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Production of interleukin-5, -10 and interferon-γ in cord blood is strongly associated with the season of birth

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Summary

Background The effect of labour and different labour-related factors on the cord blood (CB) cell cytokine production is still relatively unknown.

Objective To study the relationships between the production of IL-5, IL-10 and IFN-γ in CB samples and maternal, early neonatal and birth-related factors.

Methods Whole-blood samples were collected after birth ($n = 423$) and they were stimulated for 24 and 48 h with a combination of phorbol ester and ionomycin. Production of IL-5, IL-10 and IFN-γ was determined using ELISA. Maternal, early neonatal and birth-related variables were recorded prospectively during pregnancy, and during and after delivery.

Results After multivariable adjustment for confounders, the strongest predictor of IL-5, IL-10 and IFN-γ production in CB cell samples was the season of birth. Children born in the spring had significantly lower cytokine responses compared with those born in the fall. IL-5 production was inversely associated with female gender of the child and maternal smoking. If corrections for white blood cell (WBC) counts were not performed, IL-5 production was also significantly associated with the mode of delivery. Respectively, the production of IL-10 and IFN-γ was inversely associated with prostaglandin induction before birth.

Conclusion Environmental exposure to pollen and ultraviolet irradiation during gestation may have an effect on the cytokine profile of the offspring in CB because children born in the spring or winter showed the lowest IL-5, IL-10 and IFN-γ responses. The production of IL-10 and IFN-γ was also inversely associated with prostaglandin labour induction before birth. Other labour-related factors were not significantly associated with production of IL-5, IL-10 and IFN-γ after WBC count correction.

Keywords birth, cord blood, gender, IFN-γ, induction, IL-10, IL-5, intrapartum, leukocytosis, prostaglandin, season of birth, WBC
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Introduction

Cord blood (CB) cytokine values have been increasingly used as prognostic tests when assessing neonatal immunological development and future lifetime risk for various allergic diseases among offspring [1–4]. However, parturition both at term and preterm is an inflammatory process and is associated with an increment of different markers of inflammation such as peripheral leukocytosis, granulocytosis and increased production of various different cytokines in the maternal compartment [5–7]. The effect of mode of delivery on inflammatory mediators are not well studied in the fetal compartment, although it is known that leukocytosis is observed in CB samples after normal vaginal deliveries [8, 9] and the levels of many Th1-type cytokines such as IL-6, IL-8, TNF-α and IFN-γ are increased in CB samples after spontaneous delivery [6, 9–12].

IL-5 is a key regulator of eosinophil production in humans and has a pivotal role in the innate and acquired immune response, and is further associated with a wide variety of conditions and diseases, including asthma and atopic disorders, elevated levels of IgE and eosinophil-dependent inflammatory diseases [13]. IL-10 is a well-known anti-inflammatory and immunoregulatory

cytokine, mainly released by, and acting on cells of the immune system such as monocytes, macrophages, T cells, NK cells and B cells. During gestation, IL-10 is also widely produced by chorion, decidual and trophoblast tissues; moreover, up to term pregnancy, decidual mononuclear cells are also known to secrete IL-10 and IFN-γ spontaneously in addition to many other Th1 and Th2 cytokines, and especially after stimulation [14, 15]. Mode of birth may also have an influence on IFN-γ and IL-10 responses [12, 16].

CB samples are readily available after delivery and their significance has been evaluated in numerous immunological and epidemiological studies. Nevertheless, the effect of labour and different labour-related factors on the CB cytokine production and further on the immunological development is still relatively unknown. The aim of this study was to evaluate the associations between different maternal, fetal and birth-related obstetric factors and the fetal production of IL-5, IL-10 and IFN-γ in CB samples following cell stimulation with staphylococcal enterotoxin B (SEB), lipopolysaccharide (LPS), and with the combination of phorbol ester and ionomycin (P/I).

Methods

Study population

The first half of the study population ($N = 195$) consisted of Finnish participants in an ongoing international birth cohort study (PASTURE; Protection against Allergy – study in Rural Environments), which is evaluating the association between a farming environment and the development of allergic diseases among offspring [17, 18]. This cohort has been prospectively followed up from the third trimester of pregnancy and is reported in more detail in earlier reports [18–20]. Study population was retrieved from four different Finnish hospitals (Kuopio, Iisalmi, Joensuu and Jyväskylä), all situated in the central or eastern part of Finland.

In the second half of the cohort (the extended Finnish cohort) ($N = 228$), all pregnant women with an estimated delivery at Kuopio University Hospital between May 2004 and May 2005, were invited to the study at 32 weeks of gestation [18, 21]. There was no selection by occupation or area of abode; however, subjects living in apartment buildings were excluded to make the building stock comparable between the two parts of the cohort. Otherwise, the inclusion and exclusion criteria were the same as described as in the Finnish PASTURE. All the children were born between September 2002 and May 2005 (PASTURE study: September 2002–May 2004; Extended cohort: May 2004–May 2005).

Maternal and perinatal data

Data on maternal weight gain, smoking, education and parity were obtained from questionnaires. Maternal allergic disease was identified from pregnancy questionnaire data on doctor-diagnosed asthma, hayfever or atopic eczema at any time in life. The first questionnaire was administered during the third trimester of pregnancy, and follow-up data were collected when children were 2 months old. The details of the questionnaires have been described elsewhere [20]. Midwives collected intrapartal information during delivery. Information on clinical factors included gestational age, prostaglandin induction before delivery, the duration of labour and ruptured membranes before birth, the use of oxytocin augmentation during delivery, the quality of amniotic fluid at delivery (clear/other or meconish) and the mode of delivery (spontaneous, assisted vaginal, elective Caesarean or non-elective Caesarean). Neonatal data included gender, birth weight, 5 min Apgar scores and suspicion of congenital neonatal infection at the labour ward.

Phorbol ester and ionomycin, staphylococcal enterotoxin B and lipopolysaccharide stimulated production of cytokines in cord blood

All whole-blood samples were stimulated by using a standardized protocol at the Environmental Toxicology Unit, at the Department of Environmental Health, National Institute for Health and Welfare in Kuopio. Heparinized CB was diluted 1 : 8 with RPMI-1640 with Glutamax I (Gibco, Paisley, UK) cell culture medium [supplemented with 1% antibiotic–antimycotic from Gibco and 10% heat-inactivated FBS Gold from PAA Laboratories GmbH (Pasching, Austria)] and stimulated at +37 °C, 5% CO_2 in 24-well plates (BD Labware, Bedford, MA, USA) in duplicate wells using three different stimulation (+control) for 24 and 48 h: SEB (final concentration 100 ng/mL), LPS (100 ng/mL) and the combination of phorbol 12-myristate 13-acetate (PMA) (5 ng/mL) and ionomycin (1 μg/mL) (P/I). Stimuli for Finnish PASTURE were provided by the PASTURE central laboratory at the Department of Clinical Chemistry and Molecular Diagnostics in Philipps-University of Marburg (Marburg, Germany). For the extended Finnish cohort, stimuli were acquired from Sigma Chemicals (St Louis, MO, USA). After incubation, blood cultures were 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 IL-5, IL-10 and IFN-γ were measured immunochemically by using a sandwich 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, while the number of washes was increased). Ranges of valid cytokine measurements were as

follows: IL-5 (4.7–300 pg/mL), IL-10 (6.3–400 pg/mL) and IFN-γ (12.5–800 pg/mL). Only the linear part of the standard curve was accepted. Individual CB white blood cell (WBC) counts were determined from EDTA–blood using the Sysmex KX-21N blood cell analyzer (Sysmex Corporation, Kobe, Japan) in the Environmental Toxicology Unit in Kuopio Local Study Center. Because P/I stimulation yielded the highest number of detectable cytokine levels in general, it was chosen for further analysis.

Detectable CB cytokine values were ln-transformed, and approximate normal distribution was confirmed. Further, cytokine data were also transformed into Δ-values (stimulated cytokine values−control values) and standardized for the number of leucocytes (picograms/one million WBCs, pg/10^6 WBC) (Tables 3 and 4). This was carried out to avoid possible confounding from individual variations in baseline cytokine production.

If the stimulated value was smaller than the control value, a negative Δ-value was replaced with a value of 0 pg/mL. Both cytokine ln-transformed-values and WBC standardized cytokine Δ-values were used in analyses.

Statistical analysis

The data were analysed using the statistical package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 17.0 for Windows. Comparisons of the prevalence of different maternal and prenatal factors and proportions of two different birth cohorts in univariate analysis were performed with the χ^2-test and continuous variables by the Mann–Whitney *U*-test. Comparisons of cytokine values between different explanatory variables in univariate analysis were evaluated by the Kruskal–Wallis test. Covariance analysis was used to investigate the relationships between the production of specific cytokine values and the wide variety of different possible confounders and clinical factors, such as maternal age at delivery ($\leqslant$28, 29–33 vs. $>$33 years), maternal parity (0, 1 vs. $\geqslant$2), maternal weight gain during pregnancy ($<$12, 12–16 vs. $>$16 kg), maternal smoking during pregnancy (smoker vs. earlier, but not during pregnancy vs. never smoking), maternal allergic diseases (no/yes), maternal education (basic, middle vs. academic), gestational age at delivery (35–39, 40 vs. $\geqslant$41 gestational weeks), birth induction with prostaglandin (no/yes), oxytocin induction or augmentation (no/yes), duration of labour (0–30 min, 30 min–2 h, 2 h–6 h and $>$6 h), duration of ruptured fetal membranes ($<$2 h, 2–6 h and $>$6 h), the mode of birth (spontaneous vaginal, assisted vaginal, non-elective vs. elective Caesarean), season of birth (March–May, June–August, September–November, December–February), fetal gender (female/male), birth weight ($\leqslant$3470, 3471–3820 vs. $>$3820 g), suspicion of congenital neonatal infection at the labour ward (no/yes), Apgar scores at 5 min ($\leqslant$7 vs. $>$7), paternal farming (farmer vs. non-farmer) and the cohort (PASTURE/extended). The cohort, paternal farming and maternal allergy variable were always included in the multivariate covariance analyses. Besides this, all variables that were significant in the univariate analysis at $P<0.10$ were included simultaneously in the covariance analysis. If the proportion of missing values exceeded 5% for any case, the remaining data were included in the analysis as a separate group. The cut-off level for statistical significance was 0.05.

Results

There were significant differences in many characteristics between the study cohorts (Table 1); thus, an indicator variable for the cohort was always included in the multivariate analyses. The cytokine responses with a higher percentage of samples over the lower detection limit were included for further analysis (i.e. IL-5 after 48 h, IL-10 after 24 h and IFN-γ after 48 h) (Table 2).

Specific neonatal and maternal variables that were associated at least with bordering significance ($P<0.10$) with WBC counts and cytokine responses (median values) in the univariate analysis are shown in Table 3. Fetal gender, maternal smoking, hayfever and paternal farming were associated significantly with the IL-5 production even after controlling by WBC counts. Similarly, levels of IL-10 were associated significantly with the birth weight, maternal education and paternal farming (without WBC standardization) and levels of IFN-γ were associated significantly with the paternal farming (with WBC standardization). In contrast, maternal allergic diseases altogether or asthma or eczema separately were not related to IL-5, IL-10 or IFN-γ levels in the univariate tests. Thus, only maternal hayfever was included in the further analyses.

Table 4 shows associations ($P<0.10$) between WBC counts and cytokine levels and obstetric variables and season of birth. Children born in the spring had significantly lower cytokine responses compared with those born in the fall (Fig. 1). The mode of delivery and duration of labour were associated significantly with the production of IL-5 and IFN-γ; however, no significant association was detected longer after WBC count standardization. In separate univariate analysis of children who were delivered by vaginal route ($N=371$), the production of IL-5 and IFN-γ tended to be higher ($P<0.049$ and $P<0.069$) and the production of IL-10 tended to be lower ($P<0.073$) even after WBC count standardization among children who were delivered by assistance (mainly by vacuum delivery) compared with those who were delivered spontaneously.

In general, the median levels of IL-10 and IFN-γ after WBC count correction were lower in neonates whose mothers had had prostaglandin induction before delivery compared with those without (Table 4). No other

Table 1. Maternal and early neonatal characteristics for the two study cohorts

Characteristics	Finnish pasture (*N* = 195)	Extended cohort (*N* = 228)	*P*-value
Nulliparous	43/195 (22.1)	104/228 (45.6)	0.0001
Maternal age (years)	32.0 (4.9)	30.1 (5.0)	0.0001
Maternal smoking			
Never	102/195 (52.3)	127/228 (55.7)	0.485
Not during pregnancy	63/195 (32.3)	60/228 (26.3)	0.177
During pregnancy	30/195 (15.4)	41/228 (18.0)	0.477
Maternal weight gain during pregnancy (kg)	13.9 (5.4)	14.4 (5.5)	0.27
Maternal asthma	12/195 (6.2)	19/228 (8.3)	0.391
Maternal hay fever	43/195 (22.1)	56/228 (24.6)	0.543
Maternal atopic eczema	23/195 (11.8)	62/228 (27.2)	0.0001
Any maternal allergic disease	60/195 (30.8)	104/228 (45.6)	0.002
Father as a farmer	103/195 (52.8)	11/228 (4.8)	0.0001
Gestational age at delivery (weeks)	39.5 (1.1)	39.6 (1.2)	0.459
Mode of delivery			
Spontaneous vaginal	162/195 (83.1)	183/228 (80.3)	0.457
Assisted vaginal	8/195 (4.1)	18/228 (7.9)	0.106
Elective caesarean	13/195 (6.7)	17/228 (7.5)	0.753
Nonelective caesarean	12/195 (6.2)	10/228 (4.4)	0.415
Duration of labour (hours)*	5.9 (4.9)	8.6 (5.6)	0.0001
Prostaglandin induction	27/195 (13.8)	24/228 (10.5)	0.296
Oxytocin augmentation during delivery	103/195 (52.8)	136/227 (59.9)	0.143
Meconium in amniotic fluid	17/192 (8.9)	37/216 (17.1)	0.048
Male sex	94/195 (48.2)	122/228 (53.5)	0.277
Birth weight	3655 (470)	3640 (467)	0.754
Apgar scores, 5 min	9.0 (0.8)	9.0 (0.5)	0.466
Suspicion of neonatal infection in labor ward	4/192 (2.1)	10/227 (4.4)	0.188
Birth in spring	74/195 (37.9)	51/228 (22.4)	0.0001
Birth in summer	14/195 (7.1)	53/228 (23.2)	0.0001
Birth in fall	54/195 (27.6)	62/228 (27.2)	0.909
Birth in winter	53/195 (27.2)	62/228 (27.2)	0.998
WBC count, *10E9/L cordblood	13.0 (3.9)	14.4 (4.0)	0.0001

*Including only those undergone vaginal delivery.

Data are presented as mean (SD) or number (%).

P-values are determined by Mann–Whitney *U*-test or χ^2-test (Pearson).

WBC, white blood cell.

Table 2. Production of IL-5, Il-10 and IFN-γ after P/I stimulation and follow-up period in cord blood samples

	Total *N*	Samples within detection limits % (*N*) 24 h	Median±SD (pg/mL)	Total *N*	Samples within detection limits % (*N*) 48 h	Median±SD (pg/mL)
IL-5						
Spontaneous production	423	0 (0)	0±0	361	0 (0)	0±0
P/I	421	94.1 (396)	49.0±53.5	359	**96.1 (345)**	92.5±108
IL-10						
Spontaneous production	420	3.1 (13)	0±50.4	356	6.7 (24)	0±10.1
P/I	419	**85.7 (359)**	24.9±34.1	355	75.8 (269)	17.9±76.6
IFN-γ						
Spontaneous production	418	0 (0)	0±0	351	0 (0)	0±0
P/I	413	95.6 (395)	4320±6670	345	**97.1 (335)**	4990±11 300

Bold indicates stimulation, which were selected for further analysis.

examined mother- or birth-related factors (weight gain, gestational age, duration of ruptured fetal membranes, the use of oxytocin during labour, suspicion of congenital neonatal infection at the labour ward) were associated with the production of IL-5, IL-10 and IFN-γ in univariate analysis (data not shown).

Table 5 shows the estimated selected coefficients from the multivariate covariance analysis to predict IL-5, IL-10

Table 3. Association between selected maternal and neonatal variables on white blood cell counts and cytokine responses in cord blood

		WBC counts	IL-5		IL-10		IFN-γ	
	N	X10E9/L	48 h, pg/mL	pg/10E6 WBC	24 h, pg/mL	pg/10E6 WBC	48 h, pg/mL	pg/10E6 WBC
Neonatal factors								
Gender								
Male	216	13.2 (4.0)	108.3 (118.8)	69.0 (57.9)	25.4 (41.4)	14.4 (20.7)	5000 (11 658)	3019 (5681)
Female	207	13.8 (4.1)	80.8 (93.0)	48.0 (47.7)	24.6 (24.1)	13.6 (16.1)	4944 (10 935)	2926 (5156)
P-value		*0.135*	***0.004***	***0.0001***	*0.819*	*0.567*	*0.270*	*0.109*
Birth weight (g)								
I tertile (−3470)	142	12.5 (3.7)	83.5 (100.1)	54.5 (55.6)	19.5 (19.4)	12.6 (14.1)	4331 (13 230)	3208 (6103)
II tertile (3471–3820)	141	13.2 (4.0)	100.5 (120.2)	60.4 (57.1)	28.0 (39.6)	15.5 (19.9)	5053 (11 322)	3337 (5456)
III tertile (3821-)	138	14.6 (4.2)	95.2 (97.6)	58.7 (45.6)	25.8 (39.3)	13.9 (21.2)	4933 (8934)	2580 (4554)
P-value		***0.0001***	*0.197*	*0.483*	***0.007***	***0.072***	*0.713*	*0.104*
Apgar scores, 5 min								
⩽7	11	16.7 (4.4)	160.6 (118.8)	80.9 (57.3)	36.5 (19.6)	18.5 (11.4)	8603 (12 738)	4121 (5424)
8-	411	13.3 (4.0)	91.3 (107.3)	56.5 (53.9)	24.6 (34.4)	13.8 (18.8)	4919 (11 256)	2954 (5430)
P-value		*0.115*	***0.077***	*0.132*	***0.067***	*0.216*	***0.030***	***0.082***
Maternal factors								
Parity								
0	147	14.3 (4.0)	106.1 (126.4)	61.4 (59.4)	27.0 (21.8)	13.6 (14.7)	5829 (16 619)	3508 (7657)
1	142	13.5 (4.4)	98.0 (88.2)	58.7 (47.8)	24.4 (38.1)	14.0 (16.7)	4434 (6102)	2854 (3217)
⩾2	134	11.9 (3.4)	80.6 (105.2)	55.6 (54.9)	24.5 (40.4)	14.6 (23.6)	4450 (7528)	3015 (4163)
P-value		***0.0001***	***0.054***	*0.399*	*0.527*	*0.768*	***0.019***	***0.068***
Age (years)								
I tertile (-28)	141	14.3 (4.0)	106.1 (109.1)	60.0 (53.0)	26.8 (36.7)	14.3 (15.8)	5127 (11 926)	3030 (5615)
II tertile (29-33)	150	12.7 (3.9)	96.1 (107.7)	62.6 (53.5)	24.8 (24.5)	13.8 (16.3)	4719 (10 172)	2951 (5165)
III tertile (34-)	132	13.3 (4.1)	84.0 (106.6)	48.7 (56.0)	23.0 (40.3)	13.3 (23.4)	4353 (11 732)	2968 (5525)
P-value		***0.007***	*0.181*	*0.316*	*0.125*	*0.361*	***0.079***	*0.401*
Maternal hay fever								
No	324	13.4 (4.1)	91.1 (103.5)	54.5 (53.9)	24.6 (37.2)	13.8 (20.0)	4919 (11 785)	2941 (5675)
Yes	99	13.3 (3.7)	109.5 (120.1)	67.0 (53.8)	25.8 (21.0)	13.9 (13.1)	5060 (9729)	3386 (4634)
P-value		*0.983*	*0.115*	***0.050***	*0.251*	*0.285*	*0.261*	*0.227*
Maternal smoking								
Never	229	13.3 (4.5)	94.9 (110.6)	58.4 (55.4)	25.5 (35.1)	14.2 (21.2)	5056 (13 040)	3113 (6173)
Earlier, but not during pregnancy	123	13.7 (3.3)	108.9 (103.0)	65.4 (48.8)	23.4 (38.7)	13.7 (16.9)	5134 (8395)	3254 (4106)
During pregnancy	71	13.3 (3.5)	67.1 (105.1)	40.6 (56.4)	23.9 (18.5)	14.2 (10.1)	4030 (9610)	2605 (4883)
P-value		*0.928*	***0.010***	***0.003***	*0.404*	*0.394*	***0.062***	*0.156*
Maternal education								
Low	143	13.8 (3.5)	86.7 (101.6)	55.9 (52.0)	22.9 (37.8)	13.7 (16.9)	4318 (11 067)	2668 (5610)
Middle	195	13.2 (4.0)	91.3 (107.1)	59.5 (54.3)	24.5 (33.8)	13.9 (20.0)	5183 (12 063)	3312 (5487)
Academic	85	13.3 (4.8)	102.9 (120.1)	79.9 (57.0)	29.0 (27.6)	14.9 (18.2)	4950 (9722)	2851 (4979)
P-value		*0.888*	*0.394*	*0.358*	***0.018***	***0.088***	*0.113*	*0.117*
Father as farmer								
No	309	13.7 (4.0)	107.5 (113.8)	63.2 (56.5)	25.8 (28.4)	14.3 (16.7)	5047 (12 858)	3058 (6084)
Yes	114	12.5 (4.1)	73.5 (107.9)	45.6 (43.3)	20.2 (46.5)	12.3 (23.1)	4318 (11 312)	2872 (2491)
P-value		***0.028***	***0.0001***	***0.0001***	***0.022***	*0.127*	***0.002***	***0.006***

Cytokines are expressed as media values (SD).
P-values are estimated by Kruskal–Wallis test.
Bold indicates $P < 0.10$.

and IFN-γ production after different stimuli. In total, the production of cytokines in whole-blood samples was associated significantly with the season of birth (with *P*-values of 0.007–0.0001) and cohort (with *P*-values of 0.001), and mostly significantly with CB WBC counts (with *P*-values of 0.168–0.0001). Further, IL-5 response was also associated significantly with maternal smoking during pregnancy ($P < 0.001$) and gender of the child ($P < 0.001$). With regards to IL-10, the production was associated significantly with the prostaglandin induction ($P < 0.002$), maternal education (low vs. academic; $P < 0.04$) and birth weight (I vs. III tertiles; $P < 0.02$) in multivariate adjustments. A similar significant factor in the production of IFN-γ was prostaglandin induction ($P < 0.005$).

Table 4. Association between selected obstetric variables on white blood cell (WBC) counts and cytokine responses in cord blood

		WBC counts	IL-5		IL-10		IFN-γ	
	N	X10E9/L	48 h, pg/mL	pg/10E6 WBC	24 h, pg/mL	pg/10E6 WBC	48 h, pg/mL	pg/10E6 WBC
Induction with prostaglandin								
No	372	13.2 (4.0)	94.3 (110.0)	57.4 (55.0)	24.9 (35.5)	14.0 (19.3)	5041 (11 675)	3072 (5566)
Yes	51	14.0 (4.1)	74.3 (88.8)	55.9 (45.7)	25.5 (20.0)	10.7 (10.9)	4000 (7792)	1978 (4207)
P-value		*0.324*	*0.299*	*0.246*	*0.237*	***0.041***	***0.035***	***0.028***
Mode of delivery								
Spontaneous vaginal	345	13.5 (4.0)	94.3 (104.2)	58.4 (52.1)	25.4 (36.6)	14.0 (19.2)	5000 (10 456)	2954 (5030)
Assisted vaginal	26	15.4 (3.5)	174.9 (117.1)	81.9 (61.8)	23.4 (16.8)	12.8 (8.6)	10 917 (16 831)	7068 (7972)
Elective caesarean	30	11.0 (3.9)	65.0 (75.7)	50.6 (51.0)	20.9 (13.3)	15.3 (14.3)	4051 (3394)	3058 (2585)
Nonelective caesarean	22	13.0 (3.9)	80.7 (160.5)	42.7 (74.5)	24.9 (26.2)	13.6 (22.5)	4081 (17 730)	2516 (8603)
P-value		***0.0001***	***0.007***	*0.198*	*0.587*	*0.240*	*0.188*	*0.320*
Duration of labour								
0–30 min	35	11.2 (4.0)	70.0 (89.4)	50.6 (52.1)	21.6 (14.2)	15.2 (13.7)	4040 (3621)	3035 (2525)
30 min–2 h	134	12.3 (3.3)	78.6 (97.1)	54.5 (51.6)	24.2 (40.1)	14.2 (22.0)	4331 (7008)	2891 (3839)
2–6 h	104	13.8 (4.3)	97.3 (97.4)	60.0 (48.7)	24.0 (28.6)	12.8 (19.6)	4890 (8532)	2585 (4108)
>6 h	148	14.8 (4.0)	115.9 (124.2)	63.7 (60.0)	26.9 (35.3)	13.9 (15.6)	5614 (16 000)	3532 (7465)
P-value		***0.0001***	***0.003***	*0.186*	*0.147*	*0.633*	***0.019***	*0.139*
Quality of amniotic fluid								
Normal	352	13.3 (4.0)	90.6 (107.0)	56.5 (54.8)	25.4 (36.7)	14.4 (19.8)	770.2 (499.2)	467.7 (300.4)
Meconish or bloody	56	14.0 (3.4)	109.3 (119.2)	70.3 (51.1)	22.1 (16.4)	13.3 (11.2)	851.1 (422.9)	520.1 (305.5)
P-value		*0.370*	*0.225*	*0.240*	*0.647*	*0.333*	***0.086***	*0.195*
Season of birth								
Winter	115	13.8 (4.2)	80.7 (119.8)	55.6 (56.5)	24.1 (53.0)	13.9 (26.8)	4123 (13 585)	2610 (6462)
Spring	125	13.5 (3.9)	81.0 (100.6)	45.5 (50.3)	18.6 (19.2)	10.9 (10.3)	4329 (12 547)	2631 (5703)
Summer	67	13.2 (4.2)	94.0 (87.7)	53.5 (46.2)	28.5 (27.1)	17.7 (19.3)	5089 (8394)	3557 (4109)
Fall	116	13.3 (3.8)	113.5 (111.1)	71.1 (57.5)	28.5 (23.2)	15.9 (13.5)	5440 (8794)	3291 (4716)
P-value		*0.917*	***0.023***	***0.005***	***0.0001***	***0.0001***	***0.023***	***0.027***

Cytokines are expressed as media values (SD).
P-values are estimated by Kruskal–Wallis test.
Bold indicates $P<0.10$.

In multivariate analysis, there was a significant association between the IL-5 values and the mode of delivery (elective Caesarean vs. others) ($P<0.02$), but after correction for WBC counts, the association was no longer significant.

Discussion

Our results show that there were various non-atopy-related factors that modified the IL-5, IL-10 and IFN-γ production in whole CB samples. Among all these potential factors, season of birth had the most significant association with cytokine production. Further, there were also other factors, such as WBC counts, maternal education and smoking during pregnancy, sex of child, birth weight and prostaglandin induction that were associated with the production of these cytokines. Interestingly, most of these factors had stronger associations with the examined cytokine production in comparison with the association of possible genetic background factors such as maternal asthma, hayfever or allergy or early environmental factors such as paternal farming.

Children who were born during spring time had significantly lower production of cytokines compared with those born in the fall. Standardization of the cytokine production by WBC count and multivariate adjustment for confounders did not affect the results. This indicates that the season of birth had great relation not only to the absolute cytokine levels but also on the capability of CB samples to produce specific cytokines. Sullivan Dillie et al. [22] reported that the season of birth was related significantly with the production of IL-5, IL-10 and IFN-γ from CB monocytes; however, they measured the highest responses during summer. In contrast, another study suggested that the CB allergen cockroach-specific IL-5 responses of children born in the winter were increased to those born in other seasons [23]. Maternal allergen exposures during gestation may partly explain these results: various pollen exposures during spring and summer may drive the maternal cytokine environment more towards Th2-biased responses, which further promotes the development of similar cytokine responses in CB in particularly for those children who are born in the fall. This idea is supported by a study in which high-level birch pollen exposure during pregnancy was shown to increase

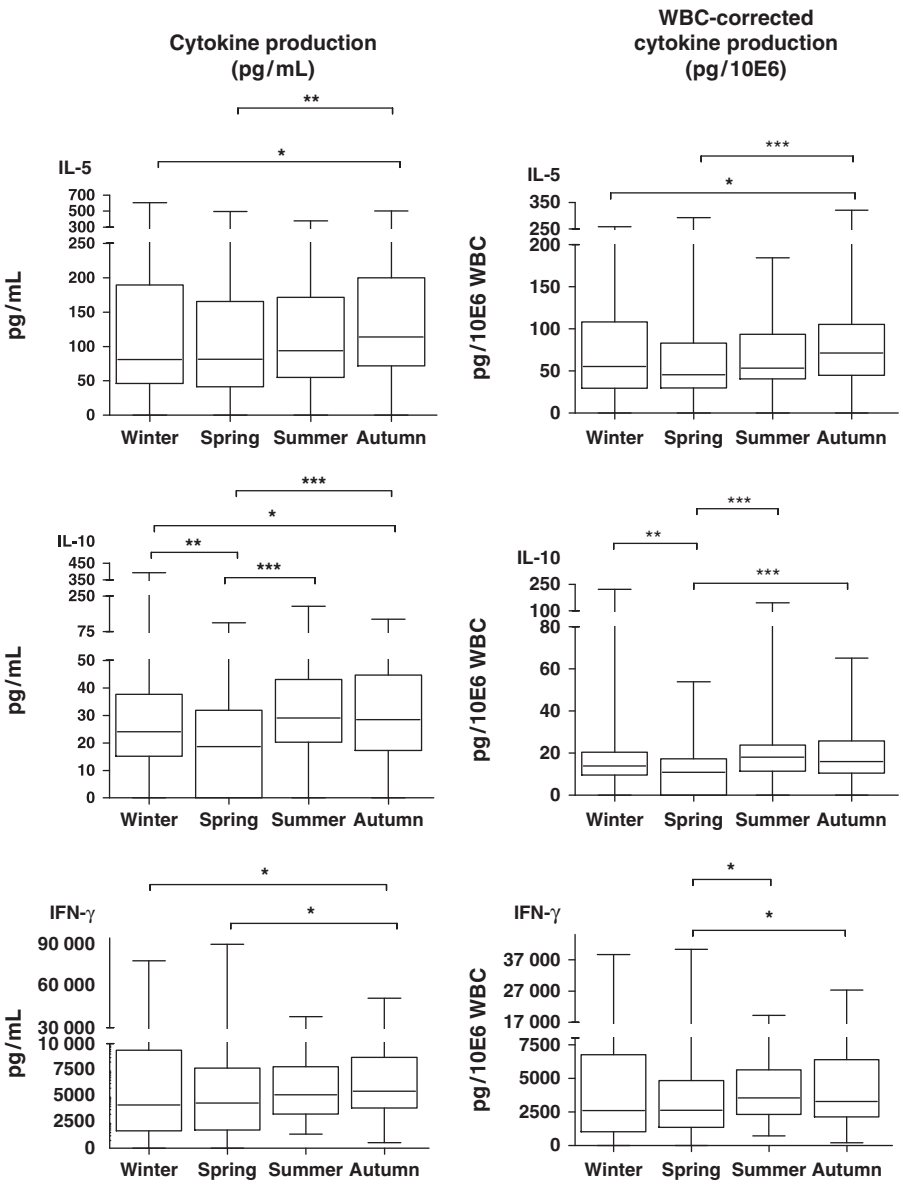

Fig. 1. Production of IL-5, IL-10 and IFN-γ following stimulation with phorbol ester and ionomycin. Box plots represent the interquartile range, median (horizontal line) and extremes (whiskers) of individual values. Statistical significances are marked as $^{*}P<0.05$; $^{**}P<0.005$; $^{***}P<0.001$.

the risk of sensitization and symptoms of atopic diseases in childhood [24]. In addition, the risk of asthma has been associated with the season of birth in some other studies, the risk being highest in those born in the fall or winter [25, 26], but the association between atopy and season of birth is more ambiguous [25–27].

Besides allergen exposure, there might also be other explanations for the observed association between season of birth and CB cytokine production. The study country and its specific photoperiodical properties related to latitude have also to be considered. For example, in our study area in Finland (at geographic latitude 63°N) the sunlight exposures vary drastically depending on the season. It is evident that serum biologically active vitamin D concentrations largely depend on skin exposure to ultraviolet (UV) light B, which is necessary for cutaneous vitamin D synthesis [28]. It is known that there are seasonal changes in maternal vitamin D status, which have a significant effect on the serum levels of vitamin D metabolites in their newborn infants [29, 30]. In addition, vitamin D is suggested to promote the expansion of the specific T cell population, regulatory T cells [31], which produce the anti-inflammatory cytokine IL-10 [32, 33]. Experimental data indicate that the vitamin D metabolite (1α,25-dihydroxyvitamin D3, known as calcitriol) effectively inhibits not only IL-12-generated IFN-γ production but also suppresses IL-4 and IL-13 expression induced by IL-4 [34]. Calcitriol also induces IL-10 receptor expression

Table 5. Multivariate adjusted associations with IL-5, Il-10 and IFN-γ production after P/I stimulation in cord blood

	IL-5*		IL-10†		IFN-γ‡	
	Coefficients	95% CI	Coefficients	95% CI	Coefficients	95% CI
Intercept	2.76	2.05 to 3.46	2.73	1.91 to 3.55	7.91	6.44 to 9.38
White blood cell count	**0.10**	**0.07 to 0.12**	**0.06**	**0.03 to 0.09**	0.03	−0.01 to 0.08
Cohort: pasture vs. extended	**−0.48**	**−0.74 to −0.22**	**−0.55**	**−0.83 to −0.28**	**−0.99**	**−1.46 to −0.52**
Season of birth						
Winter vs. fall	**−0.5**	**−0.79 to −0.22**	−0.26	−0.57 to 0.04	**−0.87**	**−1.37 to −0.36**
Spring vs. fall	**−0.4**	**−0.68 to −0.12**	**−0.77**	**−1.08 to −0.47**	**−0.52**	**−1.02 to −0.012**
Summer vs. fall	−0.31	−0.65 to 0.03	−0.31	−0.67 to 0.04	−0.27	−0.87 to 0.33
Gender: male vs. female	**0.45**	**0.23 to 0.66**	NI	NI	NI	NI
Birth weight	NI	NI			NI	NI
I vs. III tertile			**−0.35**	**−0.63 to −0.06**		
II vs. III tertile			−0.17	−0.45 to 0.11		
Apgar scores: ≤ 7 vs. >7	0.17	−0.49 to 0.83	0.15	−0.55 to 0.86	0.92	−0.26 to 2.10
Maternal parity			NI	NI		
0 vs. ≥ 2	0.12	−0.15 to 0.40			−0.14	−0.67 to 0.38
1 vs. ≥ 2	0.02	−0.25 to 0.28			−0.18	−0.65 to 0.29
Maternal age	NI	NI	NI	NI		
I vs. III tertile					0.34	−0.17 to 0.85
II vs. III tertile					0.04	−0.44 to 0.52
Mode of delivery: other vs. elective caesarean	0.25	−0.15 to 0.65	−0.17	−0.61 to 0.28	0.04	−0.72 to 0.80
Maternal smoking			NI	NI		
Never vs. smoking during pregnancy	**0.48**	**0.18 to 0.44**			0.34	−0.18 to 0.87
Earlier smoker, but not during pregnancy vs. smoking during pregnancy	**0.76**	**0.42 to 1.10**			0.54	−0.04 to 1.13
Prostaglandin induction: no vs. yes	NI	NI	**0.57**	**0.21 to 0.92**	**0.6**	**0.02 to 1.17**
Maternal education	NI	NI			NI	NI
Low vs. academic			**−0.33**	**−0.65 to −0.009**		
Middle vs. academic			−0.29	−0.59 to 0.012		
Quality of amniotic fluid: normal vs. meconish or bloody	NI	NI	NI	NI	−0.11	−0.67 to 0.46
Father as farmer: no vs. yes	0.16	−0.12 to 0.45	0.13	−0.17 to 0.43	0.21	−0.30 to 0.72
Maternal hay fever: no vs. yes	−0.25	−0.50 to 0.006	−0.17	−0.44 to 0.10	−0.29	−0.73 to 0.15
	$R^2 = 0.31$		$R^2 = 0.24$		$R^2 = 0.19$	

*In final covariance analysis included: gender, Apgar scores at 5 min, maternal parity, smoking and hay fever, mode of delivery, season of birth, white blood cell counts, paternal farming and the cohort.

†In final covariance analysis included: Apgar scores at 5 min, maternal education and hay fever, prostaglandin induction and mode of delivery, season of birth, white blood cell counts, paternal farming and the cohort.

‡In final covariance analysis included: Apgar scores at 5 min, maternal parity, age, smoking and hay fever, prostaglandin induction and mode of delivery, presence of me conium in amniotic fluid, season of birth, white blood cell counts, paternal farming and the cohort.

Bold indicates statistical significances with $P < 0.05$.

P/I, phorbol myristate acetate (PMA) and ionomycin; NI, not included in final multivariate covariance analysis.

in epidermal cells [35], and further down-regulates strongly the production of IL-10 in dendritic cells, which are differentiated in its absence [36]. Furthermore, CB IL-10 levels have been associated with vitamin D levels [37]. Specific WBC types may also respond differently to light periodicity. In hamsters, macrophages collected during long days have been shown to be more responsive to LPS challenge compared with short-day macrophages [38–40]. This association is explained by the altered photoperiods, which possibly affect hypothalamic cytokine gene expression [40]. The association between vitamin D and the production of IL-5 is thus far contradictory [41–43]. Rausch-Fan et al. [43] showed enhanced IL-5 responses in peripheral blood mononuclear cell cultures with calcitriol exposure, but in most studies, vitamin D has been associated in cell cultures or animal studies with more likely diminished IL-5 responses [41, 42, 44]. Based on our results, we suggest that the observed down-regulation of CB IL-10, IL-5 and IFN-γ levels in children born during spring may be a consequence of the differing amounts of

allergens or sunlight and UV irradiation during gestation. These findings should be evaluated in future with respect to maternal diet during pregnancy, vitamin D levels and the possible effects on the health of children later in life.

Various birth-related factors may modify the cytokine production in CB samples. The birth process initiates the production of acute phase proteins and pro-inflammatory cytokines and is always a physiologically stressful event for the neonate born through vaginal delivery [45]. The impact of labour is rarely included in analyses when CB cytokine responses are evaluated. We showed that IL-5 levels were significantly lower in univariate analysis in those children who were delivered by elective Caesarean in comparison with other modes. Also, a longer duration of labour resulted in higher CB IL-5 and IFN-γ responses. However, labour-associated IL-5 and IFN-γ increments were explained mostly by the CB leukocytosis; WBC counts associated positively with the duration of labour and taking into account WBC counts, associations between production of IL-5 and IFN-γ and mode of delivery were no longer significant. If cytokine responses measured from whole-blood samples are standardized with the WBC count, the impact of birth stress is likely to be controlled in the analysis.

In contrast to IL-5 and IFN-γ production, IL-10 production was not associated with the mode of delivery or duration of birth process, which is in line with an earlier study [46]. However, both IL-10 and IFN-γ production were inversely associated with the prostaglandin induction before delivery. There are no earlier reports of similar evaluations in clinical studies, but Boniface et al. [47] showed recently that prostaglandin differentially regulated IFN-γ production *in vitro* and at the same time inhibited the production of the anti-inflammatory cytokine IL-10. Prostaglandins have an important role in the onset of the labour process, promoting physiological inflammation and pro-inflammatory functions and they have been used for the induction of labour since the 1960s. The evaluation of prostaglandin birth induction separately from other induction methods in the significance of allergic diseases and asthma among offspring is not much studied; however, we have recently shown that prostaglandin induction before birth was likely to increase the risk of early onset persistent asthma among children in another birth cohort [48]. Prostaglandins are widely used in obstetric wards for labour induction, but their long-term health effects on offspring are not well evaluated and require further investigation.

Boys showed significantly greater CB IL-5 levels and also higher IL-5-producing capacity than girls, but production of IL-10 or IFN-γ was not affected by gender. Uekert and colleagues showed that boys had greater IL-5 and IFN-γ responses than girls at the age of 3 years, but not at birth. Further, they did not find associations between IL-10 responses and the gender of the child at birth when using mononuclear cell lines [49]. In cell cultures, testosterone has been shown to be more effective in inducing IL-5 mRNA levels in the T cell population compared with estradiol [50]. Modification of cytokine production by sex hormones has also been related to different disease susceptibility and outcomes between genders, although exact pathophysiological pathways behind this finding have remained unclear.

Strong evidence points to the conclusion that smoking during pregnancy has an influence on immune development and is an important risk factor for respiratory outcomes in children [51]. Our result of an association between decreased CB IL-5 production and maternal smoking during pregnancy are consistent with and extend those of Tsunoda et al. [52] who showed uniformly lower serum levels of cytokines (e.g. IL-4, IL-5, IL-6, IFN-γ) in smoking pregnant women. Concurrently, Thatcher et al. [53] reported recently an observation that smoking altered immune responses in a dose-dependent manner; T cell-mediated responses were inhibited in cell cultures in addition to provoking a systemic inflammatory response. Macaubas et al. [54] found decreased CB IL-4 and IFN-γ levels in children of smoking mothers. However, the production of TNF-α and IL-5, -6, -10, -12 and -13 at birth were not affected by maternal smoking [54]. On the other hand, Noakes and colleagues showed that maternal smoking increased house dust mite- and ovalbumin-stimulated IL-13 responses in CB mononuclear cell line and the trend was similar also for IL-6 protein, IL-5 mRNA and IL-9 mRNA levels. In addition, IFN-γ mRNA levels were decreased but the production of IFN-γ protein was not affected [55]. In another of their studies, the production of IL-6, IL-10 and TNF-α were found to be decreased following stimulation with different Toll-like receptor ligands in the CB monocyte cell line from children of smoking mothers [56].

In conclusion, these findings suggest that IL-5, IL-10 and IFN-γ responses in CB samples were strongly related to the season of birth. There were also other neonatal, maternal and obstetric factors that were associated with the examined cytokine production. However, these selected factors explained only 19–31% of the variation of examined cytokine production. Vaginal birth is an inflammatory process and even physiological birth stress is associated with CB leukocytosis. Thus, correction for WBC counts in whole-blood cytokine measurements give information about the functional capacity of CB cells and this would further control the effect of birth-associated leukocytosis.

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