

Maturation of cytokine-producing capacity from birth to 1 yr of age

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Little is known about the immunologic maturation in the early stages of life. The aim of this study was to investigate maturation of immune system from birth to 1 yr of age and to compare immune functions between mothers and their children. Also the effect of atopy to the immune responses of children was examined. Cord blood samples ($n = 228$) and peripheral blood samples of children ($n = 200$) and their mothers ($n = 208$) 1 yr after birth were collected. Whole blood samples were stimulated for 24 and 48 h with Staphylococcal enterotoxin B (SEB), lipopolysaccharide (LPS) and the combination of phorbol ester and ionomycin (P/I). Production of TNF- α , IFN- γ , IL-5, IL-8 and IL-10 was determined using ELISA. Significant mother-to-child correlation was detected in cytokine-producing capacity at the age of 1 yr. TNF- α (P/I, SEB and LPS stimulation), IFN- γ (P/I and SEB), IL-5 (P/I and SEB) and IL-10 (P/I, SEB and LPS) producing capacity increased from birth to 1 yr of age. In general, stimulated cytokine responses were higher in mothers' than in children's blood samples, except in the case of P/I and LPS-stimulated IL-8, which were highest at birth. Maternal inhalation atopy was associated with increased cord blood IL-5 (24 and 48 h) and IL-10 (48 h) production following P/I stimulation. Also children of food atopic mothers expressed elevated cord blood IL-10 (48 h, P/I) responses and decreased IFN- γ /IL-5 ratio (24 h, P/I). In addition, the production of IFN- γ (24 and 48 h, P/I) and the IFN- γ /IL-5 ratio (24 h and 48 h, P/I) at the age of 1 yr was lower among children with food atopic mothers. In conclusion, our results suggest that both adaptive and innate immune responses increase from birth to 1 yr of age, but are still weak in comparison to adult responses. Cytokine responses of children begin to correlate with those of their mothers during the first year of life. Although only few associations were observed between atopy and cytokine-producing capacity, our results suggest that children of atopic mothers express T_H2 -polarized cytokine pattern.

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It has been suggested that asthma and allergies begin to develop in the early stages of life (1), probably even before birth *in utero* (2). It is likely

that manifestation of allergy links closely to the immunologic events initiated in the early stages of life during the maturation process of the immune system. Thus, research on general immunologic development in children and different factors contributing to this maturation process have the potential to offer an important insights to the cascade of events leading to allergy.

Abbreviations: APC, antigen-presenting cell; PASTURE, protection against allergy: study in rural environments; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; P/I, combination of PMA and ionomycin; SEB, staphylococcal enterotoxin B; WBC, white blood cell.

There are only limited data available on the immunologic development in early childhood. Immature immune functions in infancy have been suggested to be a result of deficiencies in both innate (3) and adaptive immune responses, especially in T-cells functions (4). Poor T-cell responses in infancy may be partly due to defective antigen-presenting cell (APC) functions (3, 5). It is known that the development of T-cell memory is initiated early in life (6), maybe already *in utero* (7). Newborns immune system is naturally T-helper cell type 2 (T_h2) –skewed (7), probably as a consequence of maternal T_h2 –biased responses occurring in successful pregnancy (8). Microbial exposures among other factors may drive developing immune system towards normal (cell mediated) T_h1 –responses (9).

Activated cytokine pattern, including TNF- α , IFN- γ , IL-5, IL-8 and IL-10, plays an important role in allergic disorders and may also affect in immunologic development, because the levels of these cytokines change remarkably during the first year of life and are influenced by birth-related factors. Before functions of adaptive immunity are established, tumor necrosis factor- α (TNF- α) has a pivotal role in innate immunity by mediating acute inflammatory responses against invading microbes. The level of TNF- α in cord blood is found to be higher following vaginal delivery compared to elective caesarean section (10). In innate immunity, interferon -gamma (IFN- γ) activates macrophages to destroy phagocytosed microbes and in adaptive immunity IFN- γ promotes development of T_h1 –mediated responses and inhibits T_h2 –differentiation. At birth, IFN- γ responses are weak, but increase progressively with age (11) indicating development of adaptive T-cell responses. Interleukin-5 (IL-5) is predominantly regarded as T_h2 –cytokine, which contributes to allergic inflammations and asthma by activating eosinophils. Recent evidence is suggesting that season of birth affects the levels of IL-5 in cord blood (12). Chemokine IL-8 (also known as CXCL8) is produced not only by cells of both adaptive and innate immune system but also by other cell types including endothelial and epithelial cells. Primary biologic function of IL-8 is recruitment of neutrophils. Birth stress and mode of delivery seem to contribute to the levels of IL-8 at birth (13). IL-10, a cytokine with inhibitory properties, is secreted principally by regulatory T cells. IL-10 suppresses the functions of T cells (both T_h1 and T_h2 subsets) and also cells of innate immune system. It seems that, in comparison to adults, neonatal T cells have impaired

IL-10 producing capacity and receptor expression (14).

In the current paper, our focus is to assess the immunologic maturation by comparing the levels of cytokines at birth and at the age of 1 yr and also between mothers and their children 1 yr after birth. We also aimed to evaluate if cytokine responses differ between atopic and non-atopic children and if maternal sensitization to food or inhaled allergens is associated with cytokine responses of their children. Adaptive and innate immune responses were evaluated in whole blood culture using generally known potent immunostimulants: (i) Superantigen staphylococcal enterotoxin B (SEB), derived from Gram-positive bacteria, is a potent activator of cells in both innate and adaptive immunity, (ii) Gram-negative bacterial LPS was used to stimulate innate immune responses and (iii) the combination of mitogenic phorbol 12-myristate 13-acetate (PMA) and calcium ionophore ionomycin was used to investigate adaptive immune responses. Stimulated production of IFN- γ , IL-5, IL-10, TNF- α and IL-8 was measured from cord blood and from peripheral blood of children and their mothers 1 yr after delivery.

Methods

Study design

The study population consists of mothers and their children from ongoing Finnish prospective birth cohort study (LUKAS2). LUKAS2 is an extension of the Finnish part of the EU funded birth cohort study PASTURE investigating mechanisms of early protective exposures of allergy development (15).

Pregnant women who gave birth between May 2004 and May 2005 at the Department of Obstetrics at Kuopio University Hospital were invited to the study. All children were followed up with written questionnaires already from the last trimester of pregnancy and again at the age of 2 and 12 months. Participants were living in suburban and rural areas in middle and in eastern Finland. Only 11 mothers lived on an actively operating farm. A written informed consent was acquired from all mothers. Ethical permission was granted by the Research Ethics Committee, Hospital District of Northern Savo.

Invitation letter was sent to 1763 pregnant women. A response was received from 1370 (77.7%) women, of which 886 fulfilled inclusion criteria (maternal age of 18 yrs or older, singleton pregnancy, native language Finnish, no plans of moving from the study area and not living in an

apartment). A total of 458 were willing to participate and 228 of them fulfilled further inclusion criteria [parturition at term [≥ 37 weeks of gestation], no neonatal congenital abnormalities, successful cord blood sampling, delivery between Sunday afternoon and Thursday morning]. Seventeen babies were born by elective caesarean section and 10 children by emergency section.

Blood sampling

Cord blood samples from neonates ($n = 228$) and peripheral venous blood samples from both mothers ($n = 208$) and their children ($n = 200$) were collected 1 yr after birth in lithium-heparin (samples for cytokine analysis) and in EDTA (samples for white blood cell count) -tubes by an aspiration technique (Vacutainer, Becton & Dickinson, Plymouth, United Kingdom) at the Laboratory of Kuopio University Hospital. Temporary storage and the transportation of blood samples were performed at 4°C. Samples were processed for further analysis within 27 h.

Determination of specific IgE

Serum concentrations of specific Immunoglobulin E (sIgE) against 13 inhalant and six food allergens were determined from both children (1 yr of age) and their mothers (at delivery) by using the Allergy Screen Test Panel for Atopy (Mediawiss Analytic, Moers, Germany) (16). Test panel of aeroallergens included house dust mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), pollens (alder, birch, European hazel, grass pollen mixture, rye, mugwort and plantain) danders (cat, horse and dog), and the mold (*Alternaria alternata*) and a panel of food allergens contained hen's egg, cow's milk, peanut, hazelnut, carrot and wheat. Atopic sensitization was defined as at least one positive specific IgE result (≥ 0.35 kU/l) for measured allergens. In statistical analysis, atopic sensitization was assessed separately against inhalant (excluding food atopy) and food (excluding inhalation atopy) allergens and also for combined group (sensitization against at least one inhalation and one food allergen).

Whole blood culture and analysis of stimulated cytokine production

Heparinized blood was diluted 1:8 with RPMI 1640 with Glutamax I (Gibco, Paisley, United Kingdom) cell culture medium (supplemented with 1% Antibiotic-Antimycotic from Gibco,

Paisley, United Kingdom and 10% heat-inactivated FBS Gold from PAA Laboratories GmbH, Pasching, Austria) and stimulated at +37°C, 5% CO₂ in 24-well plates (BD Labware, Bedford, MA, USA) in duplicate wells using three different stimulation (+control) for 24 and 48 h: Staphylococcal enterotoxin B (SEB) (final concentration 100 ng/ml), lipopolysaccharide (LPS) (100 ng/ml) and the combination of phorbol 12-myristate 13-acetate (PMA) (5 ng/ml) and ionomycin (1 µg/ml) (all from Sigma Chemicals, St. Louis, MO, USA, except LPS from Research Center Borstel, Borstel, Germany). After incubation, blood culture was transferred to Eppendorf tubes for 10 min centrifugation at 800 *g*. Cell free -supernatant was collected from centrifuged blood culture and stored at -80°C for later cytokine measurements. Concentrations of TNF- α , IFN- γ , IL-5, IL-8 and IL-10 were measured immunochemically by using sandwich enzyme-linked immunosorbent assay, ELISA (OptEIA™ Human ELISA set; BD Biosciences, San Diego, CA, USA). Determination of cytokines was performed according to the manufacturer's instructions with minor modifications (optimal antibody concentrations were selected according to the in-house titration experiments and the number of washes was increased). Ranges of valid cytokine measurements were as follows: IFN- γ (12.5–800 pg/ml), IL-5 (4.7–300 pg/ml), IL-8 (15.6–1000 pg/ml), IL-10 (6.3–400 pg/ml) and TNF- α (15.6–1000 pg/ml). Only linear part of the standard curve was accepted. Cytokine concentrations below the detection limit were given a value of 0 pg/ml. If the concentration of measured cytokine exceeded the upper limit of standard curve the sample was diluted as many times as needed to get the concentration within the range. Individual leukocyte count was calculated from EDTA blood using Sysmex KX-21N blood cell analyzer (Sysmex Corporation, Kobe, Japan).

Statistical analysis

Cytokine concentrations were standardized on the number of individual leukocytes (pg/10⁶ white blood cells) and, to show the effect of stimulation, the data are expressed as Δ -values (standardized cytokine level in stimulated samples minus standardized cytokine level in control samples). The cytokine data was not normally distributed. Thus Spearman's rank correlation test was used to assess the correlations between cytokine-producing capacity and non-parametric Wilcoxon signed rank test was applied to examine differences in cytokine responses (birth vs.

child 1 yr, mother 1 yr vs. child 1 yr). Associations between cytokine responses and atopic status of the mothers and their children were examined by using Mann-Whitney test. Statistical analyses were carried out using Statistical Package for the Social Sciences (SPSS) software version 15.0.1 for windows (SPSS inc. Chicago, IL, USA). The level of $p \leq 0.05$ was considered statistically significant.

Results

P/I, SEB and LPS –stimulated production of cytokines in cord blood and in peripheral blood of mothers and their children 1 yr after birth

Unstimulated concentrations of IL-5, IL-10, TNF- α and IFN- γ were mostly below detection limits following both 24 and 48 h incubation [median: 0 pg/10⁶ white blood cells, interquartile ranges (IQR): 0–0 pg/10⁶ white blood cells, except child, 1 yr after birth, IL-10, 48 h: median 0, IQR 0–6.37]. In the case of IL-8, unstimulated values in 24 h incubation were as follows: cord blood – median 705, IQR 252–1970; child, 1 yr after birth – median 4450, IQR 1520–14 500; mother – median 2030, IQR 678–8380, and in 48 h incubation as follows: cord blood – median 731, IQR 308–1720; child, 1 yr after birth – median 3300, IQR 1490–18 400; mother – median 1750, IQR 726–5000.

Absolute levels of cytokines were principally higher following 48 h stimulation in comparison to 24 h stimulation (data not shown). Associations in cytokine responses between mothers and their children and between birth and 1 yr of age following 24 h stimulation were very similar to those in 48 h stimulation. Because of similar associations found in 24 h and 48 h stimulation and greater amount of samples exceeding the detection limits in 48 h stimulation, cytokine responses only from 48 h time-point are presented.

The combination of PMA and ionomycin (P/I) was the most efficient activator of IL-5 production (Fig. 1). P/I –stimulated production of IL-5 was significantly higher in children at the age of 1 yr when compared with IL-5 responses at birth ($p < 0.001$). Furthermore, P/I stimulation induced higher production of IL-5 in maternal samples than in children's blood samples at the age of 1 yr ($p < 0.001$). SEB-induced IL-5 responses were poor at birth and comparable between blood samples of children and their mothers 1 yr after birth. As expected, LPS –stimulated levels of IL-5 were mostly below the detection limit in all the studied blood sample types.

All the stimulants induced the production of TNF- α in cord blood and in peripheral blood of mothers and their 1-yr-old children, the most potent inducer of TNF- α being P/I (Fig. 1). Following stimulation with P/I, TNF- α responses at birth were significantly weaker than at the age of 1 yr ($p < 0.001$). In addition, P/I –stimulated TNF- α responses were higher in maternal samples than in children's samples of 1 yr after birth ($p < 0.001$). In response to SEB, associations in TNF- α producing capacity between different blood sample types were similar to those of P/I stimulation. LPS induced only minor TNF- α production in cord blood, but similar TNF- α levels in children and their mothers 1 yr after birth.

Remarkable production of IL-8 was detected following stimulation with P/I, LPS and SEB in all blood sample types (Fig. 1). Following P/I –stimulation, IL-8 responses were higher in cord blood samples than in blood samples of 1-yr-old children ($p < 0.001$). In addition, P/I –stimulated levels of IL-8 were higher in the maternal than in the children's blood samples of 1 yr after birth ($p < 0.001$), but lower than in cord blood. In SEB-stimulated blood samples, production of IL-8 was lower at birth than at the age of 1 yr ($p = 0.019$) and, furthermore, mothers had remarkably higher IL-8 responses compared to 1-yr-old children ($p < 0.001$). Like following P/I stimulation, the highest production of LPS-stimulated IL-8 was detected in cord blood.

Lipopolysaccharide was the most potent activator of IL-10 production (Fig. 1). LPS –stimulated IL-10 responses were higher at the age of 1 yr than at birth ($p < 0.001$) and mothers had higher levels of IL-10 compared to their 1-yr-old children ($p < 0.001$). Only minor production of IL-10 was measured in cord blood following stimulation with SEB and P/I. Similarly to LPS, IL-10 levels in response to SEB and P/I were higher at the age of 1 yr than at birth and in maternal blood samples than in 1-yr-old children.

As in the case of IL-5 and TNF- α , the production of IFN- γ was induced most efficiently by P/I (Fig. 1). P/I-stimulated IFN- γ responses were lower in cord blood in comparison to blood samples collected 1 yr after birth ($p < 0.001$), which in turn had notably lower IFN- γ responses compared to maternal blood samples ($p < 0.001$). Also SEB induced higher IFN- γ responses in maternal blood samples than in children's samples at the age of 1 yr ($p < 0.001$). SEB-stimulated IFN- γ responses in cord blood were weak and predictably, IFN- γ

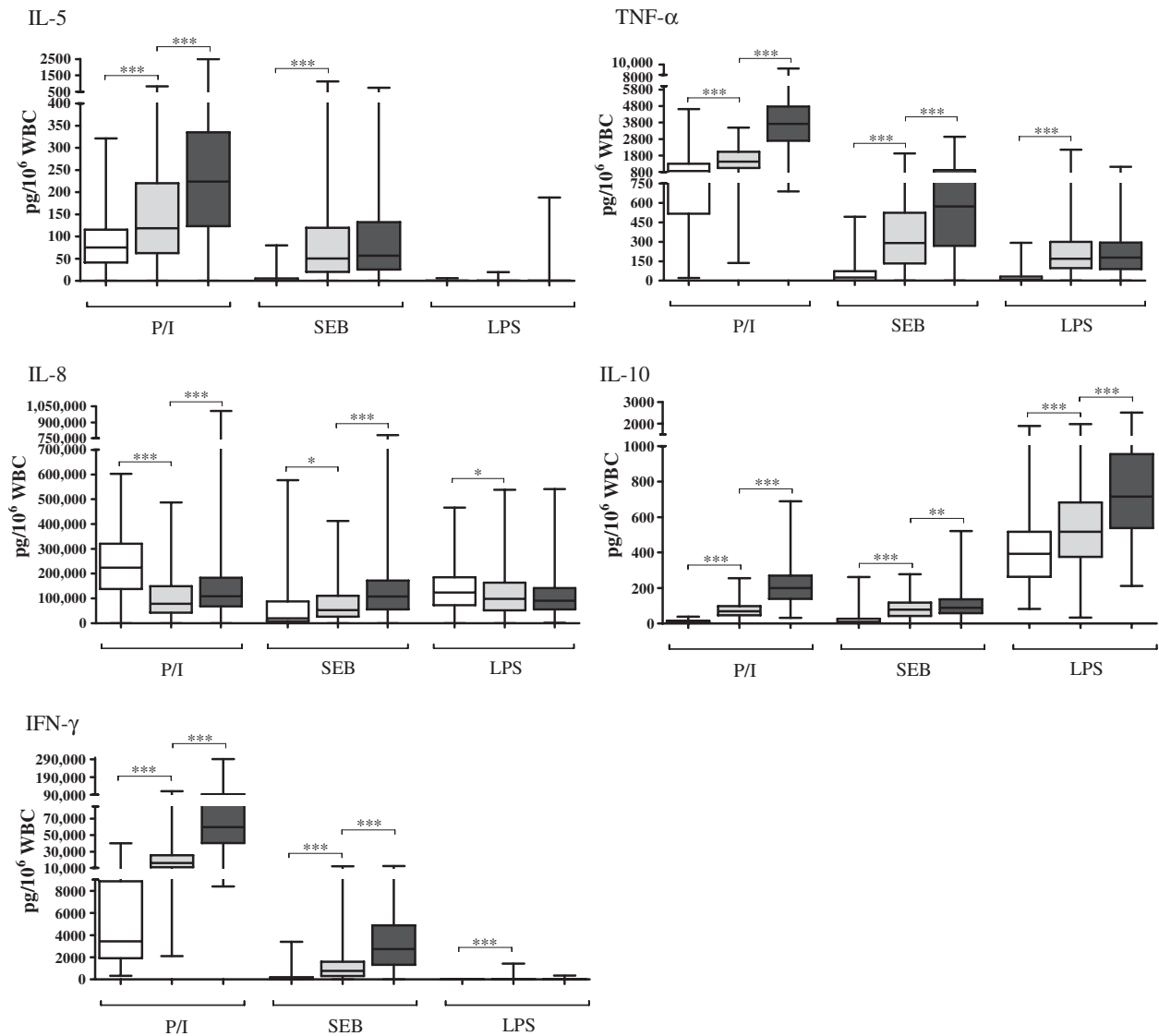


Fig. 1. Production of IL-5, TNF- α , IL-8, IL-10 and IFN- γ following 48 h stimulation with combination of phorbol ester and ionomycin, Staphylococcal enterotoxin B and lipopolysaccharide in cord blood (empty boxes) and in peripheral blood of children (light grey) and their mothers (dark grey) 1 yr after birth. Box plots represent the interquartile range, median (horizontal line) and extremes (whiskers) of individual delta values. Statistical significances are marked as *p ≤ 0.05; **p < 0.01; ***p < 0.001.

responses following LPS stimulation were mostly below detection limits in all sample types.

Correlation between cytokine responses

In general, 48 h -stimulated birth-to-1 yr cytokine correlations were weaker than mother-to-child correlations 1 yr after birth (Table 1). P/I-stimulated TNF- α , IFN- γ and IL-8 responses correlated between cord blood samples and peripheral blood samples of 1-yr-old children. Following SEB stimulation, significant birth-to-1 yr correlation was detected only in IFN- γ

production. In response to LPS, IL-10, TNF- α and IFN- γ concentrations in cord blood correlated significantly with those in peripheral blood of 1-yr-old children. Levels of IL-5 did not correlate between birth and 1 yr of age in any of the used stimulants.

Following 48 h stimulation with P/I-, SEB- and LPS, concentrations of IL-10, TNF- α , IFN- γ and IL-8 correlated statistically significantly between mothers and their 1-yr-old children (Table 1). Also levels of IL-5 in blood samples of mothers and children correlated significantly in P/I and in SEB stimulation but not in response to LPS.

Table 1. Spearman's correlation coefficients between cytokine responses at birth and at the age of 1 yr and of mothers and their children 1 yr after birth following 48 h stimulation with P/I, SEB and LPS

	P/I	SEB	LPS	P/I	SEB	LPS
	Birth vs. 1 yr of age			Child vs. mother, 1 yr after birth		
IL-5	0.14	0.08	-0.05	0.30	0.42	-0.01
Sig.	0.099	0.335	0.568	<0.001	<0.001	0.901
n	140	142	143	191	193	196
IL-10	0.04	-0.01	0.20	0.30	0.26	0.26
Sig.	0.602	0.905	0.015	<0.001	<0.001	<0.001
n	140	142	143	191	193	194
TNF- α	0.31	-0.06	0.31	0.31	0.48	0.40
Sig.	<0.001	0.459	<0.001	<0.001	<0.001	<0.001
n	140	142	142	191	193	193
IFN- γ	0.20	0.18	0.32	0.28	0.43	0.30
Sig.	0.015	0.033	<0.001	<0.001	<0.001	<0.001
n	140	142	143	191	193	195
IL-8	0.24	0.02	0.11	0.25	0.15	0.25
Sig.	0.004	0.818	0.177	<0.001	0.045	<0.001
n	140	142	143	190	189	193

SEB, Staphylococcal enterotoxin B; LPS, lipopolysaccharide; P/I, combination of phorbol ester and ionomycin.

Correlations between cytokine responses in maternal peripheral blood and in cord blood were always weaker than mother-to-child correlations 1 yr after birth. Following 48 h stimulation, maternal cytokine responses 1 yr after birth correlated statistically significantly with those of cord blood only for TNF- α in response to LPS ($r = 0.27$, $p < 0.001$), IFN- γ in response to SEB ($r = 0.27$, $p < 0.001$) and IL-8 in response to P/I ($r = 0.20$, $p = 0.013$).

Associations between cytokine responses and atopic sensitization of children

Children's sensitization against inhalant allergens was almost as prevalent as against food allergens (Table 2). Of the measured allergens, children were sensitized most frequently against cat dander (17.5%) and against cow's milk (14.5%). Mothers had notably higher prevalence of sensitization against aeroallergens than their children (52.4% vs. 21%), but the prevalence of food atopy was almost similar (20.7% vs. 23%, respectively) (Table 2).

Of the studied 200 children, 19 were sensitized against both inhaled and food allergens at the age of 1 yr. Sensitization against only food allergens was detected in 27 and against only inhalation allergens in 23 of 200 children. 131 of 200 children were not sensitized against any of the measured allergens. Associations between cytokine responses and atopy were slightly different between 24 h and 48 h stimulation. Therefore, results from both time-points are presented.

Table 2. Prevalence of sensitization (%) and median values of IgEs (kU/L) among sensitized children (1 yr of age) and mothers for each of the measured allergen separately

	Child (Total n = 200)		Mother (Total n = 208)	
	Prevalence (%)	IgE median (kU/L)	Prevalence (%)	IgE Median (kU/L)
Inhalant allergens	21		52.4	
House dust mite				
<i>Dermatophagoides pteronyssinus</i>	0.0	—	13.5	1.85
<i>Dermatophagoides farinae</i>	0.5	0.86	10.6	1.20
Pollen				
Alder	1.5	0.75	19.7	2.80
Birch	1.5	1.20	25	8.50
European hazel	2.0	0.56	19.7	2.60
Grass	1.5	0.80	17.8	3.40
Rye	1.5	2.20	14.9	4.30
Mugwort	0.0	—	7.2	1.50
Plantain	0.0	—	0.5	0.96
Animal dander				
Cat	17.5	0.91	22.6	1.20
Horse	0.5	0.59	3.4	2.40
Dog	4.0	0.71	7.7	0.83
Mold				
<i>Alternaria alternata</i>	0.0	—	0.5	1.40
Food allergens	23		20.7	
Hen's egg	4.5	0.64	5.3	1.30
Cow's milk	14.5	0.55	14.9	0.57
Peanut	5.5	0.45	4.8	1.50
Hazelnut	0.5	0.59	5.8	0.67
Carrot	0.5	0.51	1.4	1.00
Wheat flour	2.5	1.00	9.6	1.10

Children with atopy against at least one inhaled and one food allergen had significantly lower IL-5 responses following 48 h P/I stimulation at the age of 1 yr compared to their non-sensitized peers (atopic, median 69.2; non-atopic, median 132; $p = 0.04$). Ratio of IFN- γ to IL-5 following 48 h P/I and SEB stimulation at the age of 1 yr was significantly higher in children with both inhalation and food atopy than in non-sensitized children (P/I: atopic, median 308; non-atopic, median 142; $p < 0.01$ and SEB: atopic, median 28.7; non-atopic, median 12.5; $p = 0.03$). Similar finding was observed also following 24 h P/I stimulation (atopic, median 261; non-atopic, median 114; $p = 0.03$).

Inhalation atopy of the children was associated only with elevated cord blood IL-5 following 48 h stimulation with SEB (Table 3). No associations were found between inhalation atopy and cytokine responses at the age of 1 yr.

Children's sensitization against food allergens was inversely associated with IFN- γ responses in cord blood following 48 h SEB stimulation (Table 4). Also SEB-stimulated ratio of IFN- γ to IL-5 in cord blood was lower among food

Table 3. Median values of 48 h P/I, SEB and LPS-stimulated cytokine responses (pg/10⁶ wbc) of children in relation to atopic sensitization against inhaled allergens only

		Children's inhalation atopy			Maternal inhalation atopy		
	N	No 131	Yes 23	p-value	No 85	Yes 80	p-value
Cord blood							
P/I	IL-5	71.0	61.1	0.84	63.6	88.2	<0.01
	IL-10	11.0	9.68	0.15	9.87	12.6	<0.01
	TNF- α	855	953	0.30	823	898	0.36
	IFN- γ	3710	3040	0.33	2980	3770	0.31
	IL-8	207 000	249 000	0.84	251 000	209 000	0.33
SEB	IFN- γ /IL5	59.5	51.6	0.43	57.6	47.2	0.32
	IL-5	0.00	7.24	0.03	0.00	0.00	0.44
	IL-10	7.53	20.3	0.15	8.02	11.9	0.37
	TNF- α	24.1	21.9	0.83	22.1	32.5	0.16
	IFN- γ	113	133	0.26	96.5	114	0.12
LPS	IL-8	18 600	78 700	0.25	17 300	29 900	0.57
	IFN- γ /IL5	22.5	23.0	0.71	21.3	17.3	0.61
	IL-5	n.a.	n.a.		n.a.	n.a.	
	IL-10	406	313	0.15	396	369	0.92
	TNF- α	13.0	10.3	0.85	11.5	9.34	0.37
	IFN- γ	n.a.	n.a.		n.a.	n.a.	
	IL-8	140 000	109 000	0.37	140 000	119 000	0.19
	IFN- γ /IL5	n.a.	n.a.		n.a.	n.a.	
Children, 1 yr after birth							
P/I	IL-5	132	93.8	0.31	117	117	0.61
	IL-10	66.4	68.5	0.77	68.6	72.5	0.23
	TNF- α	1390	1480	0.64	1440	1420	1.00
	IFN- γ	15 100	17 200	0.57	16 400	17 100	0.82
	IL-8	82 700	80 800	0.92	84 200	68 900	0.50
SEB	IFN- γ /IL5	142	142	0.45	168	146	0.39
	IL-5	51.5	47.0	0.73	53.6	45.6	0.78
	IL-10	78.0	79.8	0.76	74.1	81.4	0.84
	TNF- α	283	379	0.23	280	309	0.78
	IFN- γ	675	1130	0.14	873	671	0.56
LPS	IL-8	47 500	79 100	0.11	56 600	54 100	0.55
	IFN- γ /IL5	12.5	20.6	0.14	13.9	11.5	0.91
	IL-5	n.a.	n.a.		n.a.	n.a.	
	IL-10	518	507	0.90	532	498	0.72
	TNF- α	169	190	0.93	197	142	0.10
	IFN- γ	0.00	0.00	0.93	0.00	0.00	0.28
	IL-8	98 100	117 000	0.35	85 900	100 000	0.78
	IFN- γ /IL5	n.a.	n.a.		n.a.	n.a.	

IFN- γ /IL5 is ratio of IFN- γ to IL-5. Subjects with atopy against food allergens excluded.

SEB, Staphylococcal enterotoxin B; LPS, lipopolysaccharide; P/I, combination of phorbol ester and ionomycin; n.a., not applicable, more than 80% of the values are zeroes. Bold indicates statistically significant at level $p \leq 0.05$.

atopic children (Table 4). Cord blood IL-8 in response to LPS (24 h) was lower among children who were sensitized against food allergens (atopic, median 67 800; non-atopic, median 84 400; $p = 0.03$). Food atopy of the children was not associated with cytokine responses at the age of 1 yr.

Associations between cytokine responses and atopic sensitization of mothers

Sensitization against both inhaled and food allergen was detected in 29 mothers, whereas sensitization against only food allergens or only

inhalation allergens was detected in 14 and 80 of 208 mothers, respectively. 85 of 208 mothers were not sensitized against any of the measured allergens. Associations between cytokine responses and atopy were slightly different between 24 h and 48 h stimulation. Therefore, results from both time-points are presented.

Maternal atopy against both inhaled and food allergen was not consistently associated with children's cytokine-producing capacity. Only 48 h LPS-stimulated levels of IL-10 in cord blood were associated with maternal inhalation and food atopy (atopic, median 516; non-atopic, median 396; $p = 0.04$). Some associations were

Table 4. Median values of 48 h P/I, SEB and LPS-stimulated cytokine responses (pg/10⁶ wbc) of children in relation to atopic sensitization against food allergens only

		Children's food atopy			Maternal food atopy		
	n	No 131	Yes 27	p-value	No 85	Yes 14	p-value
Cord blood							
P/I	IL-5	71.0	78.7	0.76	63.6	79.8	0.36
	IL-10	11.0	13.5	0.27	9.87	13.9	<0.01
	TNF- α	855	835	0.89	823	753	0.98
	IFN- γ	3710	2420	0.43	2980	2800	0.79
	IL-8	207 000	203 000	0.27	251 000	293 000	0.23
SEB	IFN- γ /IL5	59.5	39.3	0.48	57.6	39.2	0.29
	IL-5	0.00	0.00	0.97	0.00	1.63	0.20
	IL-10	7.53	7.55	0.90	8.02	16.3	0.59
	TNF- α	24.1	17.3	0.50	22.1	28.8	0.38
	IFN- γ	113	38.6	<0.01	96.5	116	0.32
LPS	IL-8	18 600	8450	0.10	17 300	16 500	0.53
	IFN- γ /IL5	22.5	1.41	0.01	21.3	3.66	0.60
	IL-5	n.a.	n.a.		n.a.	n.a.	
	IL-10	406	352	0.62	396	471	0.08
	TNF- α	13.0	0.00	0.53	11.5	15.0	0.99
	IFN- γ	n.a.	n.a.		n.a.	n.a.	
	IL-8	140 000	81 500	0.06	140 000	139 000	0.87
	IFN- γ /IL5	n.a.	n.a.		n.a.	n.a.	
Children, 1 yr after birth							
P/I	IL-5	132	192	0.16	117	159	0.54
	IL-10	66.4	71.4	0.28	68.6	71.2	0.55
	TNF- α	1390	1530	0.54	1440	1800	0.70
	IFN- γ	15 100	17 100	0.65	16 400	11 000	0.02
	IL-8	82 700	68 500	0.47	84 200	79 400	0.88
SEB	IFN- γ /IL5	142	86.3	0.41	168	61.7	<0.01
	IL-5	51.5	76.5	0.36	53.6	68.3	0.81
	IL-10	78.0	80.3	0.73	74.1	80.5	0.66
	TNF- α	283	221	0.14	280	264	0.95
	IFN- γ	675	532	0.42	873	575	0.34
LPS	IL-8	47 500	56 700	0.88	56 600	48 300	0.62
	IFN- γ /IL5	12.5	7.57	0.07	13.9	8.90	0.50
	IL-5	n.a.	n.a.		n.a.	n.a.	
	IL-10	518	531	0.37	532	635	0.06
	TNF- α	169	161	0.64	197	194	0.86
	IFN- γ	0.00	0.00	0.86	0.00	0.00	0.11
	IL-8	98 100	97 900	0.90	85 900	118 000	0.34
	IFN- γ /IL5	n.a.	n.a.		n.a.	n.a.	

IFN- γ /IL5 is ratio of IFN- γ to IL-5. Subjects with atopy against inhaled allergens excluded.

SEB, Staphylococcal enterotoxin B; LPS, lipopolysaccharide; P/I, combination of phorbol ester and ionomycin; n.a., not applicable, more than 80% of the values are zeroes. Bold indicates statistically significant at level $p \leq 0.05$.

found between maternal inhalation atopy and cord blood cytokines: In both 24 h and 48 h stimulation, P/I- induced cord blood IL-5 was higher (24 h: atopic, median 40.0; non-atopic, median 27.7; $p < 0.01$ and 48 h: Table 3) and 24 h LPS -stimulated TNF- α lower (atopic, median 70.0; non-atopic, median 99.7; $p = 0.02$) in children of mothers with inhalation atopy. In addition, cord blood IL-10 levels in response to P/I were higher in children whose mothers were sensitized to inhaled allergens (Table 3). Maternal inhalation atopy was not associated with cytokine responses of 1-yr-old children.

Maternal sensitization against food allergens was associated with increased cord blood IL-10 responses following 48 h P/I stimulation (Table 4). P/I -stimulated (24 h) ratio of IFN- γ to IL-5 in cord blood was lower among children of food atopic mothers (atopic, median 43.7; non-atopic, median 94.3; $p = 0.01$). At the age of 1 yr, decreased P/I -stimulated IFN- γ responses were found in children whose mothers were diagnosed as food atopic (24 h: atopic, median 4690; non-atopic, median 9050; $p < 0.01$ and 48 h: Table 4). Maternal food atopy was associated also with lower P/I -stimulated IFN- γ /IL-5 ratio at the age of 1 yr (24 h: atopic, median

76.3; non-atopic, median 160; $p < 0.01$ and 48 h: Table 4).

Maternal atopy against both inhaled and food allergens was inversely associated with their own TNF- α responses following 48 h SEB stimulation (atopic, median 407; non-atopic, median 660; $p = 0.03$). Decreased IFN- γ /IL-5 ratios following 24 h P/I and SEB stimulation were found in mothers with inhalation atopy (P/I: atopic, median 227; non-atopic, median 296; $p = 0.01$ and SEB: atopic, median 53.8; non-atopic, median 91.0; $p = 0.04$). Similarly, maternal food atopy was associated with lower ratios of IFN- γ to IL-5 in 24 h P/I stimulation (atopic, median 168; non-atopic, median 296; $p < 0.01$) and in SEB stimulation (24 h: atopic, median 39.0; non-atopic, median 91.0; $p < 0.01$ and 48 h: atopic, median 30.4; non-atopic, median 44.4; $p = 0.04$).

Discussion

Our findings extend the results of previous studies, but also provide new information about the development of immune responses during the first year of life when immunologic events seem to play an important role, not only in the maturation process of immune functions, but also in the development of allergic disorders (6). We found significant mother-to-child cytokine correlations at the age of 1 yr. Moreover, opposite to other measured cytokines, the stimulated levels of IL-8 decreased from birth to 1 yr of age. Our results also support previous reports suggesting, that although cytokine responses increase from birth to 1 yr of age, immune functions during the first year of life are still immature compared to adulthood. In addition, we found some associations between allergic sensitization and the cytokine-producing capacity, which suggest higher T_H2 -cytokine responses in children of atopic mothers.

To evaluate cytokine-producing capacity in children at birth and at the age of 1 yr and also in their mothers, whole blood samples were stimulated with the combination of protein kinase C activating PMA and the ionophore ionomycin (P/I) to induce adaptive responses, with superantigen Staphylococcal enterotoxin B (SEB) to provoke both adaptive and innate immune responses and with the component of the Gram-negative bacterial cell wall, LPS, to activate innate immunity.

We found that newborns' adaptive immune system responds deficiently to P/I stimulation, indicated as low production of IL-5, IL-10 and IFN- γ at birth. These responses increased during

the first year of life, but remained still weaker in comparison to adults. Although P/I is regarded mainly as T-cell stimulant it can also stimulate other lymphocytes like monocytes (17). Similarly to adaptive responses, P/I –stimulated production of proinflammatory cytokine TNF- α increased with age. Our results suggesting the increase in both adaptive and innate immune responses from birth to adulthood confirm findings from previous studies showing an intracellular IFN- γ and TNF- α expression following P/I stimulation in whole blood (11) and in lymphocytes (18) of newborns, children and adults. Weak IFN- γ responses at birth may be a result of maternal cytokine environment during pregnancy: maternal cytokine responses during gestation are T_H2 -biased because the production of T_H1 cytokines is deleterious for successful pregnancy causing fetal loss (8). It is thus possible that maternal bias away from T_H1 responses during pregnancy may result in similar cytokine pattern also in newborn indicated as a low level of IFN- γ production at birth. Interestingly, P/I –stimulated IL-8 responses were strongest at birth. T cells are not able to produce IL-8 in response to P/I even in the presence of accessory cells (17). Thus, high level of P/I –stimulated IL-8 is probably produced by other PBMCs than T cells, especially monocytes (17), which have been identified as the major source of IL-8. Levels of cord blood IL-8 are known to be increased by birth stress initiated in normal labor (13). Thus, high levels of IL-8 in cord blood in response to P/I may be due to 'pretreatment' of cord blood white blood cells with birth stress.

We found that SEB-stimulated TNF- α and IL-10 profiles in mothers and children were similar to those induced by P/I. SEB-stimulated responses of IL-5 were at the same level in both mothers and their children 1 yr after birth but weaker in cord blood. Also IFN- γ production was poor in cord blood but increased with age being the highest in maternal blood samples. Because IL-5 (T_H2), IL-10 (T_{reg} s) and IFN- γ (T_H1) are known to be produced by T cells, our results further support the view that adaptive responses are deficient at birth and that they increase with age. Our findings are also in line with the study of Smart et al. (19), which showed that the production of IL-5 and IFN- γ in response to SEB was significantly related to the age in children from birth to 16 yrs. SEB is able to activate both adaptive T-cell responses and antigen presenting cells (APCs) in innate immunity by binding to T-cell receptor (TCR) and, at the same time, to major histocompatibility class

II complex (MHC II) molecule on surface of APCs.

Our study shows that LPS-stimulated levels of IL-10 and TNF- α were highest in maternal samples and lowest in cord blood, which may indicate the development of innate immune responses from birth to adulthood. In line with our results, LPS-stimulated cord blood leukocytes are demonstrated to produce less IL-10 (20) and TNF- α than those of adults (5, 20, 21). Interestingly, cytokine responses measured in mononuclear cell lines following LPS stimulation seem to be not consistent, because opposite results for TNF- α and IL-10 production are also reported (22). However, results suggesting poor neonatal innate immune responses are supported by earlier reports (3). Deficiencies in newborn's innate immune functions may be partly due to impaired Toll-like receptor (TLR) responses to different ligands as demonstrated by Levy et al. (5). Effects of LPS are mediated via TLR4 and it seems that TLR4-mediated signaling in newborn mononuclear cells following LPS stimulation is impaired in comparison to adult cells (5, 21). Even though IL-10 is principally secreted by T_{reg}S, also cells of innate immune system like macrophages are capable of producing IL-10. It is thus possible that the cells of innate immune system are responsible for the LPS-induced production of IL-10. All together, our results suggest that compared to adults, neonatal immune system responds deficiently to stimulants originating from Gram-negative (LPS) and Gram-positive (SEB) bacteria.

In our study, significant mother-to-child correlations were detected in all measured cytokines 1 yr after birth. In line, stimulated IFN- γ and IL-10 responses have been shown to correlate between mothers and their 2-yr-old children (23). Our previous paper, showed significant mother-to-child correlations in mitogen-stimulated production of proinflammatory cytokines (TNF- α and IL-6), but not in IFN- γ responses 3 months after birth (24). Poor IFN- γ responses in infancy may be a reason why child's IFN- γ production begins to correlate with that of the mother only later in infancy. Good mother-to-child cytokine correlations in general may be partly due to similar living environments and habits of mothers and their children. Correlations between children's cytokine responses at birth and 1 yr of age were weak in comparison to mother-to-child correlation 1 yr after birth. Poor cytokine correlations between birth and 1 yr of age probably indicate changes in cytokine balance during the first year of life.

Previous studies have suggested that children with atopic heredity express T_H2-biased cytokine responses (25–27). Also in our study, depending on the type and the duration of stimulation, maternal inhalation as well as food atopy were associated with higher cord blood IL-5 and IL-10 responses in their offspring. Further, children of food allergic mothers had decreased IFN- γ responses at the age of 1 yr, and they also expressed lower ratio of IFN- γ to IL-5 at birth and at the age of 1 yr than offspring of non-allergic mothers. One should also keep in mind that it is likely that cytokine responses of children are affected not only by atopic/non-atopic heredity itself but also several different environmental and maternal factors including, smoking, nutrition, lactation, intra-uterine infections and cytokine environment during gestation.

Previous reports have shown that immune responses of atopic children are biased towards humoral T_H2-responses, away from cell-mediated T_H1-responses (6, 28, 29), but in our study IFN- γ /IL-5 ratio at the age of 1 yr was higher in those individuals who were sensitized against both inhaled and food allergens. Similarly, P/I-stimulated IL-5 responses were lower in 1-yr-old children with both inhalation and food atopy compared to their non-atopic peers. Because immune system goes through major changes during the first year of life and also at birth, it is possible that the skewing towards atopic cytokine responses continues beyond childhood as suggested by Prescott et al. (6) and thus associations between atopic status and cytokine-producing capacity from birth to 1 yr of age do not necessary describe the true atopic/non-atopic cytokine responses. Lack of associations between atopic status and cytokine responses in children in the present study may also be due to the poor predictive value of IgE levels at the age of 1 yr for later development of atopy (30). In maternal samples, food and inhalation atopy separately were associated with lower ratio of IFN- γ to IL-5. Thus it seems that in addition to considering cytokines independently, the calculation of cytokine ratios may be an appropriate additional way to interpret the functions of immune system. However, our results demonstrate that more investigation is needed to clarify how atopy affects the cytokine responses at early age.

In conclusion, stimulation of whole blood samples with three different stimulants suggests that both adaptive and innate immune responses are deficient in infancy. Although clear augmentation in cytokine production is detected during

the first year of life, it is still weaker in comparison to adulthood. Even though children had lower absolute levels of cytokines at the age of 1 yr compared to their mothers, strong mother-to-child cytokine correlations indicate that already at the age of 1 yr children begin to express similar cytokine pattern than their mothers. Few associations found between atopic sensitization and cytokine production suggest that children of atopic mothers may have stronger bias towards T_H2 responses already at birth. First year of life is extremely a crucial period for immunologic development and should be investigated in more detail in further studies.

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