Original Article

Maternal Allergy Status Has No Impact on Neonatal Immune Responses to Allergen Stimuli

(allergy / allergen / cytokine / cord blood / gene expression)

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Abstract. Due to the increasing incidence of allergic diseases, there is a strong need to identify a prognostic marker pointing to increased risk of allergy development allowing introduction of early preventive measures. Cord blood seems to be a good source for searching for such marker. The capacity of cord blood cells to respond to common allergens could point to increased predisposition to later allergy development. In our study, cytokines typical of Th1 (IFN-γ), Th2 (IL-5, IL-13) and Treg (IL-10) immune responses were followed at both the level of gene expression and cytokine secretion in cord blood cells of newborns of healthy mothers (children with relatively low risk of allergy development) and allergic mothers (children with relatively high risk of allergy development) stimulated by allergens (pollen from birch and timothy grass, house dust mite, ovalbumin). We have not observed any difference in the response of cord blood cells of neonates of healthy and allergic mothers to allergen *in vitro*. Both gene expression and secretion of cytokines in response to allergen stimulation were comparable with the unstimulated controls. It seems that early postnatal events will be more decisive for future allergy development than prenatal sensitization of the foetal immune system with allergen *in utero* in allergic mothers.

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Abbreviations: CBMC – cord blood mononuclear cells, ELISA – enzyme-linked immunosorbent assay, OVA – ovalbumin, PHA – phytohaemagglutinin, qRT-PCR – quantitative real-time polymerase chain reaction.

Introduction

Increasing incidence of allergic diseases during the last couple of decades (Platts-Mills, 2015), together with a steep raise of financial and sociological demands, brought up the need to stop this trend or even reverse it. The first choice solution would be finding a strategy for primary prevention. However, allergy is a multifactorial disease with many aspects involved in its aetiology. Although many groups intensively searched for a good prognostic marker (elevated IgE in cord blood (Prokešová et al., 2008; Peters et al., 2012), increased Th2 cytokines (Fu et al., 2013, Hrdý et al., 2018), impaired immunoregulation (Hrdý et al., 2012), increased reactivity of cord blood cells (Žižka et al., 2007), compounds of maternal milk promoting allergy origination (e.g. Th2 cytokines) (Bottcher et al., 2000)) pointing to increased risk of allergy development, it seems that the allergic status of the mother has remained the best marker until now (Wang et al., 2005; Prescott et al., 2003; Benn et al., 2004; Steinke et al., 2008).

The key factor in finding a suitable preventive marker is to understand the immunological processes during the early period before and after birth. During the early postnatal period, the immune system is exposed to a broad spectrum of microbial stimuli promoting immune system maturation. The presence of microbes in the placenta has been described, suggesting prenatal interaction of the immune system of the foetus with microbes. Similarly, the passage of allergens through the placenta has been reported, indicating that the immature neonatal system could encounter and possibly develop inappropriate immune responses to allergens *in utero* (Loibichler et al., 2002; Vance et al., 2005). In addition to that, the immune system of the new-born organism is shifted towards Th2 reactions promoting the pro-allergic phenotype. Increased immune responses to allergens have been described for cord blood cells of the new-borns of allergic mothers in comparison with the healthy ones (Contreras et al., 2003; Fu et al., 2013). However, contradictory results have been presented in the literature on

responses of cord blood cells of the new-borns at high risk of allergy development (Kim et al., 2012). Cytokines play a key role in setting the immune responses. After antigen recognition on antigen-presenting cells by TCR with appropriate co-stimulation, the milieu of cytokines present in a close proximity of naïve T cells interacting with antigen-presenting cells could skew the immune polarization either to Th1, Th2, Th17, or to Treg. Typical cytokines belonging to Th1 are represented by IL-2, IFN-γ and TNF. Th2 cytokines could be represented by IL-4, IL-5, IL-9, IL-13, IL-25, and IL-33. Typical Th17 cytokines are IL-17A, IL-17F, IL- 22 and cytokines with regulatory functions are IL-10, IL-35, TGF-β. Some of the cytokines mentioned above promote induction of the corresponding immune response.

Previous work from our laboratory showed that cord blood mononuclear cells (CBMC) from babies born to allergic mothers exerted higher responsiveness after stimulation with polyclonal activator compared to those from the healthy ones (Žižka et al., 2007). We suggest that CBMC of new-borns of allergic mothers with increased reactivity and impaired regulatory responses could easily respond to relatively innocuous environmental antigens – allergens. To test this hypothesis, CBMC of new-borns of healthy mothers (children at relatively low risk of allergy development) and allergic mothers (children with relatively high risk of allergy development) were stimulated with allergens (ovalbumin – OVA, birch pollen – Bet v1, grass pollen – Phl p5, house dust mite – Der p 2) and the capacity of CBMC to respond to allergen was characterized by gene expression and secretion of cytokines IL-5, IL-10, IL-13, and IFN-γ.

Material and Methods

Subjects

In total, 53 children were included in this study, 25 children of allergic mothers and 28 children of healthy mothers. The allergy status of the mothers was determined by clinical manifestation of allergy persisting for more than 24 months (allergy against food and respiratory allergens manifested by individual combinations of eczema, asthma, bronchitis, hay-fever, conjunctivitis, etc.). Mothers in care of an allergist were monitored for positive skin prick tests and/or positive specific IgE antibodies against allergens. Mothers using anti-allergic treatment before pregnancy were included in the study. Exclusion criteria were: multiple pregnancy, diabetes mellitus, tumour, previous transfusion, transplantation, complications during the course of pregnancy (e.g., gestation diabetes, preeclampsia, pre-term deliveries). Cord blood only from children born physiologically (vaginally) in full term was used for further analysis. Cord blood was collected immediately after the delivery. The standard procedure consisted of careful cleaning of the cord and puncture of the umbilical vein to avoid maternal blood contamination. Usually about 20 ml of cord blood was collected in sterile heparinized tubes (10 U heparin/

ml). The study was approved by the Ethic Committee of the Institute for the Care of Mother and Child, Prague, Czech Republic, and was carried out after signed written informed consent of the mothers.

CBMC isolation and stimulation in vitro

Cord blood mononuclear cells (CBMC) were isolated on Ficoll-Paque gradient (Amersham Biosciences, Freiburg, Germany) using gradient centrifugation according to the manufacturer's instructions. Cells were cultivated in concentration 10^6/ml and stimulated with phytohaemagglutinin (PHA) (Sigma-Aldrich Co. LLC., St. Louis, MO), ovalbumin (OVA) (Sigma-Aldrich Co.) allergens: nPhl p 5 from *Phleum pratense,* rBet v 1 from *Betula verrucosa* and rDer p 2 from *Dermatophagoides pteronyssinus* (kindly provided by ALK-Abelló A/S, Horsholm, Denmark) in concentration 10 μg/ml (the same concentration was used for all allergens assayed). In a pilot study, different concentrations of allergens (1 μg/ml, 10 μg/ml and 50 μg/ml) were tested without significant impact on gene expression of cytokines in CBMC (data not shown). Cells were cultured in RPMI 1640 media containing L-glutamine, HEPES (Sigma-Aldrich, Munich, Germany), gentamycin (LEK Pharmaceuticals, Ljubljana, Slovenia), and 10% FBS (Biosera, Nuaille, France) for 7 days in an incubator with regulated CO_2 (5.5%) at 37 °C.

RNA isolation

Total mRNA was isolated after 24 h cultivation using RNasyMinikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The concentration of RNA was measured using NanoDrop 2000, Thermofisher Scientific, Waltham, MA. Electrophoresis in 2% agarose gel (Type II: Medium EEO, Sigma-Aldrich) was used to check the extracted RNA for any DNA contamination and to determine RNA integrity based on the bands of 18S and 28S rRNA. The purity of RNA was assessed by the ratio of absorbance at 260 nm and 280 nm, and was in the range of 1.9 to 2.2. RNA was stored in aliquots at –20 °C until used for reverse transcription.

Real-time PCR

According to the manufacturer's instruction, the High capacity RNA to cDNA reverse transcription kit was used to transcribe the isolated RNA into cDNA (Thermofisher Scientific). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the effect of stimulation on the gene expression of cytokines in CBMC as described previously (Hrdý et al., 2010, 2017). Briefly, the PCR reaction mix consisted of TaqMan Universal PCR master mix, TaqMan Gene Expression Assays (*Ifng* Hs00174143_m1; *Il5* Hs99999031_m1; *Il10* Hs00174086_m1; *Il13* Hs00174379_m1, and *β actin* as an endogenous control/housekeeping gene Hs99999903_m1) and RNase-free water (Thermofisher Scientific). Thermal reaction scheme: 50 °C 2 min, 95 °C 10 min, 40 cycles of 95 °C 15 sec and 60 °C 60 sec. The results are expressed as a relative quantity of gene ex-

pression of the gene of interest (*Ifng*, *Il5*, *Il10*, *Il13*) to the expression of endogenous control *β actin* using the ΔΔct method ($2^{-\Delta\Delta ct}$) as described previously (Hrdy et al., 2010). Briefly, the value of the cycle where fluorescence exceeded the threshold was indicated as ct. Δ ct was calculated as ct of the gene of interest (goi) minus the ct value of the housekeeping gene (*β actin*). Next, ΔΔ ct was calculated as the difference between Δ ct of the particular sample minus average of Δ cts of unstimulated CBMC (C) of new-borns of healthy mothers. Finally, the results were expressed as $2^{-\Delta\Delta ct}$.

Determination of in vitro secreted cytokines by the ELISA method

Concentrations of IL-5, IL-10, IL-13, IFN-γ in the cell culture supernatants were quantified by an enzyme-linked immunosorbent assay (ELISA) on high-adsorption polystyrene microtitration plates (NUNC) using the instructions of antibody manufacturer (R&D Systems, Minneapolis, MN). The detection kit for IL-10 (DY217-B) and primary antibody, secondary biotinylated antibody and recombinant protein for IFN-γ (MAB2852; BAF285; 285-IF), IL-5 (MAB405; BAF6051; 205-IL) and IL-13 (MAB213; BAF213, 213-ILB-005) in this order were purchased from R&D Systems. The concentrations of cytokines were calculated from calibration curves and expressed in pg/ml.

Statistics

Differences between groups were evaluated using the nonparametric (Mann-Whitney) test based on not normal distribution of data (tested by the Shapiro-Wilcoxon test). For multiple comparison analysis, the Bonferroni correction was used to prevent false positivity. Statistical significance was set at $P \leq 0.05$. For statistical evaluation of the concentration of cytokines detected by ELISA, the values under the detection level were given as half the detection limit. Statistical and graphical evaluation was performed using GraphPad Prism software (San Diego, CA). Results are expressed as mean ± standard error of the mean (SEM).

Results

To compare the capacity of allergen to induce neonatal immune responses of new-borns of healthy and allergic mothers, CBMC were stimulated with allergen and phytohaemagglutinin (PHA) as a positive control, and gene expression of cytokines was followed at the mRNA level. The effect of allergen stimulation on gene expression of Th1 cytokine *Ifng* was negligible. As expected, PHA stimulation promoted gene expression of *Ifing*. No significant difference in the gene expression of *Ifng* by CBMC of new-borns of heathy and allergic mothers was observed (Fig. 1A). The impact of stimulation of CBMC by allergens on one of the key effector cytokines of Th2 immune response, *Il5*, was tested. Although gene expression of *Il5* was higher in a group

of new-borns of allergic mothers, it did not reach statistical significance (Fig. 1B). Gene expression of another Th2 cytokine, *Il13*, in CBMC was not affected by stimulation with any allergen used. Significantly increased gene expression of *Il13* after PHA stimulation was detected in CBMC of new-borns of both healthy and allergic mothers, with no difference between these two groups (Fig. 1C). To confirm the possibility of impaired immunoregulation after allergen encounter at the level of CBMC, gene expression of *Il10* was followed in CBMC stimulated either with allergens or with PHA. The only significant difference in the gene expression of *Il10* was observed after PHA stimulation. No effect of allergens on the *Il10* gene expression in CBMC was detected (Fig. 1D).

To confirm and evaluate the data on gene expression, the cytokines were investigated in cell culture supernatants of CBMC stimulated with allergens or PHA. Stimulation with allergens had no impact on IFN-γ secretion. Only after PHA stimulation, increased concentrations of IFN-γ were detected by ELISA in cell culture supernatants, without any difference between CBMC of new-borns of healthy and allergic mothers (Fig. 2A). The concentration of IL-5 in cell culture supernatants was not affected by allergen stimulation. Increased levels of IL-5 were determined only in supernatants of PHA-stimulated CBMC of new-borns of allergic mothers in comparison with unstimulated controls (Fig. 2B). The effect of stimulation of CBMC with allergen had no impact on IL-13 secretion. Higher concentrations of IL-13 were detected in supernatants of CBMC stimulated with PHA, without any difference between the healthy and the allergic group (Fig. 2C). Secretion of IL-10 was not influenced by allergen stimulation. Only after PHA stimulation, elevated concentrations of IL-10 were observed in cell culture supernatants of CBMC, being similar between new-borns of healthy and allergic mothers (Fig. 2D).

Discussion

The capacity of CBMC to respond to allergens could serve as a possible prognostic marker indicating increased risk of allergy development. However, we were not able to detect significantly different gene expression or secretion of cytokines by CBMC of neonates at higher risk of allergy development in comparison to CBMC of neonates at lower risk of allergy development. Allergen-stimulated CBMC of new-borns of allergic mothers on the one hand and healthy mothers on the other hand had comparable gene expression and secretion of cytokines typical of Th1 (IFN-γ), Th2 (IL-5, IL-13) and Treg (IL-10) immune responses. The only difference was observed in the concentration of IL-5 in cell culture supernatants of PHA-stimulated CBMC, where CBMC of new-borns of allergic mothers exerted higher response after polyclonal stimulation with PHA in comparison with CBMC of new-borns of healthy mothers.

Different factors (e.g., colonizing microbiota, mode of delivery, environmental factors) promoting develop-
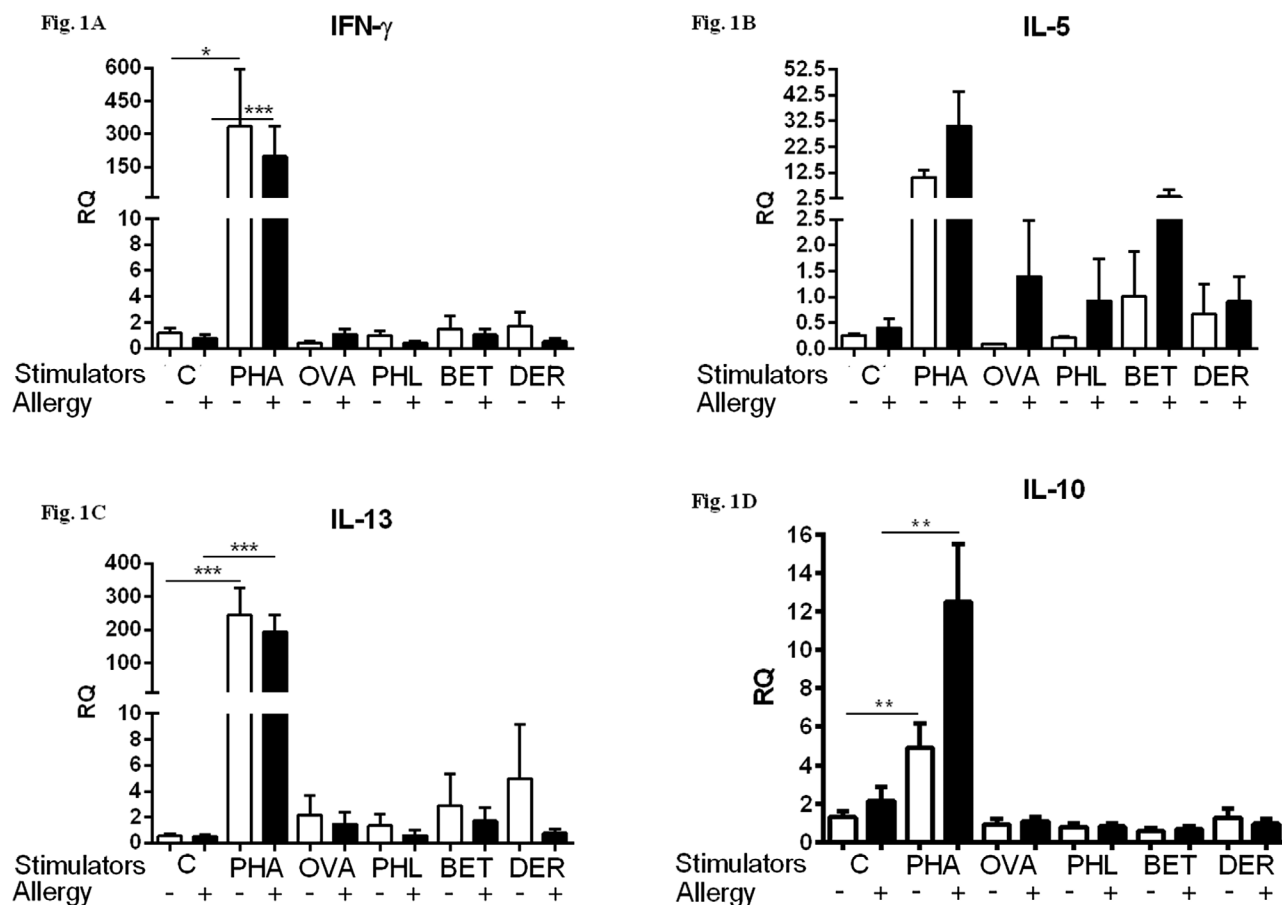
Fig. 1. The effect of allergens on cytokine gene expression by cord blood mononuclear cells of new-borns of healthy and allergic mothers measured by immunoenzymatic method ELISA
Stimulators: **C** – unstimulated negative control, **PHA** – positive control stimulated with phytohaemagglutinin, **OVA** – sample stimulated with ovalbumin allergen, **PHL** – sample stimulated with allergen nPhl p 1 from timothy grass, **BET** – sample stimulated with allergen rBet v 1 from birch, **DER** – sample stimulated with allergen rDer p 2 from house dust mite. Allergy: – CBMC of children of healthy mothers, + CBMC of children of allergic mothers. **A** – gene expression of IFN-γ. **B** – gene expression of IL-5. **C** – gene expression of IL-13. **D** – gene expression of IL-10. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. RQ – relative quantification.

ment of pro-allergic phenotype occur right after the birth. This drives the curiosity and effort to reveal the possible signals pointing to future development of allergy and to identify the early prognostic markers indicating an increased risk of allergy, allowing early introduction of some preventive measures. Maternal atopy has been reported to contribute to the risk of development of allergic disease in children more than paternal atopy (Liu et al., 2003; Moore et al., 2004; Schaub et al., 2005). It has been proposed that not only the genetic background, but also the maternal influence during intrauterine development may support later allergy appearance in the child (Jones et al., 2000). Especially, allergic sensitization of the immature immune system during the intrauterine life seems to be a good candidate for the key event responsible for pro-allergic tuning in predisposed children.

We aimed to confirm that maternal allergy predisposed new-borns to increased risk of allergy by supporting immune responses to allergens. Distinct cytokine patterns predispose infants to later asthma development

(Rothers et al., 2011). Notably, lower levels of IFN-γ and increased levels of IL-13 in response to allergen stimulation were associated with the onset of allergy (Contreras et al., 2003). In our study, no difference in secretion of IFN-γ and IL-13 in response to allergen stimulation between children of healthy and allergic mothers was observed. Similarly, no difference in IFN-γ and IL-13 secretion after PHA stimulation was observed between the cord blood of new-borns who developed atopic dermatitis and who did not (Kim et al., 2012). Previously, impaired function of Treg cells in new-borns at higher risk of allergy development has been described (Hrdý et al., 2012; Černý et al., 2018). Namely, lower levels of IL-10 were considered to be a marker pointing to increased risk of allergy development (Súkeníková et al., 2017; Suzuki et al., 2018). In addition to that, an insufficient response of Treg to microbial antigens was reported by Ismail et al. (2014).

Previously, some research groups demonstrated the ability of cord blood cells to respond to allergens by ele-
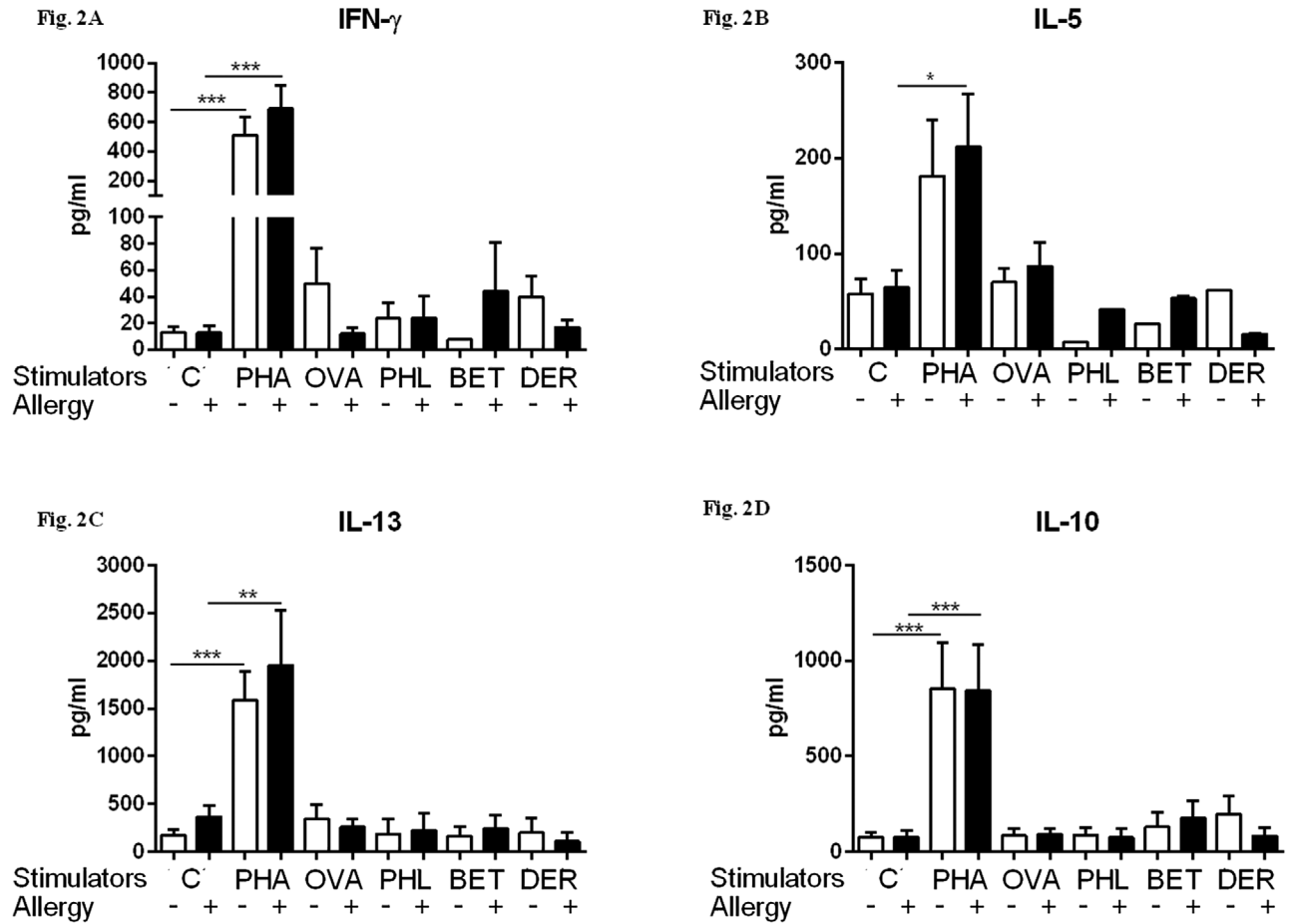
Fig. 2. The effect of allergens on cytokine secretion by cord blood mononuclear cells of new-borns of healthy and allergic mothers measured by immunoenzymatic method ELISA

Stimulators: **C** – unstimulated negative control, **PHA** – positive control stimulated with phytohaemagglutinin, **OVA** – sample stimulated with ovalbumin allergen, **PHL** – sample stimulated with allergen nPhl p 1 from timothy grass, **BET** – sample stimulated with allergen rBet v 1 from birch, **DER** – sample stimulated with allergen rDer p 2 from house dust mite. Allergy: – CBMC of children of healthy mothers, + CBMC of children of allergic mothers. **A** – concentration of released IFN-γ in pg/ml. **B** – concentration of released IL-5 in pg/ml. **C** – concentration of released IL-13 in pg/ml. **D** – concentration of released IL-10 in pg/ml. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$

vated proliferation activity (Kim et al., 2012). Following these findings, we focused on cytokine production monitored at two levels: mRNA and protein release to supernatants during cultivation. Our results point to a very weak ability of common allergens (timothy grass, birch, mite, and ovalbumin) to stimulate CBMC to produce cytokines at both the mRNA and protein levels. Although we did not observe significant differences in CBMC cytokine production (IL-5, IL-10, IL-13, IFN-γ) after stimulation with allergens between the groups of children of healthy and allergic mothers, there was a trend towards higher levels of cytokine production after polyclonal stimulation by umbilical cord blood cells of children of allergic mothers. This higher CBMC reactivity of children from the allergic group reflects the ability of the immune system of predisposed new-borns to respond more effectively to antigens than CBMC of children of healthy mothers. The increased predisposition to heightened immune responses together with impaired immune regulation could lead to easier development of inappropriate immune responses to relatively innocuous environmental allergens, resulting in development of allergic diseases.

We acknowledge that only a limited number of cytokines promoting Th1, Th2 and Treg immune responses was tested in the current study. Stimulation of CBMC by allergen can impact other cytokines playing a role either in promotion of allergy development (e.g., IL-4, IL-25, IL-33) or in down-regulating pro-allergic immune responses (e.g., IL-35 or TGF-β). Importantly, Th9, Th17, Th22 cytokines or cytokines promoting ILC2 have not been inspected at all. Nevertheless, our results indicate that the perinatal sensitization seems not to be decisive for allergy development, and that postnatal events including environmental factors and postnatal colonization with microbes play a more important role in the decision whether atopic persons become true patients suffering from allergic diseases.

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