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SNAPSHOT OF CYTOKINE PRODUCTION BY MEDITERRANEAN NEWBORNS. INFLUENCE OF SEX AND SEASON OF BIRTH

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Complete List of Authors:	Garcia-Serna, Azahara; Biomedical Research Institute of Murcia (IMIB-Arrixaca); University of Murcia, Biochemistry and Molecular Biology (B) and Immunology Morales, Eva; Biomedical Research Institute of Murcia (IMIB-Arrixaca); University of Murcia, Department of Public Health Sciences Cantero-Cano, Ester; Biomedical Research Institute of Murcia (IMIB-Arrixaca) Norte-Muñoz, Maria; University of Murcia, Biochemistry and Molecular Biology B and Immunology; Biomedical Research Institute of Murcia (IMIB-Arrixaca) Gil-Buendia, Mª Angeles; "Virgen de la Arrixaca" University Clinical Hospital, Obstetrics & Gynecology Service Velazquez-Marin, Josefa; "Virgen de la Arrixaca" University Clinical Hospital, Obstetrics & Gynecology Service Hernandez-Caselles, Trinidad; University of Murcia, Biochemistry and Molecular Biology B and Immunology; Biomedical Research Institute of Murcia (IMIB-Arrixaca) Perez-Fernandez, Virginia; Biomedical Research Institute of Murcia (IMIB-Arrixaca); University of Murcia, Department of Public Health Sciences Martinez-Torres, Antonia; Biomedical Research Institute of Murcia (IMIB-Arrixaca); Virgen de la Arrixaca University Children's Hospital, University of Murcia and ARADyAL Allergy Network, Paediatric Allergy and Pulmonology Units Garcia-Marcos, Luis; Biomedical Research Institute of Murcia (IMIB-Arrixaca), Murcia, Spain; Virgen de la Arrixaca University Children's Hospital, University of Murcia and ARADyAL Allergy Network, Murcia, Spain, Pediatric Allergy and Pulmonology Units Martin-Orozco, Elena; University of Murcia, Biochemistry and Molecular Biology B and Immunology; Biomedical Research Institute of Murcia (IMIB-Arrixaca)
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SNAPSHOT OF CYTOKINE PRODUCTION BY MEDITERRANEAN NEWBORNS. INFLUENCE OF SEX AND SEASON OF BIRTH.

Azahara M García-Serna, BSc,[1,2] Eva Morales, MD, PhD,[1,3,*] Ester Cantero-Cano, BSc,[1] María Norte-Muñoz, BSc,[1,2] Mª Angeles Gil-Buendía, BSc,[5], Josefa Velazquez-Marin, BSc,[5], Trinidad Hernández-Caselles, PhD,[1,2,6] Virginia Pérez-Fernández, PhD,[1,3], Antonia Elena Martínez-Torres, MD, PhD,[1,4], Luis García-Marcos, MD, PhD,[1,4], Elena Martin-Orozco, PhD,[1,2,6,*] and the NELA study group[**].

[1] Biomedical Research Institute of Murcia (IMIB-Arrixaca), Murcia, Spain

[2] Department of Biochemistry and Molecular Biology (B) and Immunology, University of Murcia, Murcia, Spain

[3] Department of Public Health Sciences, University of Murcia, Spain

[4] Paediatric Allergy and Pulmonology Units, Virgen de la Arrixaca University Children's Hospital, University of Murcia and ARADyAL Allergy Network, Murcia, Spain.

[5] Obstetrics & Gynecology Service, "Virgen de la Arrixaca" University Clinical Hospital, University of Murcia, Spain

[6] Network of Asthma and Adverse and Allergic Reactions (ARADyAL)

[**]Members of the NELA study group listed at the end of the article.

***Contact:**

Dr. Elena Martín-Orozco, PhD; Department of Biochemistry and Molecular Biology (B) and Immunology, University of Murcia and Biomedical Research Institute of Murcia (IMIB-

Arrixaca), Building LAIB, 4th floor, Desk 4-58, Ctra Buenavista s/n, 30120-El Palmar, Murcia, Spain. Tel: +34 868883988; E-mail: emartin@um.es.

Dr Eva Morales, MD, PhD, MPH; Biomedical Research Institute of Murcia (IMIB-Arrixaca); University of Murcia; Buenavista Rd., 30120 Murcia, Spain. Tel: +34 868883691; E-mails: embarto@hotmail.com; evamorales@um.es

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Ms. Mª Angeles Gil-Buendia: The author declares no conflicts of interest.

Ms. Josefa Velazquez-Marin: The author declares no conflicts of interest.

Dr. Trinidad Hernandez-Caselles: The author declares no conflicts of interest.

Dr. Virginia Perez-Fernandez: The author declares no conflicts of interest.

Dr. Antonia E. Martinez-Torres: The author declares no conflicts of interest.

Dr. Luis Garcia-Marcos: The author declares no conflicts of interest.

Dr. Elena Martin-Orozco: The author declares no conflicts of interest.

Author Contributions

Conception and design of the study: AGS, EM, LGM, EMO

Acquisition and analysis of data: AGS, EM, ECC, MNM, MAGB, JVM, THC, VPF, AEMT, EMO

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Impact:

- Newborns from the south-eastern Mediterranean area exhibit specific cytokine signatures influenced by sex and season of birth.
- There is a limited number of population-based studies on the immune status at birth and the influence of prenatal and perinatal factors on it.

- The characterization of newborn specific immune signatures could be associated with future clinical outcomes and will improve our understanding of immunity prenatal programming.

ABSTRACT

Background: The characterization of specific immune signatures at birth could be associated with future clinical outcomes and will improve our understanding of immunity prenatal programming.

Methods: Data come from 235 newborns from the prospective birth cohort study NELA. Production of cytokines by stimulated cord blood samples was determined using Luminex technology. Association between cytokine concentrations with sex and season of birth were examined by multivariate regression models.

Results: Cells from newborns produced high levels of inflammatory cytokines; moderate levels of Th1/Th2/Tr related cytokines; and low levels of Th17 cytokines. Male newborn cells secreted higher levels of Th2/Th17 and lower levels of Th1 cytokines in comparison to females. Also, children born during cold seasons mainly secreted innate cytokines meanwhile adaptive immunity cytokines were more frequently secreted by children born during warm seasons.

Conclusion: Newborns from the Mediterranean region showed specific cytokines signatures, which were influenced by sex and season of birth.

Introduction

Cytokine network is the main component of immunity, acting as a general coordinator/regulator of the overall immune response against pathogens and in the initiation and/or development of an immune-related disease. Thus, immunity status is reflected in the profile of cytokines produced by the cells both in absence of stimulus and after stimulation with different antigens [1–7]. Based on that principle, many studies have been conducted to identify the factors that could have an impact on immunity from the conception to birth and beyond, as well as to unravel the relationship between the cytokine signatures at birth and health outcomes later in life, including allergies, asthma, autoimmunity disorders and others [4,8–17]

Although many studies have been published on this issue, several discrepancies have been found that could be explained due to the many factors that could affect the development of immunity and the diversity of cytokines or stimuli used by the different researchers [3,4,9,18–20]. Also, the studies that have been published use cells from individuals of different origins and genetic backgrounds and exposed to different climate areas. In consequence, more studies are needed to shed light on the impact of prenatal and perinatal factors on the immune system development in humans from different climate regions of the world.

The current study is focused on the cytokines released by cord blood cells from newborns participating in an ongoing birth cohort study set up in a Mediterranean area. The cells were cultured in the presence of different stimuli and the cytokine profiles have been analyzed, and, also, the influence on them of two covariates with a role in asthma incidence and severity such as sex and season of birth [21,22]. To the best of our knowledge, this is one of the most comprehensive studies on the newborn cytokine profiles in a cohort from the south-eastern Mediterranean region since we have analyzed cord blood cells from 235 newborn children and determine the secretion of up to twenty cytokines by these cells cultured under nine different conditions. This study will contribute to the knowledge of the cytokine signatures of

Mediterranean newborns and to unravel the influence of sex and season of birth on them. The analysis of cytokine profiles will help to complete the puzzle of the influence of prenatal and/or perinatal factors in the occurrence and evolution of immune related phenotypes and diseases during the first years of life in children from this climate region of the world.

Methods

Study participants

Data come from participants of the Nutrition in Early Life and Asthma (NELA) study (www.nela.imib.es), a prospective population-based birth cohort set up in a south-eastern Mediterranean region of Spain designed to identify the early life origins and mechanism of asthma and allergy. The study protocol and recruitment details have been described previously [23]. In summary, pregnant women with Spanish Caucasian origin, 18-45 years of age, singleton pregnancy, non-assisted conception, and normal echography at 20 weeks of gestation (no major malformations) were invited to participate in the study at the time of the control ultrasound at 20 weeks of gestation at the Maternal-Fetal Unit of the Virgen de la Arrixaca University Hospital from March 2015 to April 2018. Women who suffered from an existing chronic disease, pregnancy complications (except gestational diabetes and hypertensive disorders), and not intending to deliver in the reference hospital were excluded. The study protocol was reviewed and approved by the Ethics Committee of the Biomedical Research Institute of Murcia (IMIB-Arrixaca) in accordance with the guidelines of The Declaration of Helsinki (report 9/14; 29/09/2014). Written informed consents were obtained from parents at recruitment.

Cord blood sample collection, cell stimulation and cytokine assays.

Cord blood samples were collected from the umbilical cord vein and transferred into sterile heparinized tubes. All blood samples used in this study were processed within 48 hours after delivery, based on previous studies [22]. First, a pilot experiment using an ex-vivo system was designed to measure 20 different cytokines secreted by cord blood cells from 12 newborns from the NELA cohort at two time points: 48 hours and 7 days [3,4,18]. Samples were diluted 1:7 with RPMI 1640 medium and cultured unstimulated and in the presence of 8 different stimuli

(**Supplementary Table** S1) as follows: Concanavalin A (Con A), Immunostimulatory CpG oligonucleotides (CpG-ODN), Polyinosinic-polycytidylic acid (pI:C), Peptidoglycan (PG), Lypopolisaccharide (LPS), Phytohaemaglutinin (PHA) and O*lea europaea* (olive, O) and D*ermatophagoides pteronyssinus* (mites extracts, D.p.). The culture supernatants were collected and frozen (-80 ºC) until their analysis for cytokines determination by using the Human Cytokine Multiplex-Assay-Kit according to the manufacturer's instructions (ThermoFisher, Viena, Austria), with Luminex technology. We analyzed general inflammatory response cytokines (IL-6, IFN-α, IL1-β, TNF-α, IL-8, IL-33), T helper 1 (Th1)-related cytokines (IL-12p70, IFN-γ, IL-2, IL-18, MIG), T helper 2 (Th2)-related cytokines (IL-4, IL-5, IL-13), T helper 17 (Th17)-related cytokines (IL-17A, IL-17F, IL-23), T helper 9 (Th9)-related cytokine (IL-9) and T helper 2/T regulatory (Th2/Treg)-related cytokines (IL-10, TGF-β).

Mixes of multiple standard cytokines were used to generate standard curves for each cytokine. We established a lineal range in the curve that was considered the detection range. Measurements out of the detection range were censored: values below the detection range were given a value that corresponded to a half of the lowest value of the detection range of the respective cytokine, and values over the detection range were given a value that corresponded to the highest value of the detection range. The detection limits (pg/ml) of the assay are specified on the **Supplementary Table** S1.

Based on the results of the preliminary experiment **(Supplementary Figure** S1), we decided to collect the supernatants after 7 days of culture. Also, we selected the 12 specific cytokines with the better secretion levels in the pilot experiment, to be analyzed in the supernatant of the 235 newborn samples.

Sociodemographic and clinical variables

Information about sociodemographic and clinical variables was obtained through face-to-face questionnaires administered during pregnancy and after delivery. Variables considered in this study included the following: maternal and paternal age; parity; maternal and paternal education level, maternal pre-pregnancy body mass index (BMI) based on height and pre-pregnancy self-reported weight; maternal tobacco smoking during pregnancy and paternal tobacco smoking; gestational diabetes mellitus, maternal hypertension in pregnancy, parental social class; maternal contact with farming animals during pregnancy; pets at home; heat pump at home; moisture and mold problems at home; maternal and paternal reported history of asthma and atopy. Information related to child's sex, gestational age; birth weight; season of birth; mode of delivery; and fever and use of antibiotics during labor was obtained from clinical records.

Statistical analysis

Descriptive analyses were performed using Mann-Whitney test for continuous variables and Chi-square test for categorical variables. The spearman's rank correlation coefficient with correction for multiple comparisons using the Benjamini-Hochberg method was computed to evaluate correlations between each cytokine and cytokine concentrations across the different stimuli. Distribution of cytokine concentrations was evaluated graphically using box-plot representations and non-parametric tests with correction for multiple comparisons.

Cytokine concentration distributions did not follow a Gaussian distribution and low detection rates were shown after several stimuli. Thus, according to cytokine concentrations, samples were categorized into high responders (H,>60th percentile of each cytokine concentrations) and low responders (L,<40th percentile of each cytokine concentrations). Associations between sex and season of birth with cytokine response patterns were examined using multivariate logistic

regression models. Coefficients of associations are presented as odds ratio (OR) and 95% confidence interval (CI). Variables were retained in multivariate models only if potential confounders were close to significant association ($p < 0.1$) or modified the coefficient by at least 5%. Final models were adjusted for maternal age, maternal body mass index (BMI) pre-pregnancy, maternal history of atopy, gestational age and birth weight. Due to the small sample size, season of birth was dichotomized as cold seasons (winter and autumn births) and warm seasons (spring and summer).

Data were analyzed in Stata Software (version 15.1, StataCorp, College Station, Texas, USA), RStudio (version 1.1.463, RStudio, Boston, Mass) and GraphPad Prism Software (version 8.0.2, GraphPad Software Inc., USA).

Results

Cohort description

As previously described by Garcia-Serna *et al*, among the 738 women embedded in the NELA cohort [25], umbilical cord blood samples were collected in 390 (53%) newborns and cytokine concentrations were determined in 235 (32%) newborns. Compared with excluded participants, those included in the present study had older parents, mothers with higher pre-pregnancy BMI, maternal asthma were reported more frequently, newborns were born at a more advanced gestational age and were heavier, and they were born less frequently during the summer season (**supplementary Table S2**). Overall, both groups did not differ in other main baseline characteristics.

Descriptive analysis of cytokine measurement.

Overall, for unstimulated samples, the detection rates were ≥23% for the following cytokines: IFN-α, IL-1β, IL-6, IFN-γ, IL-2, IL-4, IL-5, IL-13, IL-10, IL-23. Only for TNF-α and IL-17F the detection rates were very low, specifically 15.3 y 4.7%, respectively (**Supplementary Table** S3). For stimulated samples, most of the results were above the LOD and below the UPD for all the stimuli except for CpG-ODN and the olive extract (**Supplementary Tables** S4-S6). Specifically, for the CpG-ODN stimulus the values included between LOD and UPD were below 40 % in the case of IFN-γ, IL-13, IL-17F, IL-23, IL-4, IL-6 and TNF-α cytokines. For the olive extract, detection rates below 40 % were obtained for the following cytokines: IFN-γ, IL-13, IL-17F, IL-23, IL-4, and TNF-α. For the mite extract, only the detection rate of IL-4 was lower than 40 %.

Cord whole blood cytokines

Although unstimulated cells showed, in general, absence or low secretion of cytokines, significant cytokine levels were observed in certain newborns' samples. Regarding stimulated cells, response to mitogens and PAMPs was vigorous, and patterns of cytokine responses were stimulus-dependent (**Figure** 1). Thus, Con A, PHA, LPS, PG and pI:C stimulation of cells induced high levels of IL-1β and IL-6; intermediate concentrations of TNF-α, IFN-γ, IL-10, IL-5 and IL-13, and a lower secretion of IFN-α, IL-2, IL-4, IL-17F and IL-23. Stimulation with allergenic extracts induced high secretion of IL-6 and IL-1β; and intermediate secretion of IL-10, IL5 and IL-2. Regarding the allergenic extracts, only the D.p. extract induced a significant secretion of TNF-α, IFN-γ, IL-13, IL-17F and IL-23 cytokines. Finally, CpG-ODN only induced a significant secretion of IFN-α (**Figure** 1). Overall, mitogens and PAMPs induced high levels of inflammatory cytokines, intermediate levels of Th1/ Th2/Tr related cytokines and low levels of Th17 cytokines. Allergens, specially D.p. extract, induced an equivalent response but with less intensity since most of the above cytokines are poorly induced.

Correlation between cytokine responses

Cytokine responses showed coordinated production patterns that were common to unstimulated and stimulated cells. Thus, we found co-expression of IL-1β and IL-6 and, also, of IFN-α and IL-4. This linked production pattern incorporates additional associated cytokines in a number that depends on the type of stimulus used. As an example, IFN-α and IL-4 production were also associated with the secretion of IL-5 in cells stimulated with LPS, PG or pI:C (**Figure** 2). In the case of IL-1β and IL-6, a strong association was observed in the production of these two cytokines in all the conditions although this association included additional co-expression of TNF-α and IFN-γ in cells stimulated with PHA, LPS, PG or pI:C (**Figure** 2).

Newborn sex and cytokine signatures

Analysis of sex differences in cytokine production revealed that, in general, males were prone to show higher cytokine levels than females in response to specific stimuli (**Figure** 3). Male newborns showed increased odds of IL-6 production than females in umbilical cord blood cells stimulated with D.p (**Figure** 3A); and for IL-23, in cells incubated with pI:C. Specially relevant was the increased odds of IL-13 response to mitogens and PAMPs, except for CpG-ODN, observed in males compared to females (**Figure** 3D). By contrary, lower odds of IFN-α production in response to PG and IL-2 in response to olive were found among males compared to females (**Figure** 3A-B).

Season of birth and cytokine signatures

Cytokine responses in relation to season of birth were diverse. As an example, IL-2, IFN-γ and IL-4 responses to stimuli were independent of the season of birth. Other cytokines, such as IL-10 showed fluctuations with the season of birth only when cells were incubated in the presence of allergenic extracts. In that case, we observed significant increased odds of IL-10 release in response to the mite and olive extracts in children born during warm seasons compared to those born during cold seasons (**Figure** 4). No season-related IL-10 responses were observed for other stimuli. Also, a significant increased probability was observed for IL-17F and IL-13 production during warm seasons in response to some of the stimuli tested. Additionally, we observed a significant decreased probability of IFN-α, TNF-α, IL-6, IL-1β, or IL-5 responses in cells stimulated with PHA and/or PAMPs from children born in summer or in spring. For other cytokines tested such as IL-23, the results were dependent on the stimulus. As general rule, to be born during cold seasons was associated with a higher production of innate immunity cytokines (i.e., TNF-α, IFN-α, IL-6, IL-1β; and of the Th2 related cytokine IL-5. By contrary,

to be born during summer and spring was associated with a higher production of IL-13 and IL-17F cytokines (**Figure** 4).

Discussion

With this study, we aimed to contribute to a deeper knowledge of cytokine signatures in newborns from a south-eastern Mediterranean region, which is characterized by a Mediterranean climate and a specific genetic background. For this purpose, umbilical cord blood cell supernatants were analyzed before and after exposure to a variety of antigens and the influence of sex and season of birth on cytokines release was investigated.

Cytokine profile of newborns was characterized by a high production of inflammatory cytokines, moderate production of specific Th1, Th2 and Tr cytokines and low levels of Th17 cytokines (**Figure** 1). Our results are partially in agreement with those from previous studies. In fact, Gold *et al* [9] reported that the response to innate stimuli was in general more intense in cord blood cells, and cytokine profiles were dependent on the stimulus used to induce their release. In connection with this and in agreement with data by others [3], we found a positive correlation between IL-6 and IL-1β cytokine levels (**Figure** 2).

Another characteristic of the neonatal cytokine profile supported by several studies is that neonatal peripheral blood naïve T cells show only weak Th17 responses [26]. In this regard, in our preliminary experiments we observed scarce production of IL-17A, significant in response to PHA, along with low although significant production of IL-17F in response to PHA and pI:C (**Supplementary Figure S1**). Since both cytokines share cellular sources and function [27], it is tempting to speculate that perhaps there is a predominant bias for IL-17F to be secreted in higher levels in newborns although this pattern could change with age in favor of increased secretion of IL-17A instead. This characteristic cytokine response in neonates may explain their vulnerability to suffer certain diseases, for example mucocutaneous Candidiasis, since Th17 response is essential to limit Candida invasion at mucosal surfaces [26].

In contrast with previous findings that support that neonatal T cells are biased towards Th2 o T regulatory cell differentiation, we found moderate secretion of Th1, Th2 and regulatory

cytokines. In support of this, the discovery of fetal Th1 cells, together with the potent induction of Th1-type responses to vaccination, raises doubt about the existence of an intrinsic Th2 bias in newborns and suggests that there are mechanisms that lead to efficient Th1 cell responses during the first years of life [28]. Nevertheless, the same study describes that cord blood cells produce less IFN-α [28] and that neonatal response to CpG-ODN-TLR-9 is selectively defective. According to this finding, we have obtained a low cytokine response in newborn cells stimulated with CpG-ODN (**Figure** 1).

As supported by other studies [5], we found that cytokine concentrations at birth were generally higher in males compared to females (**Figure** 3). These increased cytokine concentrations in males reached statistical significance for IL-6, IL-13 and IL-23 in cells incubated with specific stimuli. These data were only partially coincident with those obtained by Horst *et al*. who found that in adults the production of TNF-α, IL-6 and IL-1β was higher in men after LPS stimulation in comparison to women[3]. The higher secretion of IL-13 observed in male newborns' cells stimulated with PHA or PG, could indicate a bias to secrete higher amounts of this cytokine from the moment of birth and could explain the higher predisposition of males to develop asthma or allergic rhinitis during childhood [29]. Furthermore, in comparison to females, we also observed a higher secretion of Th17 polarizing cytokines, such as IL-6 and IL-23, in newborn males (**Figure** 4) after stimulating the cells with Con A, PG, or mite extract. In this regard, IL-6 has been described as a significant predictor of subsequent asthma exacerbation risk [30] or associated with a higher risk for suffering allergic affections [4,31]. In addition, IL-23 and, also, IL-17F have been related to immune-mediated inflammatory diseases such as psoriasis or Crohn disease [32]. On the contrary, we found an increased secretion of Th1-related cytokines in newborns females; specifically, a higher release of IL-2 and IFN-α in response to olive extract and PG, respectively. These results could explain the lower prevalence of allergic disease among females during childhood [29].

Several authors support the idea that children born during spring season show a predisposition to develop autoimmune diseases [33–35]; by contrary, children born during fall or winter suffer more frequently from allergic diseases [36–38]. Nevertheless, these associations should be taken carefully, since other authors have obtained different results connecting diseases and birth seasonality [39]. In our study, we have observed a seasonal immune pattern that was stimuli- and cytokine-dependent (**Figure** 4). In general, Th1 cytokines such as IFN-α, the inflammatory cytokines IL-6, IL-1β and TNF-α and the Th2 cytokine, IL-5, were more frequently secreted by cells of neonates born during autumn or winter (**Figure** 4B). These results are in accordance with those obtained by other researchers. Thus, Dopico *et al* [40] found an increase on IL-6 receptor during the European winter season. Also, Gold *et al* [9] found higher TNF-α or IFN-γ responses against several antigens in autumn or winter, and higher IFN-α responses during cold seasons in response to PG and LPS. Furthermore, Thysen *et al*. showed that winter births were associated with higher IL-5 levels, among other cytokines [11]. Nevertheless, other researchers have reported different results, since they have described that production of TNF-α, IL-6 and IL-1β exhibited a significant peak in summer [3]. Additionally, we have observed higher production of the Th17 cytokine IL-17F, the Th2 cytokine IL-13 and the regulatory cytokine IL-10 among neonates born during warm seasons. Related to that, a study of Sullivan Dillie *et al* [41] showed an increased level of IL-13 secretion during warm seasons[41]; but, nevertheless, different results have been obtained by other authors[9]. Further studies should be performed to confirm the cytokine release pattern with each season of birth and to unravel the association of these cytokine profiles with the development of immune-related diseases during early life.

Among the strengths of this study, we found their prospective population-based study design, which allows us to get information of potential confounders. In addition, a wide panel of cytokine and specific innate and adaptive stimuli was used to characterized cytokine response of cord blood cells.

Our study has some limitations. First, our results are based on a subsample of the NELA cohort. However, differences between included and excluded participants were evaluated and included in multivariate models to strengthen the external and internal validity of the results. Secondly, the small sample size could affect the study power and precision of estimates. Cytokine concentrations were categorized due to low detection cytokine concentration rates showed on several conditions and associations between predictor variables and outcome could be affected by this classification. Nevertheless, regression results were consistent with distributions of cytokine concentrations (data not shown).

In conclusion, we have characterized the newborn immune status by analyzing unstimulated and stimulated cytokine production. Newborns from a Mediterranean area exhibited a cytokine signature that shares characteristics with the data previously published. We found a preference for certain cytokines to be released in higher amounts depending on the season of birth and the stimulus used. Overall, our results suggest that the functional status of the innate and adaptive immunity differs already at birth between females and males and can be measured by the capacity of their whole blood cells to produce cytokines by unstimulated cells or in response to different stimuli. The singularity of cytokines secretion might be affected by genetic and environmental factors that depend on the genetic background of the population and other characteristics, such as climate, diet, air pollution, and others, associated with the region of the world where these individuals are born. These factors might be determinant to skew the Th1/Th2/Th17 balance which could determine the response to microbes and allergens during the first year of life and therefore the risk of respiratory infections or allergy manifestations.

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NELA study group:

ME Candel-Torralba[1], L Garcia-Marcos (PI)[1,2,17], MJ Gimenez-Banon[1], A Martinez-Torres[1,2,17], E Morales (PI)[1,3], V Perez-Fernandez[1,3,17], M Sanchez-Solis[1,2,17], A Nieto[1,5], MT Prieto-Sanchez[1,5], M Sanchez-Ferrer[1,5], L Fernanez-Palacios[1,6], VP Gomez-Gomez[1,6], C Martinez-Gracia[1,6], P Peso-Echarri[1,6], G Ros-Berruezo[1,6], M Santaella-Pascual[1,6], A Gazquez[1,7], E Larque[1,7,18], MT Pastor-Fajardo[1,7], M Sanchez-Campillo[1,7], A Serrano-Munuera[1,7], M Zornoza-Moreno[1,7,20], P Jimenez-Guerrero[1,8], E Adoamnei[1,3], JJ Arense-Gonzalo[1,3], J Mendiola[1,3], F Navarro-Lafuente[1,3], AM Torres-Cantero[1,3], C Salvador-Garcia[9], M Segovia-Hernández[1,10], G Yagüe-Guirao[1,10], PL Valero-Guillén[1,11], FV Aviles-Plaza[1,12], J Cabezas-Herrera[1,12], A Martinez-Lopez[1,12], M Martinez-Villanueva[1,12], JA Noguera-Velasco[1,12], A Franco-Garcia[1,13], AM Garcia-Serna[1,13], T Hernandez-Caselles[1,13,17], E Martin-Orozco[1,13,17], M Norte-Muñoz[1,13], M Canovas[1,13], E Cantero-Cano[1], T de Diego[1,13], JM Pastor[1,13], RA Sola-Martínez[1,13], A Esteban-Gil[1,14], JT Fernández-Breis[1,15], MV Alcántara[16], S Hernández[16], C López-Soler[4,16]. [1]Biomedical Research Institute of Murcia, IMIB-Arrixaca,

Murcia, Spain; [2]Paediatric Respiratory and Allergy Units, “Virgen de la Arrixaca” Children's University Clinical Hospital, University of Murcia, Spain; [3]Department of Public Health Sciences, University of Murcia, Spain; [4]Paediatric Psychology Unit, “Virgen de la Arrixaca” Children's University Clinical Hospital, University of Murcia, Spain.; [5]Obstetrics & Gynaecology Service, “Virgen de la Arrixaca” University Clinical Hospital, University of Murcia, Spain; [6]Food Science and Technology Department, Veterinary Faculty of Veterinary, University of Murcia, Spain; [7]Department of Physiology, Faculty of Biology, Campus Mare Nostrum. University of Murcia, Spain; [8]Regional Atmospheric Modelling Group, Department of Physics, University of Murcia, Spain; [9]Microbiology Service, General University Hospital Consortium, University of Valencia, Spain;[10]Microbiology Service, University Clinical Hospital "Virgen de la Arrixaca", University of Murcia, Spain; [11]Microbiology and Genetics Department, University of Murcia, Spain; [12]Molecular Therapy and Biomarkers Research Group, Clinical Analysis Service, University Clinical Hospital "Virgen de la Arrixaca", University of Murcia, Spain; [13]Department of Biochemistry and Molecular Biology B and Immunology, University of Murcia, Spain; [14]Foundation for Healthcare Training & Research of the Region of Murcia (FFIS); [15]Department of Informatics and Systems, University of Murcia, Spain; [16]Paediatric and Adolescent Clinical Psychology University Research Group (GUIIA-PC), University of Murcia, Spain; [17]Network of Asthma and Adverse and Allergic Reactions (ARADyAL), Spain; [18]Maternal and Children Health Network (SAMID III), Spain; [19]Preventive Medicine Service, “Virgen de la Arrixaca” University Clinical Hospital, University of Murcia, Spain; [20]"Vistalegre-La Flota" Health Center, Health System of Murcia (SMS), Murcia, Spain.

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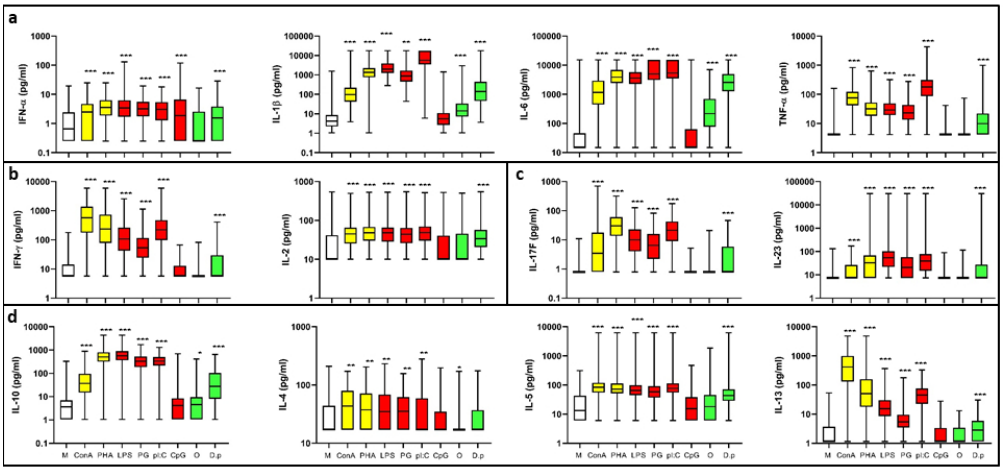

Figure 1. Cytokine concentrations and patterns of response in cord blood cells. Box-plots represent concentration levels of different cytokines produced by cells unstimulated (white filled boxes) or stimulated with different mitogens (yellow filled boxes), PAMPs (red filled boxes) or allergenic extracts (green filled boxes). Box plots represent the concentration values (pg/ml) included in the 10th to 90th percentiles. Median values are represented by the horizontal line inside each box. The whiskers represent the highest and the lowest cytokine values. P-values derived from Mann-Whitney test with correction for multiple comparisons using the Benjamini-Hochberg method. *p-value<0.05 **p-value<0.01 ***p-value<0.005. (A) Inflammatory cytokine production in cord blood cells; (B) Th1 cytokines production in cord blood cells; (C) Th17 cytokines production by cord blood cells; (D) regulatory and Th2 cytokines production by cord blood cells.

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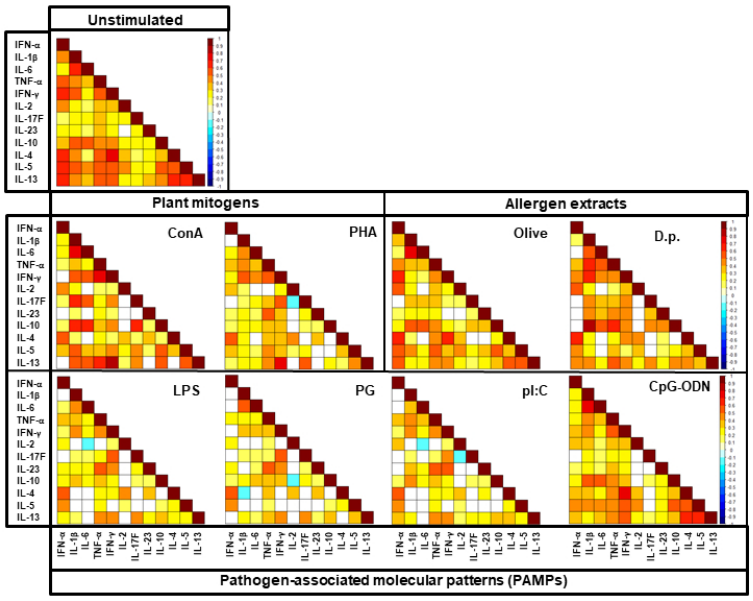

Figure 2.- Correlation matrix based on Spearman coefficients for cytokine concentrations in unstimulated and stimulated cord blood cells. Coefficients were adjusted by Benjamini-Hochberg method and non-significant coefficients (adjusted-p<0.05) were represented using white boxes.

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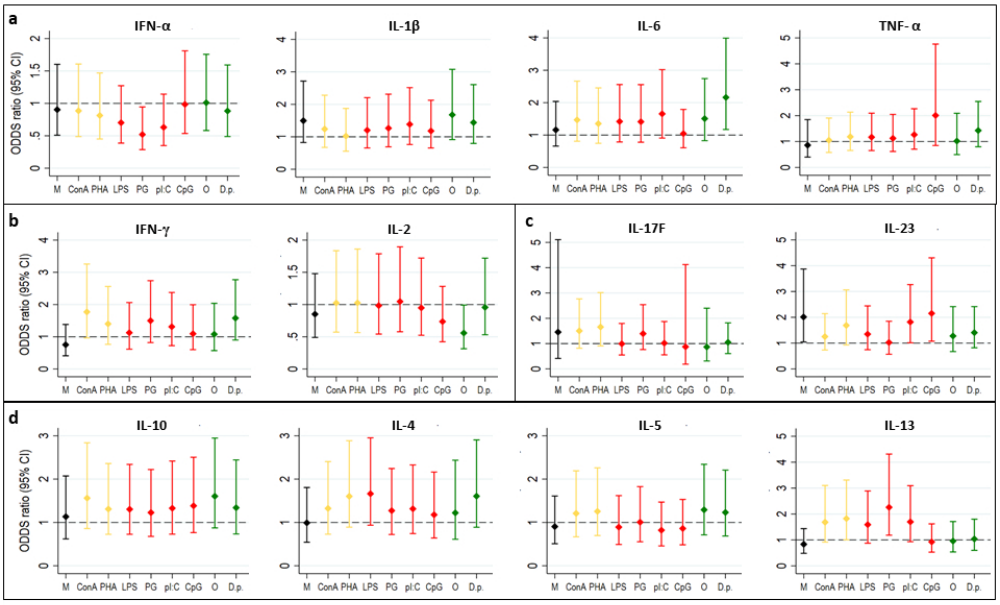

Figure 3.- Cytokine response in unstimulated and stimulated cord blood cells according to sex. Graphs show results derived from associations between production of (A) inflammatory, (B) Th1-related, (C) Th17-related and (D) Th2-related and immunomodulatory cytokines in unstimulated cells (black) and in cells stimulated with mitogens (yellow), pathogen associated molecular patterns (red) and common allergens extracts (green). Coefficients were derived from a multivariate logistic regression comparing high (>60th percentile cytokines concentrations) with low (<40th percentile cytokines concentrations) responder samples Female low responder individuals were used as the reference group. Models were adjusted by maternal age, pre-pregnancy body mass index, maternal atopy, gestational age and birth weight. Diamonds represent coefficients and horizontal bar with whiskers represent 95% confidence intervals.

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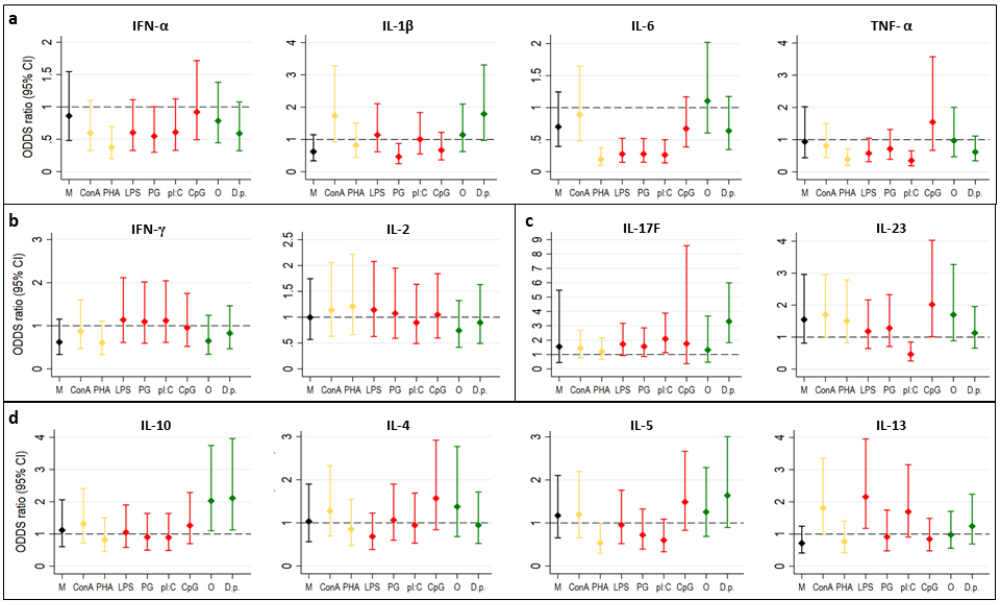

Figure 4.- Cytokine response in unstimulated and stimulated cord blood cells according to season. Association between cytokine response and season of birth. Graphs show results derived from associations between production of (A) inflammatory, (B) Th1-related, (C) Th17-related and (D) Th2-related and immunomodulatory cytokines in unstimulated cells (black) and in cells stimulated with mitogens (yellow), pathogen associated molecular patterns (red) and common allergens extracts (green) and season of birth categorized into warm (March, April, May, June, July) and cold (September, October, November, December, January, February) months. Coefficients are derived from a multivariate logistic regression comparing high (>60th percentile cytokines concentrations) with low (<40th percentile cytokines concentrations) responder samples low responder individuals born in cold months were used as the reference group. Models were adjusted by maternal age, pre-pregnancy body mass index, maternal atopy, gestational age and birth weight. Diamonds represent coefficients and horizontal bar with whiskers represent 95% confidence intervals.

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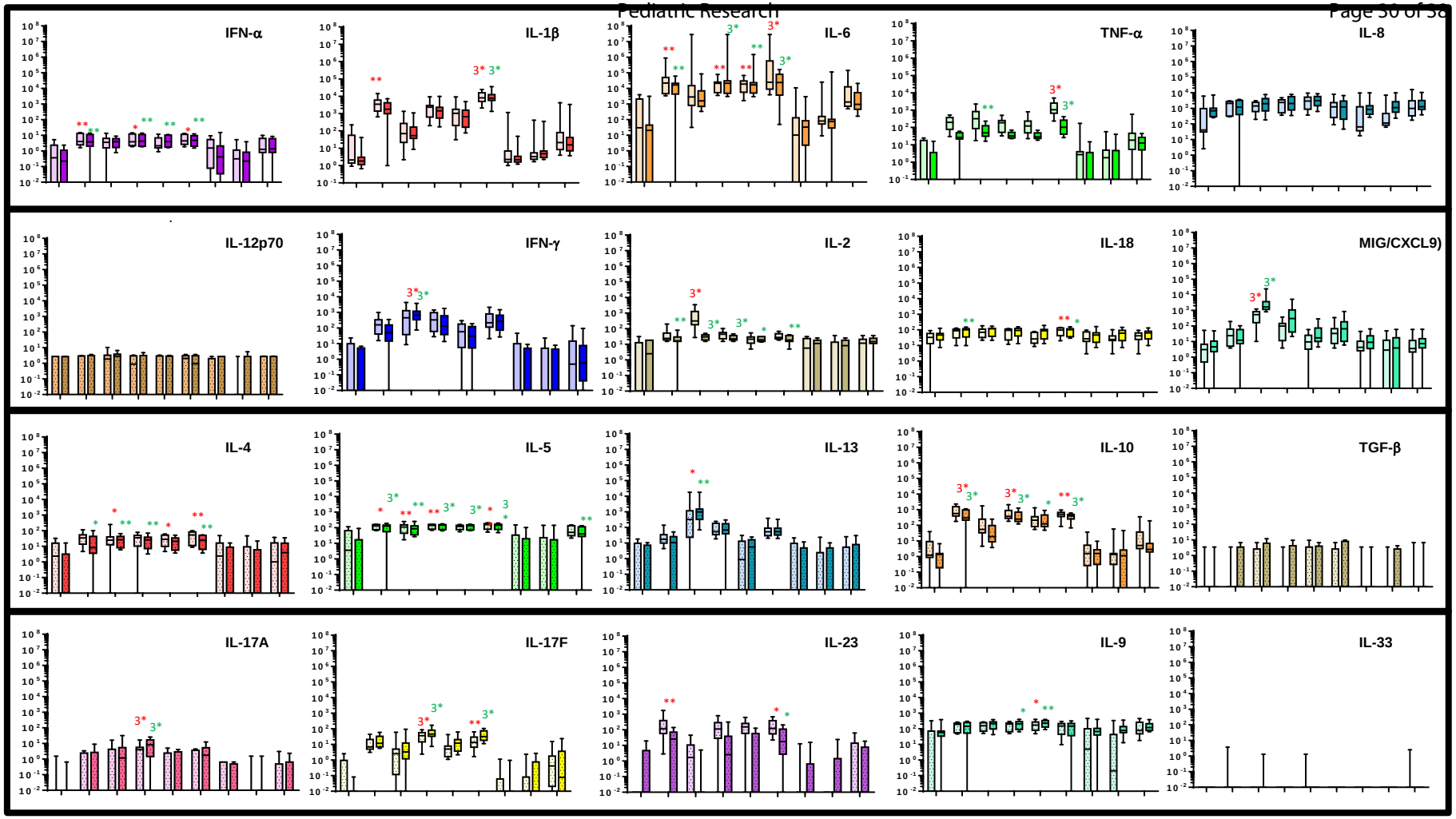
a
IFN-α
IL-1β
IL-6
TNF-α
IL-8
b
IL-12p70
IFN-γ
IL-2
IL-18
MIG/CXCL9)
c
IL-4
IL-5
IL-13
IL-10
TGF-β
d
IL-17A
IL-17F
IL-23
IL-9
IL-33
M LPS Con A PHA PG pI:C CpG Olive Dp

Supplementary Figure 1. Cytokine production by whole cord blood cells. The figure shows box and whisker plots of the cytokine concentration (pg/ml) obtained from supernatants of whole cord blood cells incubated for 48 hrs (light colors) and 7 days (dark colors) with media alone or with different stimuli. The horizontal line within the box indicates the median, boundaries of the box indicate the 25TH- and 75th -percentile, and the whiskers indicate the highest and lowest values of the results. The concentration data were analyzed by using one-way ANOVA analysis. Cytokines are classified according to their participation in innate or adaptive immune response and the Th population that mostly produce each of them: **(A)** Inflammatory cytokines (IFN-α,IL-1β, TNF-a, IL-6 and IL-8); **(B)** Th1 cytokines (IL-12p70, IFN-g, IL-2, IL-18, MIG); **(C)** Th2 and immunomodulatory cytokines (IL-4, IL-5 and IL-13; and IL-10 and TGF-β, respectively); **(D)** Th17, Th9 cytokines and alarmin (IL-17A, IL-17F and IL-23; and IL-9; and IL-33, respectively). Asterisks represent: *p<0,05; **p<0,01 and ***p<0,001. n=12. Red asterisks represent p values at 48 hours of culture (stimulated versus unstimulated cells) and green asterisks represent p values at 7 days of culture (stimulated versus unstimulated cells).

Supplementary Table S1. Stimulants used in the cytokine assays**.**

Cytokines[9]	Lower Limit of Detection (LLOD) (pg/ml)	Upper Limit of Detection (ULOD)(pg/ml)	Stimulants	Final Concentration
General inflammatory response				
IFN-α	0.5	550	Medium alone	-
IL-Iβ	2.1	8800	Mitogens	
IL-6	29.7	7625	Concanavalin A[1] (ConA).	10 μg/ml
TNF-α	8.4	2168	Phytohemagglutinin[2] (PHA).	5 μg/ml
Th1-related response			Pathogen-associated molecular patterns (PAMPs)	
IFN-γ	11.5	2950	Lipopolysaccharide[3] (LPS)	1 μg/ml
IL-2	20.1	5150	Polyinosinic:polycytidylic acid[4] (pI:C).	12.5 μg/ml
Th2-related response			Peptidoglycan[5] (PGN)	10 μg/ml
IL-4	34	8725	Cytosine-phosphorothioate-guanine oligodeoxynucleotides[6] (CpG-ODN).	1 μg/ml
IL-5	12.1	3118	Allergenic extracts	
IL-13	2.3	2400	Olive *europaea* extract[7]	10 μg/ml
Immunomodulatory response			D*ermatophagoides pteronyssinus* extract[8]	10 μg/ml
IL-10	2.1	2175		
Th17-related response				
IL-17F	1.6	1712		
IL-23	14.8	15225		

[1] Sigma-Aldrich
[2] Sigma-Aldrich
[3] Sigma-Aldrich
[4] Sigma-Aldrich
[5] Sigma-Aldrich
[6] Trilink Biotechnologies
[7] BIAL-Aristegui
[8] BIAL-Aristegui
[9] ThermoFisher

Supplementary Table S2. Comparison between included and excluded participants of the study, the NELA cohort birth.

	Included (n=235)	Excluded (n=503)	p-value*
Maternal age,years , mean (sd)	33.3 (4.3)	32.2 (4.8)	**0.002**
Mother's age ≥ 35 years	99 (42.1)	153 (30.4)	**0.002**
Nulliparus mothers, n (%)	121 (51.5)	253 (50.3)	0.763
Maternal education level			0.146
Incomplete secondary or less, n (%)	42 (17.9)	104 (20.7)	
Complete secondary, n (%)	53 (22.6)	138 (27.4)	
University, n (%)	140 (59.6)	261 (51.9)	
Maternal pre-pregnancy BMI, mean (sd), (kg/m^2)	24.4 (4.5)	23.7 (4.4)	**0.028**
Normal, n (%)	152 (64.7)	361 (71.8)	0.135
Overweight, n (%)	59 (25.1)	97 (19.3)	
Obesity, n (%)	24 (10.2)	45 (9.0)	
Maternal smoking during pregnancy, yes, n (%)	39 (16.6)	89 (17.7)	0.714
Gestational Diabetes mellitus, yes, n (%)	32 (10.0)	32 (7.1)	0.194
Hypertension in pregnancy, yes, n (%)	6 (2.6)	12 (2.6)	0.997
Paternal age, years, mean (sd)	36 (5.1)	34.4 (5.4)	**0.005**
Father's age ≥ 35 years	138.0 (58.7)	251.0 (49.9)	**0.025**
Paternal education level			0.056
Incomplete secondary or less, n (%)	57 (24.3)	161 (32.1)	
Complete secondary, n (%)	84 (35.7)	145 (28.9)	
University, n (%)	94 (40.0)	195 (38.9)	
Paternal smoking	83 (35.3)	175 (34.9)	0.918
Parental social class			0.252
I-II, n (%)	126 (53.6)	241 (47.9)	
III, n (%)	52 (22.1)	110 (21.9)	
IV-V, n (%)	54 (23.0)	137 (27.2)	
Unemployed, n (%)	3 (1.3)	15 (3.0)	
Maternal contact with farming animals	44 (18.7)	88 (17.5)	0.685
Pets at home	111 (47.2)	230 (45.7)	0.702
Heat pump at home, n (%)	203 (86.4)	435 (86.7)	0.920
Moisture and Mold Problems at home, n (%)	54 (23.0)	102 (20.3)	0.403
History of atopy			
Maternal asthma, yes, n (%)	33 (14.0)	48 (9.5)	0.068
Maternal history of atopy, yes, n (%)	107 (45.5)	203 (40.4)	0.185
Father asthma, yes, n (%)	22 (9.4)	44 (8.8)	0.778
Father history of atopy, yes, n (%)	88 (37.6)	175 (34.9)	0.469
Female newborns	119 (50.6)	244 (50.3)	0.934
Gestational age, mean (sd), weeks	39.8 (1.3)	39.5 (1.6)	**0.033**
Preterm (<37 weeks), n (%)	6 (2.6)	30 (6.0)	**0.045**
Birth weight, mean (sd), g	3275 (430.5)	3226 (494.8)	0.335
<2500 gr, n (%)	10 (4.3)	32 (6.4)	0.250
Season of birth			0.066
Autumm, n (%)	74 (31.5)	128 (26.4)	

Spring, n (%)	63	(26.8)	123	(25.4)	
Summer, n (%)	53	(22.6)	155	(32.0)	
Winter, n (%)	45	(19.2)	79	(16.3)	
Mode of starting delivery					**0.038**
Inducing labor, n (%)	94	(40.0)	161	(33.8)	
Spontaneous labor, n (%)	131	(55.7)	272	(57.1)	
Predicting Cesarean, n (%)	10	(4.3)	43	(9.0)	
Mode of ending delivery					0.822
Vaginal non-instrumental, n (%)	132	(56.2)	277	(58.1)	
Vaginal Instrumental, n (%)	48	(20.4)	98	(20.6)	
Cesarean section, n (%)	55	(23.4)	102	(21.4)	
Fever during labor, yes, n (%)	11	(4.7)	23	(5.0)	0.882
Use of antibiotics during labor, yes, n (%)	68	(30.2)	110	(26.8)	0.363

* p value derived from Mann-Whitney test for continuous variables and from Chi2 test for categorical variables.

Supplementary Table S3. Descriptive analysis of cytokine concentrations in unstimulated cord blood cells from newborns (n=235).

Cytokines	median (pg/ml)	p25	p75	LOD (pg/ml)	UPD (pg/ml)	Detection rate (%)
IFN- α	0.66	<0.5	2.4	0.5	550	52.8
IFN-γ	5.75	<11.5	14.13	11.5	2950	26.8
IL-1β	4.21	2.22	8.61	2.1	8800	76.6
IL-10	3.64	<2.1	6.97	2.1	2175	66.0
IL-13	1.15	<2.3	3.71	2.3	2400	37.9
IL-17F	0.8	<1.6	0.8	1.6	1712	4.7
IL-2	10.05	<20.1	41.47	20.1	5150	43.8
IL-23	7.4	<14.8	7.4	14.8	15225	23.0
IL-4	17	<34	43.95	34	8725	28.5
IL-5	13.26	<12.1	43	12.1	3118	52.3
IL-6	14.85	<29.7	45.83	29.7	7625	31.5
TNF-α	4.2		4.2	8.4	2163	15.3

p25: 25th percentile; p75: 75th percentile; LOD: low detection limit; UPD: up detection limit

Supplementary Table S4. Descriptive analysis of cytokine concentrations mitogen-stimulated in cord blood cells from newborns (n=235).

Cytokines	Stimuli	median (pg/ml)	p25	p75	Detection rate (%)
IFN-α	**ConA**	2.5	0.3	4.7	71.5%
	PHA	3.6	1.9	6.3	88.1%
IFN-γ	**ConA**	570.7	175.6	1380.2	91.1%
	PHA	234.9	77.9	741.2	88.5%
IL-1β	**ConA**	98.5	41.7	221.2	99.1%
	PHA	1361.3	759.1	2254.2	91.5%
IL-10	**ConA**	37.5	15	96.2	98.3%
	PHA	501.9	317.1	797.1	97.9%
IL-13	**ConA**	416.5	131.2	988.8	91.9%
	PHA	50.9	17.8	157.9	98.3%
IL-17F	**ConA**	3.5	0.8	17.6	61.3%
	PHA	30.4	13.7	60.1	95.7%
IL-2	**ConA**	45	25.5	64.9	88.9%
	PHA	48.2	30.4	67.9	93.2%
IL-23	**ConA**	7.4	7.4	26.3	40.4%
	PHA	33	7.4	66.9	66.8%
IL-4	**ConA**	43.3	17	79.5	60.0%
	PHA	37.3	17	71.7	54.9%
IL-5	**ConA**	83.3	55.4	120.1	94.0%
	PHA	71.6	51.6	113.1	95.3%
IL-6	**ConA**	1163.9	446	2931.5	83.0%
	PHA	3904.7	2441.1	6848.6	74.0%
TNF-α	**ConA**	76	42.1	122.3	97.0%
	PHA	31.9	18.3	52.9	92.3%

ConA: Concanavalin A; PHA: Phytohaemaglutinin; p25: 25th percentile; p75: 75th percentile; LOD: low detection limit; UPD: up detection limit

Supplementary Table S5. Descriptive analysis of cytokine concentrations PAMP-stimulated in cord blood cells from newborns (n=235).

Cytokines		median (pg/ml)	p25	p75	Detection rate (%)
IFN-α	**LPS**	3.4	1.7	6.3	86.0
	PG	3.1	1.3	5.5	82.6
	pI:C	3.2	1.8	5.6	83.8
	CpG-ODN	1.9	0.3	6.6	62.6
IFN-γ	**LPS**	107.9	42.7	260.8	91.9
	PG	222.7	97.9	476.7	97.0
	pI:C	53.5	24.3	116.6	83.4
	CpG-ODN	5.8	5.8	12.9	27.7
IL-1β	**LPS**	1972.9	1287.6	3926.7	89.4
	PG	5704.6	3484.5	17600	70.6
	pI:C	890	455.3	1682.3	94.9
	CpG-ODN	5.6	2.7	11.1	80.9
IL-10	**LPS**	568.8	365.1	895.9	98.7
	PG	337.7	216	496.7	99.1
	pI:C	333	185	526.7	99.6
	CpG-ODN	4.2	1.1	8.4	72.3
IL-13	**LPS**	15.3	8.6	30	95.3
	PG	45	22.5	75.4	97.9
	pI:C	5.4	3.5	9.6	83.8
	CpG-ODN	1.2	1.2	3.4	34.0
IL-17F	**LPS**	10	4	22.9	87.7
	PG	21.4	9.1	42.8	94
	pI:C	6.5	2.2	15.9	80.4
	CpG-ODN	0.8	0.8	0.8	3.0
IL-2	**LPS**	48.2	28.4	65.5	89.8
	PG	48.6	30.4	69.9	90.2
	pI:C	44.1	25.9	64.3	89.8
	CpG-ODN	10.1	10.1	40.9	46.4
IL-23	**LPS**	54.2	21.9	101.9	80.0
	PG	39.3	14.9	77.5	74.9
	pI:C	20.8	7.4	57.9	54.0
	CpG-ODN	7.4	7.4	7.4	20.4
IL-4	**LPS**	34.9	17	68.4	51.5
	PG	17	17	59	48.1
	pI:C	35.5	17	62	52.8
	CpG-ODN	17	17	34.9	26.0
IL-5	**LPS**	65.1	45.1	99.2	93.6
	PG	76.7	54.9	114.4	94.5
	pI:C	58	37	91.5	91.9
	CpG-ODN	15.5	6.1	38.1	53.6
IL-6	**LPS**	3579.6	2299	5734.1	78.7
	PG	5411.6	3529.1	15250	60.4
	pI:C	5044	3096.3	15250	65.1
	CpG-ODN	14.9	14.9	63	37.9
TNF-α	**LPS**	29.2	19.5	49.2	91.1

PG	179.6	87.4	310.1	98.3
pI:C	23.2	13.6	43.5	85.1
CpG-ODN	4.2	4.2	4.2	12.3

p25: 25th percentile; p75: 75th percentile; LOD: low detection limit; UPD: up detection limit

Supplementary Table S6. Descriptive analysis of cytokine concentrations allergen extract-stimulated in cord blood cells from newborns (n=235).

Cytokines		median (pg/ml)	p25	p75	Detection rate
IFN-α	**Olive**	0.3	0.3	2.6	47.7
	Dp	1.6	0.3	3.8	67.2
IFN-γ	**Olive**	5.8	5.8	5.8	23.0
	Dp	5.8	5.8	29.7	48.5
IL-1β	**Olive**	14.2	7.2	33.6	97.0
	Dp	142.4	46.2	437.5	99.1
IL-10	**Olive**	4.5	1.1	9.6	73.6
	Dp	27.5	8.2	103.6	93.6
IL-13	**Olive**	1.2	1.2	3.4	37.0
	Dp	2.8	1.2	5.9	58.7
IL-17F	**Olive**	0.8	0.8	0.8	7.2
	Dp	0.8	0.8	5.9	46.0
IL-2	**Olive**	10.1	10.1	45.7	49.4
	Dp	33.9	20.5	56.9	77.4
IL-23	**Olive**	7.4	7.4	7.4	23.4
	Dp	7.4	7.4	27.2	40.9
IL-4	**Olive**	17	17	17	18.7
	Dp	17	17	37.1	28.5
IL-5	**Olive**	18.2	6.1	45.5	62.6
	Dp	43.5	28.5	71.2	86.8
IL-6	**Olive**	218.6	77	698.7	88.1
	Dp	2554.9	1276.3	4907.2	80.4
TNF-α	**Olive**	4.2	4.2	4.2	16.6
	D.p	9.9	4.2	22.2	54.9

D.p: D*ermatophagoides pteronyssinus* extract