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Summary of Ph.D. thesis

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Imunoregulační vlastnosti buněk dětí alergických a nealergických matek a možnost jejich ovlivnění probiotickým kmenem *E. coli* O83:K24:H31

Immunoregulatory characteristics of immune cells of children of allergic and non-allergic mothers and the possibility of their modulation with probiotic *E. coli* strain O83:K24:H31

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Abstrakt

Pro vysokou incidenci a potenciální závažnost představují alergická onemocnění jeden z klíčových problémů imunologie 21. století. Přesto v poznání časných mechanismů, podílejících se na rozvoji alergie, existují významné mezery. Aby bylo u rizikových jedinců možné přistoupit k časným preventivním opatřením, je třeba zavést spolehlivější prediktory rizika alergie.

Podkladem alergie je nerovnováha mezi větvemi imunitní reakce, především nežádoucí převaha Th2 odpovědi. Regulační T lymfocyty (Treg) zodpovídají za jemné nastavování rovnováhy imunitního systému i periferní toleranci vůči alergenům. Časně postnatálně má na vývoj imunitního systému výrazný vliv kolonizující mikrobiota. Slibným přístupem v prevenci alergie je proto podávání probiotik za účelem podpory fyziologického vývoje mikrobioty a imunitního systému.

V naší studii jsme pozorovali snížení znaků souvisejících s regulační funkcí Treg a nižší produkci IL-10 v pupečníkové krvi novorozenců alergických matek, u této skupiny dětí byla rovněž nižší proporce Helios$^-$ indukovaných Treg. Tyto nálezy odrážejí opožděnou funkční maturaci Treg, která může vést k vyššímu riziku rozvoje alergie a pravděpodobně též zodpovídá za vyšší reaktivitu dendritických buněk (DC), kterou jsme u této skupiny pozorovali.

Ve věku 8 let jsme zaznamenali snížení incidence alergie u dětí alergických matek, kterým byl po narození podán probiotický kmen *E. coli* O83:K24:H31 (EcO83). Biologický efekt byl daný pravděpodobně normalizací produkce IL-10 a IFN-γ a podporou imunoregulačních mechanismů. V *in vitro* studiích jsme po stimulaci buněk pupečníkové krve EcO83 potvrdili zvýšení produkce IL-10 a IFN-γ a zvýšenou indukci IL-10$^+$ CD4$^+$ u T buněk kokultivovaných se stimulovanými DC.

Klíčová slova: Treg, alergie, pupečníková krev, regulace imunity, probiotika

Abstract

Due to high incidence and impact, allergic disorders are a crucial issue for 21st century immunology. Much still remains to be elucidated regarding very early processes in allergy development. In order to introduce timely preventive measures, novel predictive factors of allergy risk need to be established.

Allergy is caused by dysregulation of balance between immune response branches, chiefly unwarranted Th2 dominance. Regulatory T cells (Treg) are crucial for finely setting immune balance and inducing tolerance towards allergens. Early postnatally, interaction with microbiota modulates immune development. Supplementation with probiotic bacteria to support physiological microbiota and immune development is thus a potentially promising approach for allergy prevention.

We observed decreased presence of function-associated surface markers and lower IL-10 production in cord blood Treg of children of allergic mothers, as well as a decreased proportion of Helios$^-$ induced Treg. These findings hint at delayed functional maturation of Treg in the high-risk group, consistent with observation of increased dendritic cell (DC) reactivity of these children.

Early postnatal colonisation with probiotic *E. coli* strain O83:K24:H31 (EcO83) reduced allergy incidence in colonised children of allergic mothers at the age of 8 years, likely owing to normalisation of IL-10 and IFN-γ production. This effect may also be due to promotion of regulatory responses. Upon *in vitro* stimulation with EcO83, we observed increase in production of IL-10 and IFN-γ by CBMC, higher ability of DC to produce IL-10 and higher induction of IL-10$^+$ CD4$^+$ T cells in coculture with the stimulated DC.

Keywords: Treg, allergy, cord blood, immune regulation, probiotics

1. Introduction

Allergy represents a heterogeneous group of disorders with collectively high incidence, characterised by unwarranted immune response toward harmless external antigens, i.e. allergens. Both genetic predisposition and environmental factors contribute to allergy development.

While there are many options for symptomatic treatment of allergy, causal therapy of allergic diseases and especially effective predictive and preventive measures remain limited, with maternal or biparental allergy positivity being the strongest predictors of high risk of allergy development[1,2]. Research into the early processes involved in allergy pathogenesis thus continues to be of crucial importance in immunology.

Underlying cause of allergy is a dysregulation of immune system balance, mainly undue dominance of Th2. Over the majority of prenatal period, Th2 bias is maintained to prevent unwanted Th1 or Th17 immune reactions at the maternal-foetal interface[3]. After birth, immune system needs to rebalance to prepare the neonate for adequate reactivity towards newly encountered environmental stimuli.

Regulatory T cells (Treg) play a central role in finely setting this balance and inducing as well as maintaining tolerance towards allergens[4]. Human Treg are defined as $CD4^{+}CD25^{high}CD127^{low}$ cells with high expression of lineage-specific transcription factor FoxP3, characteristic epigenetic regulation and ability to efficiently suppress and modulate immune reactions both in local, antigen-specific manner and in a broader, more distal fashion, such as by producing IL-10 and TGF-β. Treg can be divided into various subpopulations of different tissue and biological context, chiefly into $Helios^{+}$ thymically generated natural Treg (nTreg) with self-antigen specific T-cell receptor (TCR) or $Helios^{-}$ peripherally induced Treg (iTreg) with TCR specific for environmental antigens[5]. In the context of allergy, other regulatory cell types can play important roles, including $CD4^{+}FoxP3^{-}$ type 1 Treg (Tr1) and regulatory B lymphocytes (Breg), both of which produce very high amounts of IL-10 and are indispensable for the success of allergen-specific immunotherapy, the sole causal treatment option used in allergy[6].

Interactions with external factors, most importantly microbiota, exert large influence on the developing immune system, particularly during the early postnatal "window of opportunity[7]." Factors such as composition,

diversity, metabolic activity and spatiotemporal properties of exposure to symbiotic, commensal and pathogenic microbes play a key role in the induction of or protection from numerous immune-related, inflammatory disorders, including allergy[8]. Major theories including the "hygiene hypothesis" and "old friends" hypothesis[9] have proposed dysregulation of microbiota (termed dysbiosis) as the major factor behind the extreme rise in incidence of non-communicable inflammatory diseases, including allergy, in the developed countries over the last century.

Supplementation with probiotic bacteria is considered a potentially promising approach for allergy prevention. Probiotics can promote immune maturation and support regulation in numerous ways, depending on the strain used and the particulars of its administrations. Usefulness of probiotics in allergy prevention is currently hotly debated, with combined prenatal and postnatal supplementation proposed as most likely to be useful in atopic eczema prevention[10]. In our studies, we observed the effect of probiotic bacterium *Escherichia coli* strain O83:K24:H31 on allergy development in high-risk children at the age of 8 years, as well as its effect on cells derived from cord blood of children *in vitro*.

2. Aims

2.1. First major aim was to characterise Treg cell populations involved in immune regulation during the perinatal period and to evaluate their potential usefulness in allergy prediction. In particular, we aimed to:

a) Compare proportions of $CD4^{+}CD25^{high}FoxP3^{+}$ total Treg and their subpopulations, $FoxP3^{+}Helios^{+}$ nTreg and $FoxP3^{+}Helios^{-}$ iTreg, in cord blood of children of allergic mothers and children of healthy mothers.
b) Compare surface presence of markers associated with regulatory function, intracellular presence of regulatory cytokines and suppressive function of cord blood Treg of children of allergic mothers and children of healthy mothers.
c) Evaluate cord blood Treg proportions and presence of selected function-associated markers in the context of clinical allergy development during childhood.

2.2. Second major aim was to describe the effect of probiotic strain *E. coli* O83:K24:H31 on cell populations in the context of allergy in order to uncover the possible mechanisms behind the ability of this probiotic

strain to decrease allergy incidence in the high-risk group. In particular, our aims were to:

a) Analyse the influence of early postnatal EcO83 supplementation of children of allergic mothers on allergy development and sensitization and Treg, nTreg and iTreg population proportions at the age of 8 years.
b) Evaluate the influence of early postnatal EcO83 supplementation of children of allergic mothers on IL-10 production by Treg and $CD4^{+}FoxP3^{-}$ Tr1 cells and plasma levels of key cytokines (IL-4, IL-10 and IFN-γ) at the age of 8 years.
c) Determine *in vitro* the influence of EcO83 on CBMC and CBMC-derived moDC to assess EcO83-elicited cytokine production and DC maturation.

3. Materials and methods

Subjects and sample collection

Healthy and allergic mothers with physiological pregnancies delivering at full term in the Institute for the Care of the Mother and Child in Prague were included for the study. Allergy status of the mother was determined based on clinical manifestation of allergy persisting for at least 24 months; allergy against respiratory and/or food allergens manifested by various individual combinations of symptoms (e.g. hay fever, conjunctivitis, eczema, bronchitis, asthma etc.), monitoring by an allergist, positive skin prick tests or positive specific IgE and anti-allergic treatment before pregnancy. The study was approved by the Ethical Committee of the Institute for the Care of Mother and Child, Prague, Czech Republic, and was carried out with a signed written informed consent of the mothers.

Cord blood (CB) samples (approx. 10-20 ml) were collected into sterile heparinized tubes immediately after birth for cell analysis by flow cytometry. CB plasma was obtained for cytokine detection.

Allergy status of 8-year-old children was followed by allergist (parents reports of allergy manifestation was confirmed either by positive skin prick tests and/or positive specific IgE antibodies). Peripheral blood (PB) was collected for cell analysis by flow cytometry. PB plasma was obtained for cytokine detection.

Flow cytometry analysis of regulatory T cells

Cord blood samples were prepared and stained for flow cytometry as described previously[11]. Briefly, samples of whole cord blood were stained with antibodies against Treg surface markers CD4, CD25 and CD127. Staining and sample preparation were performed according to manufacturer's instructions using human regulatory T cell whole blood staining kit (eBioscience). After fixation and permeabilization, the samples were stained with antibodies against Treg intracellular markers FoxP3 and Helios. In some experiments, non-stimulated whole blood samples were stained for the following surface markers associated with Treg function: CTLA-4, PD-1, GITR. Non-stimulated whole blood samples treated with BD GolgiPlug (Becton Dickinson) for 6 hours were permeabilized after surface staining for Treg and stained for intracellular expression of regulatory cytokines IL-10 and TGF-β. Proportion of Treg in peripheral blood of 8-year-old children was determined using TregFlowEx Kit (Exbio) optimized for detection of Treg in heparinized whole blood. Gating strategies described previously[11] were used.

Flow cytometry data were acquired on a BD FACSCanto flow cytometer using BD FACS Diva version 6.1.2 software (Becton Dickinson) and analysed using FlowJo 7.2.2. (TreeStar).

CBMC isolation, culture and *in vitro* stimulation with EcO83

Cord blood mononuclear cell fraction (CBMC) was obtained from whole cord blood by density gradient centrifugation (Histopaque-1077; Sigma-Aldrich) for downstream culture-based assays and RNA isolation. To characterize the effect of EcO83 on neonatal immune system, CBMC were stimulated by EcO83 *in vitro* and cytokines typical for Th1 (IFN-γ), Th2 (IL-4) and Treg (IL-10) were detected by qPCR and ELISA. The capacity of EcO83 to promote Treg induction was tested by flow cytometry. The capacity of EcO83 to induce immune response was compared with other probiotic strain *E. coli* Nissle 1917 (EcN) (kindly provided by Ulrich Sonnenborn, ArdeyPharm). LPS (1 μg/ml, *Escherichia coli*, Sigma) was used as a control.

Dendritic cell generation and *in vitro* stimulation with EcO83

Dendritic cells (monocyte derived dendritic cells; moDC) were derived from adherent fraction of CBMC as described previously[12]. Briefly, after 1 h cultivation of CBMC in cell culture flasks in the incubator with regulated CO_2 atmosphere, nonadherent CBMC were

washed out and adherent CBMC were cultured for 6 days with recombinant human (rh) IL-4 and rhGM-CSF. *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 of 10 bacterial cells to 1 moDC for 24 h. Maturation status after stimulation was estimated according to the presence of activation markers (CD40, CD80, CD83, CD86, MHCII) on moDC by flow cytometry.

Flow cytometry analyses of immune responses induced by moDC

Intracellular staining of transcription factors and cytokines typical for Th1 (T-bet; IFN-gamma), Th2 (GATA3; IL-4, IL-13), Th17 (RORgt; IL-17A, IL-22) and Tregs (FoxP3; IL-10) was performed after cell surface staining by antibody against CD4 for 20 min, then washed twice with PBS containing 1% BSA, followed by fixation and permeabilization (BD Pharmingen Transcription Factors Buffer Set) according to the manufacturer's recommendation.

Cytokine concentration in cord blood and peripheral blood plasma and cell culture supernatants

Cytokines in plasma of cord blood or peripheral blood of 8-year-old children and cytokines released by non-stimulated and stimulated cultured CBMC or moDC were detected by ELISA (reagents obtained from R&D Systems). The results were read from calibration curve in picograms per millilitre.

Relative quantification of gene expression

Total RNA was isolated from CBMC or *in vitro* generated moDC using RNeasy Mini Kit (Qiagen) followed by reverse transcription. Gene expression of cytokines (IL-4, IL-10, IFN-γ) and indolamine 2,3-dioxygenase (IDO) was estimated by quantitative real-time PCR, as described previously[13].

CFSE suppression of proliferation assay

A proliferation suppression assay was performed utilising coculture of magnetically isolated CFSE-stained target non-Treg cells with magnetically isolated Treg. EasySep™ Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (StemCell Technologies) was used to obtain Tregs and target cells from cord blood mononuclear cells. The target cells were stained with 5 μM CFSE, plated into 48-well plates with

or without Tregs and cultivated for 72 hours. 20 ng of recombinant human IL-2 (PeproTech), 1 μg of purified, functional grade human anti-CD3 (ThermoFisherScientific) and 1 μg of purified, functional grade human anti-CD28 (ThermoFisherScientific) were added per 10^6 target cells to stimulate proliferation. 0.5×10^6 cells in total were seeded in each well. After 72h, cells were stained for CD4 and analysed with flow cytometer.

Statistics

Data were evaluated using unpaired Student's t-test in case of data with normal distribution and non-parametric Mann-Whitney test for the rest of the data.

4. Results

Distinct characteristics of Tregs of newborns of healthy and allergic mothers.

Černý, V., Hrdý, J., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., Prokešová, L. PLoS ONE (2018) 13(11): e0207998. https://doi.org/10.1371/journal.pone.0207998

The goal of this work was to evaluate whether any differences could be found in the population and phenotypic characteristics of Treg in cord blood of children of allergic mothers and cord blood of children of healthy mothers.

Evaluation of cord blood Treg, iTreg and nTreg populations by flow cytometry

Percentage of $CD4^+CD25^+CD127^{low}FoxP3^+$ Treg in $CD4^+$ cells was significantly higher in children of allergic mothers than in the low-risk group. Furthermore, the children of healthy mothers had significantly higher proportion of $Helios^-$ iTreg among Treg. These findings might hint at a delayed immune maturation and iTreg formation in the high-risk group, which could potentially contribute to the increased risk of allergy.

Determination of selected phenotypic characteristics associated with Treg regulatory function

Significantly higher proportion of PD-1^+ Treg was observed in cord blood of children of healthy mothers, with a similar albeit non-significant trend discernible for CTLA-4^+ Treg. Furthermore, children of healthy mothers had significantly higher percentage of IL-10^+ Treg in cord blood. Taken together, markers associated with Treg function are observably

lower in the high-risk group, potentially hinting at a compromised regulatory function.

Assessment of Treg regulatory function

We performed a functional assay based on cocultivation of Treg isolated from cord blood with CFSE-stained non-Treg ($CD4^+CD25^-$) conventional T cells. We observed lower ability of cord blood Treg of children of allergic mothers to inhibit proliferation of conventional T cells, hinting at defective suppressive function of Treg in the high-risk group. Furthermore, levels of IL-10 and TGF-β were significantly lower in cord blood plasma of children of allergic mothers, further pointing to a dysregulated pattern of immune regulation in the high-risk group.

The results exposed impaired regulatory capabilities of cord blood Treg of children of allergic mothers that may play a role in the higher risk of allergy described in literature. Another interesting finding is that children from the high-risk group have lower proportion of iTreg among their Treg. We hypothesise that our data reflect dysregulated development of Treg in children of allergic mothers, potentially due to delayed maturation of their immune system. The counterintuitively higher percentage of total Treg among $CD4^+$ T cells in the high-risk group may be due to a compensatory expansion.

Value of cord blood Treg population properties and function-associated characteristics for predicting allergy development in childhood.

Černý, V., Hrdý, J., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., and Prokešová, L. (accepted for publication in CEJI, 2019)

In this follow-up study, we compared cord blood Treg population properties and phenotypic characteristics, measured at the time of birth[11], among children divided into two groups according to their allergic status at the age of 6–10 years. Healthy and allergic children were further subdivided into four groups according to the risk of allergy predicted at birth by maternal allergy status.

Proportion characteristics of cord blood Treg in allergic and healthy children

While no differences were observed in percentage of $CD4^+CD25^+CD127^{low}$ Treg among neither the two basic groups nor the four subgroups, significantly higher percentage of $CD4^+CD25^+$ T cells

(including Treg but potentially also activated T cells) was found in the group of healthy children. Upon subdivision according to maternal allergy status, the group of healthy children of healthy mothers, i.e. healthy children from the low-risk group, showed a higher percentage of these cells than both healthy children of allergic mothers and allergic children of healthy mothers.

Treg phenotypical characteristics and intracellular presence of immunoregulatory cytokines in Treg of allergic and healthy children

No difference was observed in FoxP3 MFI among the groups in the current study. Upon subdivision according to maternal allergy status, significantly higher proportion of IL-10^+ Treg was uncovered in healthy children of healthy mothers and conversely, a significantly lower proportion of TGF-β^+ cells was discernible in allergic children of allergic mothers.

Concentration of cytokines with regulatory functions in plasma of cord blood

Healthy children of healthy mothers exhibited significantly higher serum concentration of IL-10 than allergic children of allergic mothers, as well as significantly higher levels of TGF-β than both groups of children of allergic mothers.

The results of this study support the notion that analyses of functional aspects of Treg might have greater predictive value than simply determining population proportions. Differences observed between the original study and the follow-up stress the importance of maintaining sufficient cohort sizes, as limiting the analysis to a selection from a larger cohort may influence the results, contributing to numerous discrepancies observed between published studies.

Decreased allergy incidence in children supplemented with *E. coli* O83:K24:H31 and its possible modes of action.

Hrdý, J., Vlasáková, K., Černý, V., Súkeníková, L., Novotná, O., Petrásková, P., Boráková, K., Lodinová-Žádníková, R., Kolářová, L., and Prokešová, L. Eur. J. Immunol. (2018) 48(12): 2015–2030. https://doi.org/10.1002/eji.201847636

This study reports lower allergy incidence at the age of 8 years in infants of allergic mothers supplemented early postnatally with EcO83. Immunological characteristics including Treg population proportions and regulatory cytokine profiles at the age of 8 years were followed. In

addition, effect of *in vitro* stimulation of cord blood mononuclear cells (CBMC) with EcO83 was tested.

The children were originally divided into three groups: children of allergic mothers colonised with EcO83 during the first days of life; non-colonised children of allergic mothers; and non-colonised children of healthy mothers. At the age of 8 years, the three basic groups were further subdivided according to the children's allergy status determined by allergist examination into six groups in total.

Effect of EcO83 supplementation on allergy incidence and regulation

Colonised children of allergic mothers had lower incidence of allergy than non-supplemented children of allergic mothers, reaching incidence observed in low-risk group of children of healthy mothers. No difference of total Treg population was observed. However, healthy children had significantly higher proportion of $Helios^-$ iTreg, regardless of maternal allergy status. This increase was driven by higher percentage in the group of colonised children, particularly those who did not develop allergy.

Allergy-associated differences in cytokine profiles in colonised and non-colonised 8-year-old children

Quantification of plasma IFN-γ and IL-10 by ELISA revealed significantly higher levels of both cytokines in children of healthy mothers and colonised children of allergic mothers, compared with non-colonised children of allergic mothers. Generally, healthy children had highest levels of IFN-γ and IL-10; however, healthy non-colonised children of allergic mothers had lower levels than the other two healthy groups, hinting at less developed regulatory mechanisms preventing Th2 immune response activation in the untreated high-risk group even in absence of overt disease. We observed significantly higher proportion of $IL\text{-}10^+$ Treg as well as $CD4^+FoxP3^-$ cells (putative Tr1) in the blood of children of healthy mothers and colonised children of allergic mothers.

Collectively, the observed patterns of cytokine production are consistent with the known preventive effect of EcO83 supplementation and might represent a possible immunoregulatory mechanism underlying this phenomenon.

Biological effects of stimulation of CBMC by EcO83

In order to elucidate the possible mechanisms involved in EcO83 probiotic function, we investigated the effect of *in vitro* stimulation by the strain on cytokine production by mononuclear cells isolated from cord blood of children of allergic and of healthy mothers. No changes in gene

expression of IL-4 were present, regardless of the stimulation or of maternal allergy status. Gene expression of IFN-γ was significantly lower in unstimulated controls than in CBMC stimulated by EcO83, EcN and LPS; the difference remained significant even after subdividing the subjects according to maternal allergy status, except in the case of stimulation of CBMC of children of allergic mothers by EcN. Gene expression of the regulatory cytokine IL-10 determined by qPCR was significantly increased after culture with all three stimulants; importantly, stimulation of CBMC by EcO83 led to significantly higher upregulation of IL-10 expression than stimulation with LPS and EcN. This pattern was also found in the subgroup of children of healthy mothers, while in children of allergic mothers, only CBMC cocultured with EcO83 and LPS upregulated IL-10 gene expression.

CBMC cocultured with LPS and EcO83 produced significantly higher amounts of IFN-γ into the supernatant than unstimulated cells or cells stimulated with EcN. Importantly, stimulation of CBMC by EcO83 induced significantly more IL-10 than all the other conditions. After division of CBMC according to the mothers' allergic status, significantly increased IL-10 levels were detected after all three modes of stimulation in CBMC both groups. Moreover, CBMC of children of allergic mothers stimulated by EcO83 produced significantly more IL-10 than corresponding cells stimulated by LPS or EcN.

Results of this study further corroborate the potential of probiotic *E. coli* strain O83:K24:H31 for allergy prevention. Higher proportion of iTreg in the colonised group supports the hypothesis that probiotic supplementation with EcO83 promotes tolerogenic environment necessary for mitigation of allergy development in the high-risk group. While colonisation had no effect on IL-4 production, colonised children of allergic mothers exhibited significantly larger proportion of IL-10$^+$ Treg and non-Treg CD4$^+$ T cells and higher levels of IFN-γ and IL-10 in serum, reaching levels found in the low-risk group. Increased production of IFN-γ and IL-10 could help account for the long-term beneficial effects described in the study.

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.

Súkeníková, L., Černý, V., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., Prokešová, L., and Hrdý, J. Immunol. Lett. (2017) *189*, 82–89. https://doi.org/10.1016/j.imlet.2017.05.013

Responsiveness of monocyte-derived DC generated *in vitro* from cord blood of children of allergic and healthy mothers to EcO83

Surface expression of activation marker CD83 was significantly upregulated after culture with EcO83 and LPS. Furthermore, moDC from allergic mothers had significantly higher CD83 than moDC from healthy mothers, regardless of stimulation, implying higher reactivity in the high-risk group. Unstimulated moDC derived from CBMC of healthy mothers had significantly higher gene expression of both IDO and IL-10 than unstimulated moDC from CBMC of children of allergic mothers. After stimulation with EcO83, gene expression of IDO was significantly upregulated in both low-risk and high-risk group, reaching comparable levels. While the expression of IL-10 likewise increased in both stimulated groups compared to the respective unstimulated moDC, the upregulation was significantly more pronounced in moDC derived from CBMC of children of healthy mothers, possibly indicating impaired regulatory function in the high-risk group of children of allergic mothers. Levels of inflammatory cytokines TNF-α and IL-6 were increased after stimulation with EcO83 and LPS. While IL-10 was likewise significantly upregulated upon culture with LPS and EcO83 in moDC from children of both healthy and allergic mothers, the increase was significantly higher when moDC from the low-risk group were stimulated with EcO83, compared both with stimulation by LPS and with stimulation of moDC from allergic mothers by EcO83. Taken together, the data imply that moDC derived from children of allergic mothers show higher reactivity and activate upon microbial stimulation more strongly but show signs of regulatory deficiency.

Induction of Th responses by probiotic primed moDC

No difference in the percentage of $FoxP3^+$ Treg was observed upon coculture with moDC, regardless of maternal allergy status or microbial stimulation; however, moDC stimulated with EcO83 were able to induce significantly larger proportion of $IL\text{-}10^+$ $CD4^+$ T cells than non-stimulated moDC. Surprisingly, significantly higher percentage of $IL\text{-}10^+$ cells was

also present after $CD4^+$ T cell coculture with moDC derived from CBMC of children of allergic mothers compared with moDC from CBMC of children of healthy mothers.

The data in this study uncovered higher expression of activation markers, greater capacity to induce inflammatory Th responses and overall increased reactivity of moDC derived *in vitro* from mononuclear cells isolated from the cord blood of children of allergic mothers, combined with impaired expression of regulatory factors such as IDO and IL-10. Stimulation with EcO83 promoted regulatory function of moDC in a complex fashion, increasing both regulatory IL-10 and inflammatory IL-6 and TNF-α. The upregulation of tolerogenic markers was more pronounced in moDC generated from CBMC isolated from children of healthy mothers. The results indicate that DC of children of the low-risk group are better equipped to induce tolerogenic responses upon encounter with innocuous environmental stimuli, including probiotic bacteria. Moreover, we have demonstrated that EcO83 is capable of promoting the upregulation of regulatory mechanisms in DC, possibly an important mechanism of its probiotic effect.

5. Discussion

In our work, we aimed to gain more insight into early processes in allergy development by comparing the role of Treg and their subpopulations in cord blood of children with higher and lower expected risk of allergy development. We also described *in vivo* and *in vitro* effects of probiotic *E. coli* strain EcO83 to help improve our understanding of the mechanisms by which probiotic bacteria can promote immune homeostasis.

In cord blood of children of allergic mothers we observed larger total proportion of Treg. Nevertheless, this group had fewer $Helios^-$ induced Treg in cord blood than children of healthy mothers. In addition, smaller proportions of $PD\text{-}1^+$ and $IL\text{-}10^+$ Treg cells were present in cord blood of high-risk children, with similar trends noticeable for $CTLA\text{-}4^+$ and $TGF\text{-}\beta^+$ Treg. These observations hint at impaired or immature functional capacity of Treg in the high-risk group; the increase of total Treg in $CD4^+$ T cell population might result from compensatory upregulation. iTreg arise in peripheral lymphoid tissues after exposure to environmental factors and are crucial in allergy control. Higher iTreg proportion may

reflect more matured immune system of the low-risk children at birth, contributing to control of allergy.

Analysing a different cohort of older children, we compared actual allergy development with subpopulation proportions and functional properties of cord blood Treg originally described by our group in 2012[11]. Main findings include higher proportion of IL-10$^+$ Treg in healthy children of healthy mothers and lower TGF-β in allergic children of allergic mothers, reflecting the role of these cytokines in allergy control. We also observed increased proportion of CD4$^+$CD25$^+$ cells in peripheral blood of healthy children of healthy mothers, but no difference in children of allergic mothers (Černý et al., accepted for publication in CEJI, 2019). As CD4$^+$CD25$^+$ cells include both Treg and activated conventional T cells[15], this observation may point to a differential composition or functional role of this cell population between the high-risk and low-risk groups at birth. Children of allergic mothers have been described to be overall more prone to immune activation[12,16], CD4$^+$CD25$^+$ cells might thus include higher ratio of activated non-Treg cells to Treg in this group.

We observed higher expression of activation marker CD83 on DC generated from CBMC of children of allergic mothers compared to children of healthy mothers, coupled with lower expression of regulation-associated genes (IDO, IL-10) and greater reactivity of these cells to stimulation by EcO83. DC of the high-risk neonates are thus more prone to immune activation and less tolerogenic. This is in line with the hypothesis that an insufficiency of immune regulatory mechanisms contributes to the increased risk of allergy.

Various groups have attempted to elucidate the role of Treg in allergy development, with inconsistent results. Some groups report increased Treg proportions in cord blood of children with higher risk of allergy[11,17,18], while in other studies opposite trend was observed[19] or no differences were found[20]. Various factors could explain this inconclusiveness. Genetic background and environment combine to determine allergy development, therefore data from different populations will vary, particularly between studies carried out in urban[17] vs. farming environment[18].

Furthermore, for Treg identification to be comparable among studies, gating strategy and the choice of markers need to be carefully considered[11]. Identifying Treg as CD4$^+$CD25$^+$ cells is especially problematic, as CD25 is upregulated in effector T cells[15] and CD4$^+$CD25$^+$

cells will invariably include activated non-Treg. The very highest CD25 expression correlates better with the regulatory characteristics than simple CD25 positivity[21], so Treg should be identified as $CD25^{high}$ cells. Transient FoxP3 upregulation has been described in activated Tconv[15]. As we have observed higher tendency towards immune activation in children of allergic mothers[12,16], cells described as Treg in this group might in fact contain larger proportion of recently activated non-Treg $CD4^+$ T cells. Furthermore, choice of clone of monoclonal antibodies used for FoxP3 staining is of paramount importance. Using different clones can provide different estimates of Treg proportions or identify Treg subpopulations with different effectiveness[22,23].

Among Treg subpopulations, iTreg are particularly important for oral tolerance and allergy control as they arise upon harmless antigen recognition in periphery. So far, no indisputable markers have been proposed for iTreg identification. Expression of transcription factor Helios has been proposed as a marker of thymus-derived nTreg[24], with peripherally generated iTreg being $Helios^-$. The suitability of Helios as an exclusive marker of nTreg has been questioned by various studies[25,26], though the issue is yet to be resolved[27]. Alternative explanations of Helios biological role have been proposed, including its upregulation in T cells upon activation[25]. In such case, the observed higher proportion of $Helios^+$ Treg in the high-risk group might again reflect higher tendency toward immune activation and inflammation. Alternatively, Helios was proposed to control Treg development and acquisition of stable regulatory phenotype[28]. Upregulation of Helios might then in fact be a sign of the compensatory expansion of Treg.

Considering the issues mentioned above, it follows that analysis of characteristics associated with Treg function will give more reliable information than simple enumeration of these cells or their subpopulations. Treg function can be assessed indirectly by estimating presence of relevant surface markers with immunoregulatory functions or regulatory cytokines, or directly using assays based on coculture with CFSE-stained or radioactive ^{3}H-thymidine labelled target cells.

CTLA-4 and PD-1 are the most important co-inhibitor molecules involved in Treg function[29] and play an indispensable role in contact-dependent suppression mediated by Treg, evidenced by various autoimmune and lymphoproliferative syndromes associated with deficiency of these molecules[30]. CTLA-4 has been implicated in food

allergy[31] and regulating the Th1/Th2 crosstalk in asthma[32]. PD-1 has been shown to control asthma in animal models[33].

IL-10 and TGF-β are chief immunoregulatory cytokines, and their importance for allergy control is universally accepted[34]. IL-10 production by Treg and FoxP3$^-$ CD4$^+$ Tr1 cells is indispensable for the success of allergen-specific immunotherapy[35]. TGF-β supports mucosal homeostasis and generation of iTreg, playing a key part in oral tolerance[36].

Our observations (decreased expression of PD-1, lower production of IL-10, impaired suppressive ability estimated by a coculture-based assay) unveil compromised Treg function in children of allergic mothers. This defect in suppressive activity of Treg may explain our previous observations of exaggerated immune reactivity in the high-risk group[12,16] and may be a major cause of the higher risk of allergy development in this group.

Probiotic treatment offers a promising possibility of intervention, although the optimal microbial strains and mode of supplementation for such use are yet to be conclusively established. In our study, we describe the effects of early postnatal administration of probiotic *E. coli* strain O83:K24:H31 in a prospectively followed cohort of colonised children of allergic mothers, compared with non-supplemented children of allergic mothers and non-supplemented children of healthy mothers. Current report describes allergy status and relevant immune characteristics at the age of 8 years. In the supplemented group of high-risk children we observed lower incidence of allergic diseases, comparable with the incidence in the low-risk group. This was likely due to the effect on Treg: colonised children had more induced Treg than both other groups. Supplementation of high-risk children with EcO83 increased production of IL-10. The cytokine production reached the levels seen in children belonging to the low-risk group, with an equally strong upregulation observed for IFN-γ.

We also determined the effect of stimulation by EcO83 on cells isolated from cord blood. In one study, we observed increased expression of IL-10 and IFN-γ in CBMC. We further tested the effect of EcO83 stimulation on moDC generated *in vitro* from CBMC in order to see if promoting adequately polarising antigen presentation might play a role in EcO83 probiotic effect. We describe a complex stimulatory effect of EcO83 on model DC: upregulation of CD83, gene expression of IL-10

and IDO as well as release of IL-10, IL-6 and TNF-α. Importantly, DC of children of healthy mothers upregulated IL-10 production to a significantly greater degree, implying higher tolerogenic capacity in the low-risk group. EcO83 stimulated DC of the low-risk group were also able to induce more IL-10^+ $CD4^+$ T cells upon coculture, illustrating the more robust immune regulation present in the low-risk children.

Biological effects of probiotic supplementation are highly strain specific. In our study, we demonstrate that the key effect of EcO83 administration in the context of allergy is likely its ability to promote IL-10 production by Treg and Tr1 as well as Th1 response. Induction of Treg by various probiotic bacteria has been described by many studies so far[37–40]. Furthermore, the effect of EcO83 is apparently mediated through the priming of neonatal DC toward Treg induction, similar to reports of other groups[38,41].

The data regarding probiotic use in prevention of allergy remain inconclusive and further studies coupled with rigorously performed meta-analyses need to be carried out to determine most beneficial strains. Likewise, conditions such as timing, mode of application and dosage need to be optimised for maximal beneficial effect, with combined prenatal and early postnatal supplementation being proposed by current guidelines[42]. So far, the most persuasive arguments have been made for probiotic use in atopic dermatitis prevention[10,42], with the benefit of probiotics in other forms of allergy being hotly debated[43].

In this thesis, we described a broadly preventive effect of EcO83 in allergy lasting up to the age of eight years[44]. We propose that this effect is likely at least in part mediated via inducing maturation of DC, which leads to increased support of Treg and Th1 responses and a long-term increase in IL-10 and IFN-γ production in the treated neonates, helping the immune system to rebalance away from prenatal dominance of Th2 response. Supplementation with EcO83 seems like a useful approach for allergy prevention in the groups with higher risk of allergy.

6. Conclusions

6.1. We analysed population proportions and functionally-relevant characteristics of Treg in cord blood of children of allergic mothers.

a) Children of allergic mothers (with higher risk of allergy development) had larger proportion of total Treg population

among $CD4^+$ T cells. They also had larger $Helios^+$ nTreg fraction, while the percentage of $Helios^-$ iTreg among total Treg was lower than iTreg proportion among Treg of children of healthy mothers.

b) Children of allergic mothers exhibit lower percentage of PD-1^+ and IL-10^+ cells among Treg, lower cord blood plasma levels of IL-10 and TGF-β, and impaired Treg suppressive function. We observed higher expression of activation marker CD83 on moDC from children of allergic mothers, as well as higher ability to induce T cell polarization to Th1, Th2 or Th17 branches upon coculture.

c) Proportion of $CD4^+CD25^+$ T cells was higher in healthy children of healthy mothers compared with allergic children of healthy mothers, but no difference was observed between healthy and allergic children of allergic mothers, hinting at different composition or role of this population between the high-risk and low-risk group. Healthy children of healthy mothers showed increased IL-10 and TGF-β production and allergic children of allergic mothers had decreased TGF-β at birth.

Our data supports the hypothesis that children of allergic mothers have delayed functional maturation of regulatory T cells in the perinatal period, reflected in decreased presence of markers associated with function, lower cord blood plasma levels of IL-10 and TGF-β and lower numbers of induced Treg. This may be linked with the higher immune reactivity of moDC derived from CBMC of children of allergic mothers.

Analysis of function-associated markers of Treg and direct analyses of regulatory function will likely be more useful for improving our understanding of early phases of allergy development than simple analysis of Treg proportion in circulatory $CD4^+$ T cells.

6.2. We evaluated effects of early postnatal supplementation of children of allergic mothers with EcO83 on immune system at the age of 8 years. Furthermore, we analysed the effect of *in vitro* stimulation with EcO83 on CBMC and moDC.

a) We observed decreased incidence of allergy at the age of 8 years in the group colonised with EcO83. Colonised children had more $Helios^-$ iTreg at the age of 8 years As iTreg generation occurs

mostly in mucosal peripheral tissues and iTreg include cells with TCR specific for allergens, colonisation with EcO83 may favour more robust control of unwarranted reactivity against exogenous antigens, including Th2-mediated allergy.

b) Production of IL-10 and IFN-γ was substantially increased in supplemented children of allergic mothers at the age of 8 years. Concentration of IL-10 and IFN-γ reached levels found in the low-risk children.

c) Stimulation of CBMC with EcO83 strongly induced expression of IL-10 and IFN-γ in CBMC. EcO83 stimulation led to upregulation of CD83 on moDC and induced expression of regulatory effector molecules IDO and IL-10. moDC derived from CBMC of children of healthy mothers stimulated with EcO83 were able to induce production of IL-10 in $CD4^+$ T cells.

Early postnatal EcO83 supplementation induces long-term regulatory effects in the high-risk children, leading to a decrease in allergy incidence at the age of 8 years. Increased IL-10 and IFN-γ together with more appropriately polarizing antigen presentation by stimulated DC may help set up immunoregulatory conditions in the infant necessary for rebalancing immune polarization away from the prenatal Th2 bias.

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List of publications

Publications *in extenso* related to the present study:

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. Súkeníková, L., Černý, V., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., Prokešová, L., and Hrdý, J. Immunol. Lett. (2017) 189, 82–89. https://doi.org/10.1016/j.imlet.2017.05.013 IF_{2018} = 2.552

Distinct characteristics of Tregs of newborns of healthy and allergic mothers. Černý, V., Hrdý, J., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., and Prokešová, L. PLoS ONE (2018) 13(11): e0207998. https://doi.org/10.1371/journal.pone.0207998 IF_{2018} = 2.776

Decreased allergy incidence in children supplemented with *E. coli* O83:K24:H31 and its possible modes of action. Hrdý, J., Vlasáková, K., Černý, V., Súkeníková, L., Novotná, O., Petrásková, P., Boráková, K., Lodinová-Žádníková, R., Kolářová, L., and Prokešová, L. Eur. J. Immunol. (2018) 48, 2015–2030. https://doi.org/10.1002/eji.201847636 IF_{2018} = 4.695

Value of cord blood Treg population properties and function-associated characteristics for predicting allergy development in childhood. Černý, V., Petrásková, P., Novotná, O., Boráková, K., Prokešová, L., Kolářová, L., and Hrdý, J. Cent. Eur. J. Immunol. (2019), accepted for publication. IF_{2018} = 1.455

Publications *in extenso* not related to the present study:

The structure-dependent toxicity, pharmacokinetics and anti-tumour activity of HPMA copolymer conjugates in the treatment of solid tumours and leukaemia. Tomalová, B., Šírová, M., Rossmann, P., Pola, R., Strohalm, J., Chytil, P., Černý, V., Tomala, J., Kabešová, M., Říhová, B., Ulbrich, K., Etrych, T., and Kovář, M. Controlled Release 223, 1–10 (2016) IF_{2018} = 8.375