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Exposure to environmental bacteria may have differing effects on tumour necrosis factor- α and interleukin-6-producing capacity in infancy

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Summary

Background Our previous study showed an association between increased concentration of endotoxin in house dust and elevated IFN- γ responses in neonates. The impact of other microbial agents on immune responses in infancy is poorly known.

Objective To examine whether stimulated cytokine responses of mothers and their children are associated with concentrations of other microbial markers in addition to endotoxin in house dust samples.

Methods Mitogen-stimulated production of IFN- γ , IL-4, IL-6 and TNF- α was measured in cord blood and in peripheral blood of mothers ($n = 29$) and their children ($n = 29$) 3 months after birth. Gas chromatography mass spectrometric analysis was applied to measure the concentrations of ergosterol (marker of fungal biomass), muramic acid (indicating the presence of Gram-positive bacteria) and 3-hydroxy fatty acids (C_{10:0}–C_{14:0}, indicating the presence of Gram-negative bacteria) in house dust. Endotoxin was determined with Limulus assay.

Results Significant mother-to-child correlations were observed in stimulated production of TNF- α and IL-6 3 months after birth. 3-hydroxy fatty acid (C_{10:0}–C_{14:0}) levels in bed dust were inversely associated with the production of TNF- α and IL-6 in blood samples of mothers and their 3-month-old children. High concentrations of muramic acid in floor dust were related to increased production of TNF- α and IL-6 at the age of 3 months. In contrast to endotoxin, none of the other microbial markers were significantly associated with enhanced IFN- γ -producing capacity from birth to 3 months.

Conclusions Exposure to Gram-negative bacteria and their components may be associated with down-regulated immune responses in early infancy, indicated as an impaired production of pro-inflammatory cytokines following mitogen stimulation. Gram-positive bacteria and their constituents seem to have opposite effects. Of the measured markers, exposure to bioactive endotoxin appears to have the strongest impact on T-helper type 1 responses.

Keywords bacteria, endotoxin, ergosterol, 3-hydroxy fatty acid, microbial exposure, muramic acid, pro-inflammatory cytokines

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Introduction

Several studies have shown that exposure to environments rich in microbes, such as the farm environment, protects children against the development of atopic sensitization and prevalent allergic diseases like hayfever and asthma [1–8]. The search for protective agents has resulted in indications that children who grow up on farms are exposed to greater concentrations of microbial compounds

compared with their peers living in non-farming environments [9–12]. However, establishing the link between environmental microbial exposure and the development of allergies and immunological functions in early life is not straightforward. This is partly due to difficulties in determining underlying immunological mechanisms as well as protective microbial constituents and effective doses.

Several microbial agents present in a living environment may have an effect on immunological development

in children. Recent research has mainly focused on the biologically active structure, endotoxin (lipopolysaccharide, LPS), which is a major constituent of the outer cell membrane of Gram-negative bacteria. In addition to analysing bioactive endotoxin, the presence of Gram-negative bacteria can be determined by measuring chemical markers for LPS, 3-hydroxy fatty acids [3-OH FA ($C_{10:0}$ – $C_{14:0}$)] [13]. Elevated exposure to endotoxin in early childhood has been associated with decreased prevalence of allergic diseases [14–17] although results are partly contradictory [18]. On the other hand, endotoxin is a potent respiratory irritant and thus may also increase the risk of non-specific respiratory irritation [17, 19]. Another proposed agent is peptidoglycan, a cell wall component of most environmental bacteria. The amount of peptidoglycan is higher in Gram-positive bacteria (30–70% of cell wall) than in Gram-negative bacteria (< 10% of cell wall) and, therefore, the amount of peptidoglycan in environmental samples is influenced mostly by the presence of Gram-positive bacteria [20]. Increased concentration of muramic acid, a major component of peptidoglycan, in bed dust has been associated with lower prevalence of wheezing in children [9]. Also, cell wall components of fungi may offer some protection against wheeze in children [21] but may increase the risk of current asthma later in life [22, 23].

Atopic diseases begin to develop during the first years of life. Therefore, timing of the microbial exposure may play a crucial role in immunological maturation and manifestation of allergic disease [24, 25]. In addition to neonatal exposure, maternal exposure to microbial compounds during pregnancy has been shown to have a protective effect on offspring [26]. The underlying immunological mechanism by which prenatal and neonatal microbial exposures affect the maturation of the immune system requires further elucidation.

Our previous paper showed an association between increased concentration of endotoxin in house dust and elevated IFN- γ -producing capacity from birth to 3 months of age [27]. The objective of the current paper was to examine the associations between other microbial compounds present in an indoor environment and immune responses using the same study population. The concentrations of microbial markers were measured in bed dust, floor dust and vacuum cleaner's dust bag dust samples reflecting exposure to fungi (ergosterol), Gram-positive bacteria (muramic acid) and Gram-negative bacteria [bioactive endotoxin and 3-OH FA ($C_{10:0}$ – $C_{14:0}$)]. Cord blood samples from newborns and peripheral blood samples of mothers and their children were collected 3 months after birth, and stimulated with the combination of a potent activator of protein kinase C, phorbol-12-myristate-13-acetate (PMA), and a mitogen, concanavalin A (ConA), to evaluate the ability of leucocytes to produce T-helper type 1 (Th1) (IFN- γ) and Th2 (IL-4) cytokines and pro-inflammatory cytokines TNF- α and IL-6.

Methods

Study population

Twelve healthy women, who were admitted at term to the Department of Obstetrics at Kuopio University Hospital after a clinically normal pregnancy and who lived on actively operating farms, were consecutively recruited to the study during a 5-month period. During the same time period, 17 healthy women were randomly selected as non-farming controls from rural and suburban areas near the city of Kuopio. All the women gave birth to healthy newborns; 3 out of 29 children were born by caesarean section. A written informed consent was obtained from all subjects. Consent and the study protocol were approved by the Research Ethics Committee, Hospital District of Northern Savo.

Blood samples

Cord blood samples were collected at birth in lithium-heparin tubes (Vacutainer, BD, Plymouth, UK) by an aspiration technique. Peripheral venous blood samples were collected from the forearms of both mothers and children 3 months after birth (also by Vacutainer). Samples were stored and transferred at 4 °C and processed for further analyses within 24 h.

Analysis of cytokine production

To activate cytokine production in leucocytes of the studied children and their mothers, cord and peripheral blood samples were stimulated as a whole blood cell culture with the combination of protein kinase C-activating PMA and a mitogenic lectin protein ConA. Briefly, 500 μ L heparinized blood was added to 1500 μ L cell culture medium (RPMI 1640 supplemented with 1% penicillin-streptomycin mixture, 10% fetal bovine serum and 1% L-glutamine, all from Gibco, Paisley, UK). Cell cultures were stimulated with the combination of PMA (15 ng/mL) and ConA (10 μ g/mL) for 8 and 24 h (5% CO₂, 37 °C). There was enough blood available for an 8-h stimulation from 25 mothers and 24 children. Respective numbers for a 24-h stimulation were 29 mothers and 29 children. After incubation, cell cultures were centrifuged at 380 g for 10 min and the supernatants were collected in polypropylene tubes and stored at –80 °C for later cytokine analysis. Concentrations of pro-inflammatory cytokines (IL-6 and TNF- α) and Th1 (IFN- γ) and Th2 (IL-4) cytokines were measured spectrophotometrically from supernatants using ELISA (R&D Systems, Minneapolis, MN, USA). The samples were run in duplicate and the analysis was performed according to the manufacturer's instructions. In-house validated sensitivity limits were 10 pg/mL (TNF- α), 4 pg/mL (IL-6), 8 pg/mL (IL-4) and 5 pg/mL (IFN- γ).

Indoor dust collection and analysis of microbial markers

House dust samples were collected from the mother's mattress, from the rug of the mother's bedroom floor and the vacuum cleaner's dust bag as described previously [28]. Bed dust samples were available from all homes ($n = 29$), floor dust from 26 homes and dust bag dust from 27 homes. The numbers of microbial marker analysis varied slightly (as shown in Table 1) because there was not enough dust in all the samples available for the analysis of all microbial markers.

Two samples of about 5 mg of dust were accurately weighed: one for the determination of ergosterol and another for the determination of 3-OH FAs and muramic acid. The sample preparations of ergosterol and 3-OH FAs were carried out by slightly modifying the method presented by Sebastian and Larsson [29] and the preparation of muramic acid by modifying the method presented by Sebastian et al. [30]. In our methods, hexane was used instead of heptane. The ergosterol and muramic acid samples were diluted to 100 μ L of hexane and to 150 μ L of chloroform, respectively, before analysis by gas chromatography tandem mass spectrometry (GC-MS-MS). In the preparation of 3-OH FAs, no water was used in the first extraction.

The analyses of ergosterol and muramic acid were performed with a PolarisQ ion trap mass spectrometer (MS-MS) from Thermo (Austin, TX, USA) equipped with a Trace GC-ultra gas chromatograph (GC) from (Milan, Italy) with a DB-5MS fused-silica capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) from J&W Scientific. In both methods, the injection volume of the samples was 1 μ L, the temperature of the injector was 280 °C, helium was used as a carrier gas at a constant flow of 1.0 mL/min and the GC was operated in the splitless mode. In the ergosterol method, the temperature program of the GC was as follows: 160 °C for 1 min, 40 °C/min to 300 °C and holding at 300 °C for 6.5 min. Positive electron ionization was performed at an ionization energy of 70 eV and the temperature of the ion source was 250 °C. The selected reaction monitoring (SRM)

method was used to monitor transition from one fragment ion of silylated ergosterol and 7-dehydrocholesterol as an internal standard. The transitions monitored were m/z 363.3 $\rightarrow$ 157.1 for ergosterol and m/z 351.3 $\rightarrow$ 143.1 for 7-dehydrocholesterol. In the muramic acid method, the temperature program of the GC was as follows: 90 °C for 0.5 min, 40 °C/min to 290 °C and holding at 290 °C for 2.5 min. Positive electron ionization was performed at an ionization energy of 70 eV and the temperature of the ion source was 220 °C. The SRM method was used to monitor transition from the one fragment ion of muramic acid derivate, m/z 213.1 $\rightarrow$ 127.1 and one from the fragment ion of ^{13} C-labelled muramic acid derivate, the internal standard, m/z 221.1 $\rightarrow$ 132.1.

The analyses of 3-OH FAs were performed with an Agilent 5973N Inert quadrupole MS (Palo Alto, CA, USA) equipped with an Agilent 6890N GC with an HP-5MS fused-silica capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) from J&W Scientific. The injection volume of the samples was 2 μ L, the temperature of the injector was 280 °C, helium was used as a carrier gas at a constant flow of 1.0 mL/min and the GC was operated in the splitless mode. The temperature program of the GC was as follows: 90 °C for 1 min, 20 °C/min to 280 °C and holding for 280 °C for 1 min. Positive electron ionization was performed at an ionization energy of 70 eV and the temperature of the ion source was 200 °C. The selected ion monitoring method was used to monitor one fragment ion of silylated 3-OH FAs, m/z 175 and m/z 178 for internal standard. The fatty acids of shorter chain lengths ($C_{10:0}$ – $C_{14:0}$) have shown a higher correlation with endotoxin [13] and hence the sum of these fatty acids was calculated. Concentrations of bioactive LPS (endotoxin) were measured by limulus amoebocyte lysate (LAL) assay as described by Hyvarinen et al. [28].

Study subjects were divided into two groups according to the marker concentrations in the dust samples. Median values of marker concentrations were used as cut-off points when distributing subjects into two different groups: concentrations below the median indicating low exposure

Table 1. Geometric mean (GM) values, geometric standard deviations (GSD) and ranges of ergosterol (ng/mg), muramic acid (ng/mg), 3-hydroxy fatty acid ($C_{10:0}$ – $C_{14:0}$) (μ mol/mg) and endotoxin (EU/mg) in floor dust, bed dust and vacuum cleaner's dust bag dust samples in relation to farming

	Farming	Floor dust			Bed dust			Dust bag		
		<i>n</i>	GM (GSD)	Range	<i>n</i>	GM (GSD)	Range	<i>n</i>	GM (GSD)	Range
Ergosterol	Yes	11	8.5 (2.3) [‡]	2.6–39.5	12	3.8 (1.9)	1.3–11.5	12	12.5 (2.3)	2.6–47.7
	No	14	4.0 (1.6)	1.9–10.6	17	2.7 (1.7)	1.6–14.9	15	7.5 (1.8)	3.4–27.6
Muramic acid	Yes	11	29.7 (2.2)	14.9–150.4	12	26.9 (2.3)	5.3–93.0	11	25.5 (1.9) [‡]	6.9–66.9
	No	14	16.8 (2.1)	4.3–74.7	17	16.0 (2.2)	3.2–69.2	15	15.1 (1.5)	6.4–30.4
3-hydroxy fatty acid ($C_{10:0}$ – $C_{14:0}$)	Yes	11	47.7 (1.8) [‡]	16.2–117.0	12	46.9 (1.8)	16.0–119.0	11	61.7 (1.6)	35.8–154.7
	No	14	30.8 (1.5)	14.3–60.5	17	33.4 (1.4)	18.7–67.9	15	49.0 (1.7)	19.4–116.8
Endotoxin	Yes	12	19.1 (2.8) [‡]	3.0–95.0	12	7.3 (3.7)	1.0–89.0	12	25.6 (3.1)	1.0–104.0
	No	14	7.3 (3.5)	0.4–43.0	17	2.8 (4.2)	0.1–28.0	15	10.6 (4.0)	0.2–64.0

[‡] $P < 0.05$ when compared with non-farming families (*t*-test).

and concentrations above the median indicating high exposure. The median concentrations of each marker were as follows: ergosterol (bed dust 2.8 ng/mg, floor dust 4.7 ng/mg, dust bag dust 8.8 ng/mg), muramic acid (bed dust 22.9 ng/mg, floor dust 18.1 ng/mg, dust bag dust 18.0 ng/mg), 3-OH FAs ($C_{10:0}$ – $C_{14:0}$) (bed dust 38.1 μ mol/mg, floor dust 35.4 μ mol/mg, dust bag dust 51.1 μ mol/mg) and endotoxin (bed dust 4 EU/mg, floor dust 14 EU/mg, dust bag dust 24 EU/mg).

Statistical analysis

Normal distribution for cytokine data could not be obtained with standard transformations; thus, a non-parametric Mann–Whitney test was used to evaluate associations between microbial marker exposure and cytokine production separately for cord blood, for peripheral blood of mothers and their children 3 months after birth and for the change in neonatal cytokine-producing capacity from birth to 3 months of age. Change in neonatal cytokine-producing capacity was calculated by subtracting the cytokine levels measured in cord blood from concentrations detected in peripheral blood of children 3 months after birth. Cytokine concentrations below the detection limit were converted as 0 pg/mL. Correlations between the cytokine concentrations of mothers and their children were studied using Spearman's rank correlation test. Before parametric tests, the concentrations of microbial markers (3-OH FAs, muramic acid, ergosterol, endotoxin) were ln-transformed to approximate a normal distribution. To evaluate correlations between microbial marker concentrations and between dust samples, Pearson's correlation coefficients were calculated. The *t*-test was used to examine differences between marker concentrations in relation to the living environment (farming/non-farming). The SPSS statistical package version 15.0 (SPSS Inc., Chicago, IL, USA) was used for all analysis and a *P*-value < 0.05 was considered to be statistically significant. No adjustment of *P*-values for multiple testing was performed [31].

Results

Microbial markers in house dust samples

The geometric mean values, geometric standard deviations and ranges of microbial markers in relation to the

farming status of the family are presented in Table 1. The detected levels of the microbial markers, except muramic acid, were the highest in vacuum cleaner's dust bag dust samples. Furthermore, concentrations of ergosterol, muramic acid and endotoxin were higher in floor dust than in bed dust. Concentrations of measured microbial markers were higher in dust samples collected from farming homes, as compared with non-farming homes. Statistically significant differences between farming homes and non-farming homes were found in the concentrations of ergosterol, 3-OH FA ($C_{10:0}$ – $C_{14:0}$) (referred later as 3-OH FAs) and endotoxin in floor dust samples and muramic acid in dust bag dust samples.

Pearson's correlation coefficients between different dust sampling methods for ergosterol, muramic acid, 3-OH FAs and endotoxin are shown in Table 2. Concentrations of all the microbial markers in the floor dust samples correlated statistically significantly with those of bed dust samples. The results of dust bag dust samples will not be discussed any further, because microbial marker concentrations in this sample type were not associated with measured cytokines.

Correlations between microbial markers were in general slightly higher in bed dust than in floor dust samples. Concentrations of ergosterol in bed dust samples correlated with those of muramic acid ($r=0.713$, $P<0.01$), 3-OH FAs ($r=0.749$, $P<0.01$) and endotoxin ($r=0.551$, $P<0.01$). Similar to ergosterol results, levels of muramic acid correlated significantly with 3-OH FA ($r=0.653$, $P<0.01$) and endotoxin ($r=0.586$, $P<0.01$) concentrations. A significant correlation was also observed between 3-OH FA and endotoxin concentrations ($r=0.434$, $P<0.05$) in bed dust. In floor dust samples, a significant correlation was observed between muramic acid and 3-OH FA concentrations ($r=0.573$, $P<0.01$). Concentrations of endotoxin correlated with ergosterol ($r=0.463$, $P<0.05$) and 3-OH FA levels ($r=0.519$, $P<0.05$) in floor dust samples.

Stimulated production of cytokines

Optimal PMA/ConA-stimulated production of cytokines was detected after an 8-h stimulation and therefore results from this time-point are presented. The IL-4

Table 2. Pearson's correlation coefficients between studied house dust sample types

Dust type	Ergosterol		Muramic acid		3-hydroxy fatty acid ($C_{10:0}$ – $C_{14:0}$)		Endotoxin	
	Bed dust	Dust bag dust	Bed dust	Dust bag dust	Bed dust	Dust bag dust	Bed dust	Dust bag dust
Floor dust	0.581 [¶]	0.163	0.469 [‡]	0.250	0.551 [¶]	0.677 [¶]	0.522 [¶]	0.714 [¶]
Bed dust	–	0.012	–	0.021	–	0.406	–	0.428 [‡]

[‡]Correlation is significant at the 0.05 level.

[¶]Correlation is significant at the 0.01 level.

Table 3. PMA/ConA-stimulated production of TNF- α , IL-6, IFN- γ (pg/mL) in cord blood and in peripheral blood of mothers and their children 3 months after delivery (8-h stimulation)

	Cytokine	N	Minimum	25th percentile	Median	75th percentile	Maximum
Cord blood	TNF- α	25	250	1450	4960	5200	6420
	IL-6	25	82.6	912	3170	5240	6290
	IFN- γ	25	0	0	0	8.93	53.7
	TNF- α	24	75.9	159	263	970	1840
Child, 3 months	IL-6	24	12.1	27.1	103	214	580
	IFN- γ	24	0	11.4	27.7	56.3	540
	TNF- α	25	18.2	277	635	1050	5050
Mother, 3 months	IL-6	25	0	14.6	79.8	322	1060
	IFN- γ	25	15.7	126	246	535	844

PMA, phorbol-12-myristate-13-acetate; ConA, concanavalin A.

concentrations were mostly below the detection limit and were excluded from further analysis.

After an 8-h stimulation with the combination of PMA and ConA, detected concentrations of pro-inflammatory cytokines in cord blood were high (Table 3). The measured concentrations of TNF- α and IFN- γ were higher in maternal samples than in children 3 months after delivery. Instead, IL-6 levels were comparable. Correlations in stimulated cytokine production between mother and child at the age of 3 months were high for pro-inflammatory cytokines (IL-6: $r=0.480$, $P<0.05$; TNF- α : $r=0.659$, $P<0.01$), but poor for IFN- γ ($r=0.308$; $P=0.142$).

Of the studied cytokines, stimulated TNF- α responses in cord blood were significantly higher in studied boys ($n=14$) than in girls ($n=15$) ($P=0.009$). The age of the mother (above vs. below median age = 30 years), the use of antibiotics during pregnancy ($n=9$), the use of antibiotics during the first 3 months of life ($n=4$) or the number of siblings were not associated with any of the measured cytokines in cord blood or in peripheral blood at age 3 months (data not shown). Only one mother smoked during pregnancy and all mothers breastfed their babies. Cytokine responses in relation to allergic diseases of the mother and the child have been reported earlier [27] and no significant associations were found.

Associations between stimulated cytokine production and concentrations of microbial markers in bed dust samples

None of the measured markers in bed dust samples were associated with IL-6, TNF- α or IFN- γ responses in cord blood (data not shown). Three months after birth, the concentration of 3-OH FAs in bed dust samples, indicative of exposure to Gram-negative bacteria, was inversely associated with stimulated TNF- α -producing capacity in both mothers ($P<0.001$) and children ($P=0.001$) (Fig. 1). In line with TNF- α responses, high concentrations of 3-OH FAs were strongly associated with decreased IL-6 responses in both mothers ($P=0.003$) and their children ($P=0.056$). The concentration of muramic acid or endotoxin in bed

dust was not associated with any of the measured cytokines. TNF- α responses were decreased in mothers with high exposure to ergosterol (median 390 pg/mL, range 18–1070 pg/mL) as compared with those with low ergosterol exposure (median 930 pg/mL, range 56–5050 pg/mL) ($P=0.044$). In children, no associations were found between ergosterol concentrations and cytokine production (data not shown).

The change of IFN- γ -producing capacity from birth to 3 months of age in neonatal samples was statistically significantly associated with the bed dust endotoxin concentration, as we have reported previously (Fig. 2) [27]. A similar trend, however, was also detected with additional microbial markers measured in this study. The change in IL-6- or TNF- α -producing capacity was not associated with any of the measured microbial markers (data not shown).

Associations between stimulated cytokine production and concentrations of microbial markers in floor dust samples

The concentration of muramic acid in floor dust was associated with elevated IL-6-producing capacity in cord blood samples ($P=0.003$) (data not shown). No significant associations were found between other cord blood cytokines and measured microbial markers. Three months after birth, significantly increased IL-6 responses were detected both in mothers and in children when the concentration of muramic acid in floor dust was high ($P=0.030$ and 0.002 , respectively) (Fig. 3). Exposure to muramic acid was also associated with elevated TNF- α levels in children's blood samples ($P=0.002$). In contrast, high exposure to 3-OH FAs was associated with decreased TNF- α -producing capacity, even though the statistically significant difference was seen only in maternal samples ($P=0.006$). The concentration of endotoxin (Fig. 3) or ergosterol (data not shown) in floor dust was not associated with IL-6 or TNF- α production 3 months after birth.

Stimulated IFN- γ responses in mothers and children determined 3 months after birth (Fig. 3) or the change of

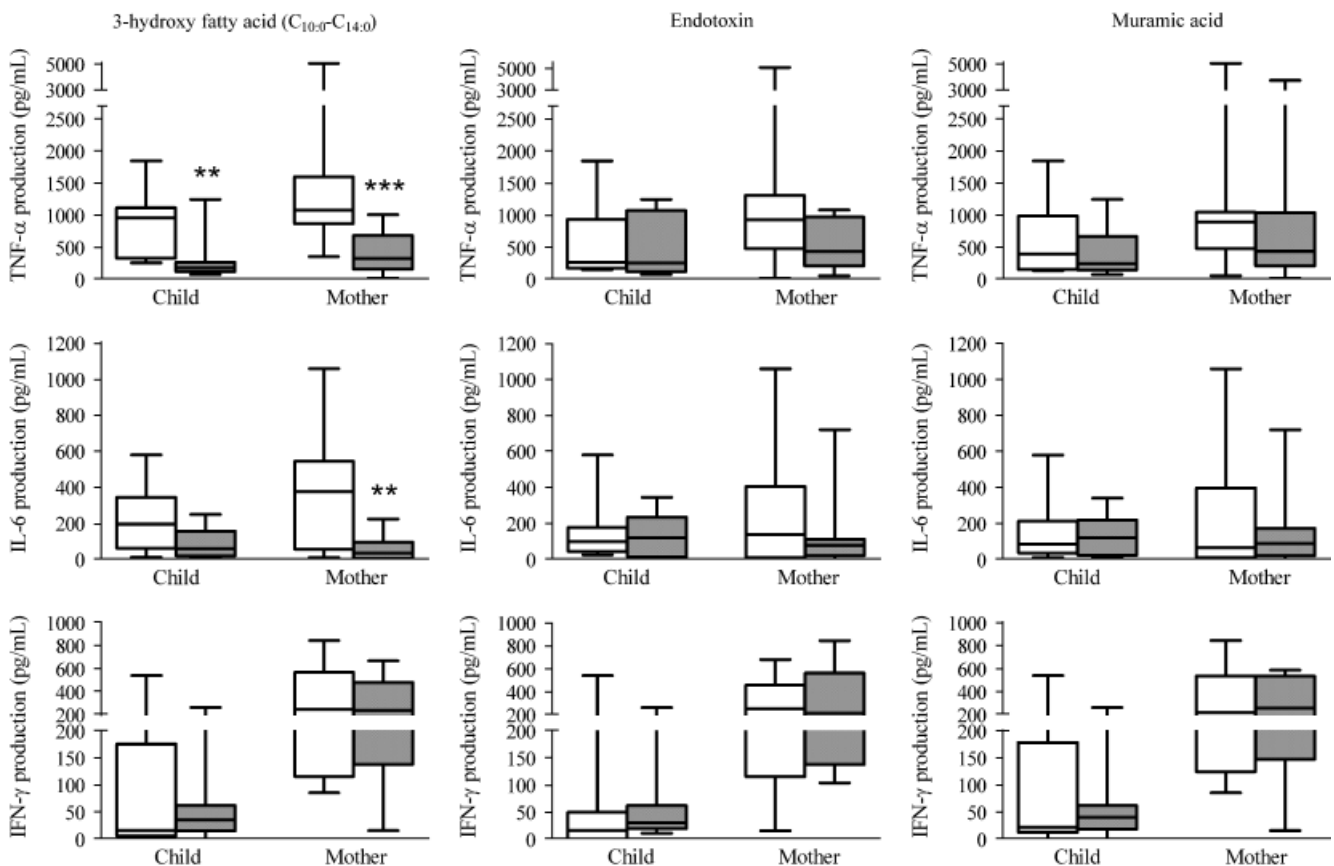


Fig. 1. Stimulated production of TNF- α , IL-6 and IFN- γ in whole blood cultures of 3-month-old children and their mothers with low (empty boxes) and high (filled boxes) concentrations of Gram-negative (3-hydroxy fatty acids and endotoxin) and Gram-positive (muramic acid) bacterial markers in bed dust. Box plots represent the interquartile range, median (horizontal line) and extreme values (whiskers). ** $P < 0.01$; *** $P < 0.001$ in comparison with the low exposure group.

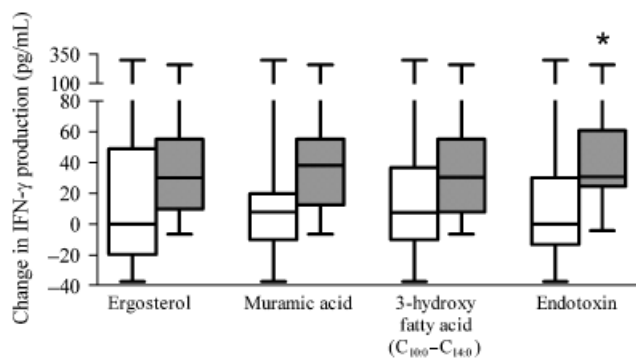


Fig. 2. Change in stimulated production of IFN- γ from birth to 3 months in whole blood culture of children with low (empty boxes) and high (filled boxes) concentrations of fungal (ergosterol), Gram-positive (muramic acid) and Gram-negative (3-hydroxy fatty acids and endotoxin) bacterial markers in bed dust. Box plots represent the interquartile range, median (horizontal line) and extreme values (whiskers). * $P < 0.05$ in comparison with the low exposure group.

neonatal IFN- γ -producing capacity from birth to 3 months age (data not shown) were not associated with any of the measured microbial markers in floor dust samples. With regard to pro-inflammatory cytokines, the

change in IL-6-producing capacity from birth to 3 months was significantly larger in children with high exposure to floor dust muramic acid (median – 4887 pg/mL, range – 915 to – 6036 pg/mL) as compared with children with low muramic acid exposure (median – 832 pg/mL, range – 37 to – 3086 pg/mL) ($P = 0.001$).

Discussion

One of the important risk factors for the increased occurrence of asthma and allergic diseases in western countries is proposed to be diminished exposure to environmental microbes [2, 3]. More specifically, exposure to microbes at an early age is thought to be an important factor in immunological maturation and in the development of these diseases. The impact of early microbial exposure to the manifestation of allergic diseases was demonstrated by Riedler *et al.* [24], who showed that children who were exposed to microbiologically rich farm environments when they were < 1 year old had a lower prevalence of hayfever, asthma and atopic sensitization than children who were 1–5 years old when exposed to farm microbes

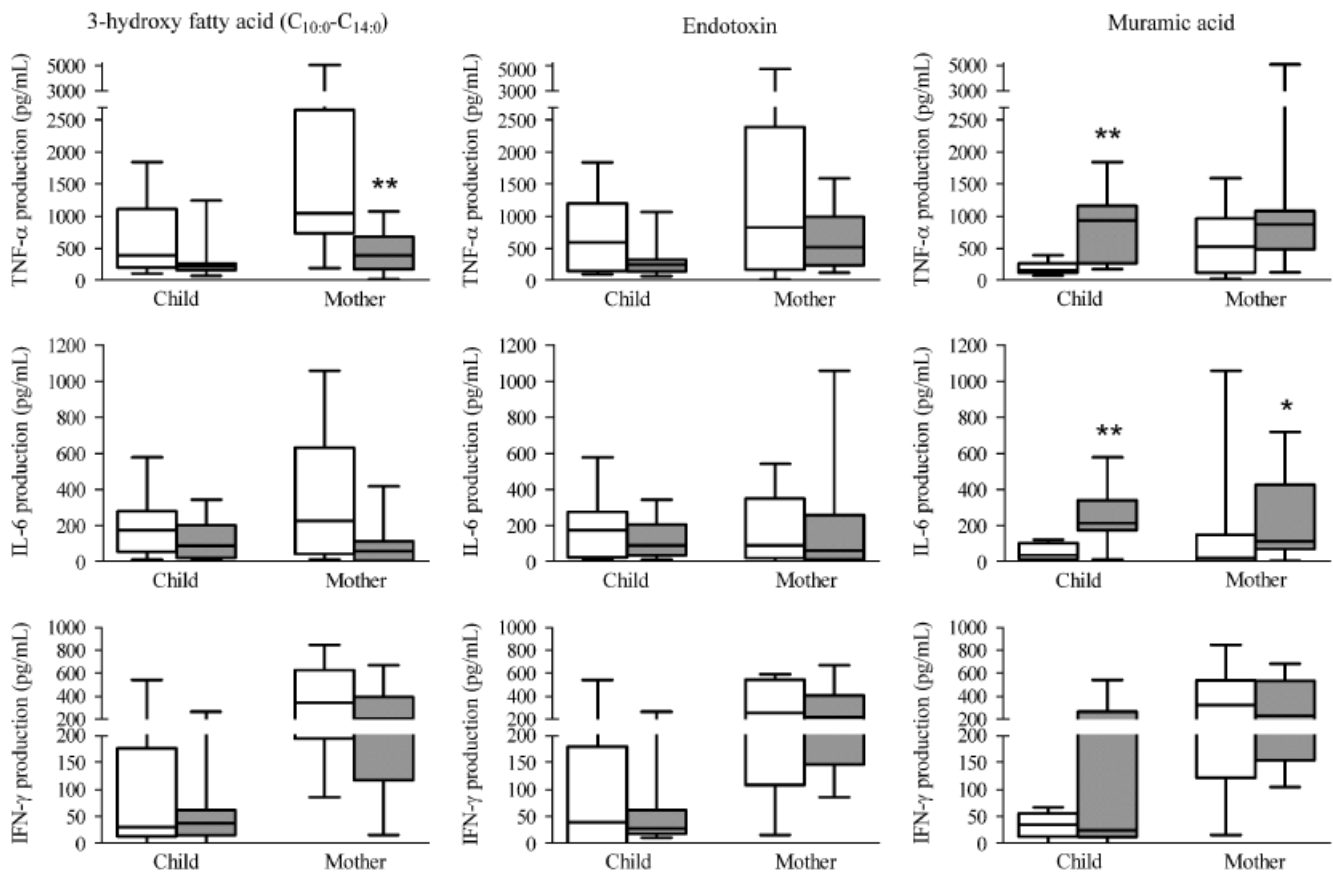


Fig. 3. Stimulated production of TNF- α , IL-6 and IFN- γ in whole blood cultures of 3-month-old children and their mothers with low (empty boxes) and high (filled boxes) concentrations of Gram-negative (3-hydroxy fatty acids and endotoxin) and Gram-positive (muramic acid) bacterial markers in floor dust. Box plots represent the interquartile range, median (horizontal line) and extreme values (whiskers). * $P < 0.05$; ** $P < 0.01$ in comparison with the low exposure group.

for the first time. However, specific microbial characteristics contributing to the immunological development in early life have not yet been identified.

A high concentration of 3-OH FAs (C_{10:0}-C_{14:0}) (considered as a chemical marker of Gram-negative bacteria and characteristic for LPS) in bed dust was associated with reduced IL-6- and TNF- α -producing capacity both in mothers and in their children 3 months after birth. In agreement with this, an earlier study has shown an inverse association between stimulated TNF- α production in children and the concentration of Gram-negative bacterial endotoxin in bed dust [16]. Interestingly, we found no significant associations between bioactive endotoxin and pro-inflammatory responses. The reasons for the different results obtained by GC-MS and LAL assays need to be clarified in future studies. However, GC-MS analysis of 3-OH FAs also detects inactive forms of LPS, suggesting that this method gives a different concept of overall exposure to Gram-negative bacteria as compared with LAL-measuring bioactive endotoxin. Altogether, our results suggest that exposure to the sources of house dust 3-OH FAs (C_{10:0}-C_{14:0}) may lead to impaired PMA/ConA-

induced pro-inflammatory responses both in children and in adults.

We have reported previously [27] that enhanced IFN- γ -producing capacity from birth to 3 months of age was associated with an increased concentration of endotoxin in bed dust. The trend was similar for additional microbial markers measured in this study but no significant associations were found. Further studies with a larger number of subjects are needed to confirm whether bioactive endotoxin is the most relevant marker of microbial exposure when Th1-related responses are studied in infancy. Altogether, these findings highlight the need to further develop analytical methods for the detection of microbial exposure, and to assess true associations between these exposures and the immunological development of exposed children.

Contrary to 3-OH FA results, we found that exposure to muramic acid, a major component of peptidoglycan and a marker of Gram-positive bacteria, may have stimulatory effects on the immune system, indicated as an increased production of pro-inflammatory cytokines in peripheral blood samples of both mothers and their

children 3 months after birth. The observed differences in the production of pro-inflammatory cytokines between muramic acid and 3-OH FAs in our study may be due to the fact that muramic acid (peptidoglycan) initiates different pathways in the innate immune system than LPS and thus elicits different immunological responses as compared with LPS. It is known that Gram-negative bacterial LPS is recognized by Toll-like receptor 4 (TLR4), whereas peptidoglycan interacts with TLR2 [32]. The study of Schaub et al. [33] showed that TLR2 agonist peptidoglycan was able to stimulate the production of TNF- α in cord blood mononuclear cells. Peptidoglycan also induced the production of IL-10, secreted, e.g. by regulatory T cells (T_{reg}), indicating that in addition to innate immune responses, exposure to peptidoglycan (or muramic acid) may also have an influence on adaptive T cell responses and contribute to T_{reg}-mediated immunoregulation. Gram-positive and Gram-negative bacteria may also have currently unknown differing effects on immune system, this point of view being supported by the study of Hesse et al., which demonstrated that Gram-positive and Gram-negative bacteria induce different patterns of pro-inflammatory cytokines in human monocytes [34].

Interestingly, the above-mentioned immunostimulatory effect of muramic acid was seen only in floor dust samples, but not in bed dust. Presumably, these two dust-sampling methods may reflect different kinds of microbial exposure. Both the quantity and the quality of sources of microbial products may be wider in floor dust as compared with bed dust. The microbial content of bed dust is probably dominated by the bacteria of human origin, while the floor dust also contains bacterial material from environmental sources. In the present study, the concentrations of muramic acid were higher in farming homes than in non-farming homes in all the dust types, being in line with the study of van Strien et al. [9].

Not only bacterial components but also fungal agents, as part of the environmental microbial burden, may have protective effects but results are partly contradictory. Douwes et al. [35] showed that high exposure to fungal extracellular polysaccharides from genera *Penicillium* and *Aspergillus* from floor dust reduced the prevalence of wheezing and asthma in early childhood whereas exposure to high concentrations of ergosterol in floor dust has been shown to increase the risk of sensitization to fungi and current asthma in adulthood [22]. Nevertheless, exposure to ergosterol was not consistently associated with the measured cytokines in our study.

With regard to cord blood samples, we found only one significant association between the concentration of muramic acid in floor dust and elevated IL-6-producing capacity. The levels of IL-6 and TNF- α at birth are strongly affected by obstetric factors [36–38], and therefore it is possible that the associations between microbial marker exposures and immune responses measured from cord

blood cannot be found in such a small study population. The effect of birth process on IL-6 and TNF- α levels may also explain why no consistent associations were found between exposure measures and the change in the production of these cytokines from birth to 3 months.

Larsson et al. [39] demonstrated strong correlations in IFN- γ production between mothers and their 2-year-old children. The poor mother-to-child correlation in IFN- γ responses in our study could be explained by the immature capability of infants' leucocytes to produce IFN- γ at the age of 3 months. In contrast to the IFN- γ finding, we found significant mother-to-child correlations in the stimulated production of IL-6 and TNF- α 3 months after birth. In addition to genetic factors, strong correlations between maternal and neonatal stimulated production of pro-inflammatory cytokines could be a result of similar living environments. Maternal exposure to microbial components during pregnancy may have immunomodulatory effects on newborns and should also be considered as a potential reason for the similar capacity of mothers and their children to produce pro-inflammatory cytokines. This view is supported by recent results showing that offspring of mothers who had regularly worked at stables during pregnancy had higher expression of TLR2 and TLR4 receptors as compared with their peers whose mothers did not undertake such work [26]. Also, maternal contact with increasing numbers of farm animal species during pregnancy was associated with up-regulated expression of those genes in their children [26]. Our results may indicate that a child's immune system responds to antigen contact by producing a similar pattern of pro-inflammatory cytokines as his mother's immune system.

In the present study, significant correlations between all the studied microbial markers were detected in bed dust. This is probably due to the homogenous composition of bed dust, while the lower marker correlation in floor dust indicates a more heterogeneous composition of floor dust. Despite rather good correlations between the measured markers, their impact on cytokine production was different. This further supports our view that different microbial exposures may have diverse effects on immunological response and the development of the immune system.

Instead of measuring cytokines from serum or plasma samples, or from leucocyte cell culture, the whole blood cell culture was applied in our study. Whole blood provides the most naturally occurring environment available for evaluating the cytokine production due to the fact that it contains both immunostimulatory and inhibitory factors, which are not present in specific cell cultures. Unfortunately, differential leucocyte count was not part of the study protocol and the exact number of leucocytes responsible for the detected cytokine responses in each individual is not known. Cytokine production was activated by stimulating leucocytes with the combination of a protein kinase activator PMA and a mitogen ConA.

Addition of the commercial LPS and peptidoglycan to the stimulation protocol would have enabled us to assess whether cytokine responses following these stimulations are associated with previous endotoxin, 3-OH FA and muramic acid exposure in the study population. It is also noteworthy that we did not measure the levels of home allergens in this study. The levels of bacterial markers may correlate with the levels of allergens, which could, at least partly, affect the cytokine responses in exposed individuals. One should also keep in mind that many other environmental and life-style factors may affect cytokine levels and they should be taken into account when assessing the true change in cytokine-producing capacity in infancy.

In conclusion, even though the number of studied children was small, the results suggest that exposure to microbial components from living environment in early life can modulate immune responses in infancy. It appears that Gram-positive bacterial compounds may have immunostimulatory effects, whereas a high content of Gram-negative bacterial 3-OH FA in house dust seems to be associated with down-regulated pro-inflammatory responses. Although the trend of Th1-related responses was similar to all the measured microbial markers, only exposure to bioactive endotoxin in house dust seems to be significantly associated with enhanced Th1 responses, as reported previously [27]. The role of various environmental microbial exposures and the immunological pathways by which they contribute to the maturation of the neonatal immune system requires further investigation.

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References

- Alfven T, Braun-Fahrlander C, Brunekreef B *et al.* Allergic diseases and atopic sensitization in children related to farming and anthroposophic lifestyle – the PARSIFAL study. *Allergy* 2006; **61**:414–21.
- Riedler J, Eder W, Oberfeld G, Schreuer M. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin Exp Allergy* 2000; **30**:194–200.
- Von Ehrenstein OS, Von Mutius E, Illi S, Baumann L, Bohm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* 2000; **30**:187–93.
- Kilpelainen M, Terho EO, Helenius H, Koskenvuo M. Childhood farm environment and asthma and sensitization in young adulthood. *Allergy* 2002; **57**:1130–5.
- Braun-Fahrlander C, Gassner M, Grize L *et al.* Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. SCARPOL team. Swiss study on childhood allergy and respiratory symptoms with respect to air pollution. *Clin Exp Allergy* 1999; **29**:28–34.
- Remes ST, Pekkanen J, Soininen L, Kajosaari M, Husman T, Koivikko A. Does heredity modify the association between farming and allergy in children? *Acta Paediatr* 2002; **91**:1163–9.
- Remes ST, Iivanainen K, Koskela H, Pekkanen J. Which factors explain the lower prevalence of atopy amongst farmers' children? *Clin Exp Allergy* 2003; **33**:427–34.
- Remes ST, Koskela HO, Iivanainen K, Pekkanen J. Allergen-specific sensitization in asthma and allergic diseases in children: the study on farmers' and non-farmers' children. *Clin Exp Allergy* 2005; **35**:160–6.
- van Strien RT, Engel R, Holst O *et al.* Microbial exposure of rural school children, as assessed by levels of N-acetyl-muramic acid in mattress dust, and its association with respiratory health. *J Allergy Clin Immunol* 2004; **113**:860–7.
- Waser M, Schierl R, von Mutius E *et al.* Determinants of endotoxin levels in living environments of farmers' children and their peers from rural areas. *Clin Exp Allergy* 2004; **34**:389–97.
- Schram D, Doekes G, Boeve M *et al.* Bacterial and fungal components in house dust of farm children, Rudolf Steiner school children and reference children – the PARSIFAL Study. *Allergy* 2005; **60**:611–8.
- von Mutius E, Braun-Fahrlander C, Schierl R *et al.* Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp Allergy* 2000; **30**:1230–4.
- Saraf A, Larsson L, Burge H, Milton D. Quantification of ergosterol and 3-hydroxy fatty acids in settled house dust by gas chromatography-mass spectrometry: comparison with fungal culture and determination of endotoxin by a Limulus amoebocyte lysate assay. *Appl Environ Microbiol* 1997; **63**:2554–9.
- Gehring U, Bolte G, Borte M *et al.* Exposure to endotoxin decreases the risk of atopic eczema in infancy: a cohort study. *J Allergy Clin Immunol* 2001; **108**:847–54.
- Gehring U, Bischof W, Fahlbusch B, Wichmann HE, Heinrich J. House dust endotoxin and allergic sensitization in children. *Am J Respir Crit Care Med* 2002; **166**:939–44.
- Braun-Fahrlander C, Riedler J, Herz U *et al.* Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002; **347**:869–77.
- Simpson A, John SL, Jury F *et al.* Endotoxin exposure, CD14, and allergic disease: an interaction between genes and the environment. *Am J Respir Crit Care Med* 2006; **174**:386–92.
- Bolte G, Bischof W, Borte M, Lehmann I, Wichmann HE, Heinrich J. Early endotoxin exposure and atopy development in infants: results of a birth cohort study. *Clin Exp Allergy* 2003; **33**:770–6.
- Park JH, Gold DR, Spiegelman DL, Burge HA, Milton DK. House dust endotoxin and wheeze in the first year of life. *Am J Respir Crit Care Med* 2001; **163**:322–8.
- Starr MP, Stolp H, Trüper HG, Balows A, Schlegel HG. *The prokaryotes, a handbook on habitats, isolation and identification of bacteria*. Berlin: Springer-Verlag, 1981; I:19.

- 21 Iossifova YY, Reponen T, Bernstein DI *et al*. House dust (1–3)-beta-D-glucan and wheezing in infants. *Allergy* 2007; **62**:504–13.
- 22 Dharmage S, Bailey M, Raven J *et al*. Current indoor allergen levels of fungi and cats, but not house dust mites, influence allergy and asthma in adults with high dust mite exposure. *Am J Respir Crit Care Med* 2001; **164**:65–71.
- 23 Matheson MC, Abramson MJ, Dharmage SC *et al*. Changes in indoor allergen and fungal levels predict changes in asthma activity among young adults. *Clin Exp Allergy* 2005; **35**:907–13.
- 24 Riedler J, Braun-Fahrlander C, Eder W *et al*. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* 2001; **358**:1129–33.
- 25 Radon K, Ehrenstein V, Praml G, Nowak D. Childhood visits to animal buildings and atopic diseases in adulthood: an age-dependent relationship. *Am J Ind Med* 2004; **46**:349–56.
- 26 Ege MJ, Bieli C, Frei R *et al*. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. *J Allergy Clin Immunol* 2006; **117**:817–23.
- 27 Roponen M, Hyvarinen A, Hirvonen MR, Keski-Nisula L, Pekkanen J. Change in IFN-gamma-producing capacity in early life and exposure to environmental microbes. *J Allergy Clin Immunol* 2005; **116**:1048–52.
- 28 Hyvarinen A, Roponen M, Tiittanen P, Laitinen S, Nevalainen A, Pekkanen J. Dust sampling methods for endotoxin – an essential, but underestimated issue. *Indoor Air* 2006; **16**:20–7.
- 29 Sebastian A, Larsson L. Characterization of the microbial community in indoor environments: a chemical-analytical approach. *Appl Environ Microbiol* 2003; **69**:3103–9.
- 30 Sebastian A, Harley W, Fox A, Larsson L. Evaluation of the methyl ester *O*-methyl acetate derivative of muramic acid for the determination of peptidoglycan in environmental samples by ion-trap GC–MS–MS. *J Environ Monit* 2004; **6**:300–4.
- 31 Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology* 1990; **1**:43–6.
- 32 Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect* 2004; **6**:1382–7.
- 33 Schaub B, Campo M, He H *et al*. Neonatal immune responses to TLR2 stimulation: influence of maternal atopy on Foxp3 and IL-10 expression. *Respir Res* 2006; **7**:40.
- 34 Hessle CC, Andersson B, Wold AE. Gram-positive and Gram-negative bacteria elicit different patterns of pro-inflammatory cytokines in human monocytes. *Cytokine* 2005; **30**:311–8.
- 35 Douwes J, van Strien R, Doekes G *et al*. Does early indoor microbial exposure reduce the risk of asthma? The prevention and incidence of asthma and mite allergy birth cohort study. *J Allergy Clin Immunol* 2006; **117**:1067–73.
- 36 Keski-Nisula L, Hirvonen MR, Roponen M, Heinonen S, Pekkanen J. Spontaneous and stimulated interleukin-6 and tumor necrosis factor- α production at delivery and three months after birth. *Eur Cytokine Netw* 2004; **15**:67–72.
- 37 Malamitsi-Puchner A, Protonotariou E, Boutsikou T, Makrakis E, Sarandakou A, Creatsas G. The influence of the mode of delivery on circulating cytokine concentrations in the perinatal period. *Early Hum Dev* 2005; **81**:387–92.
- 38 Bowen JM, Chamley L, Mitchell MD, Keelan JA. Cytokines of the placenta and extra-placental membranes: biosynthesis, secretion and roles in establishment of pregnancy in women. *Placenta* 2002; **23**:239–56.
- 39 Larsson AK, Nilsson C, Hoglind A, Sverremark-Ekstrom E, Lilja G, Troye-Blomberg M. Relationship between maternal and child cytokine responses to allergen and phytohaemagglutinin 2 years after delivery. *Clin Exp Immunol* 2006; **144**:401–8.