

ORIGINAL ARTICLE

Breastfeeding modulates neonatal innate immune responses: a prospective birth cohort study

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Abstract

Background: Neonatal Toll-like receptor (TLR) responses are biased toward Th2-polarizing responses at birth and rapidly mature toward more balanced responses during the first month of life. Postnatal TLR maturation may be guided by environmental exposure.

Aims: To determine the environmental determinants of neonatal TLR function.

Materials and Methods: A prospective birth cohort study was performed in 291 healthy term neonates. Mode of delivery, breastfeeding, birth month, siblings, pets and parental smoking were analyzed in relation to neonatal innate immune parameters at the age of 1 month. Whole blood concentrations of innate immune cells were measured by flow cytometry. *In vitro* TLR-mediated cytokine production was determined by ELISA.

Results: Breastfeeding was the major determinant of neonatal innate immunity, associated with 5 (31%) of neonatal innate immune parameters, of which the association with TLR7-mediated IL-10 production was most significant (76 pg/ml in breastfed neonates vs. 293 pg/ml in formula-fed neonates, $p = 0.001$). Of innate immune variables, TLR3-mediated IL-12p70 production was highly associated with environmental exposures (pets, breastfeeding and mode of delivery), whereas TLR9-mediated cytokine responses were not associated with any environmental factor.

Conclusion: Neonatal innate immune responses are differentially modulated by environmental exposure in the first month of life. The protective effect of breastfeeding against subsequent infections and atopy might be explained by its innate immune modulatory effects in the first month of life.

The first month of life is a challenging period for the immune system. Born from a sterile intrauterine environment, the neonate is suddenly exposed to a world full of foreign antigens. To prevent potentially harmful immune reactions toward maternal antigens *in utero* and to allow for bacterial colonization after birth, the neonatal immune system is generally biased against the generation of pro-inflammatory responses. However, this bias renders the neonate highly susceptible to infection. Infections are the second most common cause of neonatal mortality world wide, accounting for 1 million annual deaths (1). In addition, increased neonatal Th2-polarizing responses toward food and aeroantigens might induce the patterns that are associated with childhood atopy (2, 3).

We have previously demonstrated that neonatal Toll-like receptor (TLR) responses rapidly change during the first month of life, maturing from a Th2-polarizing phenotype to increased production of Th1-polarizing cytokines (4). The first month of life might thus provide a window of opportunity for interventions aimed at preventing atopy. Indeed, early life exposure to environmental factors, for example cigarette smoke (5), has been associated with increased risk of asthma or atopy, while other factors, such as siblings (6), pets (7) and breastfeeding (8), are protective. Although *in vitro* studies and animal models have shown that many of these factors have immune modulatory functions (9–11), only few studies have investigated the effects of early life environmental exposure on neonatal innate immune function in humans (12, 13). The

current study aims to provide quantitative evidence of environmental factors influencing neonatal innate immune cellularity and TLR-mediated cytokine production at the age of 1 month.

Methods

Study design and recruitment criteria

A prospective birth cohort study was performed in two urban hospitals in Utrecht, The Netherlands (5). Eligible were healthy neonates born after uncomplicated pregnancy of ≥ 37 wks after either vaginal delivery or elective cesarean section. Excluded were neonates with major congenital anomalies and neonates whose parents had insufficient knowledge of the Dutch or English language to comply with the research protocol. To avoid extensive counseling of parents just after delivery, a two-step inclusion system was used (Fig. 1). The first consent interview took place around child-birth, during which parents were informed about the study but no decision on study participation was made. One to 3 wk after delivery, parents were recontacted. During this second interview, informed consent was obtained, and a visit to the hospital was scheduled. The study was approved by the ethical review boards of the University Medical Center Utrecht and the Diaconessen Hospital. All parents provided written informed consent for study participation.

Clinical characteristics

Clinical characteristics were collected from the hospital charts and from standardized parental questionnaires performed by a physician or trained research nurse (14). Determinants assessed were mode of delivery (elective cesarean section vs. vaginal delivery), exclusive breastfeeding during the first month of life, presence of siblings or household pets and parental smoking. Birth month was included as a surrogate marker for exposure to seasonal antigens [e.g., respiratory viruses, pollen, indoor endotoxin]. For seasonality analysis, birth month was used as an ordinal variable [values 1–12]. For regression analysis, birth month was dichotomized into birth in April–September vs birth in October–March.

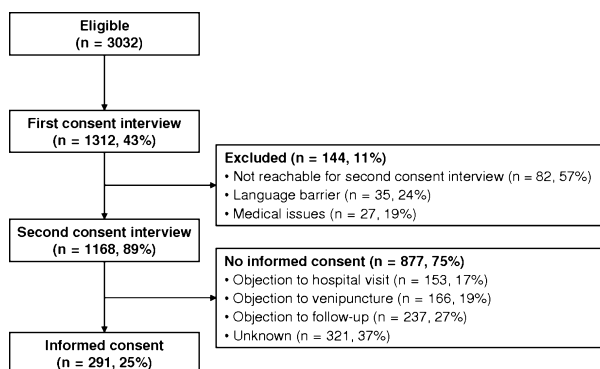


Figure 1 Flowchart of study population.

Although not directly related to environmental exposure, parental atopy was included into analyses as a marker of genetic background and because of its reported association with cord blood TLR responses (15). Parental atopy was defined as physician-diagnosed asthma, hay fever or atopic dermatitis in either parent.

Blood

At the age of 1 month, 0.5–3 ml of neonatal blood was obtained by venipuncture and collected in sterile sodium heparin tubes. None of the participants had any sign or symptoms of infectious disease (e.g., runny nose, fever) in the 2 wks prior to sampling.

Immune cell concentrations

Whole-blood concentrations of innate immune cells were determined as previously described [Table S1 and (4)]. Whole-blood concentrations of neutrophils, eosinophils and basophils were determined by total and differential leukocyte count (Cell-Dyn Sapphire haematology analyzer; Abbott Diagnostics, Abbott Park, IL, USA). Percentages of myeloid dendritic cells (mDC), plasmacytoid dendritic cells (pDC), monocytes, natural killer (NK) cells and T cells were measured by flow cytometry, using markers depicted in Table S1 and headings of figure 2 and figure S1. Percentages were converted to absolute concentrations by multiplying the percentage of cells in the lympho/monocyte gate (as determined by forward–sideward scatter properties) by the total numbers of lymphocytes and monocytes from the differential leukocyte count.

TLR stimulation

In vitro TLR stimulation was performed in 1:14 diluted whole blood, using optimal concentrations of TLR agonists and incubation times for cytokine measurements, as titrated in pilot experiments and described previously [Table S2 and (4)]. Cytokines were arbitrarily chosen based on their pro-inflammatory (TNF- α , IFN- α , IL-12p70) or regulatory properties (IL-10). TNF- α production was only measured upon TLR2-stimulation. We were unable to detect any TLR2-mediated IL-10 production.

Owing to technical reasons, data on TLR2 and TLR7-mediated cytokine responses were available for part of the cohort (n = 95 and n = 128, respectively). For most environmental determinants, this did not affect statistical power substantially. However, none of the neonates with data on TLR2-mediated TNF- α production were born after cesarean section, and no comparisons were made for this outcome parameter.

After a 4-h (TNF- α) or 24-h (IFN- α , IL-12p70, IL-10) incubation at 37°C and 5% CO₂, samples were centrifuged at 1000 g for 5 min, and supernatants were collected and stored at –80°C until further analysis. Cytokine concentrations in culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions: TNF- α , IL-10 (both from Sanquin/CLB,

Amsterdam, The Netherlands), IL-12p70 (Diacclone Research, Besançon, France) and IFN- α (Bender Medsystems, Burlingame, CA, USA). The lower limits of detection were 1.0 pg/ml (TNF- α and IL-10) and 2.0 pg/ml (IL-12p70 and IFN- α). For samples in which the cytokine concentration was below the detection limit, the concentration was arbitrarily defined as half of the detection limit.

Statistical analysis

Baseline characteristics were compared between participants and non-participants using the Student's *t*-test (gestational age, birth weight) or the χ^2 -test (gender, mode of delivery, birth season, presence of siblings). Innate immune variables were normalized by means of logarithmic transformation, and geometric means were compared between neonates with and without exposure to the environmental determinant of interest, using the Student's *t*-test. Linear regression was used to assess the weighted effects (expressed as the regression coefficient β and 95% confidence interval of β) of multiple variables on continuous outcome measures (e.g., the relative influence of breastfeeding or siblings on TLR3-mediated IL-12p70). In addition to environmental determinants, parental atopy was included in the regression analysis as a marker of genetic predisposition. To correct for multiple testing, the Bonferroni method was used, setting the threshold for statistical significance at $p < 0.0005$.

Seasonality of innate immune variables was tested by fitting logarithmically transformed cell concentrations or cytokine levels to a sine function with a period of 12 months in a non-linear regression model. Statistical significance of this seasonal distribution was determined using the *F*-test. Because this type of analysis has an inherent risk of over fitting, a more stringent cutoff was used for statistical significance ($p < 0.01$). To address the possibility that seasonal patterns were attributable to methodological factors (e.g., differences in reagents or machinery during the study period), sensitivity analyses were performed, analyzing each birth year in our cohort separately. All reported *p*-values are two-sided. Analyses were performed in SPSS 15.0 (SPSS Inc, Chicago, IL).

Results

Study population

From March 2006 to February 2010, 3032 neonates were born in participating hospitals (Fig. 1). Parents of 1168 (39%) of these neonates were asked to participate, of which 291 (25%) gave written informed consent for study participation. There were no differences in gender, birth weight, gestational age, siblings or birth season between participating and non-participating neonates (Table S3). Participating neonates were more likely to be born through elective cesarean section compared to non-participants (14% vs. 9%, $p = 0.04$). Complete perinatal data were available for all newborns, and 284 (98%) provided information on exposure during the first month of life. Characteristics of the study population are shown in Table 1.

Table 1 Characteristics of study population

Characteristic	N = 291
Gender, n (%)	
Male	141 (48)
Female	150 (52)
Gestational age, wk (SEM)	40.0 (0.06)
Birthweight, g (SEM)	3611 (27)
Mode of delivery	
Vaginal	251 (86)
Cesarean section	40 (14)
Birth season	
Spring	70 (24)
Summer	76 (26)
Fall	72 (25)
Winter	73 (25)
Any siblings, n (%)	186 (64)
Exclusive breastfeeding, n (%)	154 (53)
Pets, n (%)	92 (32)
Parental smoking, n (%)	73 (25)
Parental atopy, n (%)	160 (55)

Determinants of neonatal innate immunity

To determine the major postnatal determinants of neonatal innate immunity at the age of 1 month, univariable analysis and multivariable logistic regression analysis were performed. In univariable analyses (Figs 2–4), six environmental determinants were analyzed in relation to 16 measures of neonatal innate immunity: whole-blood concentrations of seven different innate immune cells (Fig. 2) and nine TLR-mediated cytokine responses at the age of 1 month (Fig. 3). Associations with birth month are depicted in Fig. 4. Results of multivariable analyses are shown in Tables 2 and 3 (only significant associations are shown). Of 96 combinations tested, 18 (19%) significant univariable associations were found. Breastfeeding, birth month and mode of delivery were the major determinants of neonatal innate immunity, affecting 5 (31%), 4 (25%) and 3 (19%) of neonatal innate immune parameters, respectively.

Breastfeeding

Exclusive breastfeeding during the first month of life was associated with decreased whole-blood concentrations of monocytes (3.3×10^6 vs. 4.5×10^6 /l, $p = 0.03$, Fig. 2a) and neutrophils (1.6×10^9 vs. 2.0×10^9 /l, $p = 0.01$, Fig. 2b), two-fold decreased TLR2-mediated TNF- α ($p = 0.04$, Fig. 3a), twofold increased TLR3-mediated IL-12p70 ($p = 0.002$, Fig. 3b) and fourfold decreased TLR7-mediated IL-10 ($p = 0.001$, Fig. 3g) compared formula or mixed feeding. Except for TLR3-mediated IL-12p70, all associations were independent (Table 3) and the association between breastfeeding and TLR7-mediated IL-10 remained significant after correction for multiple testing. To exclude the possibility that decreased TLR7-mediated IL-10 production in breastfed neonates was attributable to decreased numbers of pDCs or B

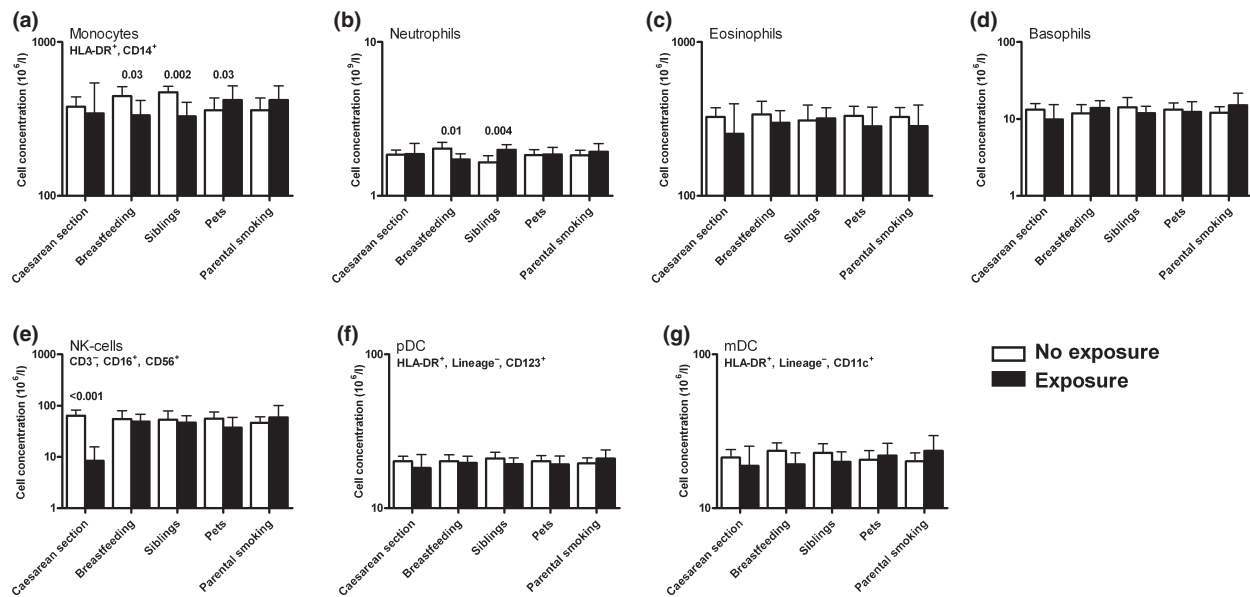


Figure 2 Associations between environmental factors and concentrations of innate immune cells at the age of 1 month. Neonatal venous blood was obtained at the age of 1 month ($n = 291$). Concentrations of neutrophils, eosinophils and basophils were determined by differential leukocyte count. Percentages of monocytes,

cells (the main cells capable of producing IL-10 upon TLR7 stimulation, we calculated TLR7-mediated IL-10 per pDC or B cell. Both TLR7-mediated IL-10 production per pDC and TLR7-mediated IL-10 production per B cell were significantly lower in breastfed neonates compared to formula-fed neonates (12.5×10^3 vs. 22.5×10^3 pg/l/pDC, $p = 0.013$ and 117 vs. 379 pg/l/B-cell, $p = 0.021$).

We next aimed to determine whether decreased TLR7-mediated IL-10 production in breastfed neonates translated to differences in adaptive immunity. Therefore, we compared absolute and relative concentrations of $CD4^+$ T cells, $CD8^+$ T cells, naïve T cells and activated T cells between breastfed and formula-fed neonates (Fig. S1). There was no association between breastfeeding and absolute concentrations of T cells, nor in their relative proportions.

Mode of delivery

Birth via elective cesarean section was associated with eight-fold lower concentrations of NK cells ($p < 0.001$) (Fig. 2e), with twofold decreased TLR3-mediated IL-12p70 ($p = 0.001$, Fig. 3b) and twofold increased TLR4-mediated IL-12p70 production ($p = 0.05$, Fig. 3d) as compared to vaginal delivery. Associations with concentrations of NK cells (Table 2) and TLR3-mediated IL-12p70 were independent and remained significant after correction for multiple testing.

Birth month

Seasonal patterns by birth month were observed for whole-blood concentrations of NK cells and mDC and for cytokine

natural killer cells, plasmacytoid dendritic cells and myeloid dendritic cells were determined by flow cytometry and multiplied by the total concentration of lymphocytes and monocytes from the complete blood count. Data are represented as geometric means and 95% confidence intervals derived from log-transformed variables.

responses to TLR3 and TLR7 (Fig. 4). Birth in winter months was associated with low TLR-3 mediated IL-12p70 production ($p < 0.001$) and high production of IL-10 upon stimulation of TLR7 ($p < 0.001$). Sensitivity analyses showed the same seasonal effects when each inclusion year was analyzed separately (data not shown). Except for mDC, all associations were independent (Table 2), and the association between birth month and TLR7-mediated IL-10 remained significant after correction for multiple testing. In multivariable regression analysis, two additional significant associations were identified, demonstrating that birth in winter months was associated with increased concentrations of monocytes ($p < 0.001$) and pDC ($p = 0.02$) compared to birth in summer months (Table 2).

Siblings, pets and parental smoking

No significant associations were found between presence of siblings, household pets and parental smoking and any parameter of neonatal immune function at the age of 1 month, neither in univariable analysis (Figs 2 and 3), nor in multivariable logistic regression analysis.

Prenatal determinants of neonatal innate immunity

To determine the role of the prenatal environment on neonatal immune function, characteristics of study participants at birth, including birth weight, gestational age and parental atopy were related to concentrations of innate immune cells and TLR-mediated cytokine responses at the age of 1 month. Birth weight and gestational age were not associ-

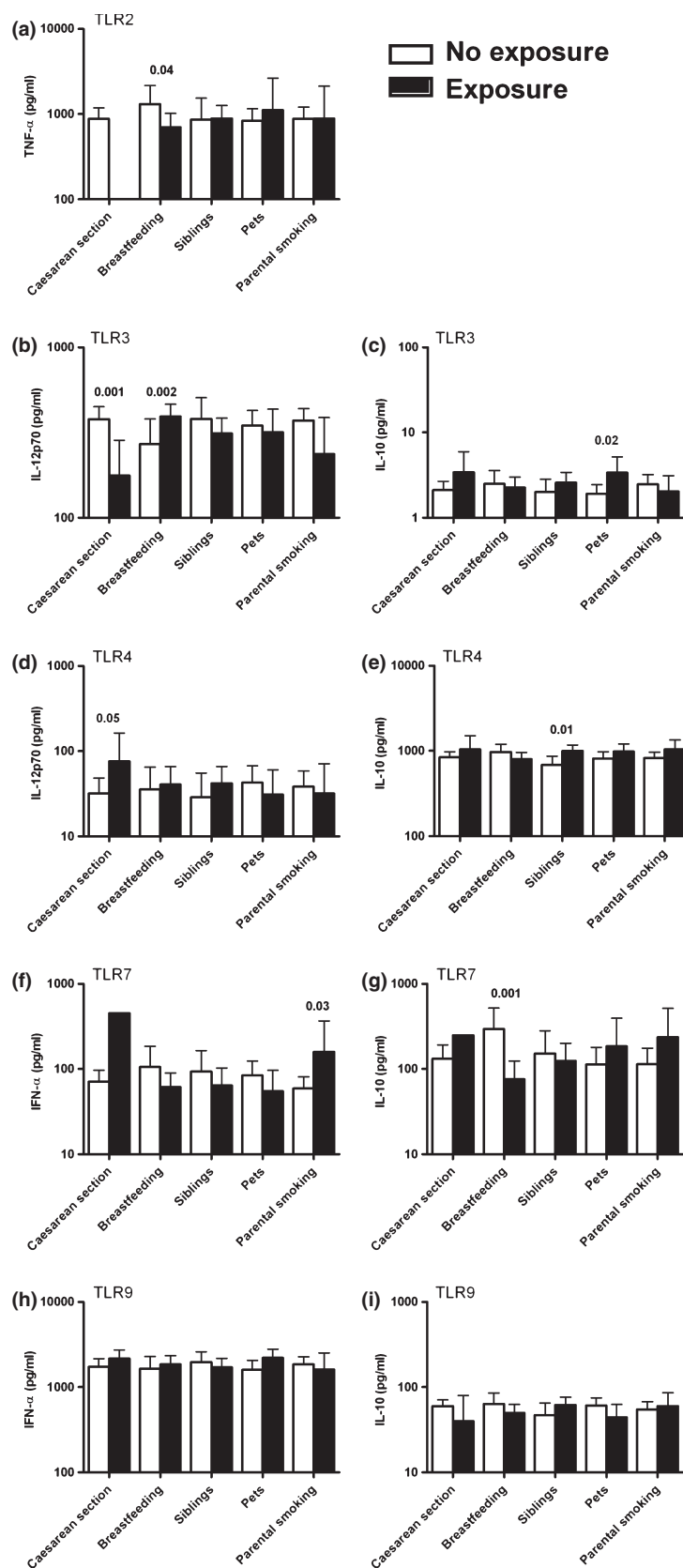


Figure 3 Associations between environmental factors and Toll-like receptor (TLR)-mediated cytokine responses at the age of 1 month. Cytokine production in neonatal venous blood at age 1 month ($n = 291$) was measured after a 4-h incubation with Pam₃Cys SSNA (TLR2, panel A) or a 24-h incubation with poly I:C (TLR3, B + C), LPS+IFN- γ (TLR4, D + E), loxoribine (TLR7, F + G) and ODN CpG (TLR9, H + I). Data are represented as geometric means and 95% confidence intervals derived from log-transformed variables.

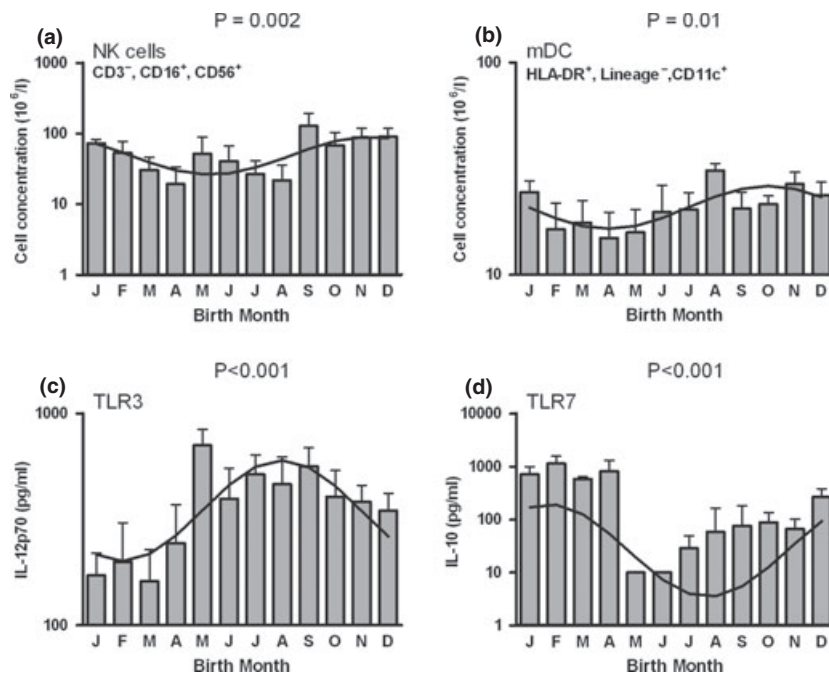


Figure 4 Seasonal patterns of neonatal whole-blood concentrations of innate immune cells and Toll-like receptor-mediated cytokine responses according to birth month. Seasonality was assessed by

fitting the data to a sinusoidal curve, and statistical significance was calculated using the *F*-test. Bars represent geometric means and 95% confidence intervals derived from log-transformed variables.

Table 2 Independent associations between environmental exposure and whole-blood concentrations of innate immune cells at the age of 1 month

	β (95% CI)	p-Value
Monocytes		
Birth April–September	−0.24 (−0.35 to −0.12)	<0.001*
Siblings	−0.14 (−0.26 to −0.17)	0.03
Neutrophils		
Breastfeeding	−0.17 (−0.13 to −0.02)	0.006
Siblings	0.19 (0.03–0.14)	0.002
NK cells		
Mode of delivery (CS)	−0.34 (−1.14 to −0.55)	<0.001*
Birth April–September	0.14 (0.04–0.44)	0.02
pDC		
Birth April–September	−0.16 (−0.14 to 0.02)	0.01

Variables included into regression analysis were parental atopy, birth in April–September, mode of delivery, breastfeeding, siblings, pets and parental smoking. Significant associations are shown.

NK, natural killer; pDC, plasmacytoid dendritic cells.

*Remains statistically significant after correction for multiple testing.

ated with neonatal innate immune cell concentrations, nor with TLR-mediated cytokine responses. Parental atopy was associated with decreased TLR3-mediated IL-12p70 (280 vs. 425 pg/ml, $p = 0.02$, both in univariate analysis (Table S3) and in multivariate analysis (Table 3). However, this association lost significance after correction for multiple testing.

Neonatal concentrations of innate immune cells and TLR responses are differentially sensitive to environmental exposure in the first month of life

When the collective effect of environmental exposure on innate immune responses at the age of 1 month was analyzed, distinct patterns emerged. For example, concentrations of monocytes were sensitive to three environmental factors (negatively associated with breastfeeding and siblings, positively associated with pets) (Fig. 2a), whereas concentrations of eosinophils, basophils and mDC were relatively inert. For TLR responses, high sensitivity to environmental influences was found for TLR3-mediated IL-12p70 (birth month, cesarean section, breastfeeding) (Fig. 3b), which was also associated with parental atopy (Table S4). In contrast to TLR3-mediated IL-12p70 production, TLR9-mediated production of IFN- α and IL-10 was not associated with any environmental factor (Fig. 3h–i).

Discussion

This study demonstrates that whole-blood concentrations of innate immune cells and TLR-mediated cytokine responses are modulated by environmental exposure in the first month of life. Breastfeeding had the highest influence on neonatal concentrations of innate immune cells and TLR-mediated cytokine responses. Concentrations of monocytes and TLR3-mediated IL-12p70 were most sensitive to environmental modulation, whereas responses to TLR9 were largely inert.

Table 3 Independent associations between environmental exposure and TLR-mediated cytokine responses at the age of 1 month

TLR2	TNF- α				
	β (95% CI)	p-Value			
Breastfeeding	-0.25 (-0.52 to -0.11)	0.04			
TLR3	IL-12p70			IL-10	
	β (95% CI)	p-Value		β (95% CI)	p-Value
Parental atopy	-0.16 (-0.31 to -0.03)	0.02	Pets	0.20 (0.05–0.46)	0.01
Mode of delivery (CS)	-0.22 (-0.54 to -0.14)	0.001			
Birth April–September	-0.22 (-0.37 to -0.10)	0.001			
Parental smoking	-0.15 (-0.35 to -0.03)	0.02			
TLR4	IL-12p70			IL-10	
	β (95% CI)	p-Value		β (95% CI)	p-Value
Parental atopy	0.13 (0.00–0.66)	0.05	Siblings	0.17 (0.04–0.29)	0.01
TLR7	IFN- α			IL-10	
	β (95% CI)	p-Value		β (95% CI)	p-Value
Parental atopy	0.18 (0.004–0.54)	0.05	Birth April–September	0.35 (0.32–0.92)	<0.001*
Parental smoking	0.24 (0.10–0.72)	0.009	Breastfeeding	-0.32 (-0.87 to -0.27)	<0.001*

Variables included into regression analysis were parental atopy, birth in April–September, mode of delivery, breastfeeding, siblings, pets and parental smoking. Significant associations are shown.

TLR, Toll-like receptor.

*Remains statistically significant after correction for multiple testing.

Previous birth cohort studies have demonstrated that dys-regulated innate immune responses at birth predispose to the subsequent risk of childhood atopy. Low TLR4-mediated IL-12p70 in cord blood is associated with enhanced allergen-induced Th2-type responses in the postnatal period (2). Moreover, neonates who subsequently develop atopic dermatitis already have increased TNF- α and IL-6 in response to TLR2, TLR3, TLR4 and TLR5 at birth (15). Parental characteristics are likely to explain part of the association between cord blood TLR responses and atopy. Two recent prospective studies in high-risk neonates have demonstrated that maternal atopy is associated with distinct cord blood TLR expression and function (15, 16). These associations are likely partially explained by genetic factors (17). In addition, increasing evidence indicates that the intrauterine environment importantly impacts on fetal immune development, thus predisposing to subsequent infections and atopy (18, 19).

We have previously identified the first month of life as a period of rapid maturation of the neonatal TLR system, offering a window of opportunity for interventions aimed at modulating innate immune development and preventing atopy. During this important period, TLR-mediated cytokine responses rapidly mature from a Th2-biased profile toward increased Th1-cell polarizing responses characteristic of later life (4). Early life exposure to TLR (ant)agonists, including microbial products, is thought to drive postnatal TLR maturation.

TLR responses at the age of 1 month thus likely reflect previous exposures that have modulated neonatal innate immune maturation. Here, we provide a comprehensive overview of environmental factors guiding postnatal TLR maturation. Our observations show that neonatal TLR responses at the age of 1 month differ according to environmental exposure during the first month of life, suggesting that strategies aimed at modulating early immune–environment interactions might be effective to prevent subsequent allergic disease.

We identify several potentially modifiable factors that modulate postnatal innate immune function. First, innate immune responses at the age of 1 month were markedly different between neonates who received exclusive breastfeeding during the first month of life and those who did not. Several explanations may account for the association between breastfeeding and low TLR7-mediated IL-10 production. First, breast milk contains multiple immune modulatory compounds that directly influence TLR-mediated immune responses, including immunoglobulins, nucleotides, oligosaccharides and antimicrobial proteins/peptides (20, 21). *In vitro*, breast milk increases monocyte production of IL-10 while decreasing production of IFN- γ in response to lipopolysaccharide, mitogen and allergen (22). Immune modulation by breast milk is TLR-specific, as breast milk suppresses IL-8 production to agonists for TLR2 and TLR3, while enhancing responses to TLR4 and TLR5 (9). To date, no studies have

investigated the direct effects of breast milk on responses to agonists for TLR7. We propose that suppression of TLR7-mediated IL-10 production may promote neonatal defense against viral infections, while induction of TLR4-mediated IL-10 production serves to promote neonatal tolerance to bacterial colonization.

Second, decreased TLR7-mediated IL-10 production at the age of 1 month might be due to decreased incidence of respiratory viral infections in breastfed neonates. Both respiratory syncytial virus (RSV) and rhinovirus, the most common causes of neonatal respiratory tract infections, are single-stranded RNA viruses that trigger TLR7 (23). Severe RSV infection is associated with increased production of IL-10 during and after infection (24). Breastfeeding protects against severe RSV infections (24) and reduces RSV-mediated immune activation (25). Reduced incidence of severe RSV infections in the first month of life may account for the lower IL-10 production in breastfed neonates. However, in our cohort, we did not find any difference in TLR7-mediated IL-10 between neonates with and without history of respiratory tract infections in the first month of life ($p = 0.96$). In addition, as RSV respiratory tract infections are relatively rare in the first 2–3 months (26), other mechanisms are likely to play a role.

Third, decreased TLR7-mediated IL-10 production (and increased TLR3-mediated IL-12p70) production in breastfed infants may also reflect more rapid transition to a Th1-polarized innate immune system. Accordingly, promotion of early life innate immune maturation might be one of the mechanisms by which breastfeeding protects against subsequent allergic disease. However, breastfeeding did not affect cytokine responses to TLR4 and TLR9. More insight into the effects of breast milk on the developing immune system is needed for the generation of immune modulatory strategies that use breastfeeding to prevent subsequent allergy.

Mode of delivery may provide a second target to modulate neonatal immune function. Consistent with findings in cord blood (27), neonates born after cesarean section had decreased NK cells and decreased TLR3-mediated IL-12p70 at the age of 1 month. Decreased neonatal pro-inflammatory responses may contribute to the increased risk of childhood asthma in infants born after cesarean section (28).

A third target for immune modulation is birth month. Birth month significantly influences subsequent neonatal innate immune function. Decreased TLR3-mediated IL-12p70 and increased TLR7-mediated IL-10 in neonates born in winter potentially indicate increased Th2-polarization, which might predispose to subsequent allergy and asthma (15). Interestingly, a recent study in cord blood demonstrates that birth in winter is associated with general hypo-responsiveness of T-cell-mediated production of Th1-type (IFN- γ) and Th2-type (IL-5) and regulatory (IL-10) responses, suggesting that part of the differences in immune responses induced by birth month are determined before birth (29). Many seasonal factor(s) might be responsible for this effect, including respiratory viruses, indoor endotoxin, seasonal allergens and vitamin D (15, 30, 31). Future studies will be needed to identify these factors and to determine their possible effects on the health of children later in life.

Finally, exposure to pets, siblings or cigarette smoke in the first month of life all left their imprint on the neonatal immune system. However, no consistent pattern toward Th1- or Th2 polarization was observed. Future studies will be needed to further delineate the interactions between environmental factors and the developing neonatal immune system, and the consequences for disease.

The effect of environmental exposure may depend not only on the type and amount of exposure but also on the relative maturational state of the innate immune system at the time of exposure. We have previously demonstrated that cytokine responses to TLR3 and TLR4 rapidly mature during the first month of life, whereas responses to TLR9 are already mature at birth (4). In the current study, responses to TLR3 and TLR4-mediated IL-12p70 were most sensitive to environmental modulation, whereas TLR9-mediated responses were inert. Thus, sensitivity to environmental triggers might differ between individual TLRs and/or cytokines, with highest sensitivity for those systems that undergo most rapid maturation after birth.

This study has several strengths. First, we included a large, unselected cohort of healthy newborns, whereas previous birth cohort studies selected children from atopic parents (11, 15). Second, we used an unbiased approach, investigating the effect of a broad panel of environmental determinants on neonatal innate immune function. Third, in addition to concentrations of innate immune cells, we also studied their functionality by measuring TLR-mediated cytokine production.

Potential limitations also deserve discussion. First, this study included multiple comparisons and is thus prone to false discovery. To minimize the possibility of type I error, we focused on general patterns that were consistent among different determinants. The high number of significant associations and the consistency with previous cord blood studies indicate that the majority represent real associations. Future studies will be needed to validate these findings. Second, the innate immune parameters measured in our study only represent part of the wide spectrum of genes that is activated upon TLR signaling. Third, we did not measure neonatal immune function in cord blood, and we cannot exclude any prenatal effects of environmental factors on neonatal immunity. Longitudinal studies, measuring neonatal immune function at multiple time points, are needed to establish the exact timing of environment-mediated immune modulation. Meanwhile, based on the rapid development neonatal immunity in the first month of life, we propose that the effect of the environmental determinants primarily affects postnatal immune development.

In conclusion, this study demonstrates that neonatal innate immune responses are associated with multiple environmental exposures, of which breastfeeding is the most important. Breast milk-mediated modulation of the developing innate immune system might create the patterns that protect from subsequent disease. Interventions aimed at modulating early life immune–environment interactions, including breastfeeding, may be effective in the prevention of infections, asthma and/or atopy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Associations between breastfeeding and concentrations of CD4⁺ and CD8⁺ T-cells at the age of 1 month.

Table S1. Markers used to identify innate immune cells by flow cytometry.

Table S2. Agonists used for *in vitro* TLR stimulation.

Table S3. Characteristics of study participants and non-participants.

Table S4. Effect of parental atopy on neonatal whole blood concentrations of innate immune cells and TLR-mediated cytokine responses at the age of 1 month.

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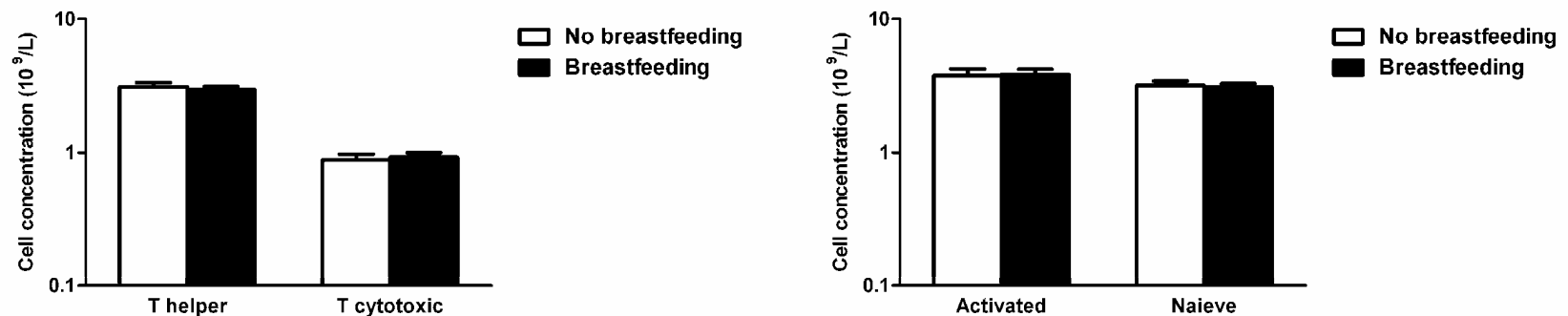
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Supplementary figure 1: Associations between breastfeeding and concentrations of CD4⁺ and CD8⁺ T-cells at the age of one month.

Neonatal venous blood was obtained at the age of one month (n=291). Concentrations of lymphocytes were determined by differential leukocyte count. Percentages CD3⁺/CD4⁺ helper T-cells, CD3⁺/CD8⁺ cytotoxic T cells, CD3⁺/CD45RO⁺ activated T cells and CD3⁺/CD45RA⁺ naïve T cells were determined by flow cytometry and multiplied by the total concentrations of lymphocytes and monocytes from the complete blood count. Data are represented as geometric means and 95% confidence intervals derived from log-transformed variables.



Supplementary table 1: Markers used to identify innate immune cells by flow cytometry

Cell type	Marker	Fluorochrome	Clone
Monocytes	HLA-DR ⁺	PerCP	L243
	CD14 ⁺	FITC	MoP9
NK-cells	CD3 ⁻	APC	SK7
	CD16 ⁺	PE	B73.1
	CD56 ⁺	FITC	NCAM16.2
Plasmacytoid DC	HLA-DR ⁺	PerCP	L243
	CD123 ⁺	PE	9F5
	Lineage ⁻	FITC	
	CD3		SK7
	CD16		3G8
	CD19		SJ25C1
	CD20		L27
	CD14		MoP9
	CD56		NCAM16.2
Myeloid DC	HLA-DR ⁺	PerCP	L243
	Lineage ⁻	FITC	
	CD3		SK7
	CD16		3G8
	CD19		SJ25C1
	CD20		L27
	CD14		MoP9
	CD56		NCAM16.2
	CD11c ⁺	APC	S-HCL-3
T cells	CD3 ⁺	APC	SK7
T helper	CD4 ⁺	PerCP	SK3
T cytotoxic	CD8 ⁺	FITC	SK1
Activated	CD45RO ⁺	PE	UCHL1
Naive	CD45RA ⁺	FITC	L48

Supplementary table 2: Agonists used for *in vitro* TLR stimulation

TLR	Agonist	Concentration	Manufacturer
2	Pam ₃ Cys SSNA	1 µg/mL	Bachem Biosciences, Weil am Rhein, Germany
3	Polyinosinic:polycytidylic acid (Poly I:C)	200 µg/mL	Invivogen, San Diego, CA
4	Ultrapure lipopolysaccharide derived from <i>E. Coli</i>	100 ng/mL	Invivogen
	Interferon-γ (IFN-γ)	20 ng/mL	Preprotech Inc, Rocky Hill, NJ
7	Loxoribine	1 mM	Invivogen
9	CpG oligodeoxynucleotide (CpG ODN)	30 µg/mL	Invivogen

Supplementary table 3: Characteristics of study participants and non-participants

Characteristic	Participants (n=291)	Non-participants (n=1021)	p-value
Gender, n (%)			0.43
Male	141 (48)	520 (51)	
Female	150 (52)	497 (49)	
Gestational age, wks (SEM)	40.0 (0.06)	40.0 (0.03)	0.97
Birthweight, g (SEM)	3611 (27)	3567 (36)	0.53
Mode of delivery			
Vaginal	251 (86)	922 (91)	0.04
Caesarean section	40 (14)	95 (9)	
Birth season			0.93
Spring	70 (24)	249 (25)	
Summer	76 (26)	263 (26)	
Fall	72 (25)	251 (25)	
Winter	73 (25)	254 (25)	
Any siblings, n (%)	186 (64)	626 (63)	0.68

Supplementary table 4: Effect of parental atopy on neonatal whole blood concentrations of innate immune cells and TLR-mediated cytokine responses at the age of one month.

Parental atopy was defined as physician-diagnosed asthma, hay fever or atopic dermatitis in either parent. Values represent geometric means and 95% confidence intervals derived from log-transformed variables.

Innate immune parameter	Parental atopy (n=160)	No parental atopy (n=131)	p-value
<i>Whole blood cell concentrations (10⁶/L)</i>			
Monocytes	379 (314-459)	377 (304-468)	0.97
Neutrophils	1825 (1666-1999)	1884 (1725-2057)	0.63
Eosinophils	315 (268-371)	315 (251-395)	0.99
Basophils	14.5 (11.5-18.3)	11.2 (8.7-14.4)	0.13
NK-cells	52.9 (37.4-74.7)	45.8 (31.8-65.9)	0.57
pDC	19.6 (18.1-21.2)	20.2 (17.9-22.9)	0.66
mDC	19.6 (16.8-23.0)	22.9 (19.7-26.7)	0.17
<i>TLR-mediated cytokine responses (pg/mL)</i>			
TLR2; TNF- α	925 (633-1351)	822 (480-1405)	0.72
TLR3; IL-12p70	280 (219-358)	425 (341-530)	0.02*
TLR3; IL-10	2.3 (1.7-2.3)	2.4 (1.7-3.3)	0.99
TLR4; IL-12p70	53.6 (32.5-88.3)	25.2 (14.2-44.6)	0.05
TLR4; IL-10	846 (691-1036)	887 (732-1075)	0.75
TLR7; IFN- α	99.1 (62.8-156)	54.0 (34.9-83.5)	0.06
TLR7; IL-10	158 (93.8-264)	98.6 (54.0-180)	0.24
TLR9; IFN- α	1750 (1365-2245)	1789 (1310-2442)	0.91
TLR9; IL-10	48.3 (37.4-62.5)	65.1 (50.7-83.6)	0.11