers were randomly included 1 in 2. Randomization was done by a blinded researcher, and the label "in" or "out" was placed in consecutively numbered closed envelopes. As a result of this selection, power analysis calculated the chance of the offspring to become atopic to be 30%.

Pregnancy complications were an exclusion criterion. Women provided informed consent. In total, 107 newborns born of 106 pregnancies were included. The study was approved by the local ethics committee.

Infants were clinically evaluated at 12 and 24 months and 6 years of age based on a detailed history and clinical examination by the same pediatrician. In January 2010, all children reached the age of 6 years. The main allergic outcomes were atopic eczema, persistent or intermittent wheeze, persistent or seasonal rhinoconjunctivitis, anaphylaxis, food allergy (positive symptoms), and allergic sensitization without symptoms. Skin prick tests (SPTs) were performed at 12 months and 6 years. In all children house dust mite, tree pollen, birch pollen, grass pollen, *Alternaria* species, cat dander, dog dander, egg white, cow's milk, peanut, and potato (Hal laboratories, Haarlem, The Netherlands) were tested, and depending on clinical history, additional SPTs (latex, tree nut, egg yellow, horse dander, and fish) or supplementary SPTs at age 24 months were performed (Aas' criteria were used to score the SPT response^{E1}). Moreover, specific IgE levels were measured at 12 and 24 months and 6 years of age to house dust mite, birch pollen, grass pollen, cat dander, dog dander, peanut, cow's milk, egg, and potato (lower detection level of 0.1 IU/mL; a kind gift of Phadia, Uppsala, Sweden). A CAP test result of greater than 0.35 IU/mL was considered positive. From birth until the second birthday, parents completed a diary at each visit of their infant to the family doctor or pediatrician.

The diagnosis of clinical allergy was based on the presence of symptoms in association with relevant sensitization. Children with any specific IgE level between 0.35 and 0.7 IU/mL but negative SPT responses for all allergens tested and no relevant symptoms were considered healthy ($n = 3$). Asymptomatic sensitization was defined as sensitization based on a positive SPT response^{E1} or specific IgE levels of greater than 0.7 IU/mL (for ≥ 1 allergen) without any relevant symptoms (excluded from analysis, $n = 3$). Those children who were already allergic before or at the age of 2 years were labeled as "early allergic." Only 1 of these children was no longer allergic at the age of 6 years. Those who had allergy between the ages of 2 and 6 years were labeled as "late allergic."

One hundred seven babies (1 twin pregnancy) were included at birth. Twenty-three children did not continue their participation in the study (mainly because of refusal of venous puncture, for personal reasons, or because they were lost to follow-up). Results are thus based on data from 84 children (32 male subjects). Cord blood was not obtained if delivery occurred by means of cesarean section or was no longer used for stimulation if transport to our laboratory took longer than 4 hours ($n = 66$ remaining). Six children completed final allergy evaluation at age 6 years, but their parents refused venous puncture at age 12 and 24 months, whereas venous puncture at age 12 months was technically not possible in 1 additional child.

Stimulation of cord blood and peripheral blood cells

T cells in CMBC and PBMC cultures were stimulated with soluble anti-CD3 mAb (UCHT-1) captured by murine P815/CD80 mastocytoma cells^{E2} at a T/P815 ratio of 1:1 in a 1-mL volume. P815 cells transfected with human CD80 were mitomycin C treated and washed 5 times before use.

In culture conditions used to measure IL-4, IL-17A, and IL-21, an anti-human IL-4 receptor α mAb (clone 25463.11 from R&D, Abingdon, United Kingdom) was used at a concentration of 2.5 μ g/mL, as reported.^{E3} This mAb blocks the bioactivity of both IL-4 and IL-13, blocks IL-4 consumption,^{E3} and avoids inhibition of T_H17 cells by T_H2 cytokines. Preliminary experiments on 4 healthy adult donors showed that *in vitro* polyclonally induced IL-17A production significantly increased, whereas IL-21 production remained unaltered when IL-4 bioactivity was blocked. Three hundred microliters of supernatant in the presence of anti-IL-4 receptor mAb was used to measure these 3 cytokines, whereas 200 μ L of separate supernatants in the absence of anti-IL-4 receptor was used to measure IL-9 and IFN- γ .

The concentrations of IL-4, IFN- γ , and IL-21 in culture supernatants were measured with a sandwich ELISA technique by using combinations of unlabeled and biotin-coupled mAbs to different epitopes of each cytokine: IL-4 capture antibody clone 8D4-8 (PharMingen, San Diego, Calif) and biotin-labeled clone mp4-25D2 (PharMingen); IFN- γ capture antibody clone 350B10G6 (Medgenix Diagnostics, Fleurus, Belgium) and biotin-labeled clone 67F12A8 (Medgenix Diagnostics); and IL-21 capture antibody clone J148-1134 (PharMingen) and biotin-labeled clone 176-539 (PharMingen). IL-17A was measured with IL-17 DuoSet (R&D Systems, Minneapolis, Minn). Capture and detection antibodies to measure IL-9 were previously reported.^{E4}

Intracellular staining procedure

Additional experiments were performed on CBMCs from 3 nonselected newborns born in 2009 ($n = 3$). Cord blood CD4⁺ T cells were isolated with the CD4⁺ isolation kit II Mac (Miltenyi Biotec, Bergisch Gladbach, Germany) by using LD columns. Purity of the cord blood CD4⁺ T cells was at least 94.5%. CBMCs and isolated CD4⁺ T cells were cultured with anti-CD3 (2 μ g/mL) and P815/CD80-transfected cells or PHA (5 μ g/mL) in the absence or presence of IL-23 (10 ng/mL) and IL-1 β (10 ng/mL). After 3 days, cells were restimulated at 37°C for 4 hours with 50 ng/mL phorbol 12-myristate 13-acetate and 0.5 μ g/mL ionomycin in the presence of brefeldin A (10 μ g/mL) in RPMI with 10% bovine calf serum. After membrane staining with anti-CD45-peridinin-chlorophyll-protein complex mAb (BD Biosciences, Mississauga, Ontario, Canada; for CBMCs only), cells were washed with PBS containing 1% FCS and fixed with Reagent A (ADG Fix&Perm, Vienna, Austria) for 15 minutes at room temperature. Cells were washed in PBS/1% FCS and resuspended in Reagent B (ADG Fix&Perm). Cells were then incubated with 10 μ L of human plasma and either anti-IgG1 FITC (BD Biosciences), anti-IgG-phycoerythrin (PE; BD Biosciences), anti-IL-17-PE (eBioscience, San Diego, Calif), anti-IFN- γ -FITC (BD Biosciences), or anti-IL-21-PE (BD Biosciences) mAb for 15 minutes at room temperature and washed twice with PBS. Analysis was done with the Cell Quest Program on FACS Sort (BD Biosciences).

Statistical analysis

Results were compared with the nonparametric Kruskal-Wallis test with the Dunn post test, Mann-Whitney U test, or paired Wilcoxon tests, 1-tailed were appropriate. A P value of .05 or less was considered statistically significant. Contingency tables were evaluated by using Fisher exact tests. Calculations, including RR assessments, were performed with Prism Graph 5 software (GraphPad Software, Inc, San Diego, Calif).

RESULTS

Cord blood intracellular cytokine responses

A small percentage of isolated cord blood CD4⁺ T cells (0.33%) in one of 3 samples had intracellular IL-17A on stimulation with anti-CD3 and CD80 (data not shown). To further increase the potential for IL-17 production, we added IL-23, the main inducer of IL-17A in human subjects,^{E5} and IL-1 β , which is essential for human T_H17 cell differentiation,^{E6} to the *in vitro* cultures, but this had no effect (Fig E1).

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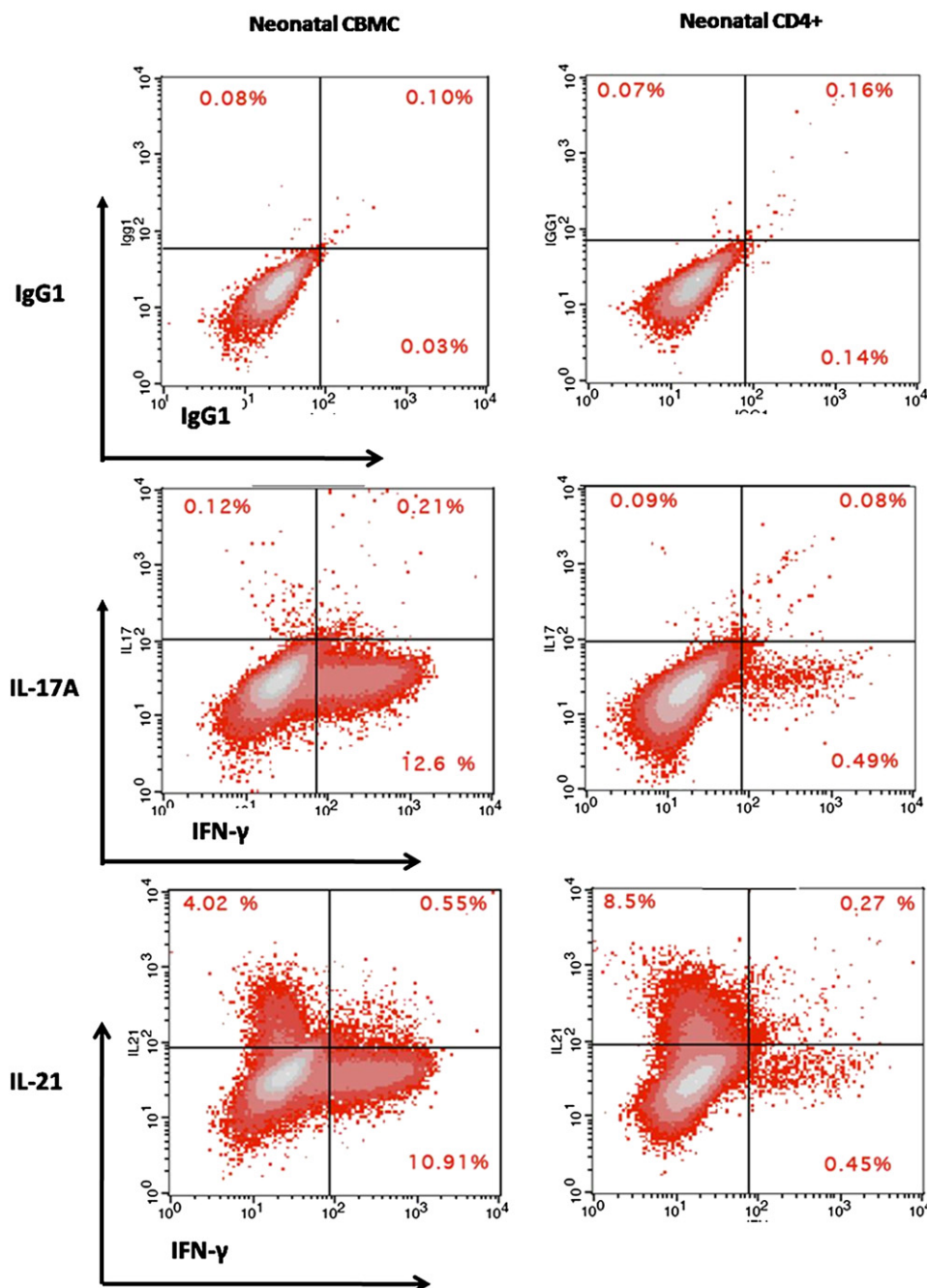


FIG E1. Intracellular cytokines in CBMCs and purified CD4⁺ cord blood T cells. Fresh CBMCs (*left column*) and purified cord blood CD4⁺ T cells (*right column*) from the same sample were stimulated with PHA, IL-23, and IL-1 β . After 3 days, cells were restimulated with phorbol 12-myristate 13-acetate and ionomycin and membrane stained with anti-CD45 mAb (CBMCs only) and stained intracellularly with anti-IL-17-PE, anti-IL-21-PE, anti-IFN- γ -fluorescein isothiocyanate or IgG1-fluorescein isothiocyanate/IgG-PE control. Results are representative of 3 independent experiments.

TABLE E1. Subjects' characteristics

	Nonallergic at 6 y of age	Symptomatic allergic before 2 y of age	Symptomatic allergic at 6 y but not yet at 2 y of age
Total no.	54	17	10
≥1 First-degree relative with atopy	47/54 (87%)	17/17 (100%)	9/10 (90%)
Infant's sex, male	17/54 (31%)	11/17 (64%)	4/10 (40%)
Neonatal parameters (mean ± SEM)			
Birth weight (g)	3404 ± 67	3473 ± 97	3275 ± 166
Head circumference (cm)	34.7 ± 0.2	35.1 ± 0.3	34.3 ± 0.6

TABLE E2. Sensitization* profiles in children with early allergy

	12 mo	24 mo	6 y
005	Egg, cat	Egg, nuts, peanuts, cat	Nuts, HDM, cat, dog, horse, birch pollen, <i>Alternaria</i> species
022	Egg	HDM	HDM, grass pollen, rabbit, <i>Alternaria</i> species
025	Potato	Negative	HDM
040	Potato	Potato	HDM, birch pollen
041	Negative	HDM	HDM, cat, dog
044	Egg, HDM	HDM, cat, birch pollen	HDM, cat, birch pollen
054	Egg	Not done	HDM, cat, dog, grass pollen
056	Egg, cow's milk, HDM	Cow's milk	HDM
065	Negative	HDM	HDM, dog, birch pollen, grass pollen
066	Egg, HDM	HDM	HDM, birch pollen
075	Egg, cow's milk	Negative	Negative
077	Egg, cow's milk, HDM	Egg, cow's milk, HDM	Egg, HDM, dog, grass pollen
086	Egg, potato	Egg	HDM, horse, rabbit, birch pollen, grass pollen
088	Negative	Peanut	Egg, HDM, grass pollen
089	Potato, cow's milk	Negative	HDM, cat, dog, birch pollen, grass pollen
097	Egg, cow's milk	Cow's milk	Cow's milk, HDM, cat, dog, grass pollen
104	Negative	HDM	HDM

HDM, House dust mite.

*Sensitization was determined as defined in the [Methods](#) section in this article's Online Repository.

TABLE E3. Sensitization* profiles in children with late allergy

	12 mo	24 mo	6 y
002	Negative	Negative	HDM, grass pollen
027	Negative	Negative	Grass pollen
045	Negative	Negative	HDM, grass pollen
046	Negative	Negative	HDM
050	Negative	Negative	HDM, dog, grass pollen, birch pollen
055	Negative	Negative	HDM, cat, dog, grass pollen
060	Negative	Negative	Peanut
068	Negative	Negative	Grass pollen
078	Negative	Negative	Birch, cow's milk
105	Negative	Negative	<i>Alternaria</i> species

HDM, House dust mite.

*Sensitization at age 6 years was determined as defined in the [Methods](#) section in this article's Online Repository.

TABLE E4. Polyclonal IL-4 and IFN- γ production by CBMCs and PBMCs at a young age

	Nonallergic*	Early allergy*	Late allergy only*	P value‡
A. CBMC IL-4 production†	15-30-55	11-55-122	10-11-30	.2
B. 12-mo PBMC IL-4 production†	104-151-219 (A vs B: $P \leq .0001$)	122-159-293 (A vs B: $P = .03$)	162-188-231 (A vs B: $P = .008$)	.7
C. 24-mo PBMC IL-4 production†	107-181-273 (B vs C: $P = .01$)	180-219-340 (B vs C: $P = .009$)	108-238-345 (B vs C: $P = .4$)	.3
D. CBMC IFN- γ production†	2,569-6,419-37,787	1,872-8,118-24,858	1,105-5,289-15,813	.9
E. 12-mo PBMC IFN- γ production†	15,238-61,317-110,345 (D vs E: $P = .0003$)	21,014-50,863-152,540 (D vs E: $P = .008$)	24,466-50,345-70,118 (D vs E: $P = .04$)	.9
F. 24-mo PBMC IFN- γ production†	13,829-39,216-88,454 (E vs F: $P = .6$)	36,720-75,726-156,400 (E vs F: $P = .8$)	4,777-47,664-58,551 (E vs F: $P = .004$)	.2

*Sensitization and clinical symptoms were evaluated as explained in the [Methods](#) section in this article's Online Repository.

†CBMCs (5×10^5) or PBMCs (5×10^5) obtained at 12 and 24 months were stimulated with anti-CD3/CD80 in the absence (IFN- γ) or presence (IL-4) of anti-IL-4 receptor mAb. Supernatants were collected after 3 days; cytokine production was measured by means of ELISA and expressed as concentrations in supernatants in picograms per milliliter. Values shown represent p25-median-p75 values for cytokines. A versus B, B versus C, D versus E, and E versus F were tested by using the paired Wilcoxon test.

‡Kruskal-Wallis test.