Charles University in Prague

First Faculty of Medicine

Summary of PhD thesis

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Preventivní použití probiotik

Immunologic Characteristics of Cord Blood in Children with Increased Risk of Allergy Development

Preventive Use of Probiotics

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Content

Abstrakt

Alergická onemocnění patří mezi jedna z nejčastějších onemocnění, proto nabývá na významu identifikace určitého včasného prognostického znaku ukazujícího na zvýšené riziko vzniku alergie.

Pupečníková krev je snadno dostupným klinickým materiálem, který může být využit pro hledání zmíněných prognostických znaků. V pupečníkové krvi dětí alergických matek (děti s relativně vysokým rizikem vzniku alergických onemocnění) a dětí zdravých matek (děti s nižším rizikem vzniku alergických onemocnění) bylo testováno proporční zastoupení Th1 cytokinů, Th2 cytokinů a regulačních cytokinů. Byla porovnána i aktivita lymfocytů, dendritických buněk (DC) a regulačních T buněk (Treg) pupečníkové krve dětí zdravých a alergických matek.

Byla zjištěna obecně vyšší reaktivita jak stimulované tak nestimulované mononukleární frakce leukocytů pupečníkové krve dětí alergických matek ve srovnání s dětmi zdravých matek. Vyšší reaktivita dendritických buněk dětí alergických matek byla detekována pouze po polyklonální stimulaci. Signifikantně nižší funkční vlastnosti Treg pupečníkové krve byly prokázány u dětí alergických matek ve srovnání s dětmi zdravých matek. Vyšší reaktivita lymfocytů a DC spolu se sníženou funkcí Treg pupečníkové krve dětí alergických matek mohou přispívat ke snazší sensitizaci/alergizaci predisponovaného jedince.

Vhodným preventivním opatřením při snižování výskytu alergických onemocnění u predisponovaných dětí se ukázalo být podávání probiotické vakcíny Colinfant New Born (*E. coli* O83:K24:H31). U osídlených dětí alergických matek byl prokázán výrazně nižší výskyt alergií srovnatelný s výskytem alergie u neosídlených dětí zdravých matek. Mechanizmus působení probiotik stále není zcela objasněn. Zlepšení funkčních vlastností Treg u osídlených dětí dovoluje předpokládat, že se jedná o jeden z účinků probiotické vakcíny Colinfant New Born.

Abstract

Allergy is one of the most common diseases. Identification of early prognostic markers pointing to an increased risk of allergy development is therefore of increasing importance.

Cord blood represents an easily attainable clinical material for searching for prognostic markers signalizing future allergy development. Proportions of Th1 cytokines, Th2 cytokines and regulatory cytokines were tested in cord blood of children of allergic mothers (children in relatively high risk of allergy development) in comparison with cord blood of children of healthy mothers (low risk children). Also the activities of lymphocytes, dendritic cells (DC) and regulatory T cells (Tregs) were compared in children of healthy and allergic mothers.

The generally increased activity of both *in vitro* stimulated and non-stimulated mononuclear cord blood leukocytes was proved in children of allergic mothers in comparison with low risk children. The increased activity of DC of high risk children was detectable only after polyclonal stimulation. Significantly less pronounced functional properties of cord blood Tregs were found in children of allergic mothers when compared with children of healthy mothers. The increased reactivity of lymphocytes and DC together with the decreased activity of Tregs can support an easier sensitization/allergisation of genetically predisposed individuals.

An early postnatal application of the probiotic vaccine Colinfant New Born (*E. coli* O83:K24:H31) appeared to be an efficient preventive measure limiting the future allergy development in predisposed children. Significantly lower incidence of later allergy in high risk children comparable with the incidence in low risk children was proved in Colinfant colonized children of allergic mothers. The mechanism of probiotic effect is not fully understood yet. It is possible to suppose the improvement of Tregs function in Colinfant colonized high risk children can be explained as one of beneficial effects of the probiotic.

1. Introduction

Allergy belongs to the most common diseases with constantly increasing incidence. One of the theories explaining such a tremendous increment of allergies is the hygiene hypothesis suggesting that lower burden of microbes, mainly in western countries, decelerates the maturation of immune system, promoting thus allergy development in predisposed individuals.

There are many immunological changes including a bias to Th2 immune responses during gestation preventing undesirable interactions of maternal organism with the antigenically different foetus [(1)]. The establishment of a new immunological balance proceeds postnatally after the encounter of a newborn with external environment. Prevalent Th2 response supports allergy development, Th1 and Th17 responses are important for anti-infection defence but their exaggeration facilitates autoimmune reactions [(2)]. Therefore, very precise regulation preventing aberrant immune responses is important after birth. Tregs play an irreplaceable role in this fine tuning and limit pathological reactions, among others allergy prone Th2 responses.

There is strong need to find some early prognostic markers indicating an increased risk of allergisation. The finding of such prognostic markers would make possible the introduction of preventive measures hampering allergy development or at least lowering its clinical outcomes.

Probiotic administration can be considered as one of the possible preventive measures. Probiotics are known to promote immune system maturation. Because of decisive role of postnatal tuning of immune system, it is necessary to apply preventive probiotic treatment early after the birth. The large differences in probiotic effect exist not only among various bacteria but also among their particular strains. In our study, *Escherichia coli* O83:K24:H31 present in probiotic vaccine Colinfant New Born was administered to allergy high risk newborns.

2. Aims

1. One of the main aims of this thesis was to follow perinatal events proceeding during the postnatal tuning of the immature immune system and having a possible effect on future allergy development.

2. The second main aim was to evaluate the beneficial effect of early postnatal colonization with the probiotic vaccine Colinfant New Born on the immature immune system of newborns in relation to allergy and an assessment of early vaccine application as a preventive measure against allergy development.

Particular aims

1a) the comparison of gene expression and production of cytokines characteristic for Th1 x Th2 x Treg cell responses in cord blood cells of children of allergic mothers (children with increased risk of allergy development) and children of healthy mothers (children with relatively low risk of allergy development)

1b) to compare the *in vitro* reactivity of cord blood cells (CBMC, DC) of children of allergic mothers and children of healthy mothers

1c) to compare the proportion and functional properties of Tregs in cord blood of children of healthy and allergic mothers

1d) the comparison of immunological properties of maternal milk of healthy and allergic mothers

2a) the evaluation of the effect of probiotic colonisation with Colinfant New Born on the cytokine production in peripheral blood of postnatally colonized children

2b) an assessment of the effect of probiotic colonisation with Colinfant New Born on the proportion and functional characteristics of Tregs in peripheral blood of postnatally colonized children

3. Materials and Methods

3.1 Subjects

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 allergy persisting for longer than 24 months (allergy to respiratory and food allergens manifested with 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.

The study was approved by the Ethical Committee of Institute for the Care of Mother and Child, Prague, Czech Republic and was carried out with the written informed consent of the mothers.

3.2. Blood sampling

Typically, 10 - 20 ml of cord blood of children was collected in sterile heparinized tubes for cell analysis (*in vitro* stimulation of CBMC, DC, Tregs).

During regular visit of the pediatrician at the Institute for the Care of Mother and Child, 1 - 2 ml of peripheral blood of 6 - 7 year old children were collected in sterile heparinized tubes for cell analysis (Tregs). A questionnaire inquiring about allergy status of the child was completed by the mother.

3.3. Cytokine concentration in sera and cell culture supernatants

Cytokine concentration was determined by ELISA using primary antibody - detection antibody pairs from R&D Systems. Cytokine values were read from calibration curves in pg/ml. Results are expressed as medians and 25%-75% percentiles.

3.4. Immunoglobulin detection

Immunoglobulin secretion by cord blood cells was measured by ELISPOT after previous *in vitro* cord blood cell stimulation.

3.5. Relative quantification of gene expression

Gene expression of cytokines and DC activation markers was estimated by quantitative real-time PCR.

3.6. Flow cytometry

Proportion and functional characteristics of Tregs in cord blood of newborns and peripheral blood of six – seven year old children were measured by flow cytometry by combination of staining of cell surface markers and intracellular markers.

Maturation stage of mDC was assessed according to the cell surface presence of CD83 on CD11c+mDC using flow cytometry.

3.7. Statistics

Data were evaluated by appropriate parametric (t-test) and nonparametric (Mann-Whitney) statistical tests according to the data distribution.

4. Results

Effect of breast milk of healthy and allergic mothers on in vitro stimulation of cord blood lymphocytes. Žižka J, Hrdý J, Lodinová-Žádníková R, Kocourková I, Novotná O, Šterzl I, Prokešová L. Pediatr Allergy Immunol. 2007 Sep;18(6):486-94.

The goal of the study was to compare the immunological characteristics of milk of healthy and allergic mothers in the effort to assess its possible effect on the immune system of breast fed children.

Effect of maternal milk on proliferation activity of cord blood mononuclear cells (CBMC)

Suppressive effect of both colostrum and milk on CBMC proliferation was observed only when colostrum/milk was added non-diluted to the culture regardless of allergy status of the mothers. On the contrary, the lower concentration of maternal colostrum/milk which corresponds better with the situation in newborn intestine has rather stimulatory effect on cord blood cells proliferation activity. Importantly, no significant difference was observed between the effect of maternal milk from healthy and allergic mothers. The proliferation rate of cord blood cells of children of allergic mothers was significantly higher in both non-stimulated and polyclonally stimulated cultures in comparison to children of healthy mothers.

Influence of maternal milk on immunoglobulin formation in cultures of cord blood cells

Only marginal production of immunoglobulins was detected in non-stimulated cord blood cells. After polyclonal stimulation by *Bacillus firmus* – BF (a polyclonal activator of B lymphocytes), immunoglobulin production was markedly increased. Immunoglobulin secretion by cord blood cells was further conspicuously increased by colostrum/milk addition to the culture. Again, we did not observe any significant difference between the effect of maternal colostrum/milk from healthy and allergic mothers.

We did not observe any difference in maternal colostrum/milk from healthy and allergic mothers on CBMC stimulation *in vitro*. From this point of view, there is no reason to be afraid of any negative influence of the milk of allergic mothers. On the other hand, we described differences between activation of CBMC of children of healthy and allergic mothers. The generally increased reactivity of CBMC of children of allergic mothers could lead to inappropriate immune responses in allergy predisposed children after allergen encounter.

Prevention of allergy in infants of allergic mothers by probiotic Escherichia coli. Lodinová-Žádníková R, Prokešová L, Kocourková I, Hrdý J, Žižka J. Int Arch Allergy Immunol. 2010;153(2):201-6.

The reports concerning the effect of probiotics in allergy prevention and treatment are rather controversial. Dr. Lodinová-Žádníková found in 2003 [(3)] that an early colonisation of newborns by probiotic vaccine Colinfant New Born (*E. coli* O83:K24:H31) significantly reduced future allergy incidence. Also in the current study, convincingly decreased allergy incidence in Colinfant New Born colonized children of allergic mothers was proven. In the effort to correlate clinical and laboratory data, cytokine levels in children sera were monitored. Due to the large individual variability and often non-detectable levels of some cytokines, it is difficult to draw any definitive conclusion. The pro-allergic cytokine phenotype seems to be evident in non-colonized children of allergic mothers starting with the 3rd day of life. Colonization of children of allergic mothers approximates gradually their cytokine profile to the non-colonized children of healthy mothers.

Significantly decreased allergy incidence in colonized children of allergic mothers when comparing with non-colonized children of allergic mothers was observed in our study. It seems, that colonization with the vaccine Colinfant New Born supports the normalization of the allergic phenotype.

Cytokine expression in cord blood cells of children of healthy and allergic mothers. Hrdý J, Zanvit P, Novotná O, Kocourková I, Zižka J, Prokešová L. Folia Microbiol. 2010 Sep;55(5):515-9.

Not all high risk children of allergic mothers develop allergy. In the effort to find some early predictive signs indicating increased risk of allergy development, gene expression of cytokines in cord blood cells and their concentrations in sera of cord blood of children of allergic mothers and children of healthy mothers were compared.

Gene expression of cytokines in cord blood cells

Significantly decreased gene expression of typical Th1 cytokines (IL-2, IFN-ɣ) in cord blood cells of children of allergic mothers in comparison to children of healthy mothers was found. Increased gene expression of IL-10, IL-13 and EGF in cord blood cells of children of allergic mothers in comparison to children of healthy mothers was detected but these differences were insignificant mainly due to the large individual differences.

Concentration of cytokines in cord blood sera

Data obtained on the protein level, did not reach such statistical significances as seen on the gene expression level. Nevertheless, a tendency to the increased levels of Th2 cytokines (IL-4, IL-13) and decreased levels

of typical Th1 cytokine - IFN-ɣ in cord blood sera of children of allergic mothers in comparison to ones of children of healthy mothers was evident.

The evidence was brought that a pro-allergic phenotype is evident already on the level of cord blood. General trend to increased levels of Th2 cytokines and decreased levels of Th1 cytokines in cord blood of high risk children is obvious on the level of both gene expression and protein secretion. It is important to keep in mind that cytokines in cord blood sera are produced not only by cord blood cells but also by many other cells namely epithelial ones. Importantly, some cytokines could be also of maternal origin. These facts explain the partial discrepancies between gene expression and protein secretion observed in our study (e.g. in the case of EGF, IL-4, IL-8, IL-10).

Cytokine expression in the colostral cells of healthy and allergic mothers. Hrdý J, Novotná O, Kocourková I, Prokešová L. Folia Microbiol. 2012May;57(3):215-9.

Maternal milk contains not only nutritional compounds but also immunologically active components both humoral and cellular. In animal studies, passage of milk leukocytes through the intestinal wall and their entrance into offspring's immune system were described. There are also indirect proofs of transintestinal passage of colostral cells in humans. Thus, certain effect of ingested colostral cells on the newborn immune system can be supposed. In the current study, we compared gene expression of cytokines in colostral cells of healthy and allergic mothers.

Colostral cells of allergic mothers could be characterized by increased gene expression of Th2 cytokines (IL-4, IL-13) and decreased expression of Th1 (IFN-ɣ) and regulatory cytokines (IL-10, TGF-beta). It remains a question to what extent can a surely small number of fully functional maternal colostral cells influence immune responses of a newborn. High concentrations of soluble colostral cytokines have certainly larger impact on the immune system of the child.

Differing gene expression of subunits of the IL-12 family of cytokines in mDC derived in vitro from the cord blood of children of healthy and allergic mothers. Hrdý J., Novotná O., Kocourková I., Prokešová L. Submitted

Differencies (very often contradictory) in many humoral components of cord blood between children of healthy and allergic mothers were described. However, the cellular components of cord blood were studied to lower extent. In our study, immunologic characteristics of mDCs derived *in vitro* from the cord blood of children of healthy and allergic mothers were compared.

Gene expression of subunits of IL-12 cytokine family

No significant difference in the gene expression of IL-12 family cytokine subunits in non-stimulated mDC was observed excepting p28 expression. The expression of p28 in non-stimulated mDC of children of allergic mothers was increased in comparison with the healthy group. Subunit p28 with its partner EBI-3 forms IL-27. Interestingly, IL-27 is capable to promote IL-4 induced IgE production. LPS stimulation in the culture increased gene expression of nearly all subunits tested. The increase was significantly higher in mDC derived from the cord blood of children of allergic mothers with the exception of p19. It points to the generally increased reactivity of mDC of children of allergic mothers.

Activation markers on the surface of mDC

The presence of activation marker CD83 on the surface of mDC was tested to evaluate the extent of mDC activation. We did not find any significant difference in the proportion of $CD83^{+}CD11c^{+}$ cells in the populations of *in vitro* non-stimulated mDC of children of healthy and allergic mothers. On the other hand, significantly increased proportion of CD83+ mDC was detected in children of allergic mothers in comparison to healthy group after LPS stimulation in the culture.

The differences in the capacity of mDC to produce cytokines of the IL-12 family and to express cell surface activation marker CD83 were proved between groups of children of healthy and allergic mothers. Especially significantly increased reactivity of mDC of children of allergic mothers after LPS stimulation could reflect the increased promptness to allergen sensitisation. We hypothesize that mDC with increased reactivity present in children of allergic mothers could easily induce the positive effector immune responses (e.g. Th2) instead of immunologic tolerance after possible allergen encounter.

Differences in immunological characteristics of Tregs in cord blood of children of healthy and allergic mothers. Hrdý J, Kocourková I, Prokešová L. submitted

In this study, we focused on the comparison of proportions and functional characteristics of regulatory T cells (Tregs) in cord blood of children of healthy and allergic mothers. Tregs are known to participate in the induction of peripheral tolerance, e.g. to relatively innocuous environmental antigens. This is quite important in the context with allergy development because allergy could be defined as an aberrant immune response to relatively innocuous environmental antigens caused by inadequate immune regulation (failing in tolerance induction).

Proportion of Tregs in cord blood of children of healthy and allergic mothers

Depending on the gating strategy and markers characterizing Tregs used (when Tregs were considered only as $CD4+CD25^{high}$) , we were able to prove the significantly increased proportion of regulatory T cells in cord blood of children of allergic mothers.

Functional characteristics of Tregs in cord blood of children of healthy and allergic mothers

We observed significantly decreased MFI of FoxP3 in Tregs in cord blood of children of allergic mothers in comparison with children of healthy group. The release of regulatory cytokines is one of the mechanisms how Tregs regulate immune responses and facilitate tolerance onset. Significantly lower intracellular presence of IL-10 and TGF-beta in cord blood Tregs of children of allergic mothers was detected.

The increased proportion of cord blood Tregs in children of allergic mothers in comparison to healthy group was found. On the contrary, functional characteristics of Tregs in cord blood of children of allergic mothers were less functionally competent. We can suppose that functional impairment of Tregs in cord blood of so far non-allergic children of allergic mothers could be at least partially compensated by their increased number. Definitely, it is important to follow not only proportion of Tregs but also their functional characteristics.

The Effect of Probiotic Colinfant New Born on Regulatory T-cells in Six Year Old Children. Hrdý J, Kocourková I, Prokešová L. Submitted

In our previous studies, we reported significantly decreased allergy incidence in high risk individuals colonized by probiotic vaccine Colinfant New Born within 48 hrs after delivery. The mechanism of possible beneficial effect of probiotics on immature immune system of newborns is still poorly understood. The participation of Tregs in these mechanisms is supposed.

Significantly decreased allergy incidence in colonized children

Probiotic colonized and non-colonized children of allergic mothers enrolled in the study are longitudinally examined and we are now able to see the significantly reduced allergy incidence in colonized children already 6-7 year old.

Proportion of Tregs in peripheral blood of early postnatally colonized children

We did not prove any significant difference in the proportion of Tregs in the peripheral blood among colonized children of allergic mothers and non-colonized children of healthy and allergic mothers. After subdivision of children according to their allergy status, significantly increased proportion of Tregs in peripheral blood of allergic children of allergic mothers in comparison to non-allergic children of allergic mothers was observed. Moreover, when children were divided according to their allergy status regardless of their colonization and allergy status of their mothers, significantly increased proportion of Tregs in peripheral blood of children suffering from allergy was detected.

Functional characteristics of Tregs in peripheral blood of non-colonized and colonized children

Significantly decreased MFI of FoxP3 in Tregs of non-colonized children of allergic mothers in comparison to non-colonized children of healthy mothers was detected. MFI of FoxP3 in Tregs of allergic non-colonized children of healthy and allergic mothers was significantly lower in comparison with non-allergic non-colonized children of healthy and allergic mothers. Significantly decreased MFI of FoxP3 in Tregs in peripheral blood of allergic children in comparison to non-allergic children regardless of their colonization and the allergic status of their mothers was observed.

Significantly lower intracellular IL-10 was observed in Tregs of allergic non-colonized children of allergic mothers in comparison to nonallergic non-colonized children of allergic mothers. Significantly decreased IL-10 in Tregs in allergic children in comparison to healthy children was observed when children were compared only according to their allergy status.

Intracellular presence of TGF-beta in Tregs of peripheral blood followed the same trend as described for intracellular IL-10 but differences were without significance.

Colonisation of newborns by probiotic vaccine Colinfant New Born significantly reduced allergy incidence in colonised children. Probiotic colonisation increased functional characteristics of regulatory T cells in peripheral blood of colonised children. Significantly decreased functional characteristics of Tregs in allergic children in comparison to healthy ones were proved. The proportion of Tregs in peripheral blood of children suffering from allergy was increased in comparison to healthy ones. It is possible to suppose impaired functional potency of Tregs of allergic children implicates increased proportion of Tregs which could partially compensates their insufficient function.

5. Discussion

Our studies were focused on investigation the several immunologic markers with the potential to serve as a prognostic signs indicating increased risk of allergy development. The issue of searching the prognostic markers is complicated by the multifactorial causes of allergy.

One of the ways we addressed this issue was the comparison of gene expression with protein level of cytokines promoting Th1 or Th2 responses and with regulatory and pro-inflammatory cytokines [(4)]. Although we proved quite convincingly allergic phenotype in children of allergic mothers on the level of cytokine gene expression (the decreased gene expression of Th1 cytokines IL–2 and IFN-γ, a trend to the increase of IL-13 and the decrease of regulatory cytokine TGF-β), the cytokine concentrations in cord blood sera do not exactly correspond

with these results (although the changes in IFN-γ, IL-13 and TGF-β concentrations were in the same sense as the changes in gene expression). When comparing our data with the literature, we could see that the most of research groups observed the decreased levels of IFN-γ in cord blood sera of high risk children in comparison to low risk children [5-9] similarly as described by Hrdý et al. [4]. The increased presence of IL-13 observed by us in cord blood of high risk newborns on the both levels – mRNA and protein formation - is in accordance with the report by Ohshima et al. [10;11].

Regulatory cytokines followed in our study and representing one of the effectors of Tregs are further indicators of changed immunological equilibrium. In our study, no significant difference in IL-10 in cord blood between children of healthy and allergic mothers was observed in concordance with Pfefferle et al. and Balossini et al. [12;13] although another study reports difference in IL-10 producing capacity [14]. In the case of the other important regulatory cytokine – TGF-β, we reported both the decreased gene expression and cord blood sera concentration in children of allergic mothers in comparison to children of healthy mothers [4]. Our observation is in accordance with several other reports (e. g. [13]). Limited capacity of high risk children to produce TGF-β could contribute to the easier allergy development because TGF-β promotes intestine maturation, induces class switching to IgA, supports induction of peripheral tolerance to allergens (e. g. by induction of regulatory T cells). Our results indicate that it would be of importance to use TGF-β as one of the prognostic markers for considering the risk of future allergy development in predisposed children.

Decreased levels of EGF (epidermal growth factor) and TGF-β in cord blood sera of children of allergic mothers could contribute to the delayed intestine maturation supporting easier allergen penetration leading possibly to allergy development in the future.

Significant differences in above mentioned cytokines (IL-4, IL-10, IL-13, IFN-γ) were described between children of healthy and allergic mothers during the first year of life by Prokešová et al., 2006 [15]. We have confirmed the importance of following the IL-4 and IFN-γ in the cord blood sera and sera of peripheral blood of children. Only these two cytokines were found to be significantly different (increased levels of IL-4 and decreased that of IFN-γ in children of allergic mothers in comparison to healthy ones) in the prospective study [16].

Evaluation of the suitability of selected cord blood cytokines as prognostic markers indicating increased risk of allergy development is complicated by the controversial data present in the literature which can be caused by the use of different methods having different sensitivity, distinct cord blood sampling, variability during pollen seasons, diverse ethnicity, considering different markers and evaluation of potential markers on different levels (mRNA level or protein

formation and secretion). However, the main obstacle is in the enormous heterogeneity of human population and diversified character and pathogeny of allergic diseases. Nevertheless, our studies indicate that IL-4, IL-13, IFN-γ and TGF-β are the most predicative cytokines.

There is no doubt about the beneficial effect of breastfeeding on the immature newborn´s immune system but it is not clear whether there are functional differences between milk of healthy and allergic mothers. Maternal milk is believed to prevent allergy development [17]. Maternal milk contains cytokines which transferred through the intestinal wall can be still fully functional. Differences in cytokine concentration between maternal milk from healthy and allergic mothers were described [15;18], but less is known about the capacity of cells present in maternal milk to produce cytokines. We tested gene expression of cytokines in colostral cells of healthy and allergic mothers. Although the only significantly increased expression was that of EGF in colostral cells of allergic mothers (Hrdý et al. [19]), the trend to an allergic phenotype was documented by the increased gene expression of IL-4 and IL-13 and decreased that of Th1 cytokine IFN-γ and regulatory cytokines IL-10 and TGF-β [19]. This is actually the first study comparing the gene expression of cytokines in colostral cells of healthy and allergic mothers. It is important to emphasize that the cross-intestinal transfer of live colostral cells was proved in animal models [20;21], and even in humans, the indirect proofs were reported [22;23]. It is questionable, however, to what extent the small number of transferred colostral cells could influence the immature newborn immune system.

To evaluate the possibly different effect of maternal milk from healthy and allergic mothers on newborn blood cells, in vitro assays were performed. The capacity of soluble components of maternal milk to influence the proliferation and immunoglobulin formation of newborn mononuclear leukocytes was tested. Humoral compounds of colostrum and mature maternal milk supported immunoglobulin formation by cord blood cells regardless of allergy status of mothers. Similarly, no difference between milk and colostrum from healthy and allergic mothers on proliferation of cord blood cells was observed. The highest concentration of colostrum/milk decreased proliferation of neonatal cells but on the contrary, diluted colostrum had rather stimulatory effect, Žižka et al. [24] which is in accordance with the other studies [17;25]. In light of data obtained, it is not necessary to be afraid of harmfull effect of the milk of allergic mothers. We did not found the possible difference in the effect of “allergic and healthy” maternal milk on newborn cells but surprisingly, we were able to detect the large difference between the proliferation rate of cord blood cells of children of healthy and allergic mothers. Significantly increased proliferation of both non-stimulated and polyclonaly stimulated cord blood cells of children of allergic mothers in comparison to children of healthy mothers was observed in our study [24]. It points

to increased reactivity of lymphocytes of high risk children which could support their future allergisation.

The fact of increased reactivity of cord blood cells of children of allergic mothers directed us to the characterisation of cord blood cells in more details. We wondered, whether the increased proliferation rate is caused by increased proportion and maturation stage of antigen presenting cells (APC) in cord blood of children of allergic mothers or by impaired function of regulatory T cells.

So, we decided to test the capacity APC to produce cytokines of IL-12 family and to express activation markers. No difference in gene expression of the subunits of IL-12 family cytokines was observed between non-stimulated mDC of children of healthy and allergic mothers with the exception of the subunit p28 decisive for the formation of IL-27. IL-27, besides promoting Th1 and suppressing Th17 immune responses, supports IgE production. The gene expression was significantly increased in LPS stimulated mDC of children of allergic mothers in all subunits tested with the exception p19 in comparison to children of healthy mothers. When testing the presence of activation marker of mDC - CD83 by flow cytometry, the significant differences between mDC in "allergic" and "healthy" groups were again found only after LPS stimulation: significantly higher both proportion of $CD11c^{+}CD83^{+}$ mDC and MFI of CD83 were observed in mDC of children of allergic mothers. This observation points to the general increase of reactivity of mDC in children of allergic mothers.

Regulatory T cells are responsible for setting and maintenance of the tolerance to environmental antigens and the homeostasis of immune system in general. Impaired function of regulatory T cells could enable setting of inappropriate effector immune responses to allergens (prevailing Th2 response) leading to the allergy development. Thus both proportion and functional characteristics of regulatory T cells in cord blood of high risk children (children of allergic mothers) and low risk children (children of healthy mothers) were compared in the effort to find some early differences in the immune system tuning signalizing later allergy development. Although no significant difference in the proportion of regulatory T cells was observed, we were able to find the quite convincing functional insufficiency of Tregs in the high risk children. The functional characteristics were evaluated according to the MFI of FoxP3. The other functional property of regulatory T cells is the release of regulatory cytokines IL-10, IL-35 and TGF-β. We have observed lower intracellular presence of IL-10 and TGF-β in Tregs in the cord blood of children of allergic mothers. In addition to Treg characteristics followed by us, other markers pointing to an impaired function of Tregs were described. Treg-specific demethylated region (TSDR) was identified as another important marker indicating functional activity of Tregs [26;27]. Hinz et al. [28] described decreased proportion of Tregs characterized by TSDR in cord blood of children who developed allergy. Further studies characterizing Tregs are needed to assess the suitability of these markers to serve

as the prognostics signs indicating the functional impairment of Tregs. The insufficient suppressive functions could support easier allergisation, even intrauterine sensitization. It seems that functional characteristics of Tregs are really promising marker indicating possible increased risk of allergy development in predisposed children.

As mentioned above, maternal allergy is at present the only really reliable marker for the evaluation of the future allergy risk. However, it is well known not all children of allergic mother attain allergy. Further early prognostic markers are needed and some of them have been already proposed. To evaluate their significance, the longitudinal observation of children of allergic mothers is necessary to compare the presence of perinatal "prognostic markers" with the later allergy development. We have the possibility to follow longitudinally the high risk children in the cooperation with the Institute for the Care of Mother and Child. We have the first results in this respect and this topic is included in our future plans which comprise the evaluation of some preventive measures as well.

One of the possible measures preventing allergy seems to be a probiotic administration. This idea is supported by hygienic hypothesis explaining the rise of allergic diseases in the developed countries by lower microbial burden early in life and thus delayed newborn immune system maturation which is necessary for postnatal balancing of Th1/Th2 immune responses because newborn immature immune system exerts proallergic phenotype with the predominance of Th2 bias which is necessary for the successful course of pregnancy [29].

Moreover, probiotics were proved to be capable to induce Tregs as indicated by Kwon et al. [30]. The positive effect of probiotics on Treg development is of particular importance during the early postnatal period when the tolerance to environmental antigens is settled and overall tuning of immune system takes part.

In our study, probiotic vaccine Colinfant New Born was administered to the newborns within 48 hrs after the delivery. This early administration ensures an effective probiotic colonisation, otherwise it is difficult for a probiotic to colonize the gut where the microbiota is already set and all the niches are occupied. The vaccine Colinfant New Born was invented by Dr. Lodinová-Žádníková, it is a monostrain preparation containing *Escherichia coli* O83:K24:H31. Its exceptional adhesive properties account for the long lasting presence in the gut of colonized children. The effect of Colinfant New Born on lowering the incidence of allergy was reported some years ago [16;31-34]. Although the beneficial effect of probiotics on immune system is well acknowledged, the mechanism of probiotic action is still poorly understood. In our present study, the proportion and functional characteristics of Tregs in peripheral blood of at birth Colinfant New Born colonized six - seven year old children were tested. As reported already before, colonized children of allergic mothers have significantly lower allergy incidence in comparison with non-

colonized children of allergic mothers. Allergy incidence in colonized children of allergic mothers was comparable with the allergy incidence in non-colonized children of healthy mothers (low risk children for allergy development).

No significant differences were proved in the proportion of Tregs. However, when comparing children only according to their allergy status regardless of probiotic colonization and allergy status of their mothers, significantly increased proportion of Tregs was found in peripheral blood of allergic children in comparison to healthy ones. Concerning the proportions of Tregs in peripheral blood of allergic individuals, the controversial data can be found in the literature. Some authors support our observation of increased levels of Tregs in allergic patients, others did not observed any difference and there are also reports on decreased proportion of Tregs in peripheral blood of humans suffering from allergy in comparison to healthy ones.

However, it is necessary to realize that the amount of Tregs alone is not decisive for effective suppression function [28]. Functional analyses of Tregs are probably more informative. Further, it is necessary to keep in mind that not all lymphocytes exerting suppressor function express FoxP3 [35]. We have observed increased functional potency of Tregs in peripheral blood of colonized children of allergic mothers in comparison with non-colonized children of allergic mothers. Both MFI of FoxP3 and intracellular regulatory cytokines IL-10 and TGF-β were increased in colonized children of allergic mothers in comparison with non-colonized children of allergic mothers clearly indicating the capacity of Colinfant New Born to modify immune function of Tregs. It remains to be resolved if the effect of the probiotic on Tregs is direct or is mediated by dendritic cells.

It is necessary to remember the large differences exist among the bacterial strains. Furthermore, it was suggested that the combination of several strains would be more efficient than a monostrain colonisation as documented on animal models [36-39].

In conclusion, in this PhD thesis the set of immunologic markers is proposed which can be considered as a potential prognostic signs indicating an increased risk of future allergy development. Probably a combination of several markers would be the most reliable. Tregs characterisation, especially that of their functional properties is of particular interest.

Probiotic administration seems to be a good preventive measure decreasing allergy incidence. We have proven the capacity of probiotic vaccine Colinfant New Born to modify functional characteristics of Tregs in peripheral blood of colonized children. Further studies are necessary to reveal which subpopulation of Tregs represents a target of probiotic action.

6. Conclusions

I. Comparison of immunologic properties of cord blood cells of children of healthy and allergic mothers

- Gene expression of cytokines in cord blood cells of children of allergic mothers has a pattern of pro-allergic phenotype (decreased Th1: IL-2, IFN-ɣ; increased Th2: IL-13) in comparison to children of healthy mothers
- Significantly increased cell surface presence of activation markers together with higher gene expression of all subunits of cytokines of IL-12 family in mDC of children of allergic mothers in comparison to children of healthy ones were proved.
- Impaired immune functions of cord blood T regulatory cells were detected in children of allergic mothers.
- Significantly increased *in vitro* proliferation of both of non-stimulated and polyclonally stimulated mononuclear cord blood cells of children of allergic mothers was evident when compared with proliferation in children of healthy mothers. in comparison to healthy ones

Pro-allergic phenotype is evident already at birth. Generally increased reactivity of cord blood cells of newborns of allergic mothers could facilitate easier allergen sensitization and development of inappropriate immune responses leading to allergy manifestation.

II. Testing of selected immunologic parameters in maternal milk from healthy and allergic mothers

- Soluble components of maternal milk influence *in vitro* stimulation of mononuclear cord blood cells. No significant difference between the effect of humoral components of milk from healthy and allergic mothers was found judged according to the effect of maternal milk on cord blood cell proliferation and immunoglobulin secretion by cord blood cells.
- Cytokine gene expression in colostral cells from allergic mothers implies their allergic phenotype (increased gene expression of Th2 cytokines and decreased that of Th1 cytokines) in comparison with colostral cells of healthy mothers. With regard to the low number of colostral cells, their possible effect in newborn organism is uncertain.

Our results do not support the possible unfavourable effect of the milk of allergic mothers.

III. Evaluation of the effect of colonization of children of allergic mothers by probiotic vaccine Colinfant New Born

- Significantly decreased incidence of allergy was observed in probiotic colonized children of allergic mothers in comparison to non-colonized children of allergic mothers.
- Substantially increased functional characteristics of Tregs in peripheral blood of 6 – 7 year old colonized children of allergic mothers were detected in comparison to non-colonized children of allergic mothers
- Generally impaired immune functions of Tregs in peripheral blood of children suffering from allergy in comparison to healthy children were proved.

The beneficial effect of the probiotic vaccine Colinfanf New Born involves the enhancement of Tregs function.

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8. Publications

Publications *in extenso* related to the present study:

Effect of breast milk of healthy and allergic mothers on in vitro stimulation of cord blood lymphocytes. Žižka J, Hrdý J, Lodinová-Žádníková R, Kocourková I, Novotná O, Šterzl I, Prokešová L. Pediatr Allergy Immunol. 2007 Sep;18(6):486-94. **IF = 2.901**

Prevention of allergy in infants of allergic mothers by probiotic Escherichia coli. Lodinová-Žádníková R, Prokešová L, Kocourková I, Hrdý J, Žižka J. Int Arch Allergy Immunol. 2010;153(2):201-6. **IF = 2.215**

Cytokine expression in cord blood cells of children of healthy and allergic mothers. Hrdý J, Zanvit P, Novotná O, Kocourková I, Žižka J, Prokešová L. Folia Microbiol (Praha). 2010 Sep;55(5):515-9. **IF = 0.982**

Cytokine expression in the colostral cells of healthy and allergic mothers. Hrdý J, Novotná O, Kocourková I, Prokešová L. Folia Microbiol (Praha). 2012 May;57(3):215-9. **IF = 0.982**

Differing gene expression of subunits of the IL-12 family of cytokines in mDC derived in vitro from the cord blood of children of healthy and allergic mothers. Hrdý J., Novotná O., Kocourková I., Prokešová L. Submitted

Differences in immunological characteristics of Tregs in cord blood of children of healthy and allergic mothers. Hrdý J., Kocourková I., Prokešová L. First revision sent to Clinical and Experimental Immunology

The Effect of Probiotic Colinfant on Regulatory T-cells in Six Year Old Children. Hrdý J., Kocourková I., Prokešová L. submitted

Publications in extenso not related to the present study:

Second-generation taxanes effectively suppress subcutaneous rat lymphoma: role of disposition, transport, metabolism, in vitro potency and expression of angiogenesis genes. Otová B, Ojima I, Václavíková R, Hrdý J, Ehrlichová M, Souček P, Vobořilová J, Němcová V, Zanardi I, Horský S, Kovář J, Gut I. Invest New Drugs. 2012 Jun;30(3):991-1002. **IF = 3.007**

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