Original Article

Gene Expression of Subunits of the IL-12 Family Cytokines in moDCs Derived *In Vitro* from the Cord Blood of Children of Healthy and Allergic Mothers

(CD83 / IL-12 family / gene expression / allergy / cord blood / dendritic cells)

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Abstract. The incidence of allergic diseases is steadily increasing an urgent need to clarify the immunologic processes which occur early in life and signal an increased risk of possible future allergy development. The ratio and maturation state of DCs together with the cytokine environment are important in directing and modulating immune responses. The maturation state (presence of CD83) of cord blood monocyte-derived dendritic cells (moDCs) of 52 children of healthy mothers and 58 children of allergic mothers was estimated by flow cytometry. The capacity of moDCs to express genes for subunits of IL-12 family cytokines was monitored using real-time PCR and protein secretion in cell culture supernatants by ELISA. The percentage of CD83⁺ moDCs was significantly higher in the allergic group after LPS stimulation (43.11 ± 4.41) in comparison to the healthy group (24.85 ± 3.37). Significantly higher gene expression of subunits of IL-12 family members was observed in

moDCs of children of allergic mothers, in comparison with children of healthy mothers. The differences were evident mainly after LPS stimulation of moDCs (healthy group: *p19*: 3.05 ± 1.24; *p28*: 14.8 ± 6.8; *p35*: 1.8 ± 0.6; *p40*: 8.0 ± 3.5; *EBI3*: 3.0 ± 1.2; allergic group: *p19*: 6.1 ± 2.7; *p28*: 61.4 ± 22.2; *p35*: 14.9 ± 6.5; *p40*: 36.4 ± 18.8; *EBI3*: 11.3 ± 3.2), with the exception of *p28*, whose expression was significantly higher in the allergic group even without stimulation (healthy group: 0.28 ± 0.12, allergic group: 0.87 ± 0.62). No significant difference between the healthy and allergic groups was found at the protein level. The observation of both increased presence of cell surface activation marker on moDCs and higher IL-12 family gene expression in LPS-stimulated moDCs of children of allergic mothers indicates a higher reactivity of these cells.**

Introduction

Although antigen-presenting cell (APC) immaturity is characteristic for the neonatal period (Velilla et al., 2006), it has been suggested that some infants may have a more significant and prolonged functional impairment of APC function, leading to a defective Th1 cell response, thus predisposing the child to future allergy development (Sandberg et al., 2009). Prescott hypothesizes that neonatal maturational deficiency is more pronounced in individuals who go on to develop atopy, but differences in IL-12 are not significant (Prescott et al, 2003). Newborns with atopic heredity show a more delayed increase in IFN-γ responses in the early postnatal period, with the tendency of IFN-γ to remain significantly reduced relative to low-risk infants (Tang et al., 1994; Warner et al., 1994; Martinez et al, 1995; Liao et al, 1996; Kondo et al, 1998). An impaired Th1 neonatal immune response, characterized by lower production of IL-12 and IFN-γ, is well known. However, it remains unresolved whether the lower IFN-γ responses are due to defective neonatal APC function. Characterization of

the monocyte-derived dendritic cell (moDC) reactivity in allergy low-risk and high-risk newborns is the topic of the present study.

In our former work concerning the immunologic characteristics of children with an increased risk of allergy, a decreased gene expression of IFN-γ in cord blood cells and its decreased level in cord blood sera were detected (Hrdý et al, 2010; Lodinová-Žádníková et al., 2010) and increased proliferation activity, both spontaneous and stimulated, was proved with cord blood lymphocytes of children of allergic mothers (Žižka et al, 2007). In addition to that, the impaired function of Tregs in cord blood of children of allergic mothers was described (Hrdý et al, 2012). The importance of dendritic cells (DCs) for T-cell function directed our interest to the possible differences between moDCs of the cord blood of newborns of either allergic or healthy mothers. Gene and protein expression of IL-12 family cytokines and surface expression of the activation marker CD83 were monitored.

In humans, two main DC populations are present (Rissoan et al, 1999; Hagendorens et al., 2003): myeloid DCs (mDCs or DC1) and plasmacytoid DCs (pDCs, DC2 or interferon-producing cells – IPCs – because of their capacity to produce huge amounts of type I interferon playing an important role in antiviral defence). The critical role of mDCs in airway allergic inflammation was shown in an experimental mouse model of asthma, whereas pDCs rather exerted suppressive functions (Mo et al., 2011; Pilette et al., 2013). On the other hand, the role of DC subsets in humans is more controversial. Increment of both mDCs and pDCs was observed after nasal allergen challenge (Pilette et al., 2013).

The IL-12 family is represented by heterodimeric cytokines formed by α (p35, p19 or p28) and β (p40 or Epstein-Barr virus-induced protein 3 – EBI-3) chains. IL-12 consists of p35 and p40; IL-23 is assembled from p19 and p40; IL-27 comprises p28 (IL-30) and EBI-3; and IL-35 comprises p35 and EBI-3, as indicated in Table 1.

Co-expression of the α and β chains is required for the secretion of a bioactive cytokine. Production of each of the four heterodimeric proteins is limited by the expression of the α chains, with β chains being produced in abundance. IL-12 family cytokines are involved in many immunological processes and, together with DC maturation stage and mDC/pDC ratio, can prevent or promote allergy development. IL-12, IL-23, and IL-27 were initially described as pro-inflammatory cytokines, promoting T-cell proliferation and cytokine production.

Table 1. Subunits of heterodimeric cytokines of the IL-12 family

	α chain	β chain
IL-12	p35	p40
IL-23	p19	p40
IL-27	p28	EBI-3
IL-35	p35	EBI-3

IL-12 is known for its capability of Th1 activation and maintenance of Th1 response, while blocking Th2 (Goriely and Goldman, 2008). IL-23 is involved in Th1 activation and induction of IFN-γ production, Th17 polarization, and proliferation. IL-27 promotes Th1 cell proliferation by blocking Th17 (Goriely and Goldman, 2008; Yoshimoto et al., 2007). Like IL-12, IL-27 can directly inhibit the secretion of IL-4 and antagonize IL-2 production, hence limiting Th2 cell differentiation and promoting T-cell proliferation and IFN-γ production by naïve T cells. Both IL-12 and IL-27 are capable of inducing T-bet, IFN-γ, and IL-12Rβ2 expression in naïve T cells (Cao et al., 2008; Goriely and Goldman, 2008). Besides this Th1-promoting function, a marginal effect of IL-27 on naïve B-cell switch to IgE production was described in humans (Boumendjel et al., 2006; Eijnden et al., 2006). Although IL-35 belongs to the IL-12 family, it serves a regulatory function by inhibiting T-cell proliferation. IL-12, IL-23, and IL-27 are all primarily produced by antigen-presenting cells; in contrast, IL-35 is mainly produced by regulatory T cells (Bettini and Vignali, 2009).

To compare the capacity of moDCs from children of healthy and allergic mothers to produce cytokines of the IL-12 family, we used DCs generated *in vitro* from adherent cord blood mononuclear cells by incubation in the presence of IL-4 and GM-CSF. Because of the heterodimeric character of cytokines, with some subunits present in two different cytokines, the gene expression of all individual subunits was measured. Cytokine production was tested by ELISA using antibodies specific for individual dimers.

Material and Methods

Subjects

Children were divided into two groups according to maternal allergy status. Healthy and allergic mothers with a physiological pregnancy and children delivered physiologically (vaginally) in full term were included in the study. Diagnostics of allergy in mothers was based on the clinical manifestation of an allergy persisting for longer than 24 months (allergy against respiratory and food allergens manifested by various individual combinations of hay-fever, conjunctivitis, bronchitis, asthma, eczema, etc.), monitoring by allergist, positive skin prick tests, or positive specific IgE antibodies and anti-allergic treatment before pregnancy. Fifty-eight children of allergic mothers and 52 children of healthy mothers were comprised in the study. The study was approved by the Ethics Committee of the Institute for the Care of Mother and Child, Prague, Czech Republic; and was carried out with the written informed consent of the mothers.

Cord blood

Cord blood from children of both healthy and allergic mothers was collected immediately after the delivery,

with the usual procedure including careful cleaning of the cord and puncture of the umbilical vein to avoid maternal blood contamination. Typically, 20 ml of cord blood was collected in sterile heparinized tubes (10 U heparin/ml).

Generation and maturation of moDCs

Cord blood mononuclear cells (CBMCs) were acquired by gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Freiburg, Germany), according to the manufacturer's instructions. Up to 6×10^6 CBMC in 15 ml of MEM (Sigma Aldrich s.r.o., Prague, Czech Republic) were incubated for 2 h in 75 cm^2 plastic culture flasks (NUNC, Schoeller Pharma Praha, Prague, Czech Republic). After removal of the nonadherent fraction of CBMCs by washing with MEM (Sigma), the adherent fraction was incubated in 15 ml of RMPI 1640 (Cambrex Corporation, East Rutherford, NJ), supplemented with L-glutamine (2 mM, Sevapharma, Prague, Czech Republic), HEPES (2 mM, Sigma), gentamicin (40 mg/l), 10% foetal bovine serum (Cambrex), recombinant IL-4 (20 ng/ml, Peprotech, Rocky Hill, NJ), and recombinant granulocyte-monocyte colony-stimulating factor – GM-CSF (500 U/ml, Leucomax) at 37 °C in a 5% CO_2 atmosphere for five days. The purity and activation state of generated moDCs were examined by flow cytometry (CD11c$^+$CD14$^-$ cells were evaluated as moDCs). The minimal yield and purity of cells used for further analyses was $2-4 \times 10^6$ moDCs from a single culture flask (purity in the range of 90 %–95 %), with up to 10 % of matured moDCs (CD11c$^+$CD83$^+$). The maturation of moDCs was induced by their cultivation (1×10^6 cells/ml in 24-well plates) with LPS (1 µg/ml, *Escherichia coli*, Sigma) for 24 h. For detection of cytokine secretion in culture supernatants, moDCs were cultivated with LPS for three days.

FACS analysis

DCs generated *in vitro* from cord blood were subjected to flow analysis using a BD FACS Canto II in BD FACS Diva version software 6.1.2. (Becton Dickinson, Franklin Lakes, NJ) and the data were analysed using FlowJo 7.2.2. (TreeStar, Ashland, OR). Cytometer setup & tracking beads were used to check the instrument performance and to assure its stability and validity of data analysed on different days. After five days of cultivation, cells were incubated with APC-conjugated CD11c mAbs (Miltenyi Biotech, Bergish Gladbach, Germany), PerCP-conjugated CD14 mAbs, and PE-conjugated CD83 mAbs (both Becton Dickinson) for 20 min, then washed three times with a FBS staining buffer (Becton Dickinson), and immediately acquired. The maturation state of moDCs was checked both immediately and after 24 h of stimulation of cells by LPS by the presence of maturation marker CD83 on CD11c$^+$CD14$^-$ cells. Both the number (%) of positive cells (CD83$^+$CD11c$^+$) and median of fluorescence intensity (MFI) of CD83 in CD11c$^+$ moDCs were followed.

RNA isolation

Total intracellular moDCs' RNA was isolated with the RNeasy Minikit (Quiagen, Valencia, CA) according to the manufacturer's instructions. RNA integrity was determined by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. 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. The total RNA concentration was estimated by spectrophotometric measurement at 260 nm, assuming that 44 µg of RNA per millilitre equals one absorbance unit. RNA was stored in aliquots at -20 °C until used for reverse transcription.

Real-time PCR

Isolated mRNA was converted to cDNA using reverse transcription reagents (Applied Biosystems/Life Technologies, Prague, Czech Republic), according to the manufacturer's instructions. A reaction mix for quantitative real-time polymerase chain reaction (qPCR) was made with a TaqMan Universal PCR master mix, RNase-free water, and Assays on Demand gene expression products for p19 (Hs00413259_m1), p28 (Hs00377366_m1), p35 (Hs00168405_m1), p40 (Hs01011519_m1), and EBI-3 (Hs00194957_m1); all Applied Biosystems. Cyclophilin A (peptidylprolyl isomerase A, Hs99999904_m1) was used as an endogenous control (Applied Biosystems). Cyclophilin A was selected as the most suitable endogenous control (with stable expression even after LPS stimulation) after testing the set of human endogenous controls (TaqMan Express Plate Endogenous Control, cat. No. 4391590, Applied Biosystems). The efficiency of reactions for all assays was very similar to the efficiency of the endogenous control.

PCR reactions were run in the 7300 real-time PCR system (Applied Biosystems) using standard conditions. A no-template control contained water instead of cDNA. The total amount of cDNA in the reaction was 100 ng. Expression of all genes was normalized to the mRNA loading for each sample and used as an internal standard (endogenous control). The quantity of mRNA (relative quantification, RQ) was given as $2^{-\Delta\Delta ct}$. $\Delta\Delta ct$ was calculated as follows: $\Delta\Delta ct = \Delta ct$ (moDCs of children of allergic mothers) - Δct (moDCs of children of healthy mothers). $\Delta ct = ct$ (concrete IL-12 family subunit) - ct (endogenous control/cyclophilin A). The ct value is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. The gene expression of subunits of the IL-12 family cytokines in moDCs of children of allergic mothers was expressed relative to the mean of gene expression of the subunit of the IL-12 family in moDCs of children of healthy mothers, using GenEx software (MultiD, Göteborg, Sweden).

Determination of in vitro secreted cytokines by ELISA method

Concentrations of IL-12, IL-23, IL-27 and IL-35 in cell culture supernatants after 3-day cultivation of

moDCs (nonstimulated or LPS-stimulated) were quantified by ELISA on high-adsorption polystyrene microtitration plates (NUNC) using the following reagents and producer recommendations: IL-12 – primary antibody (MAB 611), secondary antibody (BAF 219) and standard by R&D (detection limit 15 pg/ml); IL-23 – Duoset BMS2023/2MST (Bender MedSystem, San Diego, CA) (detection limit 80 pg/ml); IL-27 – Duoset DY2526 by R&D (detection limit 130 pg/ml); IL-35 – Detection kit E92008Hu by Uscn (Life Science Inc., St Petersburg, FL) (detection limit 15 pg/ml). The results were read from calibration curves in pg/ml.

Statistics

Differences between groups were evaluated using the paired and unpaired t-test for normally distributed data (comparing stimulatory indices, MFI of activation markers and gene expression of subunits of cytokines of the IL-12 family); the non-parametric Mann-Whitney test was utilized for comparing cytokine concentrations in cell culture supernatants). Statistical significance was set at $P \leq 0.05$. For statistical evaluation of ELISA results, the values under the detection level were given as half the detection limit. Results are expressed as mean ± standard error of the mean.

Results

Activation/maturation state of moDCs

The maturation state was evaluated in both non-stimulated and LPS-stimulated cord blood moDCs of 58 children of allergic mothers and 52 children of healthy mothers. CD83 surface expression, measured by flow cytometry, was used as an activation marker. Because of a large individual variability, the differences between nonstimulated moDCs of children of healthy and allergic mothers did not reach statistical significance – either in cell proportions or in MFI (mean ± SEM of proportion and MFI of CD11c$^+$CD83$^+$ in nonstimulated moDCs of children of healthy mothers 10.56 ± 1.6 and 6652 ± 1130, respectively; proportion and MFI of moDCs of children of allergic mothers 12.82 ± 2.64 and 6826 ± 1099, respectively). However, a higher proportion of CD83$^+$ cells (43.11 ± 4.41) and higher MFI (17160 ± 2890) in LPS-stimulated cultures of children of allergic mothers, in comparison to the healthy group (24.85 ± 3.37 and 10065 ± 1285, respectively), was quite evident and significant, and pointed to a more intensive response to LPS in the allergic group – Fig. 1a-d.

Evaluation of gene expression of the IL-12 family

Gene expression was tested in moDCs derived from CBMC of 12 newborns of allergic mothers and 12 newborns of healthy mothers. Selection criteria were the quality (260/280, 260/230 ratios) and quantity of RNA isolated from moDC cultures. Results obtained by expression analysis of IL-12 family subunits in non-stimulated moDCs revealed indistinctly increased expression of *p19*, *p28*, *p35*, and *p40* in infants of allergic mothers in comparison with that of healthy mothers; only the difference in *p28* was significant. An adverse trend was detected in *EBI3* expression. In contrast to non-stimulated moDCs, we discovered a substantially higher gene expression of all IL-12 family subunits after LPS-stimulation, with a significantly higher increase in *p28*, *p35*, *p40*, and *EBI3* expression in moDCs of children of allergic mothers, as compared with moDCs of children of healthy mothers. Only a hint of increase in *p19* gene expression was seen in comparison to non-stimulated moDCs of children of both healthy and allergic mothers – Fig. 2 a-e. The response to LPS stimulation is evidently higher in the allergic group. The obvious expression of all α chains (p35, p19 and p28) represents the prerequisite for IL-12, IL-23, IL-27 and IL-35 formation in neonatal period of life.

Cytokine secretion in vitro

The quantity of individual cytokines secreted *in vitro* after a 3-day cultivation of moDCs with or without LPS was measured immunoenzymaticaly using antibodies against specific epitopes of the heterodimeric molecules under study. Non-stimulated moDCs secreted distinct amounts of cytokines only in the case of IL-12. Secretion of IL-27; IL-23 and IL-35 was under the reliable detection limit. Production of all cytokines was substantially increased after stimulation with LPS, as was also the gene expression, but the differences between the allergic and the healthy group were not significant, while gene expression of all subunits tested except for *p19* was significantly higher in the allergic group after LPS stimulation – Fig. 3 a-d. IL-35 is known as a suppressive cytokine produced by Tregs. Here we show for the first time its intensive synthesis also by stimulated moDCs.

Discussion

The study was performed to evaluate moDC reactivity in children of healthy and allergic mothers, in an effort to elucidate the background of the increased risk of allergy development in children of allergic mothers. Paternal allergy was not taken into consideration in this study because the maternal impact on future allergy development of offspring is much stronger due to the 9-month contact of the foetus with the maternal immune system (Gabrielson et al., 2001; Sandberg et al., 2009; Takadi et al., 2009). In our previous experiments, we documented a higher proliferation activity of cord blood lymphocytes (Žižka et al., 2007), decreased gene expression of Th1 cytokines in cord blood cells (Hrdý et al., 2010) and impaired function of Tregs (Hrdý et al., 2012) in newborns of allergic mothers in comparison with children of healthy mothers. It drew our attention to the possibly increased reactogenicity of the immune system of children with increased allergy risk, which could cause easier stimulation after the encounter with potential allergens. Because DCs as antigen-presenting cells decide on the character of oncoming immune re-
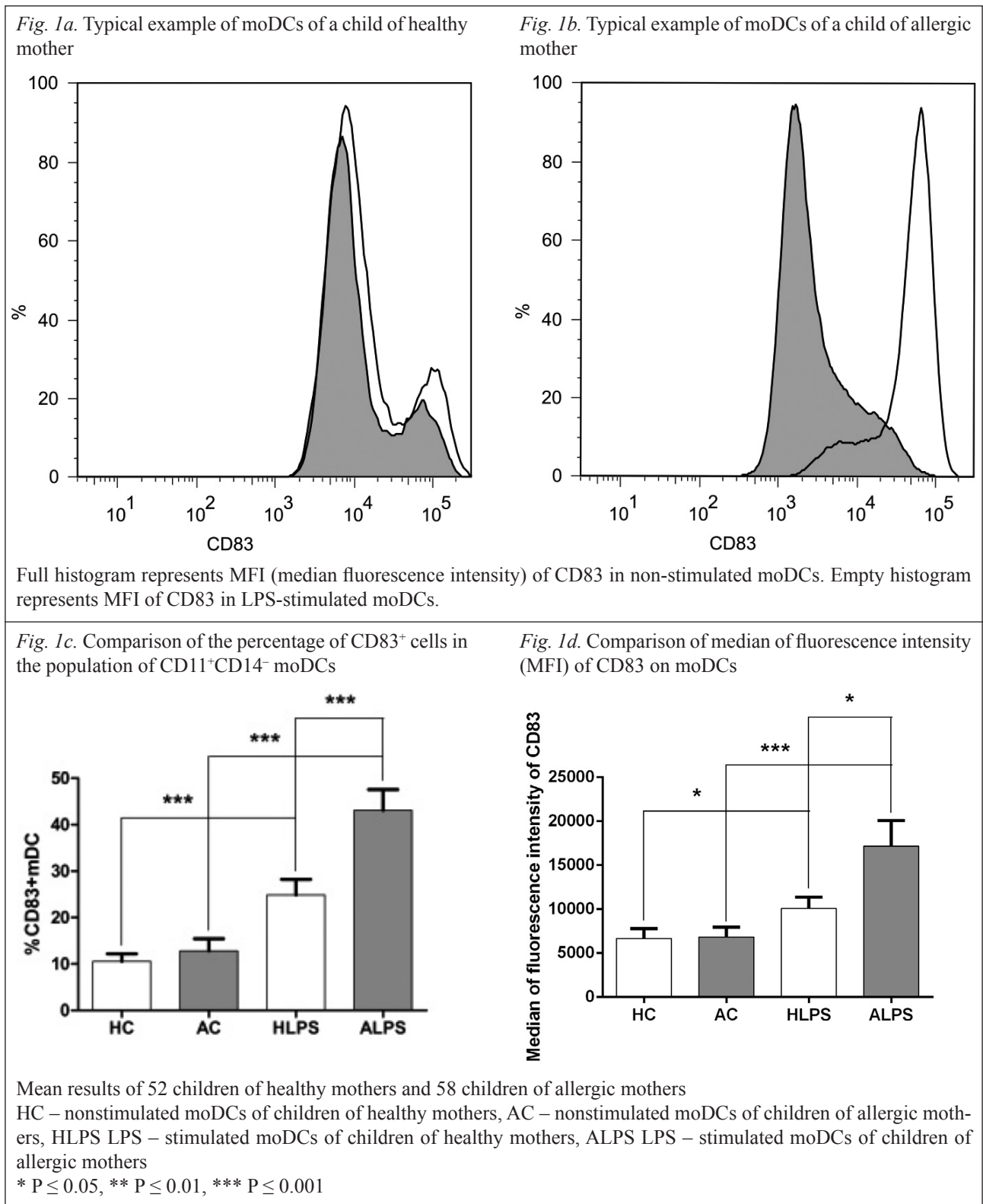
Fig. 1a. Typical example of moDCs of a child of healthy mother

Fig. 1b. Typical example of moDCs of a child of allergic mother

Full histogram represents MFI (median fluorescence intensity) of CD83 in non-stimulated moDCs. Empty histogram represents MFI of CD83 in LPS-stimulated moDCs.

Fig. 1c. Comparison of the percentage of CD83+ cells in the population of CD11+CD14− moDCs

Fig. 1d. Comparison of median of fluorescence intensity (MFI) of CD83 on moDCs

Mean results of 52 children of healthy mothers and 58 children of allergic mothers
HC − nonstimulated moDCs of children of healthy mothers, AC − nonstimulated moDCs of children of allergic mothers, HLPS LPS − stimulated moDCs of children of healthy mothers, ALPS LPS − stimulated moDCs of children of allergic mothers
* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001

Fig. 1. Surface expression of the activation marker CD83 on nonstimulated and LPS-stimulated moDCs derived from cord blood of children of healthy and allergic mothers. Flow cytometry.

sponse, we tried to follow the activation properties of moDCs generated from cord blood mononuclear leukocytes. Previous studies focused mainly on comparing the *IL12* gene expression of both p40 and p35 subunits in healthy adults and newborns, with the conclusion that the expression of *IL12* is lower in children than in adults (Goriely et al.; 2001; Prescott et al., 2003; Koga et al., 2008; Lebre et al., 2008; Renneson et al., 2009). Differences in IL-12 genes and mainly in protein expression between children with positive or negative family history of allergy were tested with contradictory results (Yang et al., 2000; Gabrielson et al., 2001;
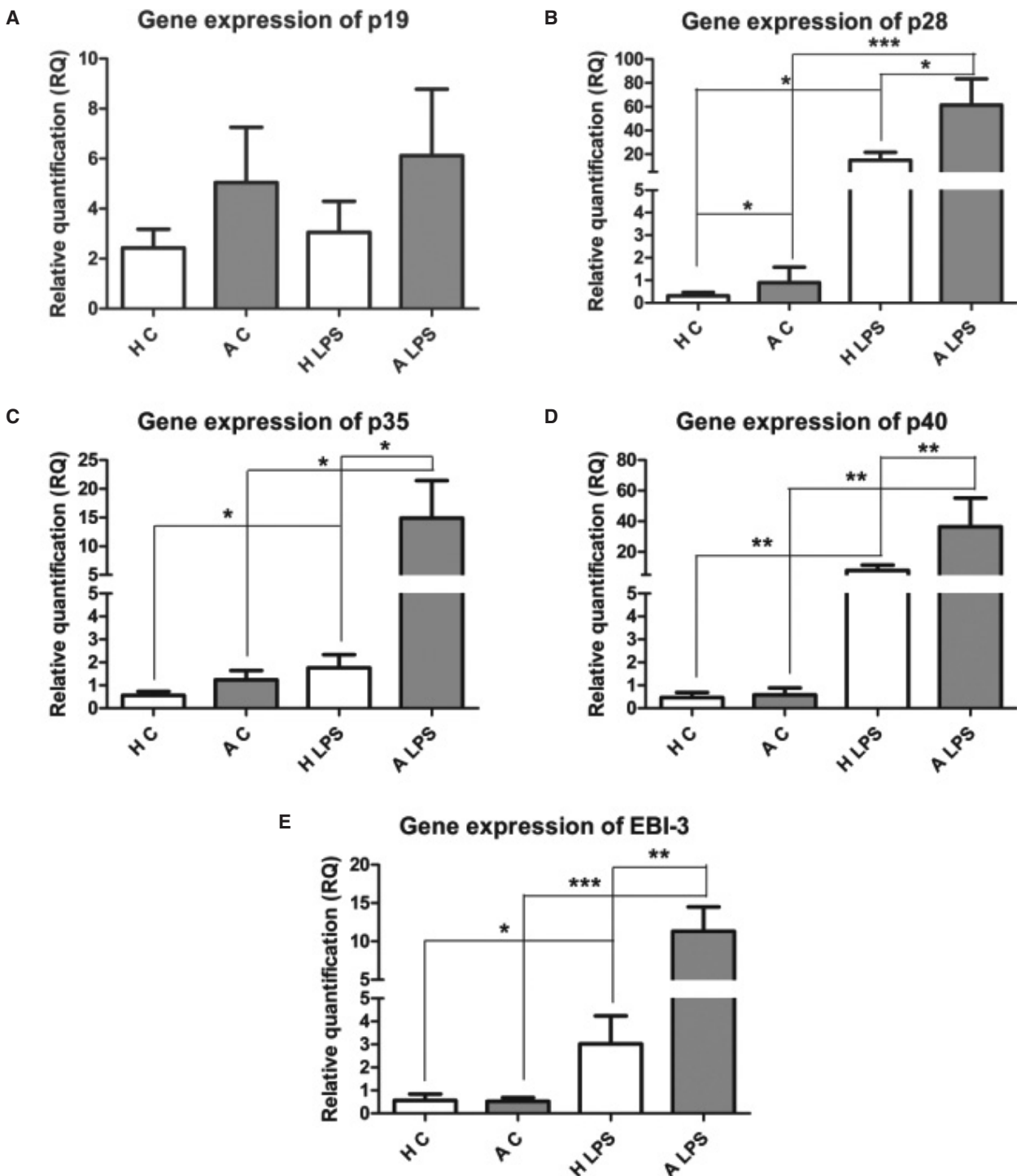
A Gene expression of p19

B Gene expression of p28

C Gene expression of p35

D Gene expression of p40

E Gene expression of EBI-3

Fig. 2. Gene expression of IL-12 cytokine family subunits in moDCs derived from cord blood detected by qPCR. Results expressed as relative quantification (RQ).

Gene expression was compared in nonstimulated and LPS-stimulated moDCs of 12 children of healthy mothers and 12 children of allergic mothers.

HC – nonstimulated moDCs of children of healthy mothers, AC – nonstimulated moDCs of children of allergic mothers, HLPS LPS – stimulated moDCs of children of healthy mothers, ALPS LPS – stimulated moDCs of children of allergic mothers

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

Prescott et al., 2003; Tadaki et al., 2009). To the best of our knowledge, there is currently no information available about the gene expression of all of the so far described members of the IL-12 family in the mDCs of high-risk newborns (infants of allergic mothers) or the low-risk ones (children of healthy mothers).

The reactivity of mDCs derived from cord blood after cultivation of mononuclear cells with IL-4 and GM-
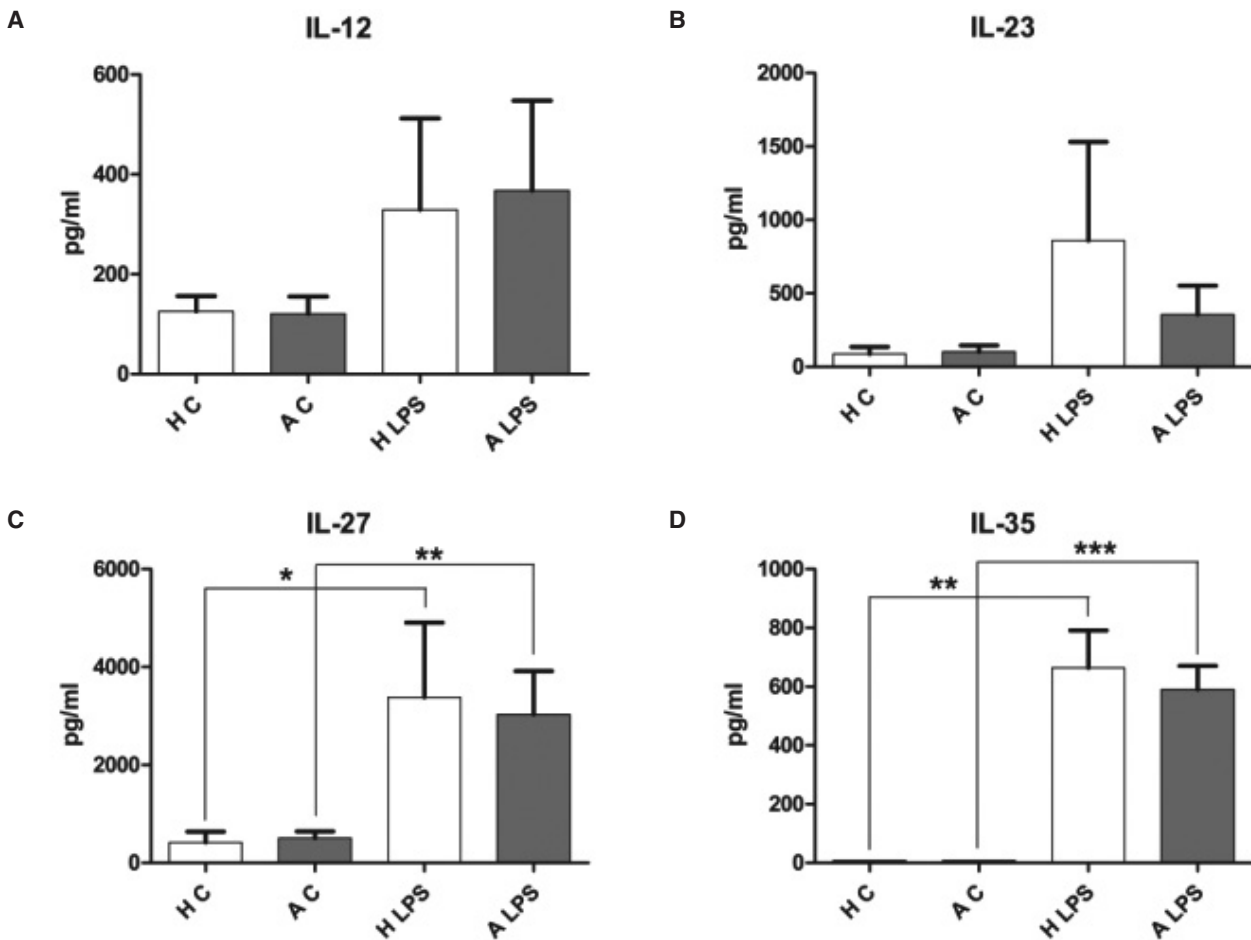
A IL-12

B IL-23

C IL-27

D IL-35

Fig. 3. Protein secretion of IL-12 family cytokines by moDCs derived from cord blood detected by ELISA in cell culture supernatants after 3-day cultivation (pg/ml)
HC – nonstimulated moDCs of children of healthy mothers, AC – nonstimulated moDCs of children of allergic mothers, HLPS LPS – stimulated moDCs of children of healthy mothers, ALPS LPS – stimulated moDCs of children of allergic mothers
* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$

CSF was assessed by testing the activation marker surface expression, by measuring gene expression of all subunits of the heterodimeric IL-12 family and by detection of IL-12 family cytokine secretion *in vitro*. The absolute majority of mDCs obtained were non-mature (non-activated); all their functional properties detected were therefore of very low, often hardly detectable, level and it was difficult or impossible to compare these data between the allergic and healthy group. However, after *in vitro* stimulation of moDCs with LPS, it was possible to follow and compare the reaction promptness of these cells. We have found increased reactivity of moDCs of children of allergic mothers. Substantial significance was reached both in gene expression of the IL-12 family and surface expression of activation marker CD83. The gene expression of IL-12 family subunits was reflected in *in vitro* synthesis of corresponding cytokines, but significant differences between allergic and healthy groups were not evident at the protein level. It is possible to imagine that the potency of increased immunological reactivity of high allergy risk newborns is evi-dent at the gene level, but this potency is not yet exploited. Differences in secretion are probably influenced by further additional factors, mainly environmental factors coming to the fore only after birth. The differences between the sensitivities of testing gene expression (qPCR) and protein secretion (ELISA) should also be kept in mind.

We followed IL-12 family cytokines as important effectors of mDCs, which are responsible for the bias of Th cells to anti-allergic Th1 population (IL-12, IL-23, IL-27). Another member of this family – IL-35 – is a suppressive cytokine which could damp inappropriate allergic responses. We therefore speculated that the formation of this cytokine could be defective in allergy-prone newborns. However, the opposite is true. The gene expression of both IL-12 subunits is very well pronounced in newborn mDC even when IL-12 secretion is not very intensive. It has been suggested that IL-23 or IL-27 could substitute for the insufficient production of IL-12 in small children (Eijnden et al., 2006; Krumbiegel et al., 2008). Indeed, we were able to prove very convinc-

ing, both gene and protein, expression in the case of IL-27; however, the formation of IL-23 in the perinatal period seems not to be so very important, mainly on the basis of modest expression of p19. We are looking for a reasonable explanation of the increased IL-12 family gene expression in the allergic group. It is widely accepted that children of allergic mothers are at increased risk of future allergy development. However, the potential allergy is not yet manifested at birth. An increased support of Th1 cells and potentiation of suppressory mechanisms (IL-35) by IL-12 family cytokines in high-risk newborns could be assumed to serve as a compensatory mechanism in genetically disadvantaged children, which can keep the equilibrium of the immune system for a certain time but may be insufficient later in life when additional environmental factors influence the predisposed organism. Increased reactivity of various cells of the immune system can then facilitate allergy outbreak after an encounter with allergens under appropriate conditions.

We can conclude that certain phenotypic differences pointing to increased reactivity of the immune system are evident in children with increased risk of allergy already at birth. However, the allergy is such a multifactorial process (Tadaki et al., 2009) that it is not possible to consider the change in one immunological characteristic as a predictive marker for future allergy development.

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