



Different capacity of *in vitro* generated myeloid dendritic cells of newborns of healthy and allergic mothers to respond to probiotic strain *E. coli* O83:K24:H31

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ABSTRACT

Allergic diseases belong to one of the most common diseases with steadily increasing incidence even among young children. There is an urgent need to identify a prognostic marker pointing to increased risk of allergy development enabling early preventive measures introduction. It has been shown that administration of selected probiotic strains or mixtures could prevent allergy development. In our study, we have tested the capacity of probiotic strain *Escherichia coli* O83:K24:H31 (*E. coli* O83) to promote dendritic cell (DC) maturation and polarisation of immune responses. Increased presence of activation marker CD83 was observed on DC stimulated by *E. coli* O83 and DC of newborns of allergic mothers have significantly more increased cell surface presence of CD83 in comparison to children of healthy mothers. Increased gene expression and secretion of IL-10 was detected in DC stimulated with *E. coli* O83 being higher in DC of newborns of healthy mothers in comparison to allergic ones. Generally, increased presence of intracellular cytokines (IL-4, IL-13, IFN- γ , IL-17A, IL-22, IL-10) was detected in CD4⁺ T cells cocultured with DC of children of allergic mothers in comparison to healthy ones. *E. coli* O83 primed DC significantly increased IL-10 and IL-17A in CD4 T cells of newborns of healthy mothers in comparison to the levels detected in CD4 T cells cocultured with control non-stimulated DC. We can conclude *E. coli* O83 induces dendritic cell maturation and IL-10 production in DC. Newborns of allergic mothers have generally increased reactivity of both DC and CD4 T cells which together with decreased capacity of DC of newborns of allergic mothers to produce IL-10 could support inappropriate immune responses development after allergen encounter.

1. Introduction

Allergic diseases present the most common illnesses with steadily increasing incidence over the last three decades especially in western countries [1]. Tremendous increment of allergy was observed even among young children. Identification of some prognostic markers pointing to increased risk of future allergy is highly desirable for introduction of early measures leading to allergy prevention or at least lowering the significance of clinical outcomes. Cord blood seems to be an ideal source of such prognostic markers because it is easily available at the time of delivery in a sufficient amount.

Different researcher groups tried to propose some immunological characteristics of cord blood as prognostic markers pointing to increased risk of allergy development (e.g. IgE levels [2], cytokines

present in cord blood sera [3–5], the capacity of cord blood mononuclear cells (CBMC) to release cytokines [5], number and activity of T regulatory cells (Treg) [6–8]). Nevertheless, contradictory conclusions exist in the literature and it is therefore difficult to find a consensus on one or a group of reliable prognostic markers in cord blood pointing to increased allergy development. It seems that the most reliable marker indicating increased risk of future allergy development is the allergic status of the mother [9].

With growing knowledge of the effect of microbiota on immune system, it seems that modification of microbiota composition and function could be exploited in allergy prevention strategies. On the other hand, disturbances in microbiota development early in life by antibiotic administration lead to impaired immune responses and allergy development, as shown on murine model of contact hypersens-

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sitivity [10]. Research of bilateral interaction between microbiota and host immune system is now in the centre of current research [11]. Pioneering bacteria colonizing relatively sterile newborns are important for the induction of proper development of immature newborn immune system [12,13]. Newborns immune system is known for its Th2 predominance. Balance among immune responses (Th1, Th2, Treg) is highly desirable and several reports suggest that bacteria colonizing newborn intestinal tract are able to facilitate the equilibrium [14,15]. Prolonged Th2 immune response together with delayed maturation of infant immune system could be the underlying mechanism leading to allergy origination [16].

Administration of specific bacterial strains with the capacity to promote immune system seems to be reasonable in terms of allergy prevention. There are many reports of probiotic administration either to pregnant women [17,18] or children [19] with the aim to prevent allergy development. Unfortunately, there are works with the opposite results [20,21]. It is important to mention that a broad spectra of microorganisms are used as a probiotic in prevention of allergic diseases – various strains of lactic acid bacteria [22], bifidobacteria [23], or *E. coli* [24]. It seems that the strain selection together with the dose and time of administration play a critical role. We have reported on a protective effect of early *E. coli* O83:K24:H31 administration in allergy prevention [24,14] but the mechanism of its action is still unknown.

In the current study, we have used a simplified model to try to propose a mode of action of this strain on immature immune system of newborns. We focused on the capacity of *E. coli* O83:K24:H31 to promote maturation of monocyte derived dendritic cell and subsequent induction of immune responses (Th1xTh2xTh17xTreg).

2. Material and methods

2.1. Subjects

Healthy and allergic mothers without any substantial complication during the course of pregnancy and children delivered by caesarean section at full term in the Institute for the Care of Mother and Child in Prague, Czech Republic from August 2015 to December 2016 were included into the study after signed written informed consent. Mothers involved in the current study were from the capital and suburban areas. The age was not significantly different between non-allergic mothers (NA) (32.8 ± 4.8 years) and allergic mothers (A) (32.7 ± 3.7 years). The length and birth weight of newborns were not different either (on average 49.4 ± 2.3 cm A, 49.9 ± 2.4 cm NA; 3.488 ± 508.5 kg A, 3.227 ± 395.1 kg NA). The length of the pregnancy was similar as well (39 w $3/7 \pm 0.5$ days A and 39 w $1/7 \pm 8.6$ days NA). Other factors possibly influencing cord blood mononuclear cell reactivity, like smoking, diet (vegetarian, raw, etc.) did not differ between healthy and allergic groups. The diagnosis of allergy in mothers was based on the clinical manifestation of 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 or possible other allergic manifestation), monitored by an allergist, positive skin prick tests or positive specific IgE antibodies and anti-allergic treatment before pregnancy. Detailed list of type or combination of allergies of allergic mothers involved in the study is provided in Table 1. Only two women used anti-allergic drugs during the whole course of pregnancy (from Table 1 patient 2 used Claritin and patient 16 used Zyrtec (cetirizine)). The mothers with previous miscarriages, diabetes, transplantation, transfusion, serious surgery and any health problem during the course of pregnancy were excluded from the study. The study was approved by the Ethical Committee of the Institute for the Care of Mother and Child (Prague, Czech Republic). A total of 65 maternal–child pairs were included in the current study. Based on maternal allergy status, the children were divided into two groups: 43 children NA (22 females, 21 males) and 22A (11 females, 11

Table 1

List of mothers included in allergy group and kind of confirmed allergy.

Allergic mother	Type of allergy/combination of allergies
1	Drugs (antibiotics), food allergy, pollen
2	Pollen, dust, mites
3	Drugs (antibiotics), pollen (grass)
4	Pollen, dust, dander (cat)
5	Atopic dermatitis, pollen (spring season)
6	Food allergy, metals, contact dermatitis
7	Metal
8	Pollen, mites, dander (cat, dog)
9	Pollen, dust, mites
10	Dust, pollen, insect venom (bee)
11	Pollen, cat dander
12	Pollen
13	Dust, pollen (grass)
14	Pollen, bee venom, drugs
15	Mites, feathers
16	Food, allergy, mites, pollen, bee venom
17	Insect
18	Dust, pollen
19	Pollen
20	Pollen
21	Dust, mites
22	Drugs, pollen

males).

2.2. Cord blood sampling

Approximately 15 ml of cord blood was collected after thorough cleaning of the cord in sterile heparinized tubes (10 U heparin/ml) for further analyses and cord blood cell separations.

2.3. Cord blood cell isolation and dendritic cell generation

Cord blood mononuclear cells (CBMC) were isolated by gradient centrifugation using Histopaque (Sigma-Aldrich). Dendritic cells (monocyte derived dendritic cells; moDC) were derived from adherent fraction of CBMC as described previously [25]. Briefly, after 1 h cultivation of CBMC in cell culture flasks in the incubator with regulated CO₂ atmosphere, nonadherent CBMC were washed out and adherent CBMC were cultured for 6 days with rhIL-4 and rhGM-CSF.

2.4. Induction and characterisation of maturation of moDC

In vitro generated moDC were seeded on day 6 at a concentration of 1×10^6 cells/ml in 12-well plates and stimulated with LPS (1 µg/ml, *Escherichia coli*, Sigma), or probiotic bacteria *E. coli* O83:K24:H31 in the ratio: 10 bacterial cells: 1 moDC for 24 h.

2.4.1. Flow cytometry analyses of maturational status of moDC

Maturational status was estimated according to the presence of activation markers on moDC by flow cytometry. moDC were cultivated with *E. coli* O83 or LPS for 24 h and then stained with anti-CD40 (cat. no. 1F-416-T100); anti-CD80 (PC-287-T100), anti-CD83 (1P-677-T100), anti-CD86 (1A-531-T100), anti-MHCII (343310, BioLegend) anti-CD11c (T7-529-T100), all Exbio, plc. and analysed by BD FACS Canto II flow cytometer with BD FACS Diva version software 6.1.2. (Becton Dickinson, Franklin Lakes, NJ). To ensure the quality and reproducibility of data, Cytometer setup & tracking beads were used to check the instrument performance and to assure its stability and validity of data analysed on different days.

2.4.2. Detection of cytokines produced by moDC

Cytokines released by nonstimulated and stimulated moDC during 24 h of incubation were detected by ELISA in culture supernatants. Reagents for IL-6 (primary antibodies – cat. no. MAB 206, biotinylated

antibodies BAF 206, recombinant proteins 206-IL), IL-10 (MAB 2174, BAF 217, 217-IL), TNF-alpha (MAB 610, BAF 210, 210-TA) detection were purchased from R&D System. The results were read from calibration curves in pg/ml.

2.4.3. Gene expression of cytokines

Total RNA was isolated with the RNeasy Minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of RNA was assessed by the ratio of absorbance at 260 nm and 280 nm, and was in the range of 1.9–2.2. The total RNA concentration was estimated by spectrophotometric measurement at 260 nm, assuming that 40 µg of RNA per millilitre equals one absorbance unit. RNA was stored in aliquots at –20 °C until used for reverse transcription. 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 IL-6 (Hs00174131_m1), IL-10 (Hs00174086_m1), TNF-alpha (Hs00174128_m1), indolamine 2, 3-dioxygenase (IDO) Hs00984148_m1; all Applied Biosystems. Cyclophilin A (peptidylprolyl isomerase A, Hs99999904_m1) was used as an endogenous control (Applied Biosystems). PCR reactions were run and data analysed as described by Hrdý et al. [25].

2.5. Characterisation of immune responses

Bacteria primed moDC were cocultured with naïve CD4+ T lymphocytes for 7 days. CD4+ T cells were isolated from non-adherent fraction of CBMC by CD4+ T Cell Isolation Kit (130-096-533) from Miltenyi Biotec according to the manufacturer's recommendation. moDC: CD4 ratio was 1: 10. On day 4, anti CD3 (16-0037-85) and anti CD28 (16-0289-85), both eBioscience, were added at the final concentration 1 µg/ml. Additional stimulation of cells and prevention of extracellular transportation was performed by Leukocyte Activation Cocktail (LAC; 550583 BD Biosciences) which was added on day 7. After 5 h, the cells were harvested for further FACS analysis.

2.5.1. Flow cytometry analyses of induced immune responses

Intracellular staining of transcription factors and cytokines typical for Th1 (T-bet, 561266; IFN-gamma, 560704, both BD Biosciences), Th2 (GATA3, 653804, IL-4, 500824, IL-13, 501907, all BioLegend), Th17 (RORgt, 563621, IL-17A, 560799, both BD Biosciences, IL-22, 366704, BioLegend) and Tregs (FoxP3, 1A-601-C100, Exbio, Plc., IL-10, 501420, BioLegend) was performed after cell surface staining by antibody against CD4 (T4-359-T100, Exbio, Plc.) for 20 min, then washed twice with PBS containing 1% BSA, followed by fixation and permeabilization (BD Pharmingen Transcription Factors Buffer Set, 562574) according to the manufacturer's recommendation. Cells were immediately acquired using BD FACS Canto II. (Becton Dickinson, Franklin Lakes, NJ).

2.6. Statistics

Differences between groups were evaluated using the unpaired *t*-test for normally distributed data (age of the mothers, birth weight and length of newborns, length of pregnancy, activation markers and gene expression in moDC); the non-parametric Mann-Whitney test was utilized for comparing cytokine concentrations in cell culture supernatants and Wilcoxon signed rank test was employed for statistical analyses of intracellular presence of cytokines and transcription factors detected in CD4 T cells. 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 of the detection limit. Results of data with normal distribution are expressed as mean + standard deviation (SD) and data with non-normal distribution are displayed by box plots

showing median with minimum and maximum values of 95% confidence interval (CI) (2.5–97.5 percentile).

3. Results

3.1. moDC

The capacity of probiotic strain of *E. coli* O83 to induce maturation and cytokine expression in moDC of newborns of non-allergic mothers (children with low risk of allergy development – DC (NA)) and newborns of allergic mothers (children with relatively high risk of allergy development – DC (A)) was tested.

3.1.1. Detection of activation markers

The effect of *E. coli* O83 on induction of moDC maturation was observed in both DC (A) (40.5 ± 34.4 vs. $23.5 \pm 25.8\%$ CD83 + CD11c+ moDC, respectively, $p = 0.0486$) and DC (NA), (17.9 ± 18.3 vs. $11 \pm 14.8\%$ CD83 + CD11c+ moDC, respectively, $p = 0.0115$) in comparison to control moDC. LPS induced maturation of DC (A) (40.9 ± 32.7 vs. $23.5 \pm 25.8\%$ CD83 + CD11c+ moDC, respectively, $p = 0.0182$) and DC (NA) (17.6 ± 17.7 vs. $11 \pm 14.8\%$ CD83 + CD11c+ moDC, respectively, $p = 0.0328$) as well. Significantly higher presence of CD83 on DC (A) in comparison to DC (NA) was detected even on nonstimulated moDC (23.5 ± 25.8 vs. $11 \pm 14.8\%$ CD83 + CD11c+ moDC, respectively, $p = 0.0101$). More increased surface expression of CD83 was detected on LPS (40.9 ± 32.7 vs. $17.6 \pm 17.7\%$ CD83 + CD11c+ moDC, respectively, $p = 0.00093$) and *E. coli* O83 (40.5 ± 34.4 vs. $17.9 \pm 18.3\%$ CD83 + CD11c+ moDC, respectively, $p = 0.00979$) stimulated DC (A) in comparison to DC (H) (Fig. 1a). Only nonsignificant changes were observed for the other activation markers (data not shown).

3.1.2. Gene expression in moDC

Stimulation of moDC with *E. coli* O83 increased gene expression of indol-amine 2, 3-dioxygenase (IDO) in both DC (NA) (50.3 ± 62.82 vs. 2.9 ± 3.9 , respectively, $p = 0.0001$) and DC (A) (68.8 ± 49.1 vs. 0.1 ± 0.1 , respectively, $p = 0.0008$). Gene expression of IDO was lower in control DC (A) in comparison to control DC (NA) (0.1 ± 0.1 vs. 2.9 ± 3.9 , respectively, $p = 0.0488$) (Fig. 1b).

IL-10 was significantly decreased in control DC (A) in comparison to DC (NA) (0.5 ± 0.4 vs. 7.2 ± 14.7 , respectively, $p = 0.0228$). Stimulation with *E. coli* O83 promoted expression of IL-10 in both DC (NA) (40.3 ± 45.5 vs. 7.2 ± 14.7 , respectively, $p = 0.0001$) and DC (A) (5.4 ± 3.5 vs. 0.5 ± 0.4 , $p = 0.0079$) but substantially lower expression increase was measured in stimulated DC (A) in comparison to DC (NA) (5.4 ± 3.5 vs. 40.3 ± 45.5 , respectively, $p = 0.0161$) (Fig. 1c).

3.1.3. Secretion of cytokines by moDC

Significantly increased concentration of IL-10 in supernatants of *E. coli* O83 (985.0 (696.8 – 1273) pg/ml vs. 13.6 (10.26 – 16.9) pg/ml, respectively, $p = 0.0001$) and LPS (322.8 (178.2 – 467.3) pg/ml vs. 13.6 (10.26 – 16.9), respectively, $p = 0.0023$) stimulated DC (NA) and *E. coli* O83 (376.6 (216.5 – 536.7) pg/ml vs. 13.6 (1.9 – 25.2) pg/ml, respectively, $p = 0.0013$) and LPS (361.1 (228.2 – 494) pg/ml vs. 13.6 (1.9 – 25.2) pg/ml, respectively, $p = 0.0021$) stimulated DC (A) was observed in comparison to control DC (NA) and DC (A), respectively, Fig. 2a. IL-10 was more increased in supernatants of *E. coli* O83 stimulated DC (NA) in comparison to LPS stimulated DC (NA), (985.0 (696.8 – 1273) pg/ml vs. 322.8 (178.2 – 467.3) pg/ml, respectively, $p = 0.0003$). Of note, supernatants of *E. coli* O83 stimulated DC (NA) contained significantly higher amount of IL-10 in comparison to allergic group (985.0 (696.8 – 1273) pg/ml vs. 376.6 (216.5 – 536.7) pg/ml, respectively, $p = 0.0007$), Fig. 2a.

No difference in the capacity of DC (NA) and DC (A) to produce TNF-alpha was observed but *E. coli* O83 (985 (542.8 – 1227) pg/ml vs.

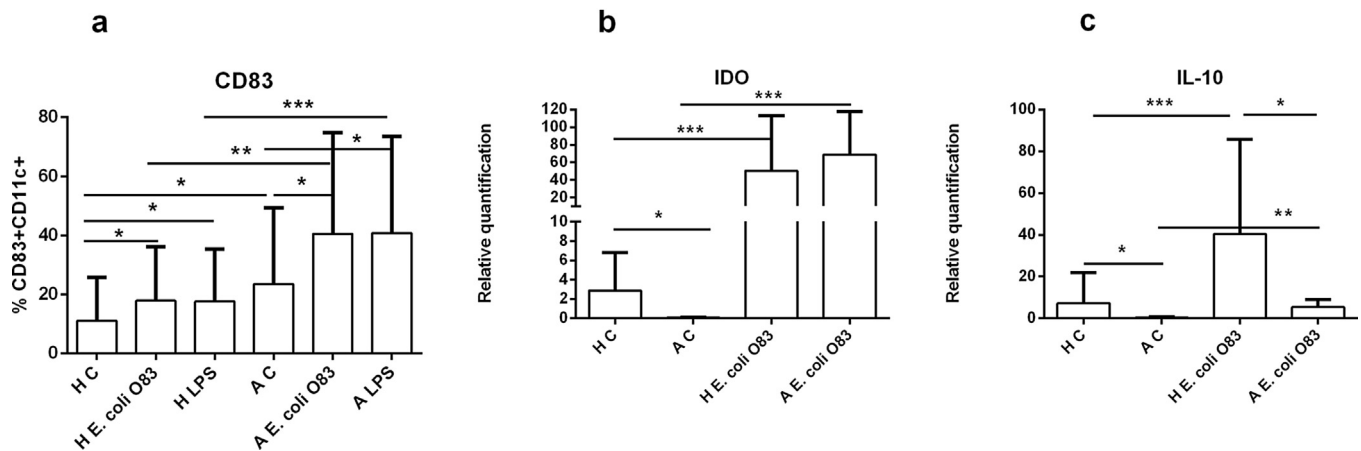


Fig. 1. The capacity of probiotic strain to induce maturation and gene expression in moDC. Activation markers indicating maturation status of moDC were followed by flow cytometry after 24 h stimulation. Gene expression of indolamine 2, 3- dioxygenase (IDO) and IL-10 in moDC was measured after 5 h stimulation by quantitative real-time PCR (qPCR).

a) cell surface presence of CD83 on moDC of newborns sorted according to the maternal allergy status
 b) Gene expression of IDO in moDC of newborns separated according to allergy status of their mothers
 c) Gene expression of IL-10 in moDC of newborns separated according to allergy status of their mothers
 HC – nonstimulated moDC of newborns of nonallergic mothers – DC (NA)
 H *E. coli* O83 – DC (NA) stimulated with *E. coli* O83
 H LPS – DC (NA) stimulated with LPS
 A C – nonstimulated moDC of newborns of allergic mothers – DC (A)
 A *E. coli* O83–DC (A) stimulated with *E. coli* O83
 A LPS – DC (A) stimulated with LPS
 * $p \leq 0.05$
 ** $p \leq 0.01$
 *** $p \leq 0.001$

67.33 (7.8–97.7) pg/ml, respectively, $p = 0.0001$ and LPS (956.9 (481.2–1198) pg/ml vs. 67.33 (7.8–97.7) pg/ml, respectively, $p = 0.0001$) promoted TNF- α secretion by DC (NA) and similarly, *E. coli* O83 (941.2 (589.5–1227) pg/ml vs. 53.6 (15.3–163.7) pg/ml, respectively, $p = 0.0001$) and LPS (908.7 (481.2–1198) pg/ml vs. 53.6 (15.3–163.7) pg/ml, respectively, $p = 0.0001$), increased TNF- α production by DC (A) (Fig. 2b).

The capacity of DC to secrete IL-6 in response *E. coli* O83 or LPS was not different between healthy and allergic group. Concentration of IL-6 was increased in supernatants of *E. coli* O83 (842 (696.1–1754) pg/ml vs. 110.7 (81.4–170.6), respectively, $p = 0.0001$) and LPS (1107 (768.8–1587) pg/ml vs. 110.7 (81.4–170.6), respectively,

$p = 0.0001$) stimulated DC (NA) and *E. coli* O83 (856 (693.5–1827) pg/ml vs. 118.1 (36.9–160.5) pg/ml, respectively, $p = 0.0005$) and LPS (1107 (769.8–1587) pg/ml vs. 118.1 (36.9–160.5) pg/ml, respectively, $p = 0.0001$) stimulated DC (A), Fig. 2c.

3.2. Induction of immune responses by probiotic primed moDC

To analyse the effect of *E. coli* O83 stimulated moDC on priming of immune responses, two panels were created. One was focusing on mutual regulation of Th1 and Th2 immune responses and the other one targeted Th17 and Tregs relationship. No difference was observed between the capacity of *E. coli* O83 stimulated and control moDC to

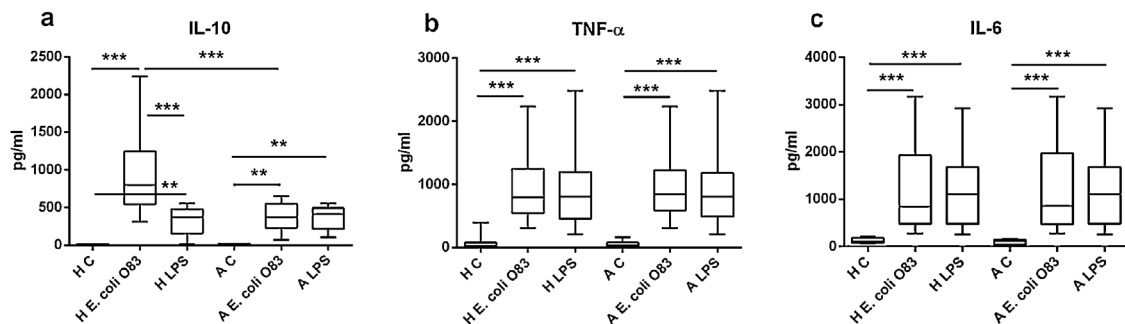


Fig. 2. Detection of cytokines in cell culture supernatants of moDC. To evaluate the capacity of *E. coli* O83 to promote secretion of cytokines, moDC were stimulated 24 h and cytokines were detected by ELISA.

a) IL-10 concentration in moDC supernatants after division of moDC according to maternal allergy status of newborns (MASN).
 b) TNF- α concentration in moDC supernatants after division of moDC according to MASN.
 c) IL-6 concentration in moDC supernatants after division of moDC according to MASN.
 HC – nonstimulated moDC of newborns of nonallergic mothers – DC (NA)
 H *E. coli* O83 – DC (NA) stimulated with *E. coli* O83
 H LPS – DC (NA) stimulated with LPS
 A C – nonstimulated moDC of newborns of allergic mothers – DC (A)
 A *E. coli* O83–DC (A) stimulated with *E. coli* O83
 A LPS – DC (A) stimulated with LPS
 ** $p \leq 0.01$
 *** $p \leq 0.001$

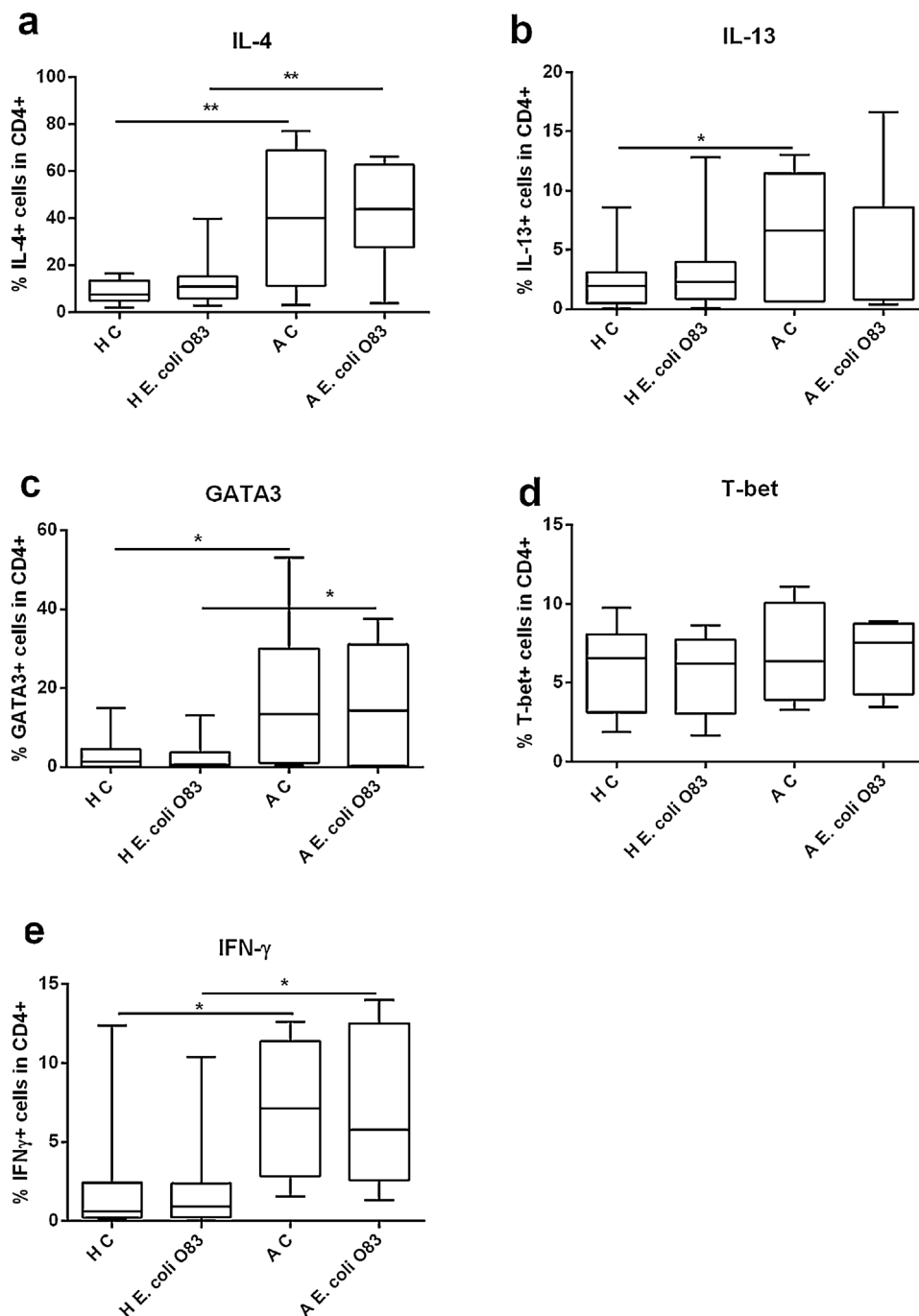


Fig. 3. Evaluation of the capacity of *E. coli* O83 stimulated moDC to induce Th1 or Th2 immune responses. Control or stimulated moDC were cocultured with naïve CD4 T cells for 7 days. On day 7, Leukocyte Activation Cocktail was added and the presence of intracellular cytokines and transcription factors was detected by flow cytometry.

a) Percentage of IL-4 positive CD4 T cells according to maternal allergy status (MASN)

b) Percentage of IL-13 positive CD4 T cells according to MASN

c) Percentage of GATA3 positive CD4 T cells according to MASN

d) Percentage of T-bet positive CD4 T cells according to MASN

e) Percentage of IFN-gamma positive CD4 T cells according to MASN

HC – nonstimulated moDC of nonallergic mothers (DC (NA)) cocultivated with naïve CD4 T cells of newborns of healthy mothers (CD4 (NA))

H *E. coli* O83–*E. coli* O83 stimulated DC (NA) cocultivated with naïve CD4 (NA)

AC – nonstimulated moDC of allergic mothers (DC (NA)) cocultivated with naïve CD4 T cells of newborns of allergic mothers (CD4 (A))

A *E. coli* O83–*E. coli* O83 stimulated DC (NA) cocultivated with naïve CD4 (A)

* $p \leq 0.05$

** $p \leq 0.01$

induce Th1 (T-bet, IFN-gamma) and Th2 (GATA3, IL-4, IL-13) responses (data not shown). When the data were compared according to the maternal allergy status, increased values of Th2 (IL-4, 40.1 (19–61.2) vs. 7.7 (5.8–11.6) % IL-4 + CD4 T cells, respectively, $p = 0.0076$; IL-

13, 7.5 (3.5–9.9) vs. 2 (1–3.9) % IL-13 + CD4 + T cells, respectively, $p = 0.0478$; GATA3, 13.4 (6.1–30.9) vs. 1.3 (0.6–4.8) % GATA3 + CD4 + T cells, respectively, $p = 0.0423$, Fig. 3a–c) immune response were detected in CD4 + T cells co-cultured with control DC (A) in

comparison to control DC (NA). Heightened intracellular presence of IL-4 (44.2 (25.9–56.1) vs. 10.9 (7–18.7) % IL-4 + CD4 + T cells, respectively, $p = 0.0056$) and GATA3 (14.4 (6.5–25.1) vs. 0.6 (0.6–4.9) % GATA3 + CD4 + T cells, respectively, $p = 0.0333$) was detected in T cells cocultured with *E. coli* O83 stimulated DC (A) in comparison to DC (NA), Fig. 3a; c.

No significant increment of characteristic Th1 transcription marker T-bet was detected (Fig. 3d) but elevated IFN-gamma was observed in CD4 T cells cocultured with control DC (A) in comparison to control DC (NA) (Fig. 3e, 7.1 (2–12.3) vs. 0.6 (0.5–4.7) % IFN-gamma + CD4 + T cells, respectively, $p = 0.0412$) and increased values of IFN-gamma was measured in CD4 T cells cocultured with *E. coli* O83 primed DC (A) in comparison to *E. coli* O83 primed DC (NA) (5.5 (1.6–12.3) vs. 0.9 (0.6–4.6) % IFN-gamma + CD4 + T cells, respectively, $p = 0.498$) (Fig. 3e).

When the presence of intracellular cytokines and transcription factors typical for Th17 and Treg was evaluated according to the allergy status of mothers, generally increased percentage of IL-10 (8.7 (3.3–16.7) vs. 2.9 (2–4.7) % IL-10 + CD4 + T cells, respectively, $p = 0.0128$), IL-17A (2.4 (0.9–3.6) vs. 1.2 (0.8–1.7) % IL-17A + CD4 + T cells, respectively, $p = 0.0254$), IL-10 + IL17A + (2.7 (0.6–4.1) vs. 1.1 (0.6–1.7) % IL-10 + IL-17A + CD4 + T cells, respectively, $p = 0.0316$) and IL-22 (4.5 (1.7–5.4) vs. 1.2 (0.6–2.5) % IL-22 + CD4 + T cells, respectively, $p = 0.0071$) cells was detected in T cells cocultured with control DC (A) in comparison to control DC (NA) (Fig. 4a–d). *E. coli* O83 primed DC (NA) were able to promote IL-10 (20.6 (3.8–37.4) vs. 2.9 (2–4.7) % IL-10 + CD4 + T cells, respectively, $p = 0.0102$) and IL-17A (3.6 (1.3–5.8) vs. 1.2 (0.8–1.7) % IL-17A + CD4 + T cells, respectively, $p = 0.0215$) in comparison to control DC (NA). Raised RORgt was detected in CD4 T cells cocultured with control DC (A) in comparison to control DC (NA) (4.8 (3.8–5.5) vs. 1.1 (0.7–1.8) % RORgt + CD4 + T cells, respectively, $p = 0.0007$). *E. coli* O83 primed DC increased induction of RORgt + T cells only in healthy group (3.2 (1.3–4.6) vs. 1.1 (0.7–1.8) % RORgt + CD4 + T cells, respectively, $p = 0.0361$), Fig. 4e. The presence of FoxP3 was not significantly changed either between healthy and allergy group or after coculture with *E. coli* O83 primed DC, Fig. 4f.

4. Discussion

Dendritic cells are connecting innate and adaptive immune responses, processing antigens and setting appropriate immune responses to a broad spectrum of antigens including allergens and microbial stimuli [26–28]. Therefore the effect of probiotics on dendritic cells is of interest. We have demonstrated an increased presence of activation markers on moDC stimulated with *E. coli* O83. The activation marker the most responsive to stimulation was CD83 pointing to the key role of CD83 on DC in priming of immune responses [29–31]. Detection of increased presence of CD83 on DC (A) in comparison to DC (NA) is in keeping with our previous observations of the increased reactivity of CBMC [32], and gene expression in moDC [25] of children of allergic mothers. Also Lisciandro et al. described increased responses of DC of Australian newborns (considered to be at higher risk of allergy development) in comparison to newborns from Papua New Guinea (children with low risk of allergy development) [27]. Of note is the fact that we have observed a higher amount of activation markers on nonstimulated moDC of newborns of allergic mothers than on corresponding cells in healthy group, suggesting that moDC of newborns of allergic mothers have generally elevated tendency to initiate immune responses.

In addition to that, lower expression and secretion of IL-10 in *E. coli* O83 stimulated moDC of newborns of allergic mothers indicate insufficient regulatory function which could support the onset of inappropriate immune responses. The capacity of probiotic bacteria to induce regulatory cytokine IL-10 was described previously by Niers et al. [33]. Reduced secretion of IL-10 from Treg of the offspring of

allergic mothers was reported by other authors [8,34] including our group [6]. In addition to that, we have detected increased expression of indoleamine 2, 3-dioxygenase (IDO) in moDC stimulated with *E. coli* O83 but nonstimulated moDC of newborns of allergic mothers have lower expression of IDO suggesting inferior regulatory potential of these cells. The importance of IDO in controlling allergic inflammatory responses was proposed [35–37]. It is possible to speculate that DC of newborns of allergic mothers are more prone to initiate inadequate immune responses to relatively innocuous environmental antigens due to the decreased regulatory responses.

At the time of delivery, immature immune responses together with Th2 predominance are characteristic for newborns. Therefore, we hypothesized that the beneficial effect of *E. coli* O83 could be mediated by equilibration of Th1 and Th2 immune responses. In contrast to our hypothesis that *E. coli* O83 primed moDC would preferentially lower Th2 and support Th1 immune responses, no difference was observed. To our surprise, increased levels of both Th1 and Th2 cytokines were observed in CD4 T cells of newborns of allergic mothers. Our results are partly in agreement with other works [38,39] where limited capacity of CBMC of newborns to release cytokines was positively correlated with lower allergy incidence. On the other hand, our observation of increased levels of IFN-gamma in CD4 T cells cocultured with DC (A) is contradictory with previously published observations (e.g. [16,40], including ours [4]) concluding that increased capacity to secrete IFN-gamma is associated with lower risk of allergy development. It is important to emphasize that detection of IFN-gamma in other studies was either on the level of cord blood sera, mRNA or stimulated CBMC and was not tested by flow cytometry in CD4 T cells cocultured with moDC. In cord blood sera, IFN-gamma could be of maternal origin and in studies using CBMC, CD8 T cells could substantially contribute to the total milieu of IFN-gamma.

Increased functional capacity of Treg in peripheral blood of probiotic supplemented children was described in our previous work [14]. We therefore decided to test the capacity of *E. coli* O83 primed moDC to induce Treg and/or Th17. Increased levels of IL-10 in CD4 T cells cocultured with *E. coli* O83 stimulated moDC were detected but no significant increase in transcription factor FoxP3 responsible for the phenotypic properties of Treg was observed. We can only speculate that although there were similar numbers of FoxP3 + CD4 T cells in *E. coli* O83 stimulated and nonstimulated cultures, the stimulated ones have increased regulatory capacity as documented by the increased presence of regulatory cytokine IL-10. It is important to consider other cell populations not expressing FoxP3 but exerting regulatory function, e.g. Tr1 which are emphasized as a key cell subpopulation mediating tolerance to allergens after successful allergen immunotherapy [41]. In contrast to Th1 and Th2 cytokines, Th17 and Treg cytokines were increased in CD4 T cells cocultured with *E. coli* O83 stimulated moDC. Th17 cells play a dual role – they can fight against bacterial and yeast infections but they support autoimmune disease development and progression as well. IL-17A can contribute to asthma [42]. Contradictory results on Th17 in newborns exist. Some studies failed to detect Th17 immune response on the level of cord blood and some papers report it [43,44]. In our work, we were able to induce Th17 cells from CD4 T cells after the coculture with moDC. Because Th17 is important for fighting against bacterial infection we believe that its induction is beneficial early after the delivery but their activity should be thoroughly regulated.

We acknowledge that we used a simplified artificial model to demonstrate the potential mechanisms of action of probiotic strain *E. coli* O83. It is necessary to consider that maternal allergies were heterogeneous. We have tried to correlate the results on moDC and cocultures with specific kind of allergy but we were not able to demonstrate any statistical difference, probably due to the low number of individuals in each subgroup. Further studies will be needed to confirm and extend our observation. Moreover, the effect of probiotics is strongly strain dependent and it is therefore not possible to draw any

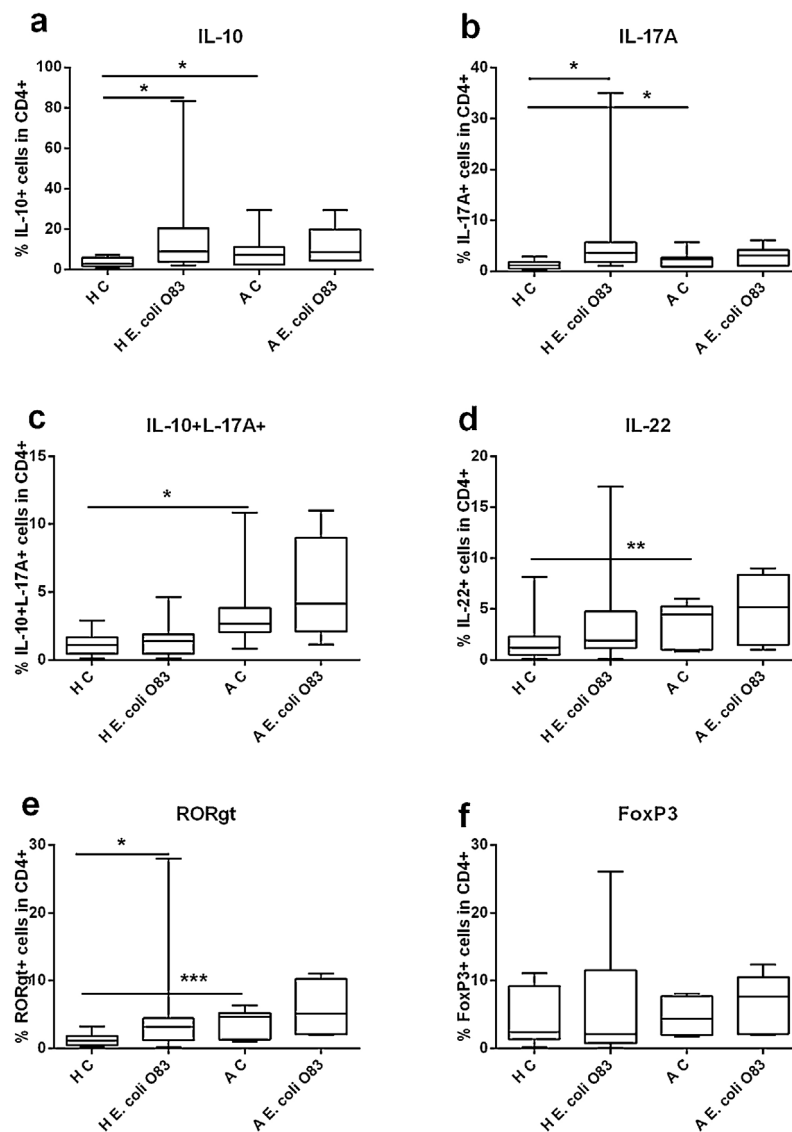


Fig. 4. Evaluation of the capacity of *E. coli* O83 stimulated moDC to induce Th17 or Treg immune responses. Control or stimulated moDC were cocultured with naïve CD4 T cells for 7 days. On day 7, Leukocyte Activation Cocktail was added and presence of intracellular cytokines and transcription factors were detected by flow cytometry.

a) Percentage of IL-10 positive CD4 T cells according to maternal allergy status (MAS)

b) Percentage of IL-17A positive CD4 T cells according to MASN

c) Percentage of double positive IL-10 + IL-17A + CD4 T cells according to MASN

d) Percentage of IL-22 positive CD4 T cells according to MASN

e) Percentage of RORgt positive CD4 T cells according to MASN

f) Percentage of FoxP3 positive CD4 T cells according to MASN

H C – nonstimulated moDC of nonallergic mothers (DC (NA)) cocultivated with naïve CD4 T cells of newborns of healthy mothers (CD4 (NA))

H *E. coli* O83–*E. coli* O83 stimulated DC (NA) cocultivated with naïve CD4 (NA)

A C – nonstimulated moDC of allergic mothers (DC (NA)) cocultivated with naïve CD4 T cells of newborns of allergic mothers (CD4 (A))

A *E. coli* O83–*E. coli* O83 stimulated DC (NA) cocultivated with naïve CD4 (A)

* $p \leq 0.05$

** $p \leq 0.01$

*** $p \leq 0.001$

general conclusion about how the probiotic bacteria work.

5. Conclusions

Probiotic strain *E. coli* O83 promotes moDC maturation, gene expression and secretion of regulatory cytokine IL-10. Increased presence of regulatory cytokine IL-10 and Th17 immune response was observed after coculture of naïve CD4 T cells with moDC stimulated with *E. coli* O83. Generally higher reactivity of CD4 T cells together with impaired capacity of moDC to produce IL-10 in newborns of allergic mothers could lead to the development of inappropriate

immune responses to antigens (allergens) instead of tolerance induction.

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