

Determination of T-cell subpopulations and intracellular cytokine production (interleukin-2, interleukin-4, and interferon- γ) by cord blood T-lymphocytes of neonates from atopic and non-atopic parents

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This report describes the results of a prospective study on immunological markers in cord blood for the prediction of allergic diseases in children. First we evaluated methodological aspects of the flow cytometric technique on cord blood cytokine measurements. Subsequently, the T-cell subsets and percentage of cytokine-producing cord blood T-helper (Th) and T-suppressor/cytotoxic lymphocytes of neonates from atopic and non-atopic parents were compared. A group of 33 healthy, full-term newborn infants of whom 23/33 were at risk for atopy (i.e. having at least one parent with one or more atopic symptoms and positive specific immunoglobulin E [IgE] to at least one common inhalant allergen) was studied. A flow cytometric technique was used to analyze cord blood T-cell subsets and to determine the percentage of interleukin (IL)-2-, IL-4-, and interferon- γ (IFN- γ)-producing cord blood Th and T-suppressor/cytotoxic lymphocytes following stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. The percentage of CD3⁺ (T lymphocytes), CD3⁺ CD4⁺ (Th lymphocytes), CD3⁺ CD8⁺ (T-suppressor/cytotoxic lymphocytes), CD19⁺ (B lymphocytes), CD3⁺ CD4⁺ CD45RO⁺ (memory Th lymphocytes), and CD3⁺ CD4⁺ CD45RA⁺ (naive Th lymphocytes) cells was unrelated to parental atopic status. PMA stimulation augmented the percentage of IL-2- and IFN- γ -producing Th and T-suppressor/cytotoxic lymphocytes, whereas the number of IL-4-producing T lymphocytes remained very low or undetectable. No differences in the percentage of IL-2-, IL-4- and IFN- γ -producing Th and T-suppressor/cytotoxic lymphocytes were found between neonates from atopic and non-atopic parents. These results will be re-evaluated when the atopic status of the children at the age of 1 and 2 years can be assessed.

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Over the last 25 years, the prevalence of atopic diseases has increased considerably in developed countries, especially in children and young adults (1). Atopy is associated with a T-helper 2 (Th2) cytokine profile (interleukin [IL]-4 and IL-5), as

opposed to the T-helper 1 (Th1) profile (IL-2 and interferon- γ [IFN- γ]), which is a feature of non-atopics (2–4). It has been accepted that the development of atopic diseases is influenced by the interaction between environmental exposure

to allergens and non-specific adjuvant factors, such as tobacco smoke, air pollution, and infections, in genetically predisposed individuals (5). The first year of life seems to be a vulnerable period for sensitization (6). Recently, lymphoproliferative responses of cord blood, as well as fetal blood lymphocytes, to specific food and inhalant allergens have indicated that initial priming of the T-cell system against environmental allergens is initiated *in utero* (7–9). The availability of predictive markers could be of considerable interest, as prevention of atopic diseases might be possible by reducing exposure to allergic and non-allergic triggers (10). Both elevated serum immunoglobulin E (IgE) levels in cord blood (11) and a positive family history of atopic diseases (12) have been shown to be risk factors for subsequent atopic diseases. Unfortunately, the sensitivity of these early markers is low (13).

The aim of the present report was to evaluate the capacity of stimulated cord blood T lymphocytes from newborns to produce IL-2, IL-4 and IFN- γ at single cell level. In the first part of this report, the intra- and inter-test reproducibility of the flow cytometric technique for intracellular cytokine measurement was evaluated. Next, the influence of the time lapse between sampling and analysis of cord blood on intracellular cytokine levels was investigated. In the clinical part of this report, cord blood T-cell sub-populations and T-lymphocytic cytokines of neonates from atopic and non-atopic parents were compared. As the included neonates are enrolled in a prospective study, a second evaluation will follow, in which the immunological markers on cord blood will be related to the atopic status of the children at the age of 1 and 2 years.

Patients and methods

Study population

After informed consent was obtained from the parents, cord blood was collected at birth in EDTA tubes (Becton-Dickinson, Meylan, France) by needle aspiration from the placental end of the umbilical vein. Samples were taken from full-term babies (gestation ≥ 37 weeks) born in 1998 after uncomplicated pregnancies and normal (vaginal or Caesarean section) deliveries. Cord blood samples were obtained from 56 babies within 6 h of birth. All of these samples were used in the first part of this study, evaluating flow cytometric techniques on cord blood. Only a sub-group of the samples ($n = 33$) was enrolled in a prospective study on risk factors

for allergic diseases in children: nine parents gave no permission for peripheral blood sampling for total and specific IgE determination; five cord blood samples were not available within 4 h of collection (see the Results); and nine neonates were excluded because they could not be clearly defined as being at high or low risk for atopic diseases.

Cord blood serum IgE and parental allergen-specific IgE

The ImmunoCAP test (Pharmacia & Upjohn, Brussels, Belgium) was used for the measurement of total IgE in cord blood serum (detection limit 0.35 kU/l). Elevated total cord blood IgE was defined as a concentration of > 0.9 kU/l (14). Specific IgE to seven common inhalant allergens (*Dermatophagoides pteronyssinus*, cat, dog, birch pollen, timothy grass pollen, *Artemisia vulgaris* [weed pollen], and *Cladosporium herbarum*) was determined in peripheral blood serum from the parents. Specific IgE > 0.35 kU/ml was considered as elevated.

Classification of neonates

Neonates were defined as being at high risk for atopic diseases ($n = 23$) when one or both parents had a clear history of allergic symptoms in combination with elevated specific IgE to one or more common inhalant allergens. A standardized questionnaire for allergic diseases was used (15). In short, parents were asked about their history of atopic dermatitis, allergic rhinitis, allergic conjunctivitis, and bronchial asthma. Additional information about seasonal or perennial occurrence of symptoms and history of symptoms after contact with animals was also obtained. When both parents had no allergic symptoms and showed no positive specific IgE, the newborn was defined as being at low risk for atopy ($n = 10$). When the parents were IgE positive but without any symptoms, or if they had a positive questionnaire without associated specific IgE, the newborns were not included in the study ($n = 9$) as they could not be clearly identified as being at high or low risk for atopy. The two groups showed no statistically significant differences in birth weight, gestational age, Apgar score (at 1, 5, and 10 min), cord blood IgE, and time between birth and the start of the cell separation. In contrast to the high-risk group, where boys and girls were equally distributed, the majority of babies included in the low-risk group were male, but this difference was not statistically significant (Table 1).

Table 1. Characterization of the two study groups (median values and range)

	Low-risk group	High-risk group
No	10	23
Sex (M:F)	8 : 2	10 : 13
Birth weigh (g)	3237 (3090–4390)	3350 (2750–3960)
Gestational age (weeks)	39.0 (38.0–40.0)	39.3 (37.0–41.0)
Apgar score (1 min)	9 (6–9)	9 (7–10)
Apgar score (5 min)	9 (9–10)	9 (8–10)
Apgar score (10 min)	10 (9–10)	10 (9–10)
Cord blood IgE > 0.9 kU/l	1/10	2/23
Maternal smoking during pregnancy	0/10	3/23
Birth by Caesarean section	2/10	0/23
Delay sampling/analysis (min)*	45 (25–230)	80 (5–220)

*Delay (in min) between sampling and the start of cell separation.

Cell separation

Before cell separation, the number of white blood cells and eosinophils were enumerated using routine hematological procedures. Cord blood was diluted 1 : 2 with phosphate-buffered saline (PBS, pH 7.4; Life Technologies, Gent, Belgium), subsequently layered on 13 ml of Nycoprep (density = 1.077 mg/ml; Nycomed, Oslo, Norway), and centrifuged at room temperature for 20 min at 1000 *g*. Cord blood mononuclear cells (CBMC) were collected and washed twice with PBS for 10 min at 200 *g*. Cells were counted and concentrated at 5×10^6 cells/ml in AIM-V culture medium (Life Technologies).

Flow cytometric analysis of lymphocyte subsets

For cell surface marker analysis, 50 μ l of whole blood was incubated for 15 min in the dark at 4°C with 10 μ l of three-color monoclonal antibody panels (CD3-peridinin chlorophyll protein [PerCP]+CD4-fluorescein isothiocyanate [FITC]+CD45RO-phycoerythrin [PE]; CD3-PerCP+CD4-PE+CD45RA-FITC; CD3-PerCP+CD4-FITC+CD8-PE; CD45-PerCP+CD3-FITC+CD19-PE; Becton-Dickinson Immunocytometry Systems). Subsequently, the red blood cells were lysed with 2 ml of a lysing solution (Becton-Dickinson Immunocytometry Systems) for 20 min at room temperature. The remaining cells were pelleted at 400 *g* for 10 min, fixed with 1% paraformaldehyde in PBS, and analyzed by fluorescence-activated cell sorter flow cytometry (FACScan; Becton-Dickinson) within 24 h. By gating on sideward scatter and CD3⁺ or CD45⁺ cells, the different lymphocyte subsets were determined.

Cell culture

As described previously (16), the isolated CBMC were cultured in AIM-V culture medium (Life

Technologies) for 6 h at 37°C in an atmosphere of 5% CO₂. Preliminary tests showed that a stimulation period of 6 h induced maximal cytokine production by T lymphocytes. One part of the cells was stimulated in the presence of 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO), 1 μ g/ml of ionomycin (Sigma) and 2 μ M monensin (Sigma). The other part of the cells remained unstimulated, in the presence of 2 μ M monensin.

Three-color flow cytometry for intracellular cytokine staining

After stimulation, cells were washed for 5 min at 1000 *g* at room temperature with 1 ml of PBS and resuspended in PBS at a concentration of 10^7 cells/ml. Cells were labeled with antibodies against CD3 and CD8 surface antigens (anti-CD3-PerCP, anti-CD8-FITC; Becton-Dickinson Immunocytometry Systems) for 15 min in the dark at room temperature. Afterwards, cells were fixed with 4% paraformaldehyde in PBS (Sigma). Cell membranes were permeabilized with 0.3% saponin (Sigma) in PBS and incubated with anti-cytokine antibodies (mouse anti-Hu IL-2-PE, mouse anti-Hu IL-4-PE, mouse anti-IFN-PE; Fast Immune; Becton-Dickinson Immunocytometry Systems) for 15 min in the dark at room temperature. Cells were centrifuged for 5 min at 1000 *g* and resuspended in PBS.

Flow cytometric analysis

Ten thousand cells were analyzed on a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems) and processed using WinMDI 2.7 software (Windows Multiple Document Interface Flow Cytometry Application, version 2.7 (Joseph Trotter 1993–98). Analysis gates were set on lymphocytes according to forward and sideward scatter properties. Th cells were defined as CD3⁺ CD8[−] and T-suppressor/cytotoxic cells as CD3⁺ CD8⁺. According to PerCP and FITC emission, CD3⁺ CD8[−] and CD3⁺ CD8⁺ lymphocyte populations were gated out in a compensated fluorescence plot. Within this gate, intracellular cytokine production (PE emission) was measured in CD3⁺ CD8[−] and CD3⁺ CD8⁺ lymphocytes. Markers were set on the 99th percentile, using isotype-matched irrelevant antibodies (PE-immunoglobulin G₁ [IgG₁] mouse antibodies; Serotec, Oxford, UK) as a reference. Results were expressed as the percentage of cytokine-producing cells.

Statistics

Inter- and intra-observer variability was analyzed using the Bland-Altman plot (17). Differences of T-cell subset distribution and intracellular cytokine production between neonates at low and high risk for atopy were assessed using the Mann-Whitney *U*-test, as the data were not normally distributed. Correlations were calculated using the Spearman rank correlation coefficient. A *p*-value < 0.05 (two-tailed) was considered statistically significant.

Results

Inter-observer and intra-observer variability

Ten samples were analyzed twice by two independent observers. The mean differences between the measurements were in all cases near zero and only one outlier exceeded more than two standard deviations (Fig. 1a,b,c,d).

Influence of cell preservation on cytokine production

The influence on cytokine production of preservation of the separated mononuclear cells for 24 h was investigated by comparing the production of IL-2 on days 1 and 2 in four samples. For each sample, after cell separation, one-half of the sample was incubated in AIM-V medium at 4°C in the dark and stimulation and analysis was postponed for 24 h. The remaining cells were incubated and analyzed immediately, as described above. The viability of the cells, measured with propidium iodide (PI), on days 1 and 2 was > 95%. In all samples, a clear decrease, varying between 17 and 58%, in IL-2 production by Th cells was found on day 2 (results not shown).

Delay between sampling and start of analysis

The effect, on IL-2 production, of the delay between sampling and the start of cell separation (Fig. 2) was evaluated in 56 samples: a negative correlation was found ($r = -0.41$; $p = 0.001$). Based on these results, only cord blood samples less than 4-h old were used for enrollment in the prospective study. The lymphocyte subset analysis was not affected by this delay period (correlation coefficients for total number of lymphocytes, T lymphocytes, B lymphocytes, Th lymphocytes, memory Th lymphocytes, naive Th lymphocytes, and T suppressor/cytotoxic lymphocytes were, respectively, 0.12, 0.11, 0.13, 0.05, 0.20, 0.01, and 0.13, all $p > 0.05$).

Parental atopic status

Only two neonates defined as being at high risk for atopy had two allergic parents. The remaining 21 either had an atopic mother ($n = 7$) or an atopic father ($n = 14$). Most (20 of 25) of the atopic parents had specific IgE to more than one allergen. Most of the parents with elevated specific IgE presented with symptoms of rhinitis ($n = 7$) or rhino-conjunctivitis ($n = 15$), and less frequently with asthma ($n = 7$) or eczema ($n = 5$).

Distribution of lymphocyte subsets

The distribution of circulating lymphocytes and lymphocyte subsets was similar between neonates from atopic and non-atopic parents with respect to CD3⁺ (T lymphocytes), CD3⁺ CD4⁺ (Th lymphocytes), CD3⁺ CD8⁺ (T-suppressor/cytotoxic lymphocytes), CD19⁺ (B lymphocytes), CD3⁺ CD4⁺ CD45RO⁺ (memory Th lymphocytes), and CD3⁺ CD4⁺ CD45RA⁺ (naive Th lymphocytes) cells (Table 2).

Cytokine-producing T lymphocytes of cord blood in relation to parental atopic status

The percentage of cord blood Th and T-suppressor cytotoxic lymphocytes that spontaneously produced IL-2, IL-4 or IFN- γ was low and comparable between neonates from atopic and non-atopic parents. In both groups, the percentage of IL-2- and IFN- γ -producing Th and T-suppressor/cytotoxic lymphocytes increased statistically significantly after stimulation. With respect to IL-4 production, only the percentage of IL-4-producing Th lymphocytes was significantly higher after stimulation for the neonates from non-atopic parents. The capacity of cord blood Th and T-suppressor/cytotoxic cells to produce IL-2, IL-4, and IFN- γ after mitogenic stimulation was unrelated to parental status (Table 3).

Cord blood total IgE and number of eosinophils in relation to cord blood T-cell subsets and cytokine production

Cord blood IgE concentrations ranged between 0.35 kU/l and 1.68 kU/l, and the number of eosinophils between 0 and 1491 10⁶/l. There was no significant relationship between cord blood total IgE or the number of eosinophils and T-cell subsets or cytokine production.

Discussion

In this study, flow cytometry was used for both identification of T-cell sub-populations and detection of intracellular cytokine production

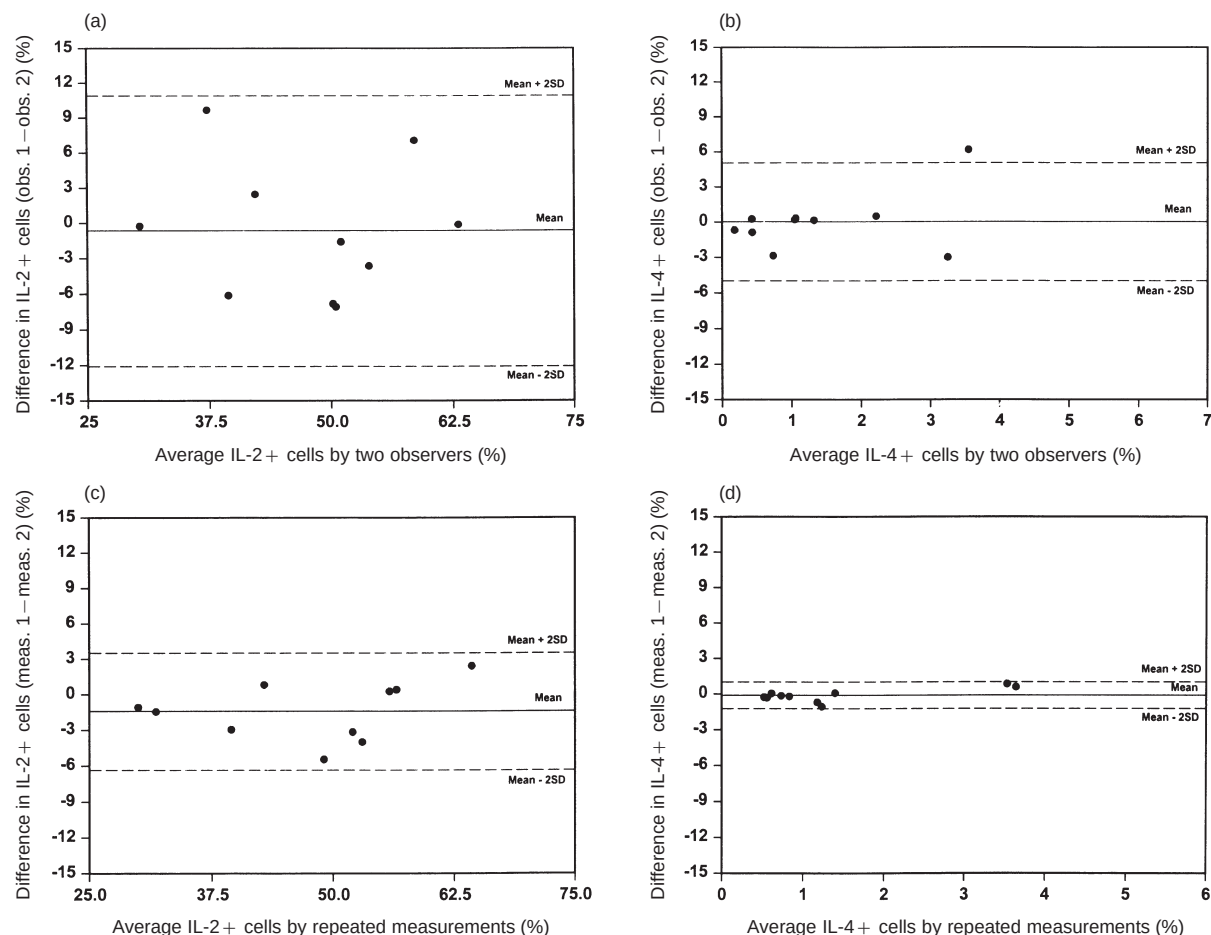


Fig. 1. Inter-observer and intra-observer variability. Inter-observer variability for the percentage of (a) interleukin (IL)-2- and (b) IL-4-producing T-helper (Th) lymphocytes. Intra-observer variability for the percentage of (c) IL-2- and (d) IL-4-producing Th lymphocytes. IL-2 + cells, IL-2-producing Th lymphocytes; IL-4 + cells, IL-4-producing Th lymphocytes; Obs, observer; Meas, measurement.

(IL-2, IL-4, and IFN- γ) in cord blood T lymphocytes. In contrast to cytokine measurements in supernatants of mononuclear cells, where it remains impossible to identify the producing cell type, the intracellular detection of cytokine production makes it possible to clearly identify the proportion of IL-2-, IL-4-, and IFN- γ -producing Th and T-suppressor/cytotoxic lymphocytes. As the flow cytometric technique is mainly used on peripheral blood of children and adults (3), and less frequently on cord blood (18), a number of preliminary tests were performed. As, for technical reasons, cord blood samples of neonates born in the afternoon could not be fully processed, the effect of overnight preservation of the separated CBMC was determined. However, when stimulation of the CBMC was postponed, a considerable decrease in cytokine production was observed, and thus immediate handling and stimulation of the isolated CBMC was necessary. Cytokine

production also diminished when the delay between cord blood sampling and the start of cell separation was more than 4 h. Available studies on cord blood have either not discussed this issue in any detail, or stated a time period that was quite long (19). As, in the present study, all these different factors of potential influence were considered, and the intra- and inter-test variability was acceptable, the results can be regarded as reliable.

Although it is generally accepted that the prevalence of allergic disorders is increasing in the industrialized Western world, there is still a need for standardized definitions of atopy and allergy (20,21). In this study, a combination of clinical and laboratory findings was used to define atopy/allergy; this is in contrast to previous cord blood studies where only the parental history for allergic diseases, sometimes in combination with total IgE on cord blood, was used to assess the risk for atopic disorders in the neonate. We did

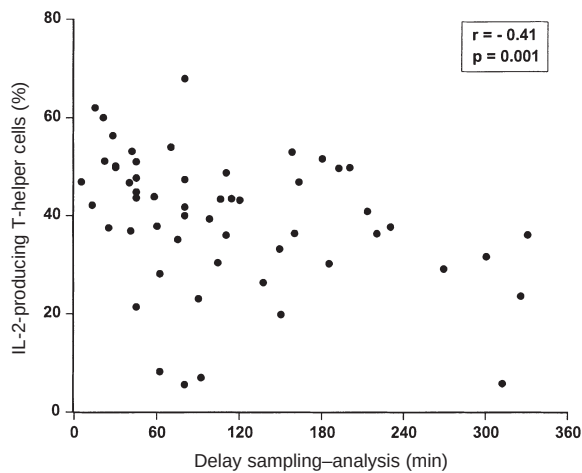


Fig. 2. Effect of delay between sampling and the start of cell separation on interleukin-2 (IL-2) production (expressed as percentage of IL-2-producing helper T cells, after stimulation with phorbol 12-myristate 13-acetate [PMA]).

not solely use the parental history to assess the atopic status, as the presence of rhino-conjunctivitis, asthma, and eczema is not exclusively related to atopy. The neonates included in the low-risk group were limited in number but highly selected: only newborns whose parents did not show any allergic symptoms or did not have IgE elevation were enrolled.

No association was found between the percentage of circulating lymphocytes or T-cell subsets at birth and parental atopic status. These results are in agreement with others (18,22,23).

In this study, the capacity of T lymphocytes to respond to mitogenic stimulation with IL-2, IL-4, and IFN- γ production was examined in relationship to the parental atopic status. There was no difference detectable in IL-2, IL-4, and IFN- γ production by cord blood T lymphocytes, assessed at single cell level, between newborns from atopic and non-atopic parents. Studies by others on cord blood have shown contrasting results with respect to the relationship between cytokine production and parental history for atopy. A decreased production of IFN- γ by CBMC of newborns from atopic parents has been described (18,23,24), but could not be confirmed

by others (9,25–28). A possible explanation for the discrepancies between the different studies is the use of different kinds of allergenic and mitogenic stimulation. Only Pohl et al. (18) also used PMA and ionomycin stimulation, but in that study a pre-incubation with bacterial products was performed. The lack of difference in the percentage of IL-2-producing T lymphocytes of neonates from atopic and non-atopic parents is concordant with most other studies (23–25). Several other groups have already reported very low or undetectable levels of IL-4 in cord blood, both after different types of allergenic (9,27,28) and mitogenic (23,24,27) stimulation, which suggests a maturational delay in IL-4 synthesis at birth. In these studies, IL-4 was only detectable when a reverse transcription–polymerase chain reaction (RT-PCR) technique was used. One other cord blood study (18) attempted to assess the IL-4 production at intracellular level in relation to parental atopic status, but no IL-4-producing naive or memory Th lymphocytes could be identified after pre-incubation with bacterial products and restimulation with PMA and ionomycin.

In this study, no differences were found in T-cell sub-populations and cytokine production (IL-2, IL-4, and IFN- γ) by cord blood T lymphocytes among neonates from atopic and non-atopic parents. Although the numbers of neonates in this study were rather small, there might be a number of reasons for considering the findings of the present study as appropriate. First, the included neonates were highly selected: only newborns that could be clearly defined as being at high or low risk for atopy were enrolled. Second, cytokine production was assessed at single cell level by flow cytometry, in contrast with most other studies on cord blood in which supernatants are used for cytokine determination. Indeed, the diminished level of IFN- γ , detected in supernatants from CBMC of neonates at risk for atopy, can be the result of either a diminished capacity of T lymphocytes to secrete IFN- γ or of a diminished number of IFN- γ -producing cells.

Table 2. Distribution of lymphocyte subsets in low- and high-risk groups (median values and range)

	Low-risk group (n = 10)	High-risk group (n = 23)
Total white blood cells($10^9/l$)	15.0 (12–23)	14.0 (8–22)
Lymphocytes ($10^9/l$)	4.7 (3.2–10.4)	4.8 (2.6–7.8)
CD3 ($10^9/l$)	2.9 (1.9–5.6)	3.1 (1.4–4.9)
CD19 ($10^6/l$)	515.0 (288–1656)	481.0 (127–2546)
CD4 ($10^6/l$)	2127.0 (1362–4259)	2489.0 (1004–3590)
CD8 ($10^6/l$)	501.0 (317–1094)	565.0 (338–1308)
CD3 CD4 CD45RO ($10^6/l$)	146.0 (21–390)	150.0 (25–1073)
CD3 CD4 CD45RA ($10^6/l$)	880.0 (464–3737)	1252.0 (432–2255)

Table 3. Cytokine production, expressed as percentage interleukin (IL)-2-, IL-4-, and interferon- γ (IFN- γ)-producing T-helper lymphocytes (Th) and T-suppressor/cytotoxic lymphocytes (Ts/c)

	Low-risk group (n = 10)	High-risk group (n = 23)
Th basal production		
IL-2	0.02 (0.01–0.96)	0.07 (0.01–0.81)
IL-4	0.08 (0.01–0.49)	0.07 (0.00–0.87)
IFN- γ	0.21 (0.04–0.60)	0.28 (0–2.58)
Th after PMA stimulation		
IL-2	38.82 (8.2–50.13)	46.79 (19.83–56.32)
IL-4	0.26 (0.15–1.52)	0.57 (0.08–3.68)
IFN- γ	4.95 (1.26–13.11)	4.11 (0.93–18.39)
Ts/c basal production		
IL-2	0.53 (0.23–1.51)	0.73 (0.01–3.54)
IL-4	0.76 (0.10–1.28)	0.77 (0.01–3.38)
IFN- γ	0.85 (0.26–2.48)	0.94 (0.24–3.72)
Ts/c after PMA stimulation		
IL-2	11.14 (1.75–17.85)	12.34 (3.3–22.62)
IL-4	1.35 (0.17–3.03)	1.11 (0.18–6.20)
IFN- γ	5.80 (1.68–17.76)	5.92 (1.04–17.79)

Results are expressed as median values (range).
PMA, phorbol 12-myristate 13-acetate.

The application of this intracellular technique strongly suggests an intrinsic IFN- γ secretion defect, as hypothesized by others (29,30).

Our results, using flow cytometry, confirm that all neonates have a reduced percentage of IFN- γ -producing T cells compared with adults (4,16). This is in concordance with the present hypothesis that successful pregnancy is associated with a Th2 cytokine profile (9) and that repeated contact with antigens (amongst other infections) during the post-natal period probably redirects this fetal Th2 profile towards a Th1 pattern in non-atopics, whereas in atopics this Th2 immunity is enforced (30).

In our search for markers to predict atopy and allergic diseases in children, at this moment no parameter other than the atopic status of the child itself can be used to evaluate the predictive power of the potentially new markers for atopy. Tang et al. (19) and Warner et al. (31) have already shown that a diminished IFN- γ production by CBMC was related to subsequent development of allergic diseases and positive skin prick tests. Thus, in view of the broad range of the study results, further clinical follow-up will be necessary to elucidate the usefulness of these immunological parameters on cord blood in the prediction of allergic disorders.

In conclusion, this study emphasizes the need for prompt analysis of cord blood samples for cytokine determination. We were unable to demonstrate differences in lymphocyte sub-populations and in numbers of IL-2-, IL-4-, and IFN-

γ -producing T lymphocytes between infants from atopic and non-atopic parents. Further clinical follow-up of the enrolled patients will validate the eventual predictive capacity of these immunological parameters on cord blood.

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