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Imunologické vlastnosti pupečníkové krve u dětí se zvýšeným rizikem vzniku alergie Preventivní použití probiotik

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

PhD thesis

Supervisor: prof. MUDr. Ludmila Prokešová, CSc.

Praha, 2012

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Dedication

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Abstrakt

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

Pupečníková krev je snadno dostupným klinickým materiálem, který může být využit pro hledání prognostických znaků signalizujících budoucí vznik alergie. 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, DC a 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 ve srovnání s neosídlenými dětmi alergických matek 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.

Klíčová slova: pupečníková krev, alergie, probiotika, regulační buňky, dendritické buňky, cytokiny, kolostrum

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 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.

Key words: cord blood, allergy, probiotic, regulatory T cells, dendritic cells, cytokines, colostrum

Content

Abbreviations

APC antigen presenting cells
BF – *Bacillus firmus*
CBMC – cord blood mononuclear cells
CTLA-4 - cytotoxic T- lymphocytes antigens 4
DAG - diacylglycerol
DC dendritic cells
E. coli – Escherichia coli
FAO - United Nations Food and Agricultural Organization
FoxP3 - forkhead box P3
GITR - glucocorticoid-induced TNF-related protein
GM-CSF – granulocytes monocytes-colony stimulating factor
IBD – inflammatory bowel disease
ILT - immunoglobulin-like transcript
IP3 – inositol 1,4,5-triphosphate
IPEX - immune disregulation polyendocrinopathy, enteropathy, X-linked syndrome
ITAM - immunoreceptor tyrosine-based activation motif
iTreg – induced regulatory T cell
nTreg – natural regulatory T cell
LAG-3 - lymphocytes activation gene-3
ldDC – low differentiated dendritic cells
MAPK - mitogen-activated protein kinase
mDC – myeloid dendritic cells
MFI – median of fluorescence intensity
NEC – necrotizing enterecolitis
NIMA – noninherited maternal antigens
PD-1 - program death domain
pDC – plasmacytoid dendritic cells
PLA2 – phospholipase A2
PKC – protein kinase C
rDC – regulatory dendritic cells
tDC – tolerogenic dendritic cells
SIT – specific allergen immunotherapy
TLR – toll like receptor
TSDR – Treg-specific demethylated region
TSLP - thymic stromal lymphopoietin
cAMP – cyclic adenosine monophosphate
PUFA – polyunsaturated fatty acid
WHO - World Health Organization

Introduction

Immune relationship between mother and foetus in human

Immune system of foetus is influenced by genetic information from both mother and father. Except from genetic information, mother influences immune system of foetus during the nine months course of pregnancy. Not only nutrients but also immunologically active substances are transmitted from mother to foetus during this close coexistence of actually two individuals. Intrauterine environment is the first environment which can influence development of immune system of the foetus. Thus, foetal immune system development is influenced by maternal factors consisting of both exogenous environmental exposures and endogenous factors [(1)].

Mother is during pregnancy exposed to a number of environmental antigens so called non-inherited maternal antigens (NIMA) constantly traversing the placenta with the capacity to set immune responses in the foetus[(2)]. Immune system of foetus is substantially influenced by mother's immune system not only during the intrauterine life but also postnatally - mainly by breastfeeding. Maternal environmental exposures including dietary factors [(3;4)], cigarette smoke [(5;6)] and microbial exposure [(7-9)] can modify neonatal immune responses. Immunomodulatory properties are well acknowledged for PUFA (poly unsaturated fatty acid) [(10)] where lower consumption of PUFA by mother during pregnancy is associated with increased risk of children allergy development [(11-16)]. The effect of maternal antioxidant and vitamin treatment is also documented [(17-20)].

Father's genetic information can be reflected in foetus HLA. Mother´s recognition of foreign HLA present on the foetus cells can lead to the development of immune response ending by foetus damage. Foetus could be considered as an allograft and therefore complex immunological mechanisms have been developed to make possible the coexistence of two genetically different individuals. Changes in maternal immune system are necessary for the successful growth and development of the foetus. Generally, mother´s immune system is biased Th2 which is reflected by clinical improvement of pregnant women suffering from autoimmune diseases [(21;22)]. Pronounced Th2 immune response downregulates Th1 immune responses to foetal antigens [(23)]. Although, suppressed Th1 immune

response is necessary for successful pregnancy its insufficient function is connected with increased susceptibility of newborns to infections.

Regulatory T cells (Tregs) represent another mechanism important for successful development of the foetus. General increase of Tregs during normal pregnancy is important for prevention of alloreactivity against foetus. [24]. Tregs promote the immunological tolerance to foreign antigens transmitted to the foetus via placenta [25]. Forkhead box P3 (FoxP3)+ Tregs are attracted to the materno-foetal interface by human gonadotrophin [26].

It was discovered that maternal cells trafficing in large numbers to foetal lymph nodes induce CD4+CD25+FoxP3+ cells with suppressive functions preventing the development of antimaternal immunity. This effect lasts until early adulthood. Tregs constitute a high proportion of total lymphocytes in the foetal lymph nodes. These data challenge the preconceived notion that foetal lymphocytes are incapable of the development of immune responses [27]. Actually, the authors of the study proved that foetal lymphocytes produced high levels of cytokines in response to NIMA when Tregs had been specifically removed. In a study using mouse model of anti-OVA tolerance in mothers, a transfer of OVA and TGF-beta via breastfeeding into suckling neonates induced suppressive CD4+ T cells. Generation of these regulatory T cells was dependent on the presence of a functional TGF-beta receptor [28]. Another animal study demonstrated the transfer of maternal tolerance to antigens achieved during pregnancy to offspring but this tolerance persisted only if the offsprings were nursed by the tolerized mother [29]. Another documentation of active transfer of cells on maternal-foetal interfaces represents transfer of delayed type of hypersensitivity to TBC tested by tuberculin reaction [30]. Interestingly, T lymphocytes of newborns of silicone breast implant recipients responded to specific stimuli by silicon dioxide even in non-breastfed infants which documented transplacental transfer of such immunogens as silicone [31]. Transplacental passage of cells was reviewed by Schöder [32], this passage is bidirectional [33]. We can distinguish both maternal and foetal microchimerism depending on whether maternal cells were transferred into foetus or foetal cells into mother [34]. Foetal cells transferred via placenta were found in various maternal tissues and persisted even over three decades [35;36]. These cells can provide even some benefits to the mother, e.g. in the protection against some pathogens, by increased immunologic repertoire but on the other hand, they could lead

also to autoimmunity and cancer development [34]. Antigens transferred via placenta and milk can effectively induce Tregs leading to toleration of these antigens. As stated by Ray et al. [2], these findings not only underscore the importance of Tregs in tempering immune responses in the developing foetus but also highlight a window of opportunity for development of novel therapeutic strategies. This could be exploited for development of novel therapeutic strategies in early life to ward off a disease (e.g. allergic diseases) [37;38].

Another mechanism supporting a normal pregnancy is the expression of HLA-G on trophoblast. The HLA-G antigen is predominantly expressed on immune-privileged tissues such as extravillous cytotrophoblast of the placenta and on embryonic cells, and is thought to have a functional role in inhibiting natural killer cell-mediated lysis and in influencing cytokine expression to maintain the mother's immune tolerance to the genetically foreign foetus [39].

It is important to notice that immune system of newborns is generally immature. At the time of delivery, antibody production is defective and immunoglobulins (IgG) are obtained from the mother transplacentally or by breastfeeding (mainly IgA) after the birth. The levels of IgG and IgM antibodies in sera of two years old children are nearly comparable with that of adults but the production of IgA reaches adult levels only during adolescence.

The immune system of human foetus starts to develop already in the 3rd week of pregnancy by the development of pluripotent hematopoietic stem cells in yolk sack. During the 5th week of gestation, this pluripotent hematopoietic stem cells migrate to foetal liver (actually the first hematopoietic organ of the foetus) and transiently to spleen. During the 8th – 11th week of gestation, stem cells reach the bone marrow, spleen and lymph nodes via embryonal circulation.

The foetus is supplied by IgG from mother by transplacental transfer beginning the 22nd week of gestation.

Foetal thymus is populated by pro-thymocytes which originate in the liver by the 10-12th week of gestation [40]. This lymphocytes are capable to response to polyclonal stimulation [41]. At the same time, allogeneic graft versus host reactivity was also described [42] documenting readiness of foetal lymphocytes to exert immune functions. Surprisingly, Jones et al. documented allergen specific responses as early as at 22nd week of gestation [43]. This finding could indicate that allergic sensitization can take part even during intrauterine life. Thorton et al. [44] described

that encounter of lymphocytes residing the foetal thymus with antigens led to an induction of Tregs. Therefore, the setting of tolerance to environmental antigens early in life could prevent allergy development later in life. Actually, several research groups tried to exploit this capability of newborn lymphocytes to respond to allergens for searching some early prognostic markers indicating an increased risk of allergy development [45-51]. Assessment of different markers pointing to pro-allergic phenotype of newborns is reviewed by Prescott [52;53]. The responsiveness of CBMC (cord blood mononuclear cells) to allergen was tested by Joerink et al. [54] but it was not possible to draw any conclusion concerning the prognostic value of results obtained [55]. Thymocytes have to pass positive and negative selection before they can migrate to the secondary lymphoid organs. In newborns, there is general lymphocytosis in infancy [56]. The cord blood cells were characterized by a low proportion of CD3+ T-cells, increased CD4/CD8 and CD45RA/CD45RO ratios, minimal expression of HLA-DR, increased proportion of CD5CD19 double positive B-cells, while CD3- CD8+ and CD3- CD7+ subsets, not usually found in adult peripheral blood, were detected [57]. This study emphasizes immature phenotype of cord blood cells.

B-lymphocytes in cord blood do not produce the complete spectrum of antibodies. At the time of delivery, active production of all Ig is negligible in the offspring. IgM is the first antibody produced after antigen stimulation. IgM is even released by non-fully matured B cells. The lowest total level of antibodies is reached during the 3^{rd} – 4^{th} month of postnatal life, when the most of transferred maternal IgG is catabolised and the own newborn IgG production is still not well developed. Newborn´s B cells are capable of the production of specific antibodies against the protein antigens already at the time of delivery but antibodies against polysaccharide structures are not formed until 2 years of life.

The immaturity of neonatal immune system is reflected in antigen presenting cells as well. Schaub et al. described insufficient function of antigen presenting cells, Th1 and Tregs at birth [58]. In relation to allergy, most research was focused on Th1/Th2 with the conclusion, that newborns had generally lower Th1 immune response with comparison to adults and that children more prone to allergy development (children of allergic mothers) had decreased production of IFN-gamma.

Colostrum (early milk) and milk are not only the source of optimal nutrition for a newborn but also the supply of immunologically active components. There is

no doubt about beneficial effect of breastfeeding but is questionable whether milk from healthy and allergic mothers is of the same quality or whether there exist differences in immunologic characteristics depending on allergy status of the mother [59]. Both cellular and humoral components of the immune system are present in colostrum/milk. In human colostrum, live cells are present in the concentration up to 10^7/ml, the number is decreased in mature milk. These cell are immunologically active and can protect the newborn through the mother's previous immune experience and by the supply of active cytokines, which can support the postnatal development of both Th1 and Th2 immune responses [60]. The uptake of living colostral cells from the intestinal tract was well described in animals: in cows [61], in sheep [62], in pigs [63;64]. Colostral cells entered the offspring's immune system [65;66] and preferentially settled down in neonatal liver [67]. In humans, it can be speculated whether colostral lymphocytes can cross intestinal barrier. There are some indirect proofs in this respect (e.g. transfer of reactivity to tuberculin from the mother to the newborn [68;69]. It is questionable to what extent can small number of transferred maternal lymphocytes influence the immature immune system (e.g. could the lymphocytes from an allergic mothers skew the immature immune system of her newborn to more prolonged predominance of Th2 immune responses and to increased potency for allergen sensitisation). There is limited number of studies dealing with the capacity of these lymphocytes to release cytokines [60]. Most of studies were focused on comparison of cytokines present in colostrum and mature milk of healthy and allergic mothers. [70-77]. Transfer of functional cytokines from colostrum/milk through the intestinal wall into offspring was also well documented [78;79]. Nguyen et al. [79] documented the capacity of these transferred cytokines to modulate immune responses of the offsprings (e.g. increased IgA production, suppression of neonatal immune responses). The proportion of colostral/milk cytokines transferred through intestinal wall in humans and their possible role in modulation of newborn immune system remains to be determined. This question is of particular importance because of known differences in cytokines concentration between maternal milk of healthy and allergic mothers [80-83].

It can be concluded that many internal and external factors influence the prenatal and early postnatal development of immune system and the history of the early development can have lasting implications for subsequent disease

development [1]. On the basis of epidemiological studies, it has been suggested that gene – environmental interactions during pregnancy could induce permanent changes in physiological processes and disease susceptibility [84]. This interactions involve changes in gene expression and disease predisposition via epigenetic mechanisms [85].

Allergy

Allergy can be defined as an aberrant immune response to relatively innocuous antigens from the environment. The name of allergy comes from Greek; allos – other and ergon – work and it was introduced by Viennese paediatrician Clemens von Pirquet. Later, it was discovered that different mechanisms are involved in immunological hypersensitivity which led to postulation of the new classification of immunopathologic conditions by Philip Gell and Robin Coombs in 1963. They suggested four main hypersensitivity reactions.

Type I – immediate hypersensitivity based on IgE antibodies – most common type of allergy. A milestone in the understanding of mechanism of allergy represented the discovery of IgE by Kimishige Ishizaka in 1960.

Type II – cytotoxic type based either on IgG and IgM antibodies and complement activation or on cytotoxic cells (phagocytes, NK)

Type III – immunopathologic reaction based on forming of antigen-antibody complexes (immunocomplexes) activating complement system with subsequent inflammation (neutrophil attraction and mast cell activation)

Type IV – late immunopathologic reaction (cell based delayed type hypersensitivity - DTH), activation of T cell and macrophages

It should be distinguished between atopy and allergy. Atopy is more general term meaning hereditary predisposition to the development of an immediate hypersensitivity reaction against common environmental antigens whereas allergy represents already manifested hypersensitivity of immune system.

"Atopic march" represents the theory supposing that predisposed individuals develop firstly mild atopic dermatitis then food allergy and still later allergic rhinitis and allergic asthma [86;87]. However, not all children follow this trend and develop allergic asthma [12]. Continuous increment of allergy diseases mainly in western countries is well documented in last three decades. Such an increase of allergy can be caused by lower microbial burden in industrial countries. This opinion is reflected

by popular hygienic hypothesis suggesting that a lack of early childhood exposure to microbes increases susceptibility to allergic diseases by the delay of neonatal immune system maturation. At the time of delivery, newborn immune system preferentially develops Th2 immune response [88] which supports allergy development. Th1 immune response is generally suppressed during pregnancy because of the prevention of maternal immune reaction to foetus alloantigens. Some studies emphasize the excessive suppression of Th1 immune responses [89-91], Tregs [92;93] and innate immunity [7;94-96] in neonates who later develop allergic diseases. Different research groups have tried to find out some early prognostic markers indicating an increased risk of later allergy development. Such prognostic markers could enable the introduction of preventive measures leading to limitation of allergy development or at least lowering clinical significance of allergy manifestation. Cord blood relatively easily available in sufficient amount is a good source of clinical material for searching such prognostic markers. Some investigators focused on comparison of cytokines present in cord blood sera of high risk newborns (newborns of allergic mothers) and low risk newborns (newborns of healthy mothers) [97-100].

Other researchers tested the capacity of cord blood cells to release individual cytokines after stimulation by various antigens (e.g. polyclonal stimulators such as phytohaemaglutinine, concanvalin A or G+/- bacteria, specific allergen stimulation) [101-103]. Cytokines were determined by various methods in different experimental settings which contribute to ambiguous results.

Total IgE levels in cord blood sera were supposed to be a good prognostic marker but unfortunately no convincing relationship between IgE level and future allergy development was proved. The testing of specific IgE (IgE antibodies against allergens) is more promising [104;105]. Recently, it was described that allergens and IgE can cross the placenta and bind to placental Hofbauer cells [106-108].

All these studies looked for some pre-symptomatic differences in the immune responses of newborns who develop allergic diseases later (reviewed e.g.by Prescott [52;53]). It was thought initially this markers could reflect the inherited genetic risk for allergy development [1]. As stated by Prescott, so far the only reliable marker indicating increased risk of allergy development is allergy status of the mother. However, strongly increasing incidence of allergic diseases [109] leads to the new hypothesis explaining the raise of allergic diseases as a complex alternation

of immune gene expression conferred by gene-environment interaction in utero. Thus, at least some of the environmental effects driving the rise in allergic disease may begin already in utero, and the differences in the neonatal immune functions may be the first signs of this increasing allergic predisposition [1].Recently, scientific interest is focused on epigenetic changes in perinatal period [110]. There is an increasing evidence that epigenetic modification can influence both innate and adaptive immune responses [111]. Changes in DNA methylation and histones acetylation regulate transcription activity of target genes via changed DNA conformation and altered accessibility of promotor regions for transcription factors. The evidence exists T cell development is under epigenetic regulation [112] including Th1/Th2 differentiation [113-117], Th17 [118] and Tregs and its transcription factor FoxP3 expression [119-121]. The epigenetic changes play a role in well documented lower level of IFN-gamma in cord blood sera in comparison to adults. Promoter of ifn-gamma gene is hypermethylated that leads to decreased transcription activity. Progressive demethylation last until adulthood [122]. Other mechanisms of epigenetic control involve histone acetylation and histone deacetylation mediated by histone acetyl transferase and histone deacetylase, respectively. Generally, histone deacetylation leads to gene silencing whereas histone acetylation supports transcription activity. It was described that events inhibiting histone deacetylase (e.g. oxidative stress) upregulated Th2 cytokine (IL-13, IL-5) and GATA-3-mediated T-cell responses [123;124]. Capacity of epigenetic regulation of immune responses could be possibly exploited for the treatment of allergic diseases by histone acetylation (promoting gene expression of genes supporting Th1 and Tregs development) and DNA methylation (suppression of gene expression of genes involved in Th2 mediated immune responses and IgE production) in the future. In murine model, maternal diet rich in methyl donor was described to enhance susceptibility to allergic diseases by increased DNA methylation [125].

Allergic reaction is triggered by binding of allergens to IgE antibodies bound on the surface of mast cells or basophils accompanied by crosslinking of high affinity IgE receptors (FcεRI) on these cells leading to degranulation of cells and release of vasoactive ammines (including histamine), lipid mediators (e.g. prostaglandin D, platelet activating factor, leukotrienes), chemokines (CXC chemokine ligand 8 – CXCL-8, CXCL-10, CC chemokine ligand 2 – CCL-2, CCL-4, CCL-5) and cytokines such as IL-4, IL-5, IL-13 causing manifestation of type 1 immediate

hypersensitivity reaction [126]. Degranulation of mast cells and basophils is facilitated by complex sequential events including protein and lipid kinases and phosphatases and also cytoskeleton rearrangements. Protein tyrosine kinases Lyn belonging to Src family is associated with the intracellular part of FcεRI in mast cells even before FcεRI activation. Cross-linkage of FcεRI activates Lyn, which in turn phosphorylates the tyrosines in the ITAM (immunoreceptor tyrosine-based activation motif) on the β and γ chains, initiating series of phosphorylation events involving phosphorylation of phospholipase C. These phosphorylation events induce the production of a number of second messengers, (e.g. inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG)) which facilitate degranulation of mast cells and basophils. IP3 increases Ca^{2+} levels, and DAG, together with Ca^{2+}, activates protein kinase C (PKC). Both activation of PKC and increase of intracellular concentration of Ca^{2+} activate degranulation. Higher membrane fluidity together with Ca^{2+} channels manifestation are results of methylation of various membrane phospholipids occuring within 15 seconds after cross-linkage of FcεRI. The peak of Ca^{2+} is reached already 2 minutes after FcεRI cross-linkage. This large increase of Ca^{2+} is due both to the uptake of extracellular Ca^{2+} and to a release of Ca^{2+} from intracellular stores in the endoplasmic reticulum. Changes in concentration of intracellular Ca^{2+} influence cytoskelet; namely by promoting the assembly of microtubules and the contraction of microfilaments, both of which are necessary for migration of granule to the cytoplasmatic membrane where degranulation occurs after the fusion of the granule with the membrane [127]. In addition, the Ca^{2+} increase, along with the induction of a mitogen-activated protein kinase (MAPK), result in both cytokine production and the activation of the enzyme phospholipase A2 (PLA2). Membrane phospholipid hydrolysis by PLA2 causes the formation of arachidonic acid, which is then converted into two classes of potent lipid mediators: prostaglandins and leukotriens. The key role of the Ca^{2+} increase in mast-cell degranulation is exploited for drug development (e. g. currently available disodium cromoglycate), preventing the Ca^{2+} influx as a treatment for allergies [127]. At the same time of phospholipid methylation and Ca^{2+} increase, there is a transient increase in the reactivity of membrane-bound adenylate cyclase producing cyclic adenosine monophosphate (cAMP). cAMP reaches the peak already about 1 minute after the cross-linkage of FcεRI. cAMP activates cAMP-dependent protein kinases, which targets are proteins on the granule membrane. Result of these phosphorylation

events is the increase of the permeability of the granules to water and Ca^{2+} leading to the swelling of the granules which facilitates their fusing with the plasma membrane followed by the release of their contents. Transient increase in cAMP is followed by a decrease of cAMP to levels even below the baseline. This drop in cAMP appears to be necessary for degranulation to proceed; when cAMP levels are further increased by certain drugs, the degranulation process is blocked which is exploited for allergy treatment [127]. Products released by mast cells and basophils activate secondary effector cells such as esosinophils, neutrophils, T lymphocytes, monocytes and platelets. Factors mediating effector allergic reaction are divided on the basis of their stepwise release and time sequence of their function on primary and secondary. The primary mediators are already present in granules before degranulation. To primary mediators belong histamine, proteases, eosinophil chemotactic factor, neutrophil chemotactic factors and heparin. The secondary mediators are produced after cell activation or breakdown of membrane phospholipids during the degranulation process. The secondary mediators include platelet-activating factors, leukotrienes, prostaglandins, bradykinins and various cytokines and chemokines.

Histamin is a dominant compound present in the granules of mast cell representing 10% of granule weight. Binding of histamine to its appropriate receptors leads to the contraction of intestinal and bronchial smooth muscles, increase venules permeability and induction of mucus production by goblet cells [127].

There is a broad spectrum of cytokines released during allergic reaction. Mast cells produce IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, GM-CSF, TNF. Some of them act in neutrophil and eosinophil attraction, especially IL-5. IL-4 and IL-13 are well-known for induction and promoting Th2 immune response. Furthermore, IL-4 is responsible for IgE class switching. High levels of TNF-alpha could lead to systemic anaphylaxis. After 6-12 hrs late phase of allergic reaction occurs due to the migration of allergen specific T cells, which are reactivated and clonally expand under the influence of chemokines and other cytokines present at the site of allergen exposure. The cellular chronic late phase response, which is mostly driven by allergen specific T cells activated by continuous allergen exposure, is the driving force for the persistent inflammation and/or tissue remodelling response for the chronic symptoms of allergic disease[126].

Dendritic cells (DCs) as a main antigen presenting cells are responsible for setting the tolerance or inducing various immune responses including Th1, Th2, Th9, Th17, Th22, Tregs (Th3, Tr1). After encounter with antigens in periphery, dendritic cells migrate to T cell rich areas of regional secondary lymphoid tissue to exert effector functions. Two main subpopulations of DCs are recognized in human: myeloid DCs (mDCs) originated from common myeloid progenitor cells are capable of preferential priming of Th1 immune responses in certain cytokine milieu. mDCs express Toll like receptors (TLR) 2, TLR6, TLR 8. After bacterial stimulation, mDCs release IL-12. Second one, plasmacytoid DCs (pDCs), originate from common lymphoid progenitor cells and preferentially support theTh2 immune responses. pDCs are called also IPC (interferon producing cell). Indeed, these pDCs release a huge amounts of interferons type I playing a critical role in antiviral defense. TLR typical for pDCs are TLR3, TLR7 and TLR9 important for virus recognition.

Another subsets of DC are present in sites of inflammation in the skin – typical Langerhans cells inducing Th1 response and inflammatory dendritic cells supporting the Th2 response [(126)].

DC are a heterogenous cell population with a variety of subpopulation described recently (e. g. rDC – regulatory DC [(128)], tDC - tolerogenic DC [(129)], intersticial DC, ld DC - low differentiated DC with increased presence in newborns [(130)]).

According to maturational status, DCs are capable of effective priming of different immune responses or preferential tolerance setting. The role of DCs and resulting immune response are influenced by type, dose and way of administration of antigen and by cytokine milieu.

T cells

Previously well accepted Th1/Th2 paradigm is now challenged by several new Th subsets described (Th9, Th22, Th17) and we can expect discoveries of further subsets of Th subpopulations. IL-12 is main cytokine responsible for Th1 induction with typical transcription factor T-bet and Th1 cytokines (IL-2, IFN-γ). Presence of IL-4 is critical for Th2 polarisation. Th2 cells can be characterised according to dominant transcription factor GATA3 and cytokines production of IL-4, IL-5, IL-13, IL-25, IL-31, IL-33.

Th1 and Th2 cells can be further characterized by chemokines secretion and presence of appropriate cell surface chemokine receptors. Th1 CXCR3, Th2 OX40, thymic stromal lymphopoietin (TSLP).

Peripheral T cells clones differentiate into these subsets using self reinforcing transcriptional circuitries which involve major transcriptional regulators: T-box expressed in Th1 cells (transcriptional factor T-bet), trans-acting T-cell specific transcription factor (GATA-3) in Th2 cells, forkhead box P3 (FoxP3) acting in Tregs, and retinoid related orphan receptor (RORgt/RORa) in Th17. Presence of IL-4 is necessary for inducing Th2 immune response. Allergen specific Th2 cells induce IgE class switching via release of IL-4 and IL-13 [126]. Th2 cells mediate IgE responses and defence against parasites. It should be emphasized that even Th1 cells are involved in the pathogeny of allergic diseases – Th1 response plays a critical role in asthma and atopic dermatitis [131;132].

Newly described subpopulation of Th9 is induced by IL-4 and TGF-beta and secretes IL-9 and IL-10. Th17 is induced by IL-6, TGF-beta, IL-21 and IL-23. Characteristic transcription factor for Th17 is RORγt. Th17 can be characterized by release of IL-6, IL-8, IL-17A, IL-17E, IL-22, IL-26. Th22 is characterized by IL-22 secretion. Induction of Th22 is supported by IL-6 and TNF-α.

Furthermore, very recently described population of nuocytes have a strong potential for promoting allergic reaction via huge secretion of IL-13 [133].

Regulatory T cells play an important role in prevention and regulation of allergy. It is supposed that deficit in numbers or function of Tregs is associated with various diseases including allergy [134]. It is well documented in individuals with FoxP3 mutations (IPEX – immune disregulation polyendocrinopathy, enteropathy, X-linked syndrome) who are more prone to development of allergic diseases [135;136]. Involvement of Tregs in allergy changed the Th1/Th2 paradigm. Tregs are responsible for setting an homeostasis, tolerance to certain antigens, controlling immune responses, inhibition of allergen specific effector T cells [126]. The imbalance between particular Th subsets accounts for different disease development including allergy. Regulatory T cells play a critical role in prevention of autoimmune diseases as documented in various animal models where rescue of number and/or function of Tregs prevent or reverse autoimmune diseases [137].

Three basic mechanisms of action of Tregs are supposed. Release of cytokines with regulatory properties: IL-10, IL-35, TGF-β. IL-10 and TGF-beta can support Ig

class switching and production of non-inflammatory antibodies IgG4 and IgA, respectively. Release of anti-inflammatory cytokines IL-10 and TGF-beta is one of the underlying mechanisms of specific allergen immunotherapy (SIT) [138-141].

The second mechanism suggested is mediated by cell-to-cell contacts via cell surface molecules present on the Tregs surface: CTLA-4 (cytotoxic T- lymphocytes antigens 4), LAG-3 (lymphocytes activation gene-3), PD-1 (program death domain 1), OX40, GITR (glucocorticoid-induced TNF-related protein). Binding of CTLA-4 to CD80 or CD86 on antigen presenting cells transmits an inhibitory signal to T cells[142]. CTLA-4 on the surface of Tregs dowregulates CD80 and CD86 on the DCs [143]. Similarly, ligation of PD-1 (another member of CD28/CTLA-4/ family) with PD-L1 (typically present on DC and macrophages, T and B lymphocytes) and PD-L2 (present only on DC and possibly in several cancer cells) inhibit immune responses [144;145]. LAG-3 was firstly described on activated T and NK cells but later also on pDC, NKT, B cells, TIL, exhausted CD8 and Tregs. LAG-3 shows similarity with CD4. LAG-3 is natural ligand for MHC II. LAG-3 could be defined as cell intrinsic inhibitory molecule [145]. Ligation of OX40 on T cells and OX40 ligand on APC sustain immune response and develop immune memory but less is known about the effect of OX40 on Tregs [146]. Nevertheless, ligation of OX40 molecule present on cell surface of Tregs with OX40L on mast cells leads to the mast cell degranulation inhibition [147]. Interaction of GITR (glucocorticoid-induced TNFR-related protein) with its ligand present mostly on APC and endothelial cells suppresses Tregs and activates effector T cell responses [148].

The presence of alpha subunit of high affinity receptor for IL-2 (CD25) is supposed to implicate the third mechamism. IL-2 attracted by these Tregs with high affinity receptor is unavailable in sufficient amount for the growth and differentiation of effector T cells. Tregs compete for specific antigens with other T cells.

Regulatory T cells a very heterogenous population. Two main subgroups of Tregs can be recognized; naturaly occuring Tregs (nTregs) developing in the thymus and induced/adaptive Tregs (iTregs) induced in periphery. Recently, two subtypes of nTregs have been described according to the presence of ICOS; ICOS- and ICOS+ which exert their immunosuppressive function via production of regulatory cytokines TGF-beta and TGF-beta, IL-10, respectively [149]. Several subsets of iTregs more or less characterized by certain phenotypes were described using combination of cell surface and intracellular markers. iTreg subsests known

so far include Tr1, Th3, Th1 like regulatory cells [150], TS1, TS2, iTr35. Other cells with regulatory properties exist: tolerogenic DC termed DC-10. DC-10 are CD14+CD16+CD11c+CD11b+CD83+HLA-DR+CD1a-CD1c, display mature myeloid phenotype and express high levels of tolerogenic markers immunoglobulin-like transcripts ILT-2, ILT-3, ILT-4 and HLA-G. DC-10 produce large amount of IL-10, low amount of IL-12 and are potent inducers of Tr1 [134].

Tr1 produce high levels of regulatory cytokines IL-10 and lower amount of TGF-beta. Tr1 arise in periphery when naïve T cells are activated by tolerogenic antigen presenting cells in the presence of IL-10 [134].

FoxP3 is transiently expressed also in activated effector cells [151;152] therefore it is unreliable to characterize Tregs only on basis of FoxP3 detection.

Surprisingly, increased proportion of IL-10 producing macrophages was detected in allergic patients in comparison to healthy control [153].

Tr1 induction plays an important role in protection of beekeepers against bee venom. This subpopulation of Tregs varies during the season [154].

The current therapy of hypersensitivity relies mostly on antihistamine drugs. In severe hypersensitivities, corticosteroids are administered leading to general decrease of immune responses. Capacity of corticosteroids to induce Tregs was described [155]. Specific allergen immunotherapy (SIT) is a promising way for treatment of some types of allergy (e.g. pollen allergy). SIT actually represents the only really specific therapeutic approach for allergy treatment. The induction of specific allergen tolerance is the essential immunologic mechanism of SIT [156]. Successful therapy is reflected by increased ratio of specific IgG4/IgE. Also induction of regulatory T cells (iTregs) both CD25+FoxP3+ and Tr1 is well documented. It seems that induction of Tr1 during the SIT is the most important goal [139-141;157]. Maybe increased presence of IgG4 antibodies is mediated by Tregs because Tregs are known by its production of regulatory cytokines IL-10, IL-35 and TGF-beta and IL-10 promotes IgG4 formation while TGF-beta supports IgA class switching. IgA can decrease allergen concentration by immune exclusion. All cytokines mentioned above generally suppress immune responses thus potentially decreasing pathologic Th2 immune response. Changed allergen memory T cell and B cell responses supporting the specific allergen tolerance development were described after SIT [138;158;159].

The possible use of probiotics in allergy treatment or prevention is intensively discussed in last years.

Probiotics

FAO (United Nations Food and Agricultural Organization) and WHO (World Health Organization) defined probiotics as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001).

Probiotics are capable to stabilize gut microbiota. Microbial colonization of newborn intestine plays an important role in early ontogeny. Mucosal microbiota supports the maturation of newborn immune system. It is important to keep in mind that there is 500-1500 different bacterial species residing human gastrointestinal tract. Most of them are non-cultivable bacteria and are identified only on the gene level according to 16S rRNA detected by powerful tool of pyrosequencing. This technique enables us to distinguish particular strains, reveal proportion of different bacteria and imagine the complexity of gut microbiota.

Prebiotic is non-digestible food ingredient promoting growth of probiotic. The term symbiotics stands for combination of probiotics and prebiotics.

History of probiotics goes back to the beginning of the 20th century when Elie Metchnikoff suggested the possibility of modifying gut microbiota by replacing harmful microbes with useful ones. He noticed the Bulgarian peasants and people from Russian steppes living largely on milk products fermented by lactic acid bacteria are exceptionally long living.

Henry Tissier was the first who isolated bifidobacteria from the breast fed infants and named them Bacillus bifidus communis (later renamed to Bifidobacterium). Alfred Nissle isolated probiotic Escherichia coli from faeces of a soldier who did not suffer from shigellosis during the 1st world war. In 1935, Lactobacillus acidophilus was acknowledged for beneficial effect in humans suffering from chronic constipation.

Actually, the term of probiotic was introduced by Werner Kollath in 1953 meaning that probiotics are microbial factors promoting the growth of other microorganism. In 1989, Roy Fuller suggested the present definition of probiotics.

Nowadays, there is a broad spectrum of different probiotic preparations from Gramm-positive (lactobacilli, bifidobacteria) and Gramm-negative (*E. coli*). The probiotic effect is strictly strain specific [160] and it seems that a combination of several bacteria or strains have increased impact [161-163].

Beneficial effect of probiotics is being described in various disorders including atopic dermatitis [164;165], allergy [166;167], eczema [168], inflammatory bowel diseases [169], Helicobacter pylori caused gastritis [170] common cold infections [171] and reduction of side effect of antibiotics[172]. Furthermore, probiotic administration is highly recommended in preterm neonates for prevention of necrotising enterocolitis (NEC) as reviewed by Deshpande at al. [173] and nosocomial and gastrointestinal infection [174].

Although beneficial effect of probiotics on human immune system is well acknowledged, the mechanisms of action is still poorly described. Probiotic administration is in an agreement with hygienic hypothesis. The majority of works concerning hygienic hypothesis emphasise the imbalance between Th1 and Th2 immune responses as the main mechanism of various health disorders [175]. Th1 and partly Th17 responses dominate in autoimmune diseases (e.g. multiple sclerosis, type I diabetes mellitus) and inflammatory bowel diseases (IBD). Diseases caused by immunopathologic Th2 immune response are represented by allergy, asthma, atopic dermatitis. Exposure of newborns´ Th2 skewed immune system [88] to microbial antigens supports its maturation, the onset of optimal Th1/Th2 balance and the protection against allergic diseases [58]. Tregs are also supposed to play a key role in setting the immunological homeostasis. Mechanisms of probiotic effects include TLR signaling pathways [176]. It is supposed both proportion and function of dendritic cells and T cells are influenced. It is still not clear whether probiotic interact with DC and activated DC promote then the development of regulatory T cells or whether these processes are parallel.

Uterus environment is believed to be sterile therefore microbial colonization of newborns occurs only after the delivery. The first encounter with microbes takes place during the passage through birth canal. The effect of the mode of delivery (vaginal versus caesarean section) on microbial colonization of the child and the risk of later allergy development was studied [177-181]. Data obtained were quite inconsistent but the increased risk of development of IgE mediated food allergy in children born by caesarean section was quite obvious [181]. Bifidobacteria are

pioneering bacteria colonizing neonatal gut therefore they might be the most important ones involved in immune system maturation and gut development.
Some studies describe correlation between composition of microbiota and allergic diseases [182-186].

It is generally accepted the highest effect of probiotics can be seen after their early postnatal administration or after the use of antibiotics. There is emerging evidence of intrauterine effects of probiotics [187]. As shown in epidemiologic studies, maternal exposures to high microbial burden (e.g. in farming families) is associated with lower allergy incidence in children [7;188]. Other studies documented increased incidence of allergy in newborns of mothers who moved from less developed countries (with generally increased exposure to more variable microbial burden) to developed countries [189-191]. Probiotic bacteria can hardly compete with the about 10^{14} bacteria of intestinal microbiota in the adhesion to mucosal membranes. Therefore, probiotic bacteria are usually not able to survive in GIT longer time and have to be applied repeatedly.

It is necessary to consider the need of the optimal dose of probiotic for reaching the optimal effect. Only the sufficient amounts of live probiotic bacteria are capable to influence the host immune system. Orally administered probiotic bacteria have to overcome the action of gastric acid, bile and competing microbiota [192;193].

Probiotics can influence immune system both directly and indirectly. They adhere to intestinal epithelial cells, thus modulating composition and activity of intestinal microbiota and competing with pathogenic bacteria for nutrients and space [194]. Probiotics can stimulate both antigen presenting cells and regulatory T cells, the release of immunoregulatory cytokines and immunological tolerance onset.

Colinfant Newborn

The development and registration of Czech probiotic vaccine Colinfant New Born containing Escherichia coli strain O83:K24:H31 was based on long positive experience of Dr. Lodinová-Žádníková [195-197] with administration of this bacterial strain to preterm neonates for preventing diarrhoea and nosocomial infections. The choice was based on the observation that this strain is harmless for newborn precolostral piglets. The strain has several convenient properties, especially excellent epithelial adhesion, long survival in intestine, absence of plasmids and sensitivity

to antibiotics. Retrospectively, it was discovered that Colinfant colonized children suffered significantly less from allergic diseases [195]. This led to the new currently on-going study trying to elucidate the mechanism of beneficial effect of Colinfant on immature newborn immune system. Several immununologic markers were compared among colonized children of allergic mothers and non-colonized children of healthy and allergic mothers [198;199].

This PhD thesis is based on the following papers:

1. 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

2. 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

3. 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

4. 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

5. 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

6. Differences in immunological characteristics of Tregs in cord blood of children of healthy and allergic mothers.

Hrdý J, Kocourková I, Prokešová L. submitted

7. The Effect of Probiotic Colinfant on Regulatory T-cells in Six Year Old Children.

Hrdý J, Kocourková I, Prokešová L. submitted

Aims

Main aims

1. One of the main aims of this thesis was to follow perinatal events playing a role in the tuning the immature immune system of newborns with the possible effect on future allergy development.

2. The second main aim was to evaluate the beneficial effect of probiotic vaccine Colinfant New Born on the immature newborn immune system 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 Tregs cells responses in cord blood cells of children of allergic mothers (children with increased risk for allergy development) and children of healthy mothers (children with relatively low risk for allergy development).

1b) to compare the *in vitro* reactivity of cord blood cells (CBMC, DC) of children of allergic mothers (children with increased risk for allergy development) and children of healthy mothers (children with relatively low risk for allergy development)

1c) to compare the proportion and functional properties of Tregs in cord blood of children of allergic mothers (children with increased risk for allergy development) and children of healthy mothers (children with relatively low risk for allergy development)

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

Results

Tremendous increment of allergic diseases has become one of the main health problems nowadays. Allergy represents not only medical issue but also and socio-economical problems. Therefore, there is urgent need to find some early prognostic markers indicating increased risk of allergy development. Finding the suitable prognostic markers could enable us introducing preventive measures hindering the allergy development. Cord blood seems to be a suitable biological source for searching such a prognostic marker.

Early probiotic administration to high risk infants is believed to be a good preventive precaution for lowering the risk of later allergy development. Significantly lowered allergy incidence in children colonized postnatally with probiotic vaccine Colinfant New Born was reported. Unfortunately, mechanisms of beneficial effect of probiotic on newborn immature immune system are still poorly understood.

The study is based on the following of 3 groups of children: children of allergic mothers (i.e. children with high risk of allergy) postnatally colonized or non-colonized with probiotic vaccine Colinfant New Born and the control group of non-colonized children of healthy mothers. Immunological characteristics were tested at birth by analysing the cord blood. Children were then longitudinally followed in the course of several years. The work was made possible by our close cooperation with the Institute for the Care of Mother and Child where the children were born and continuously monitored by paediatricians.

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.

(Attachment 1)

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. We evaluated the effect of maternal

milk from healthy and allergic mothers on proliferation activity and immunoglobulin production capacity of cord blood mononuclear cells of children of healthy and allergic mothers. We tested the hypothesis that maternal milk from allergic mothers could be less beneficial in comparison to milk from healthy mothers for breastfed infants. Any significant differences between effects of colostrums/milk of healthy and allergic mothers were found. Beyond expectation, we revealed the higher in vitro reactivity of CBMC of high risk newborns (children of allergic mothers).

Effect of maternal milk on proliferative activity of cord blood cells

We tested the effect of non-cellular components of colostrum (early milk) and mature milk from healthy and allergic mothers on *in vitro* proliferative activity of cord blood cells of children of healthy and allergic mothers. We observed that effect of colostrum and milk is dependent on concentration used. Specifically, suppressive effect of both colostrum and milk was observed only when milk was added non-diluted regardless of allergy status of mothers. On the contrary, the lower concentration of maternal milk/colostrum which is more probably present 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. Surprisingly, we proved significantly increased proliferation rate of cord blood cells of children of allergic mothers in comparison to children of healthy ones in non-stimulated and polyclonally stimulated cell cultures regardless of effect of maternal milk from both healthy and allergic mothers.

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

The capacity of maternal milk from healthy and allergic mothers to influence *in vitro* immunoglobulin secretion by cord blood cells of children of healthy and allergic mothers was tested. There was only marginal production of immunoglobulins by non-stimulated cord blood cells. After polyclonal stimulation by *Bacillus firmus* – BF (a polyclonal activator of B lymphocytes), immunoglobulin production was markedly increased, mainly by cells of children of allergic mothers. Immunoglobulin secretion by cord blood cells was further conspicuously increased

by colostrum/milk addition. We did not observed significant difference between the effect of maternal milk/colostrum from healthy and allergic mothers on immunoglobulin formation. The increment of immunoglobulin production was observed only in polyclonally stimulated cultures, the adding colostrum itself to otherwise non-stimulated cell cultures increased basal cord blood cells immunoglobulin production only marginally. Colostrum of both healthy and allergic mothers stimulated slightly better Ig formation in healthy group but differences between healthy and allergic group were not significant.

We did not observed any difference in maternal milk/colostrum from healthy and allergic mothers on immunoglobulin formation and proliferation activity of cord blood cells. From this point of view, there is no occasion to be afraid of any negative influence of the milk of allergic mothers. On the other hand, striking difference was described in the proliferation activity of cord blood cells. We described significantly increased proliferation activity of both non-stimulated and polyclonally stimulated cord blood cells of children of allergic mothers in comparison to children of healthy ones. This could reflect generally increased reactivity of the immune system of children of allergic mothers. We hypothesise that increased reactivity could lead to inappropriate immune responses in allergy predisposed children after eventual 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.

(Attachment 2)

It is believed that probiotic administration could influence immune system and importantly, it was reported that probiotic bacteria could reduce allergy incidence. It was described in the previous study of Dr. Žádníková-Lodinová that early colonisation of newborns by probiotic vaccine Colinfant New Born (*E. coli* O83:K24:H31) significantly reduced future allergy incidence. In the current study, further groups of newborns have been colonized and several immunological

parameters were followed in the effort to elucidate the mechanisms of the beneficial effect of Colinfant New Born in the prevention of allergy development.

Significantly decreased allergy incidence in colonized children

Convincingly decreased allergy incidence in Colinfant New Born colonized children of allergic mothers was proven. Although several earlier reports described a decrease of allergy incidence after probiotic administration, in most studies only marginal effect was achieved. There are two main reasons why in our study the significant effect of probiotic colonisation was recorded. Firstly, children were colonized within 48 hrs after delivery which facilitated E. coli to be one of the pioneer bacteria colonizing newborn intestine. The niches are not yet occupied by other microbiota. The early probiotic administration is supported by popular hygienic hypothesis assuming that lower microbial burden in perinatal life could lead to improper maturation of newborn immune system facilitating allergy development. Secondly, *E. coli* O83:K24:H31 used was equipped with fimbriae type 1 and had strong adherent properties enabling its long persistence in the intestine which is not common with other probiotic bacteria. It was possible to detect our *E. coli* strain in children intestine even several years after the administration. It is important to emphasise the large differences exist not only among different probiotic bacteria but also among the particular strains

Cytokines in peripheral blood of colonized children of allergic mothers and non-colonized children of healthy and allergic mothers

Due to the huge individual variability and often non-detectable levels of some cytokines, it is difficult to draw any firm conclusion which cytokines are the most influenced by probiotic colonisation. The pro-allergic 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 approximate 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 (Praha). 2010 Sep;55(5):515-9.

(Attachment 3)

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-gamma) 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 allergic mothers in comparison to healthy ones was detected but these differences were insignificant mainly due to the huge individual differences.

Concentration of cytokines in sera of cord blood

On protein level, data did not reached such statistical significances as seen on gene expression level. Though, a tendency to the increased levels of Th2 cytokines (IL-4, IL-13) and decreased levels of typical Th1 cytokine - IFN-gamma in sera of cord blood of children of allergic mothers in comparison to children of healthy ones was evident. Of note, significant differences were observed in IL-13 and EGF (epidermal growth factor). Furthermore, we can see a tendency to the lower levels of TGF-beta in cord blood of children of allergic mothers. Interestingly, decreased levels of both TGF-beta and EGF in sera of cord blood of children of allergic mothers could contribute to the delayed maturation of newborn intestine thus leading to a prolonged permeability increase of intestine for antigens enabling easier allergen sensitisation with possible future allergy development.

The evidence was brought that pro-allergic phenotype is already evident on the level of cord blood. General trend to increased levels of Th2 cytokines and decreased levels of Th1 cytokines in cord blood of children of allergic

mothers in comparison to children of healthy ones is quite obvious on the level of gene expression and protein secretion. It is important to keep on mind that cytokines in cord blood sera are not entirely produced by cord blood cells but also by many other cells namely epithelial ones. Interestingly, some cytokines could be also of maternal origin. These factors could contribute to the partial discrepancies between gene expression level and protein levels 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 (Praha). 2012 May; 57(3):215-9.

(Attachment 4)

It is well known that maternal milk contains not only nutritional compounds but also immunologically active components both humoral and cellular. Beneficial effect of breastfeeding is well acknowledged but it still remains unresolved whether maternal milk from allergic mother is of comparable quality with maternal milk from allergic mothers. In animal studies, passage of milk leukocytes through the intestinal wall and entrance into offspring immune system were described. There are also indirect proofs of transintestinal passage of colostral cells in humans. Thus, it is possible to suppose certain effect of ingested colostral cells on the newborn immune system. In the current study, we compared gene expression of cytokines in colostral cells of healthy and allergic mothers.

We focused only on colostrum because there is a substantially higher count of live leukocytes in colostrum in comparison with mature milk where death and epithelial cells are predominant. More pronounced pro-allergic phenotype of colostral cells from allergic mothers was convincingly documented by increased gene expression of typical Th2 cytokines (IL-4, IL-13), decreased Th1 cytokines (IFN-gamma). Furthermore, gene expression of regulatory cytokines (IL-10, TGF-beta) in colostral cells of allergic mothers were found to be lower in comparison with gene expression in colostral cells of healthy mothers.

Colostral cells of allergic mothers could be characterized by increased gene expression of Th2 cytokines and decreased expression of Th1

and regulatory cytokines. It remains questionable, to what extent could the surely small number of fully functional maternal colostral cells skew immune responses of immature neonatal immune system. High concentrations of soluble colostral cytokines have certainly larger impact on the immune system of the offspring.

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

(Attachment 5)

Differences in humoral components of cord blood between children of healthy and allergic mothers were described by numerous authors although with contradictory results. However, 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. Dendritic cells are the most potent antigen presenting cells deciding about the both quantity and quality of the immune responses.

Gene expression of subunits of IL-12 family of cytokines

The capacity of non-stimulated and LPS stimulated mDC to express IL-12 family cytokine genes was tested. Cytokines of IL-12 family belong to heterodimeric cytokines and all their particular subunits have to be tested. Cytokines of IL-12 family play a key role in promoting Th1 immune response (IL-12, IL-23, IL-27). Furthermore, IL-23 supports Th17 immune response as well on the contrary, IL-27 inhibits Th17 response. Last member of the IL-12 family IL-35, has rather regulatory function. We have not observed any significant differences in the gene expression of IL-12 family cytokine subunits in non-stimulated mDC excepting p28. The expression of p28 in non-stimulated mDC of children of allergic mothers was increased in comparison with healthy group. P28 with its partner EBI-3 form IL-27. Interestingly, IL-27 is capable to promote IL-4 induced IgE production.

After LPS stimulation, we observed significantly increased gene expression of nearly all subunits tested - with the exception of p19 in mDC of children

of allergic mothers was proved. It points to the generally increased reactivity of mDC of children of allergic mothers.

Secretion of cytokines of IL-12 family by mDC of children of healthy and allergic mothers

Gene expression of IL-12 family subunits was compared with the production of appropriate heterodimeric cytokines in cultures supernatants of mDC. In the time of gene expression estimation (after 24 hrs stimulation), no cytokines were detectable. Three days of cultivation seems to be an optimal time for the detection of IL-12 family cytokines in cell culture supernatants. Almost no cytokines were detectable after cultivation of non-stimulated mDC. After LPS stimulation, we succeeded in detection of all cytokines but with less significant differences compared with gene expression. For the first time, we reported the secretion of heterodimeric IL-35 by mDC in the culture. So far, it was not clear whether DC are capable to produce IL-35 although the genes for both subunits, p35 and EBI-3, are expressed because these subunits can constitute also the part of another heterodimeric cytokines, IL-12 and IL-27, respectively.

Activation markers on 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 mDC of children of healthy and allergic mothers which were not artificially stimulated in the culture. Significantly increased proportion of CD83+ mDC was detected in cord blood of children of allergic mothers in comparison to healthy group after LPS stimulation. The increased proportion of CD83+ mDC in the group of children of allergic mothers further support our observation of increased reactivity of mDC in allergy high risk children.

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 reactogenity present in children

of allergic mothers could easily induce the positive effector immune responses (e.g. Th2) instead of tolerance after eventual 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

(Attachment 6)

This study represents a continuation and an extension of our previous studies comparing immunologic characteristics of cord blood cells of children of allergic mothers (children with high risk for allergy development) and children of healthy mothers (children with relatively low risk for allergy development). In the current study, we focused on the comparison of proportions and functional characteristics of regulatory T cells (Tregs) in cord blood. Regulatory T cells are known to be responsible for the induction of peripheral tolerance, e.g. 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.

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

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

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

In order to evaluate the functional ability of Tregs, median of fluorescence intensity (MFI) of FoxP3 and intracellular presence of regulatory cytokines (IL-10 and TGF-beta) were tested in cord blood cells. MFI of FoxP3 positively correlates with the suppressive capacity of Tregs. 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. Furthermore, significantly lower intracellular presence of IL-10 and TGF-beta in Tregs in cord blood of children of allergic mothers was detected.

The increased proportion of Tregs in cord blood of 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 are less functionally competent. We can suppose that functional impairment of Tregs in cord blood of 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 functional characteristics of these cells.

The Effect of Probiotic Colinfant New Born on Regulatory T-cells in Six Year Old Children.

Hrdý J, Kocourková I, Prokešová L.

Submitted

(Attachment 7)

In our previous studies, we reported significantly decreased allergy incidence in high risk individuals colonized by Colinfant New Born within 48 hrs after delivery. The mechanism of beneficial effect of probiotic 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

In the article discussed above, a decreased incidence of allergy in five years old children colonized immediately after birth with the Colinfant New Born was found. The children 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 colonized children

We did not prove any significant difference in the proportion of Tregs in peripheral blood among colonized children of allergic mothers and non-colonized children of healthy and allergic mothers. After subdivision of children according to allergy status into the six subgroups, 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 mother allergy status

and eventual probiotic colonisation, significantly increased proportion of Tregs in peripheral blood of children suffering from allergy was detected.

Functional characteristics of Tregs in peripheral blood of colonized children

Tregs were characterized by their functional characteristics, MFI of FoxP3 and intracellular presence of regulatory cytokines using flow cytometry. When MFI of FoxP3 of Tregs in peripheral blood of colonized children of allergic mothers and non-colonized children of healthy and allergic mothers was compared, 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 colonized children of allergic mothers was comparable with MFI of FoxP3 in Tregs of non-colonized children of healthy mothers. 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 nonallergic children regardless of the allergic status of their mothers and eventual probiotic colonisation was observed.

Insignificant differences in intracellular presence of IL-10 in Tregs in peripheral blood of children were observed when colonized children of allergic mothers and non-colonized children of healthy and allergic mothers were compared. The lowest intracellular presence of IL-10 was detected in non-colonized children of allergic mothers. Significantly lower IL-10 was observed in 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 comparing only according to their allergy status.

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

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. Surprisingly, the proportion of Tregs in peripheral blood of Tregs in children suffering from allergy was increased in comparison to healthy ones. It is possible to suppose impaired functional potency of Tregs of allergic children implicate increased proportion of Tregs which could partially compensate their insufficient function in allergic children.

Discussion

Allergy is one of the most common diseases with still increasing incidence. Such tremendous increment of allergies mainly in developed countries is starting to be termed pandemic [200]. Therefore, there is an urgent need to identify suitable prognostic markers indicating soon after birth an increased risk of allergy development in children. Finding such a prognostic markers enable us an early introduction of preventive measures. Cord blood seems to be a good source of clinical material for an assessment of such markers because it is abundant and relatively easily attainable. Cord blood taking does not represent any health risk neither for the mother nor for the newborn and it is not connected with further medical intervention.

Most of the studies compared immunologic parameters in cord blood of children of healthy mothers (supposed to be children of low risk of allergy development) with children of allergic mothers (children with relatively high risk of allergy development). It is generally accepted that maternal immune system has more pronounced impact on offspring's allergy status. Although both mother and father bring the same amount of genetic information to the offspring, maternal immune system interacts with the foetal organism during the 9 months of pregnancy. Various immunologically active components could be transferred via placenta and amniotic fluid and influence the tuning of newborn immune system. Further, mother can influence the immune system of child by breastfeeding. Maternal milk does not only represent the optimal source of nutrition but also supplies immunologically active components which could exert their activity in the newborn gastrointestinal tract or possibly pass undamaged through the intestinal wall and influence even the systemic immunity.

The issue of searching the prognostic markers indicating an increased risk of allergy development is complicated by the multifactorial causes of allergy. Different research groups have suggested various prognostic markers. The data in literature are inconsistent and often contradictory. This is caused by different methods used having different sensitivity, distinct cord blood sampling, variability during pollen seasons, diverse ethnicity, considering different markers (e.g. for Tregs characterization) and evaluation of promising markers on different levels (mRNA level by gene expression, protein presence). However, the main obstacle is

in the enormous heterogeneity of human population and diversified character and pathogeny of allergic diseases.

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 [100]. Although we proved quite convincingly allergic phenotype in children of allergic mothers on the level of gene expression of cytokines (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 does 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 [201-205] similarly as described by Hrdý et al. [206]. 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. [207;208]. Decreased gene expression of IL-4 in high risk infants in comparison with low risk ones was rather surprising for us but similar observation was noticed by Prescott et al. [88] who was not able to find any reasonable explanation for this anomaly as well. On the other hand, trend to the increased presence of IL-4 in cord blood sera of high risk children was reported [100]. We could conclude that IL-13 gene expression and its concentration in cord blood sera represent the more reliable predicative makers pointing to proallergic phenotype than IL-4 does.

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. [209;210] although another study reports difference in IL-10 producing capacity [211]. 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 healthy ones [100]. Our observation is in accordance with several other reports (e. g. [210]). 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.

It is important to notice that cytokine expression in cord blood cells need not fully correspond to cytokine concentration in cord blood serum for several reasons. Firstly, cytokines present in cord blood sera could be partly of maternal origin and could be transferred from the mother via placenta or by engulfing amniotic fluid whose constituents are partially of maternal and partially of foetal origin [(100)]. Furthermore, cytokines in cord blood sera are not exclusively produced only by blood leukocytes but also by many others mesenchymal and non-mesenchymal cells.

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 [(212)]. We have confirmed the importance of following of 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 [(199)].

Evaluation of the suitability of selected cytokines followed in cord blood as a prognostic marker indicating increased risk of allergy development is complicated by the controversial data present in literature. Nevertheless, our studies indicate that IL-4, IL-13, IFN-γ and TGF-β are the most predicative ones.

There is no doubt about beneficial effect of breastfeeding on the immature newborn´s immune system but less is known about the possible differences between milk of healthy and allergic mothers. Maternal milk is believed to prevent allergy development [(213)]. 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 [(212;214)], 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 gene expression of EGF in colostral cells of allergic mothers was observed by Hrdý et al. [59], the trend to allergic phenotype was documented by the increased gene expression of IL-4, IL-13 and decreased that of Th1 cytokine IFN-γ and regulatory cytokine IL-10 and a slight decrease of TGF-β [59]. This is actually the primary study comparing the gene expression of cytokines in colostral cells of healthy and allergic mothers. It is important to emphasize that in the animal models, the cross-intestinal transfer of live colostral cells was proved [215;216], and even in humans, the indirect proofs were reported [67;217]. 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 [218] which is in accordance with the other studies [213;219]. From the point of view 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 huge difference between the proliferation rate of cord blood cells of children of healthy and allergic mothers. Significantly increased proliferation of both non-stimulated and polyclonally stimulated cord blood cells of children of allergic mothers in comparison to children of healthy ones was observed in our study [218]. It points to increased reactivity of lymphocytes of high risk children which could support their future allergisation.

The notion 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 of cord blood cells 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. To evaluate the gene expression of IL-12 family cytokines, we had to test the both subunits of the members of IL-12 family cytokines because these cytokines are heterodimeric. To make the situation more complicated, some of the subunits are present not only in one heterodimer of the IL-12 family.

No difference in gene expression of the subunits of IL-12 family cytokines was observed between unstimulated 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 of all IL-12 family subunits in non-stimulated cord blood mDC of both children of heathy and allergic mothers was quite low which could be explained by general immaturity of newborn immune system. On the contrary, substantial gene expression of all subunits of cytokines of IL-12 family was recorded after LPS stimulation. The gene expression was significantly pronounced in mDC of children of allergic mothers in all subunits tested with exception to p19. The expression increase of the IL-12 family subunits after LPS stimulation was significantly lower in 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 median of fluorescence intensity (MFI) of CD83 were observed with mDC of children of allergic mothers. This observation points to the general increase of reactivity of mDC of 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 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. There is a positive correlation between the amount of FoxP3 and suppressive capacity of Tregs [220]. The other functional property of regulatory T cells is the release of regulatory cytokines IL-10, IL-35, 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 impaired function of Tregs were described. Treg-specific demethylated region (TSDR) was identified as another important marker indicating functional activity of Tregs [102;221]. Hinz et al. [222] described decreased proportion of Tregs characterized by TSDR in cord blood of children who develop 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 study of Treg function is embarrassed by their large heterogeneity and unsettled nomenclature. It will be necessary to clarify which subpopulation of Tregs is most handicapped in allergy high risk individuals to target effectively the possible preventive measures compensating regulatory insufficiency. It is possible to distinguish between nTregs and iTregs according to the expression of transcription factor Helios but no study dealing with this topic in cord blood is available till now. Actually, evaluation of nTregs and iTregs is of particular importance for the deeper characterization of immaturity of newborn immune system. The antigenic stimulation is very limited during intrauterine life. Low number of iTregs could be supposed. On the other hand, the increased number of iTreg in cord blood could reflect the intensity of possible intrauterine sensitisation. 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 further supported by hygienic hypothesis explaining the rise of allergic diseases in the developed countries by lower microbial burden and thus delayed newborn immune system maturation which is necessary for balancing Th1/Th2 immune responses because newborn immature immune system exerts proallergic phenotype with the predominance of Th2 bias. Actually, the increased Th2 immune response is necessary for the successful course of pregnancy [(223)].

Moreover, probiotics were proved to be capable of induction of Tregs as indicated by Kwon et al. [(224)]. The positive effect of probiotic on Treg induction 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 ensure effective probiotic colonisation otherwise it is difficult for a probiotic to colonize the gut where the microbiota is 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 to 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 [(195-199)]. 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 proportion of Tregs after subdivision of children into the subgroups according to their allergy status

except to significantly increased proportion of Tregs in allergic non-colonized children of allergic mothers in comparison with healthy non-colonized children of allergic mothers. However, when comparing children only according to their allergy status regardless of allergy status of their mothers and probiotic colonisation, significantly increased proportion of Tregs was found in peripheral blood of allergic children in comparison to healthy ones. Concerning the proportion 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. This perplexity is particularly caused by different set of markers used for characterisation of Tregs, estimation of Tregs in the whole blood or in isolated PBMC. In addition to that, the using of different clones of FoxP3 antibodies could lead to different values of Tregs ratio [225-227]. The different clones of FoxP3 antibodies can detect the different Treg subpopulations. In our early experimental setting, we used two antibody clones (PCH101 – eBioscience, 259D/C7 – Becton Dickinson) with appropriate buffers. Increased proportion of FoxP3+ Tregs was detected using PCH101, which was then used for further experiments. However, it is necessary to realize that the amount of Tregs alone is not decisive for effective suppression function [221]. 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 [228]. 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 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 there 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 [229-232].

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.

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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PEDIATRIC ALLERGY AND IMMUNOLOGY

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. Effect of breast milk of healthy and allergic mothers on *in vitro* stimulation of cord blood lymphocytes.
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Maternal milk has beneficial effects on the development and function of the newborn's immune system. Whether the milk of allergic mother has the same effects as the milk of healthy mothers is not yet quite clear. To contribute to the characterization of its immunomodulatory action, we tested the effect of milk of healthy and allergic mothers on the proliferation and immunoglobulin formation in cultures of cord blood mononuclear leucocytes (CBML) of newborns of healthy and allergic mothers. CBML proliferation was tested by ^{3}H-thymidine incorporation, IgM, IgG and IgA production by reverse ELISPOT. CBML response was examined in unstimulated cultures and after stimulation with polyclonal activators in the presence or absence of colostrum or milk. The cells of children of allergic mothers have a significantly higher proliferative activity than those of children of healthy mothers. Maternal colostrum/milk in high doses markedly suppresses cell proliferation after stimulation with polyclonal activators, whereas lower milk doses in the cultures have no such effect and exert a rather stimulatory action. Immunoglobulin production by cord blood lymphocytes is also different in the two groups of children. Low basal immunoglobulin formation is increased after stimulation with a strong polyclonal activator of B cells – *Bacillus firmus*, CBML of children of allergic mothers produce more IgA than those of children of healthy mothers. The stimulated production of all immunoglobulin classes in cells of children of healthy mothers is still enhanced by colostrum/milk. Children of allergic mothers show a markedly increased production of only IgM and IgA. The effect of healthy and allergic colostrum and milk on cell proliferation and immunoglobulin production is similar. The lymphocytes of children of allergic mothers differ from the lymphocytes of children of healthy mothers in their proliferative activity and the ability to form immunoglobulin already at birth.

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Key words: cord blood lymphocytes; cell proliferation; immunoglobulin formation; maternal colostrum and milk; healthy and allergic mothers

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Breast feeding represents not only an optimum food for infants in the early postnatal period, but also affects the development of the newborn's immune system. Colostrum and milk contain a number of cytokines, growth factors, antibodies and other soluble components regulating the immune response, and several cell types (macrophages, neutrophils, lymphocytes, epithelial cells) that take part in immune responses (1–3).

We can assume that the immunoregulatory components of colostrum and milk act not only locally in the gastrointestinal tract of the newborn, but can also cross the mucosal barrier and affect

both mucosal and systemic immunity because macromolecular substances are less degraded in the immature newborn's intestine; the possibility that intact proteins pass through the intestinal wall without losing their biological activity was repeatedly proved (4–6). The passage of cytokines with preserved biological activity was proved in animal models (7). The intestine of newborns has been found to contain live cells from the milk (8) and their passage through the intestinal wall was documented in sheep and calves (9, 10) and in baboons (11); it has been indirectly implicated also in humans (transfer of reactivity to tuberculin from the mother to the newborn; 12, 13). General consensus also exists about the beneficial effect of breast feeding on the infants; breast feeding is stated to lower the probability of later allergies, infections and some other pathological conditions (14, 15). This holds undoubtedly for the breast feeding of an infant by a healthy mother while there is some doubt as to the effect of breast feeding by allergic mother. Children of allergic mothers have an enhanced genetic predisposition for the development of allergy. Certain differences in the composition of milk of healthy and allergic mothers have been described (16–19); a question can be raised if the milk of an allergic mother could not affect the development of the newborn's immunity in a way different from that exerted by the milk of a healthy mother. Since an adverse effect of allergic milk has not been unambiguously documented, while the positive benefit of breast feeding is clear, allergic mother are not recommended to reduce the breast feeding (14). In this study, we investigated and compared the effect of soluble components of colostrum and milk of healthy and allergic mothers on the stimulation of cord blood mononuclear leucocytes (CBML) collected from children of both healthy and allergic mothers in order to contribute to solving the question whether the milk of allergic mothers affect the immune system of the newborn in a way different from that exhibited by the milk of healthy mothers.

Methods

Cord blood cells and maternal milk

Two groups of mothers and children according to mothers' clinical statement were used as donors: (i) Healthy individuals (H) – non-allergic mothers and newborn children of non-allergic mothers. (ii) Allergic individuals (A) – allergic mothers and newborns of allergic mothers. Cord blood was obtained from 17 children of healthy mothers and 16 children of allergic mothers. Colostrum was given by 14 healthy and nine allergic mothers, milk by seven healthy and 10 allergic mothers. All samples were obtained after informed agreement of the mothers.

Diagnostics of allergy in mothers was based on clinical manifestation of allergy persisting for more than 24 months (allergy against respiratory and food allergens manifested by various individual combinations of high fever, conjunctivitis, bronchitis, asthma, eczema, etc.), monitoring by an allergologist, positive skin prick tests or positive specific IgE antibodies and antiallergic treatment before pregnancy.

Colostrum and milk. Colostrum and milk samples were collected on the third day and in the sixth month after delivery, respectively. Mothers expressed their breast milk manually into sterile polypropylene tubes and stored it at 4°C for 1–2 h; the fat layer and cell pellet were then removed by centrifugation (8000 ***g***, 25 min) and soluble fraction was stored in aliquots at −20°C until processed. The effect of colostrum/milk on *in vitro* stimulation of CBMC was tested. CBMC could not be cultivated with the colostrum/milk of donor's own mother because it was not possible to obtain sufficient amount of colostrum at term and mature milk was taken 6 months after delivery. In each experiment, CBML were treated in parallel by both healthy and allergic colostrum/milk and one colostrum/milk sample was used for various CBML cultures.

Cord blood cells. Cord blood samples were collected in sterile heparinized tubes (10 U heparin/ml) immediately after delivery with the usual procedures including careful cleaning of the cord and strict puncture of umbilical vein to avoid maternal contamination. Cord blood mononuclear leucocytes were isolated on Ficoll-Paque, washed three times in MEM medium, incubated for 60 min to liberate cell-bound immunoglobulin (37°C, 5% CO_2), and again washed three times in MEM medium. Isolated CBML were used for testing proliferation and immunoglobulin formation *in vitro*.

Cell cultures

Cell cultures were performed in RPMI-1640 medium supplemented with L-glutamine (2 mM), HEPES – Sigma (0.002 M; Sigma-Aldrich, Munich, Germany), gentamicin (40 mg/l) and human transferrin (20 mg/l). The medium was further supplemented with 5% fetal calf serum (FCS) for proliferation assay or with 10% FCS

and 2-mercaptoethanol (25 μM) for immunoglobulin formation. Cultivation proceeded at 37°C in humid atmosphere with 5% CO_2.

Proliferation assay. Cord blood mononuclear leucocytes were cultured in triplicate (2×10^5 cells per culture) in 96-well polystyrene plates. Cells were cultured alone (controls), with various doses of colostrum/milk (HC, HM – colostrums and milk of healthy mothers; AC, AM – colostrums and milk of allergic mothers), with polyclonal activators – Phytohaemagglutinin Sigma (PHA; 10 μg/ml culture), Concanavalin A Sigma (ConA; 5 μg/ml culture), heat-inactivated *Escherichia coli O86* – EC (10^9 bacteria/ml culture), Nocardia Delipidated Cell Mitogen (NDCM; 20, 21; 100 μg/ml culture) kindly donated by Prof. H. Tlaskalová, and formaldehyde-inactivated *Bacillus firmus* CCM 2212 (BF; 22, 23; 100 μg/ml culture) and with polyclonal activators + colostrum/milk. The cultures were incubated for 48 h. After 48 h, the cells were pulsed with 1μCi per well of ^{3}H-thymidine and 18 h later harvested on glass fibre Filtermat A (Wallac, Turku, Finland) by Harvester 96 Mach 3 (Tomtec, Orange, CT, USA) and counted in 1450 MicroBeta (Wallac) using melt-on scintillator 1450-441 Meltilex A (Wallac). Results are expressed as counts per minute (cpm).

Immunoglobulin formation. CBML were cultured in duplicate in polystyrene tubes (10^6 cells in 1 ml culture) in supplemented RPMI-1640 medium as described above. Cells were cultured alone (controls), with colostrum/milk in a final dilution of 1:100, with polyclonal activators of B lymphocytes (EC, BF), with Pokeweed mitogen Sigma (PWM; 1 μg/ml) and with polyclonal activators + colostrum/milk. Immunoglobulin-producing cells were detected by reverse ELISPOT (24). After 6-day cultivation, the cells were washed. 96-well ELISPOT plates with nitrocellulose bottom (Millipore MultiScreen$_{HTS}$; Millipore, Bedford, MA, USA) were coated overnight with 100 μl of goat antihuman immunoglobulin antibodies (anti-μ, anti-γ or anti-α antibodies; Immunotech, Birmingham, UK) in 0.1 M sodium carbonate buffer pH 9.6, washed and blocked 30 min with cultivation medium containing 10% FCS. Then, washed cells were applied (10^5 cells per well) in triplicate and cultivated overnight. On the next day, the cells were discarded and the plates were processed in a similar way as in the ELISA method but with 3-amino-9-ethylcarbazole (AEC) as substrate. Spots were counted by binocular stereomicroscope (Motic, Wetzlar, Germany). Results are expressed as the number of spots (corresponding to immunoglobulin-producing cells)/10^6 cells.

Statistics

The data are presented as mean ± s.e.m. Differences between groups were evaluated using nonparametric Mann–Whitney *U*-test. A probability level $p \leq 0.05$ was considered significant.

Results

Proliferation of cord blood mononuclear leucocytes

The processes to be studied included spontaneous proliferation of cells alone, proliferation of cells cultured with different dilutions of colostrum/milk, cells stimulated with polyclonal activators and cells cultivated with polyclonal activators + colostrum/milk. The cultures were supplied with milk undiluted and in four 10-fold dilutions so that the resulting milk dilutions in the cultures were from 1:5 to 1:50,000. Unstimulated cord blood cells in culture exhibit a very low spontaneous activity and its change caused by colostrum/milk is inconspicuous and difficult to evaluate. We therefore stimulated the cells in culture by polyclonal activators and studied the effect of milk on this activation.

Proliferation of unstimulated cells and cells after stimulation with polyclonal activators. Cord blood cells of children of allergic mothers, when cultivated in a culture without stimulation (spontaneous proliferation), incorporate significantly more ^{3}H-thymidine than cells of children of healthy mothers. After stimulation with polyclonal activators, the cells of children of allergic mothers also proliferate much more intensively than cells of children of healthy mothers. This points to a higher reactivity of lymphocytes from children with a higher risk of allergic affliction (Table 1).

Effect of colostrum and milk on proliferation. Cultivation of cord blood cells from children of healthy and allergic mothers with colostrum or milk does not cause statistically significant change of the proliferation, although there is a perceptible tendency of higher milk doses to lower the proliferation while lower doses increase it.

No statistically significant differences were found between the effect of colostrum of healthy and allergic mothers. However, the proliferation is clearly affected in the presence of polyclonal activators. The presence of colostrum/milk in the culture in a final dilution of 1:5 very markedly

Table 1. *In vitro* proliferation of CBML after stimulation with polyclonal activators tested by ^{3}H-thymidine incorporation

	Proliferation (cpm)				
	CBML(H)		CBML(A)		
Stimulator	cpm	s.e.m.	cpm	s.e.m.	p-Value
0 (control)	3619	2045	7267	3304	0.0026116
PHA	140,384	40,075	251,467	69,113	0.0000017
ConA	103,810	48,042	209,278	60,761	0.0000015
BF	9962	4480	25,571	8863	0.0000001
EC	4846	2493	14,190	7972	0.0000204
NDCM	17,185	15,198	50,688	21,940	0.0000072

Comparison of children of healthy and allergic mothers.
CBML, cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; PHA, phytohaemagglutinin; ConA, concanavalin A; BF, *B. firmus*; EC, *Escherichia coli*; NDCM, Nocardia Delipidated Cell Mitogen; cpm, counts per minute; p-Value, significance of differences between CBML of children of healthy and allergic mothers.
Mean values of 12 experiments with CBML(H) and 11 experiments with CBML(A).

decreases the stimulation with polyclonal activators. The decrease was observed in some cases even at a colostrum/milk dilution of 1:50. Lower dilutions of milk and colostrum tend to increase the cell stimulation but the differences are not significant. However, even in this case no significant difference was shown between the effect of colostrum/milk of healthy and allergic mothers (Fig. 1). The significance of differences between the stimulation with polyclonal activator alone and with polyclonal activator together with maternal colostrum/milk at 1:5 final dilution is given in Table 2.

Ig formation by cord blood lymphocytes

Production of immunoglobulin of individual classes by CBML was detected by reverse ELISPOT.

Ig formation by unstimulated lymphocytes and after stimulation with polyclonal activators. Spontaneous production of immunoglobulins of all classes by unstimulated cord blood cells is minimal and only isolated spots can be detected. In our experimental set up, EC does not stimulate immunoglobulin formation and PWM increases the number of IgM-producing cells only in children of healthy mothers. The most potent stimulator of all was *B. firmus*, which markedly increases the production of IgM in both healthy and allergic group ($p = 0.005$ and $p = 0.001$, respectively), does not affect significantly IgG formation and perceptibly increases IgA formation in the healthy group ($p = 0.002$), and still more conspicuously, in the allergic group ($p = 0.00004$). The difference in IgA stimulation between children of healthy and allergic mothers is significant at $p = 0.0043$ (Table 3).

Effect of colostrum/milk on Ig formation. Cultivation of cord blood cells from children of both healthy and allergic mothers with colostrum alone (healthy or allergic) does not perceptibly affect the basal formation of immunoglobulin. When using colostrum/milk together with a strong stimulator – *B. firmus* – all individual analyses of cells of children of healthy mothers show pronounced increase in the number of cells producing immunoglobulin of all classes while only IgM formation is markedly increased in children of allergic mothers. The results are highly convincing although they are not significant in all cells/milk combinations in view of the large individual variability and low number of examined samples. The changes have the same trend in all individual cases. There is no difference between the action of colostrum of healthy and allergic mothers (Fig. 2). In stimulations with *E. coli* and PWM the numbers of immunoglobulin-producing cells are very low and the effect of colostrum is therefore difficult to assess. When stimulating the cells from children of allergic mothers, both normal and allergic colostrum causes a less marked increase of the effect of polyclonal activators than in the stimulation of cells from children of healthy mothers. 6-month milk affects immunoglobulin formation in the same way as colostrum but the effect is smaller.

Discussion

The direction of the perinatal development of the immune system is partially determined by the character of interactions of newborn's cells with regulatory factors acquired from the mother by transplacental transfer or through the maternal milk, or prenatally through the amniotic fluid. Several groups have described the differences,

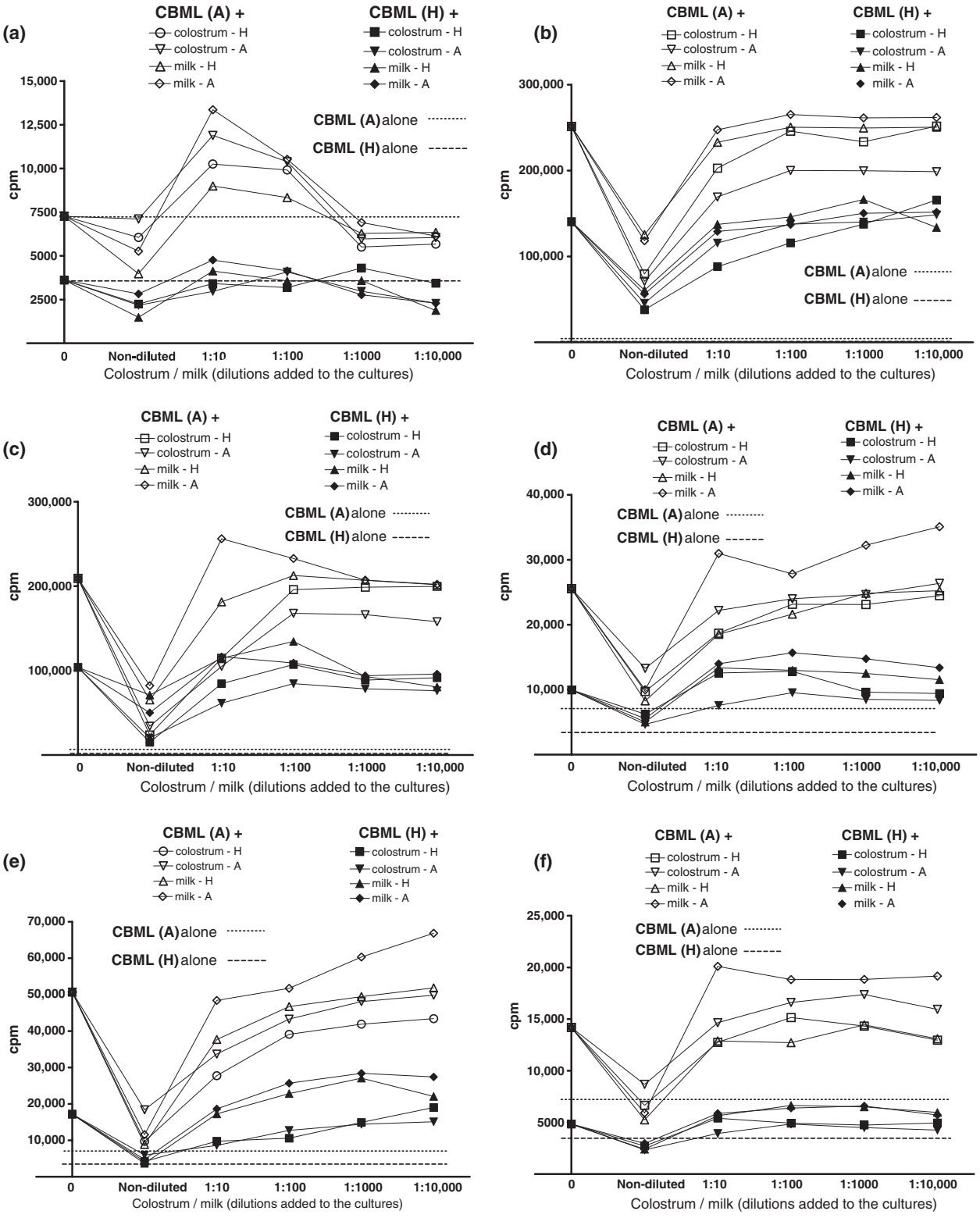

Fig. 1. Effect of various concentrations of maternal colostrum/milk on *in vitro* proliferation of cord blood mononuclear leucocytes stimulated by polyclonal activators. (a) Non-stimulated controls cultivated with various concentrations of milk. (b) Stimulation with phytohaemagglutinin. (c) Stimulation with concanavalin. (d) Stimulation with *Bacillus firmus*. (e) Stimulation with Nocardia Delipidated Cell Mitogen. (f) Stimulation with *Escherichia coli*. CBML: cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; colostrum/milk H, A: colostrum/milk of healthy, allergic mothers, respectively; dashed line: proliferation of non-stimulated CBML of children of healthy mothers; dotted line: proliferation of non-stimulated CBML of children of allergic mothers. Each curve represents mean of seven experiments.

Table 2. Effect of non-diluted colostrum and milk (final dilution in culture = 1:5) on *in vitro* proliferation of CBML stimulated by polyclonal activators

	Statistical significance (p-value)							
	CBML(H)				CBML(A)			
Stimulator	Colostrum(H)	Colostrum(A)	Milk(H)	Milk(A)	Colostrum(H)	Colostrum(A)	Milk(H)	Milk(A)
PHA	0.000009	0.000003	0.006120	0.001032	0.000003	0.000006	0.005821	0.000049
ConA	0.000003	0.000002	0.415916	0.002174	0.000002	0.000002	0.000011	0.000007
BF	0.112838	0.017339	0.027936	0.005546	0.000106	0.000073	0.000002	0.000005
EC	0.054754	0.009555	0.008591	0.078014	0.011916	0.050505	0.003497	0.001280
NDCM	0.000287	0.011489	0.000374	0.000912	0.000007	0.000314	0.000001	0.000002

Statistically significant differences between proliferation of cells stimulated with polyclonal activator alone and with activator + colostrum/milk.
CBML, cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; PHA, phytohaemagglutinin; ConA, concanavalin A; BF, *Bacillus firmus*; EC, *Escherichia coli*; NDCM, Nocardia Delipidated Cell Mitogen.
Mean values of seven experiments in each combination [CBML(H) + col(H); CBML(H) + col(A); CBML(H) + milk(H); CBML(H) + milk(A); CBML(A) + col(H); CBML(A) + col(A); CBML(A) + milk(H); CBML(A) + milk(A)].

Table 3. Immunoglobulin-forming cells detected by reverse ELISPOT after *in vitro* stimulation of CBML by polyclonal activators

	Spots/10^6 CBML								
	IgM			IgG			IgA		
Stimulator	CBML(H)	CBML(A)	p-Value	CBML(H)	CBML(A)	p-Value	CBML(H)	CBML(A)	p-Value
0 (control)	2.50	3.08	0.8818	12.22	1.58	0.4264	14.25	5.50	0.1919
EC	1.83	1.00	0.4255	27.78	1.75	0.6827	3.25	3.50	0.5395
BF	205.75	353.08	0.1939	26.22	22.50	0.8307	86.75	220.33	0.0043
PWM	37.25	1.42	0.0136	14.89	3.92	0.5129	5.83	7.83	0.7490

CBML, cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; EC, *Escherichia coli*; BF, *Bacillus firmus*; PWM, pokeweed mitogen; p-value, statistical significance of differences between CBML of children of healthy and allergic mothers.
Mean values of five experiments with CBML(H) and five experiments with CBML(A). Immunoglobulin-producing cell estimation in each experiment was carried out in triplicate.

though not always unequivocal, in the composition of the milk of healthy and allergic mothers (16–19). The question therefore arose whether the milk of allergic mothers does not enhance the risk of a later development of allergic afflictions in children of allergic mothers (although genetic factors are known to play a decisive role).

We tried to contribute to the characterization and comparison of immunomodulatory activity of milk of healthy and allergic mothers by following the effect of milk on *in vitro* activation of cord blood cells. These experiments cannot reflect precisely the *in vivo* situation, but we do not see presently any other possibility how to address this problem. As in all other experiments, *in vitro* tests are not equivalent to *in vivo* situation and we were not able to obtain a sufficient amount of newborn cells in another way than from the cord blood. Besides, the effect of maternal milk on the proliferation of normal mononuclear leucocytes from peripheral blood and cord blood and on the production of cytokines by these cells was studied also by others (25–28). Differences between CBML of children of healthy and allergic parents have previously been followed, albeit without testing the effect of milk (29, 30). We studied the stimulation of CBML from children of healthy and allergic mothers and its change under the action of colostrum and mature milk of healthy or allergic mothers, with the use of different combinations of cell and milk samples. CBML is a heterogeneous population of cells – this is similar to the *in vivo* situation where co-operation of various cell types is necessary for induction of the majority of immune reactions. Like in peripheral blood cells (27, 28), we showed in cord blood cells a lowering of proliferation by high concentrations of colostrum and milk. According to the literature, maternal milk has an immunosuppressive activity and helps thereby to induce oral tolerance to a number of food antigens and environmental antigens in breast-fed children (31–33). It lowers the probability of induction of IgE antibody response by these antigens in genetically predisposed individuals. However, we do not assume that milk, after being diluted in the gastrointestinal tract and

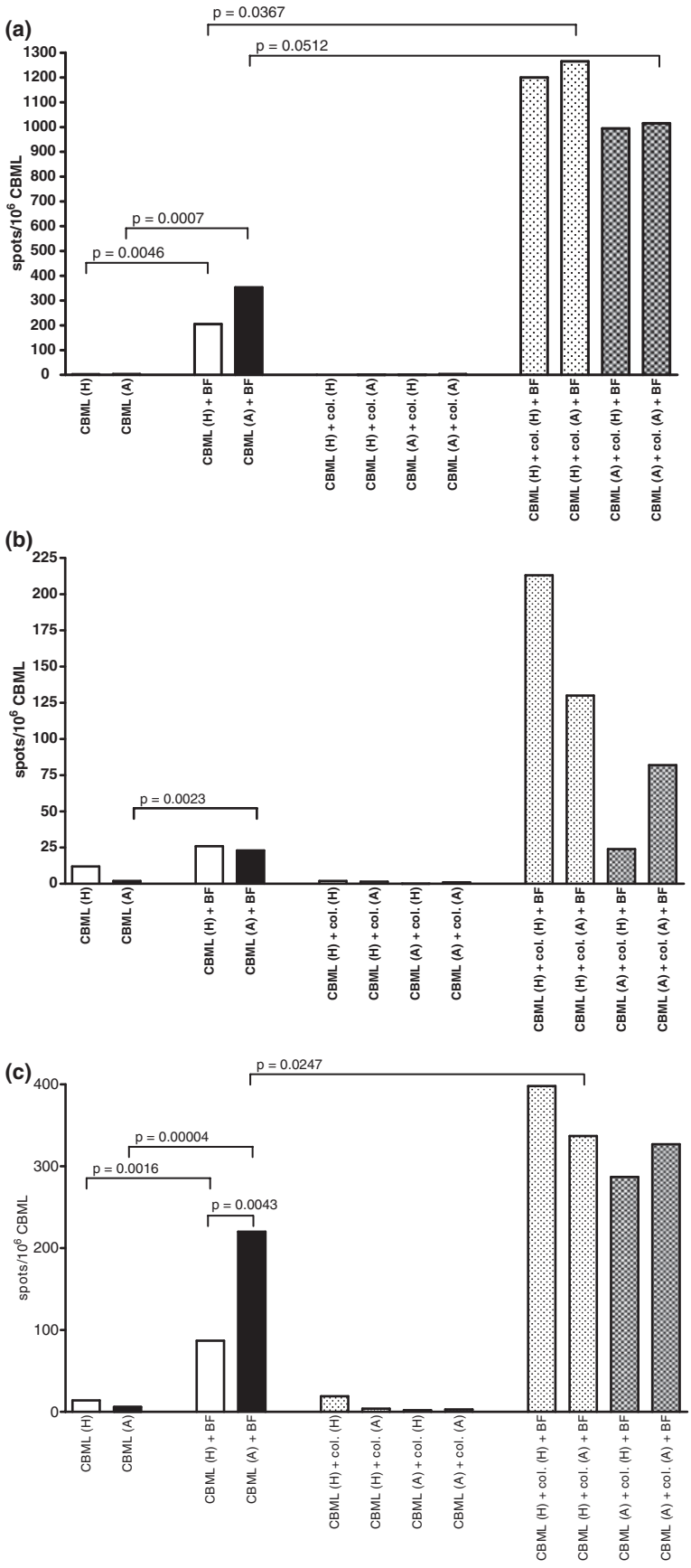

Fig. 2. Effect of maternal colostrum on the number of IgM-, IgG- and IgA-forming cells in cultures of cord blood mononuclear leucocytes stimulated by *Bacillus firmus* (measured by reverse ELISPOT). (a) Effect of maternal colostrum on the number of IgM-forming cells. (b) Effect of maternal colostrum on the number of IgG-forming cells. (c) Effect of maternal colostrum on the number of IgA-forming cells. CBML: cord blood mononuclear leucocytes of children of healthy (H) and allergic (A) mothers; col: colostrum of healthy (H) and allergic mothers (A); BF: *B. firmus*. Mean values of four experiments in each combination [CBML(H) + col(H); CBML(H) + col(A); CBML(A) + col(H); CBML(A) + col(A)].

passing through the intestinal wall, could be present in the intercellular space in an amount that would cause significant suppression *in vivo*. Milk in higher dilutions, which could be encountered *in vivo*, has a rather stimulatory effect on proliferation. The low significance of this stimulatory effect is probably due to large individual differences, the overall trend is, however, appreciable in all individual experiments. We found no substantial differences between the action of allergic and healthy milk and our results do not support the apprehension of breast feeding in allergic mothers. It is in keeping with the results of Böttcher et al. (34) who did not find any correlation between cytokine composition of milk of healthy and allergic mothers on one hand and future allergy manifestation in their children on the other hand.

An important result of these experiments was the finding of a significantly higher proliferation activity, both spontaneous and after stimulation with polyclonal activators, of cells from children of allergic mothers. The higher reactivity of cells from these children allows us to assume that these cells could also be more reactive after encounter with allergens. The question remains if these cells cannot be prestimulated already during the intrauterine development. It should be useful to follow the development of cell reactivity in postnatal ontogeny but it is difficult to perform it for ethical reasons.

Differentiation of cord blood cells into immunoglobulin-producing cells was tested after cultivation of cells by reverse ELISPOT. The cells of children of both healthy and allergic mothers were again stimulated and the stimulation was affected by the colostrum/milk of healthy and allergic mothers. In view of the above-mentioned low probability of a contact of newborn's lymphocytes with high milk concentrations, we used milk in a final dilution of 1:100 for influencing immunoglobulin production. Spontaneous immunoglobulin formation in cord blood cells is very low and, among the polyclonal activators, it was markedly stimulated only by BF, which has previously been described to exert a strong stimulatory action on B lymphocytes in both humans and mice (22, 23). The present results show that BF stimulates well also IgM and IgA production by cord blood lymphocytes, whereas stimulation of IgG formation is less significant. The effect of milk could best be assessed with a simultaneous stimulation with this activator. In general, colostrum/milk in the above dilution has a stimulatory effect on immunoglobulin formation after polyclonal activation. While the number of experiments may not be large enough to provide sufficiently massive support for this conclusion, the fact that all possible cells/milk combinations tested [CBML(H) + colostrum(H); CBML(H) + colostrum(A); CBML(H) + milk(H); CBML(H) + milk(A); CBML(A) + colostrum(H); CBML(A) + colostrum(A); CBML (A) + milk(H); CBML(A) + milk(A)] yielded very similar results represent a serious piece of evidence. Like with blast transformation, we found no distinct differences between the action of healthy and allergic colostrum/milk. Milk acts in the same way as colostrum but its effect is less pronounced, probably due to the lower concentration of immunologically active components in mature milk. We again showed differences in the reactivity of cord blood lymphocytes of children of healthy and allergic mothers. IgA production after stimulation with BF is increased much more in children of allergic mothers than in children of healthy mothers. Addition of colostrum to cultures of cells from children of healthy mothers stimulated with BF markedly increases the production of immunoglobulin of all three classes; in cells from children of allergic mothers this increase is lower and is clearly seen only in IgM. It therefore seems that the ability of spontaneous and stimulated IgM formation is comparable in children of healthy and allergic mothers. The higher ability of IgA formation after polyclonal activation of cells of children of allergic mothers could represent a physiological compensatory mechanism in children with a higher risk of allergy. IgA in the intestine contributes to the elimination of antigens and is assumed to reduce thereby the probability of allergy induction (35).

In conclusion, it can be stated that maternal milk in concentrations that are likely to occur *in vivo* has stimulatory rather than inhibitory effects on newborn's lymphocytes. No differences were found in the action of colostrum/milk of healthy and allergic mothers but substantial differences were found in the reactivity of cord blood lymphocytes of children of healthy and allergic mothers.

Acknowledgments

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Prevention of Allergy in Infants of Allergic Mothers by Probiotic *Escherichia coli*

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Key Words
Allergy in infants · Allergy prevention · After-birth oral colonization · Probiotic *E. coli*

Abstract
Background: The objective is to study the effect of after-birth oral colonization by a probiotic *Escherichia coli* strain in infants of allergic mothers to reduce occurrence of allergy later in life. ***Methods:*** In a controlled clinical trial, 158 infants were randomly divided into groups of (i) 56 colonized infants of allergic mothers, (ii) 57 control infants of allergic mothers, and (iii) 45 control infants of healthy mothers. Incidence rates of bacterial pathogens in stool and levels of anti-*E. coli* immunoglobulins and some cytokines in serum were determined, and secretory IgA was monitored in stool filtrates and maternal milk. Clinical check-ups of infants aged 4 days, 3 and 6 months, 2, 3 and 5 years were carried out and clinical symptoms of allergy were monitored. One milliliter of the probiotic *E. coli* strain was administered to infants of allergic mothers at first within 48 h after birth and subsequently 3 times a week over a period of 4 weeks. Control infants of allergic and healthy mothers were monitored in these intervals as well. ***Results:*** Presence of the *E. coli* strain was monitored in stool samples throughout the study. At the conclusion of the study, allergy symptoms were found in 14 infants of control allergic mothers, 7 infants of healthy mothers, and in 2 colonized infants of allergic mothers. Colonization affected levels of several cytokines and specific anti-*E. coli* antibodies. ***Conclusions:*** After birth, targeted colonization of the intestine by a probiotic *E. coli* strain can be an effective means of allergy prevention in infants of allergic mothers.

Introduction

Physiological intestinal flora is an important modulator of intestinal and other immune functions [1]. Changes in intestinal microbial composition have been observed in infants with allergy and in those who developed allergies later in life [2–5]. Significant differences in cytokine levels IL-4, IL-10, IL-13 and IFN-γ have been found in healthy and allergic groups of mothers and their children [6].

Attempts have been made to substitute randomly acquired intestinal flora by intentional colonization using probiotic bacterial strains. Lactobacilli and *Bifidus* bacteria have been used worldwide with no adverse effect but providing only limited short-term, or no protection. Administration of *Lactobacillus acidophilus* had no effect on food allergy [7] and asthma [8], supplementation of *Bifidobacterium lactis* did not affect intestinal microbiota in preterm infants [9], and there is no reported effect of

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Table 1. Characteristics of the test groups

Group (n)	Weeks of gestation	Birth length cm	Birth weight, g	Breastfeeding, weeks		Allergic fathers	Allergic siblings	Smoking mothers	Pets
				range	median				
H (45)	39 (38–42)	50 (48–53)	3,200 (2,800–3,900)	3–72	29	6 (15%)	5 (12%)	10%	38%
A (57)	38 (37–42)	51 (47–53)	3,300 (2,990–4,000)	2–80	28	21 (46%)	35 (62%)	12%	46%
AC (56)	39 (38–42)	50 (48–52)	3,100 (2,880–3,900)	6–88	31	28 (50%)	15 (27%)	8%	42%

H = Healthy mothers; A = allergic mothers; AC = allergic mothers of colonized children.

treatment of infantile colic using *Lactobacillus reuteri* [10]. *Lactobacillus GG* had no effect on primary prevention of sensitization in infants [11]. A significant reduction of infant eczema has been reported by Kalliomaki et al. [12], Isolauri et al. [13] and Abrahamson et al. [14]. Osborn and Sinn [15] and Kukkonen et al. [16] describe an insignificant effect of lactobacilli administration on overall allergy incidence rates in infants. Several studies describe positive effects of infant intestinal colonization by non-pathogenic *E. coli* strains. Schulze and Sonnenborn's group [17] emphasize the renaissance of the use of probiotics and Poisson et al. [18] and Lari et al. [19] recommend their use in intensive care units and in newborn and premature infants. The results of Cukrowska et al. [20] of colonizing premature infants by *E. coli* Nissle show a specific proliferative and antibody response.

Our previously published results show that the unique probiotic *E. coli* strain, administered orally after birth, remains dominant in the intestine over several months. Its prolonged presence in the gut stimulates production of serum and local antibodies [21] and reduces the presence of bacterial pathogens in the gut and on other mucous surfaces, and, according to results of a questionnaire, reduces the incidence of nosocomial infections and allergies 10 and 20 years after its administration [22]. These favorable properties of the *E. coli* strain, particularly the prevention of nosocomial infections in high-risk infants, have been recognized in clinical practice [23].

This study shows the effect of after-birth colonization of infants of allergic mothers by a probiotic *E. coli* (Colinfant®) on: (1) the incidence rate of allergies within the first 5 years of life; (2) IgE antibodies against common allergens in sera of colonized and non-colonized infants and their mothers; (3) cytokine levels of IL-4, IL-5, IL-6, IL-13, IFN-γ, TGF-β and anti-*E. coli* immunoglobulins in serum, and (4) secretory IgA levels in stool filtrates and milk.

Test Groups and Methods

A total of 158 studied full-term infants (average birth weight 3,050 g, birth length 50 cm) was randomly divided into groups of: (1) colonized infants of allergic mothers, the *'allergic colonized'* group AC (n = 56); (2) non-colonized control infants of allergic mothers, the *'allergic'* group A (n = 57), and (3) control infants of healthy mothers, *'healthy'* group H (n = 45).

In an open, controlled, randomized, clinical study, the average age of mothers was 30 years in group H, 32 years in group A, and 33 years in group AC. All infants were full-term and born vaginally. Characteristics of studied test groups are given in table 1.

Conditions for Inclusion of Allergic Mothers in the Study

The following criteria were applied in the selection of allergic mothers: (a) absence of other illnesses; (b) clinical manifestation of allergy for more than 24 months; (c) monitoring of allergy by an allergologist (via a written report); (d) positive skin test, positive specific IgE, and (e) positive reaction to antiallergic treatment. Informed and signed consent was obtained from all participating mothers.

Probiotic Treatment of Infants

The *E. coli* strain, patented in 1992 (No. 264572) and registered in 1997 in the Czech Republic as Colinfant, is produced by Dyntec Co. (Terezín, Czech Republic). One dose, containing 0.8×10^9 of lyophilized *E. coli* in 1 ml, was administered orally within 48 h after birth, and subsequently 3 times weekly over a period of 4 weeks.

Characteristics of the Strain

The strain was obtained from the international collection of *E. coli* and *Klebsiella* strains in Copenhagen, Demark. According to its biochemical properties it belongs to normal human intestinal *E. coli* strains, serotype O83:K24:H31, and occurs very rarely as a human spontaneous type. It is sensitive to most antibiotics and has no plasmid. Its detailed characteristics are described in our previous studies [21, 23]. The strain has not changed any of its properties over 30 years, including sensitivity to antibiotics, absence of plasmid and genetic characteristics.

Test Samples

Samples of maternal blood, cord blood, infant venous blood and infant stool were taken prior to administration of the vaccine and at the ages of 3–4 days, 3 and 6 months, 1, 2, 3 and 5 years.

Table 2. Detection of *E. coli* vaccine strain in colonized infants

	3 days (n = 56)	3 months (n = 52)	6 months (n = 51)	1 year (n = 35)	2 years (n = 22)	3 years (n = 17)	5 years (n = 16)
O83+	22 (39%)	34 (65%)	27 (53%)	17 (49%)	6 (27%)	3 (18%)	2 (13%)
O83	18 (32%)	13 (25%)	7 (14%)	5 (14%)	3 (14%)	3 (18%)	2 (13%)
Total	71%	90%	67%	63%	41%	36%	26%

O83+ = *E. coli* O83 vaccine strain with other *E. coli* strains; O83 = *E. coli* O83 vaccine strain only.

Table 3. Incidence of allergy during the follow-up periods

Group (n)	3 months	6 months	1 year	2 years	3 years	5 years
H (45)	4% (2/45)	11% (5/45)	15% (7/45)	16% (7/42)	15% (7/44)	16% (7/42)
A (57)	9% (5/57)	14% (8/57)	21% (12/57)	28% (14/50)*	28% (14/49)*	31% (14/45)*
AC (56)	2% (1/56)	2% (1/56)	2% (1/56)	2% (1/50)	2% (1/48)	4% (2/46)

* Significant differences relative to group AC are expressed as $p < 0.05$.

Colostrum and milk samples were collected during the breastfeeding period. Blood sera, colostrum and milk samples (after centrifugation and fat removal) and children's stool filtrates were stored at –20°C.

Infants' stool filtrates were prepared as 1 g of stool suspended in 1 ml of PBS with 1 mM phenylmethylsulfonyl fluoride, centrifuged at 8,000 *g*, and supernatants used for analysis. The *E. coli* strain was detected on blood agar, Endo agar and MPA agar in stool of colonized infants. The specificity of the strain was confirmed by agglutination with specific rabbit antiserum. Levels of IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TGF-β were quantified by ELISA on high-adsorption polystyrene microtitration plates (Nunc). For paired antibodies where the second antibody was biotinylated, a R&D System (Wiesbaden, Germany) was used according to the manufacturer's recommendations. Results were read from the calibration curves of the recombinant cytokine R&D System using Genesis software. Detection limits for 1:10 diluted samples were 10 pg/ml for IL-4, IL-5, IL-6 and IFN-γ, and 30 pg/ml for IL-10, IL-13 and TGF-β. Specific IgE was estimated immunoenzymically by RISA (Ring-Immuno-Sorbent Assay) using enzyme allergosorbent test (Doverton) kits and Dynex allergen rings (FX-4) with a mixture of the most common food allergens (egg white, cow's milk, flower, peanut and soya bean antigens), or Dynx-1 with the most common respiratory allergens (timothy grass, ray, birch wormwood, mite, hair and mould antigens). Results were obtained from the calibration curve and expressed as kU/l and RAST classes. Totals of sIgA in colostrum, milk and stool filtrates were determined by radial immunodiffusion using binding site kits.

Statistics

Overall distribution of duration of allergy incidence was compared between studied groups using Kaplan-Meier analysis and Mantel-Haenszel (log-rank) test. Differences in distribution of cytokines among groups were compared using the Mann-Whitney U test (Wilcoxon rank sum test). $p < 0.05$ was considered significant. Results are shown as time-varying medians and percentiles 25–75%.

Table 4. Types of allergy

Group	Mothers					Infants			
	n	respiratory	skin	both	others	n	respiratory	skin	food
H	45	–	–	–	–	7	2	6	1
A	57	26	5	66	2	14	2	10	3
AC	56	34	2	78	–	2	1	2	1

Results

All infants were born by vaginal delivery and all were breastfed. Differences of duration of breastfeeding among groups were statistically insignificant (table 1). The *E. coli* strain O83:K24:H31 was never found in infant stool samples before its administration. The strain was detected in some colonized infants during the entire span of the study, starting on day 3 (table 2). At the time of conclusion of the study, results of the Kaplan-Meier analysis show

Table 5. Serum cytokine levels in infants until the age of 3 months (percentage values in parentheses)

Cytokines	Group H			p H vs. A	Group A			p H vs. AC	Group AC		
	median	25th–27th percentile	positive samples total		median	25th–27th percentile	positive samples total		median	25th–27th percentile	positive samples total
Cord blood											
IL-4	221	169–367	23 (55)/42	0.6635	248	172–545	36 (65)/55	0.4672	294	193–700	45 (78)/58
IL-5	143	116–195	16 (38)/42	0.1176	189	144–251	29 (52)/56	0.4030	196	162–238	42 (72)/58
IL-6	211	131–1,817	14 (33)/42	0.3594	179	144–200	15 (27)/55	0.4644	181	157–212	23 (40)/58
IL-10	306	210–464	20 (56)/36	0.8943	321	197–448	43 (83)/52	0.2187	373	227–566	39 (70)/56
IL-13	943	289–2,609	29 (76)/38	0.1666	2,011	556–3,521	43 (80)/54	0.2744	1,391	703–2,727	48 (86)/56
IFN-γ	1,068	614–2,178	20 (49)/41	0.0355	486	195–854	22 (40)/55	0.9156	432	209–860	42 (72)/58
TGF-β	70,008	39,911–103,040	42 (98)/43	0.1358	42,356	2,412–88,889	54 (100)/64	0.3218	67,752	24,353–102,374	55 (100)/55
3 days											
IL-4	197	146–252	20 (50)/40	0.0167	268	177–715	45 (67)/67	0.8808	305	162–696	48 (75)/64
IL-5	172	128–287	17 (42)/40	0.9220	202	159–234	36 (54)/67	0.0604	225	163–304	46 (72)/64
IL-6	224	167–1,705	14 (33)/42	0.1669	197	160–235	20 (30)/67	0.4647	176	127–241	26 (41)/64
IL-10	314	209–640	19 (51)/37	0.9571	363	221–480	50 (78)/64	0.3944	341	234–939	39 (63)/62
IL-13	598	444–1,286	26 (72)/36	0.2920	945	431–2,529	53 (80)/66	0.8803	1,229	319–2,925	52 (84)/62
IFN-γ	622	318–1,835	18 (43)/42	0.1304	467	143–1,029	40 (62)/64	0.5370	403	180–1,236	47 (73)/64
TGF-β	67,707	35,775–109,968	43 (100)/43	0.2515	53,736	28,922–82,148	64 (100)/64	0.6407	54,006	29,382–89,181	61 (100)/61
3 months											
IL-4	173	132–199	20 (47)/43	0.0021	314	188–478	45 (70)/64	0.9577	295	159–702	49 (78)/63
IL-5	199	158–321	16 (37)/43	0.4263	179	121–311	38 (59)/64	0.2532	204	161–311	38 (60)/63
IL-6	165	144–718	12 (28)/43	0.3664	164	128–245	22 (34)/64	0.4147	154	129–185	18 (29)/63
IL-10	368	287–504	22 (47)/47	0.3831	297	201–623	41 (67)/61	0.4293	248	196–459	37 (29)/61
IL-13	1,020	341–2,100	32 (82)/39	0.0436	2,020	597–3,253	48 (76)/63	0.1031	1,170	413–2,891	48 (79)/61
IFN-γ	509	220–1,056	28 (65)/43	0.0226	476	191–1,070	30 (51)/59	0.9347	495	264–1,039	37 (62)/60
TGF-β	71,880	39,540–116,323	44 (100)/44	0.0277	42,077	15,030–85,892	62 (98)/63	0.0725	64,245	23,773–109,559	62 (100)/62

reduction of allergy incidence in group AC as compared to group A. Clinical signs of allergies were found in 7 (16%) infants of healthy mothers (group H), 14 (31%) in group A (allergic controls), but only in 2 (4%) infants of the colonized group (AC). This confirms significant differences between groups A and AC ($p < 0.001$). The allergy incidence reduction in group AC compared to group H was not significant. A summary of allergy incidence within specified intervals and types of allergies is shown in tables 3 and 4. Laboratory data do not fully reflect the clinical situation. Differences in total secretory IgA between groups in stool and mother milk's were not statistically significant.

Specific IgE levels in serum were elevated in both groups of allergic mothers (88% in group AC and 92% in group A). Milk samples showed increased levels of IgE in 18% of group AC and 22% of the allergic groups of mothers. Infant serum of allergic mothers revealed increased levels of IgE in 7% of colonized and 20% of non-colonized controls at 1 year of age. Correlation with clinical signs of allergy was proven only in 3 infants of allergic control mothers.

Comparison of cytokine levels among groups was difficult due to great individual differences. Samples with levels under detection limits were considered as negative. Slightly higher levels of IL-4 and significantly higher levels of IL-5 ($p = 0.0071$) were detected in sera of allergic mothers as compared with serum levels of healthy mothers. Values in children's sera of up to 3 months are shown in table 5. Levels of IL-4 and IL-13 were higher in infants of allergic mothers, and IFN-γ and TGF-β were higher in infants of healthy mothers compared to allergic mothers. The allergic phenotype can be recognized at 3 months of age and no substantial differences were found later.

Colonization with the Colinfant vaccine did not substantially influence cytokine levels in serum, infant stool filtrates and mother's milk. Serum IgA and IgG were significantly higher in group AC than in group H at 3 months, 6 months and 1 year of age ($p < 001$). Levels of IgM were higher in group AC only at the age of 3 months.

Discussion

The *E. coli* strain used in this study has been studied in our former work since 1965. Colonization of the intestine was effective for prevention of nosocomial infections in premature infants and decreased the numbers of pathogens and the necessity to use of antibiotics. Our understanding of mechanisms responsible for positive effects of colonization with probiotic *E. coli* strains is incomplete. The presence of type 1 fimbriae and its excellent colonizing ability are its favorable features. The first colonizer also benefits from quantitative dominance. However, the overall mechanism is rather complex.

In developed countries, slow colonization of the intestine with enterobacteria may reduce exposure of the developing immune system to lipopolysaccharides. Lipopolysaccharide deficiency may impede the ability of the infant's immune system to distinguish between harmful and harmless antigens (e.g. lack of development of tolerance against food antigens). Probiotics can modify the immune development [5, 26, 27]. Details of intestinal colonization with *E. coli* administered orally to infants can be found in our earlier studies [21, 23]. Comparison of the effect of *E. coli* and lactobacilli shows that *Lactobacilli* administration resulted only in a significant reduction of atopic eczema [13], but no other differences in occurrence of allergic diseases were found [15].

This study covers a period between birth and the age of 5 years. All infants were full-term, born by vaginal delivery. During the 5-year period, clinical symptoms of allergy were found only in 2 infants of allergic mothers colonized after birth with the probiotic *E. coli* strain compared to 14 infants of allergic control mothers. The difference is statistically highly significant. Seven cases of allergy occurred in infants of healthy mothers. It is evident that colonization with probiotic *E. coli* suppresses development of allergy.

The used *E. coli* strain had additional positive effects on studied infants. Levels of pathogenic and potentially pathogenic bacteria *(Klebsiella, Pseudomonas aeruginosa, Staphylococcus epidermidis)* found in stool samples of colonized infants of allergic mothers were significantly lower during the first 3 months of age than in infants of allergic and healthy control mothers. These pathogens are some of the most dangerous in the first months of life [24].

Numerous studies report on values of cytokines in serum and mother's milk of healthy and allergic mothers and in their infant serum. Results are rather controversial. It seems that levels of IL-13 and IL-5 have greater significance and reflect a better Th2 polarization than IL-4. Both increased and decreased production of IFN-γ was described to be associated with allergic phenotype [27]. In our study it was difficult to compare cytokine levels among the infants studied due to the magnitude of observed differences and a large number of observations found under the detection limit. Allergic phenotype, higher IL-4 and IL-13 and lower IFN-γ and TGF-β, dominated in the allergic group but the values observed were not quantitatively different. The allergic phenotype is evident in the first 6 months of life, later there are no substantial differences between children of healthy and allergic mothers. Cytokine levels do not reflect the pronounced clinical effect of colonization with probiotic *E. coli* used in our trial. The clinical effect of the Colinfant strain is complex and bacterial colonization of the intestine does not influence levels of mucosal cytokines in a significant way. Higher levels of IgA in serum of colonized infants can increase the defense ability of the infants against enteral infections due to possible cross-reactions between the antibodies.

Conclusions

To conclude: (1) Oral administration of probiotic *E. coli* after birth resulted in effective colonization of the infant's intestine and maintained dominant presence in the stool over several weeks. (2) Early colonization of the intestine decreased significantly clinical symptoms of allergy in infants of allergic mothers. Cytokine levels in several body fluids are not markedly influenced by colonization. (3) The replacement of natural, incidental and at times pathogenic colonization of the intestine by controlled oral administration of *E. coli* after birth may be a therapeutic option to reduce and possibly prevent allergies later in life.

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Cytokine Expression in Cord Blood Cells of Children of Healthy and Allergic Mothers

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ABSTRACT. To determine some early signs connected with the increased risk of future allergy development, gene expression and production of selected cytokines were tested in children of allergic mothers and compared with newborns of healthy mothers. Expression of IL-1β, IL-2, IL-4, IL-8, IL-10, IL-13, IFN-γ, TNF-α, TGF-β and EGF was tested in cord blood cells using real-time PCR and production of these cytokines was evaluated in cord sera by ELISA. Gene expression of IL-2, IL-4, IL-8, IFN-γ, IL-1β, TNF-α and TGF-β was decreased and that of IL-10, IL-13 and EGF increased in children of allergic mothers in comparison with those of healthy mothers. Significant differences in sera of healthy and allergic groups were only in IL-10 and EGF. Different relationship among serum cytokine levels reflects the fact that the cytokines are not produced only by blood cells. Significantly decreased production of EGF in newborns of allergic mothers could negatively influence maturation of mucosal membranes of these children and support thus their easier allergization. Allergic phenotype pointing to the bias to T_H2 response and to possibly impaired intestine maturation was apparent already on the level of cord blood and could serve as a predictive sign of increased allergy risk.

Abbreviations

AM	allergic mothers	CBML	cord blood mononuclear leukocytes	HM	healthy mothers
CBC	cord blood cell(s)	CV	coefficient of variance	RT	real-time (PCR)

Allergic diseases are in the centre of interest of current medicine mainly because of their large and steadily increasing incidence. It is commonly accepted that genetic predisposition is the main risk factor for allergy development and manifestation (Benn *et al.* 2004; Hauer *et al.* 2003; Peden 2005; Prescott *et al.* 2003; Steinke *et al.* 2008; Wang *et al.* 2005). However, the effect of other factors should not be forgotten. Besides environmental impact, mainly prenatal and early postnatal influence of maternal immune system on the development and tuning of offspring's immune reactions has to be kept in mind. A child obtains many biologically active substances from the mother – prenatally *via* placental transfer or amniotic fluid and postnatally by breast-feeding. Already at birth, immunological milieu and immunological reactivity of the newborn could point to the increased risk of future allergy development.

We were able (Žižka *et al.* 2007) to prove a striking difference in the CBC reactivity of newborns of HM and AM in spontaneous and polyclonally stimulated proliferation. Here we should like to reveal the difference in immunological tuning in "healthy" and "allergic" groups of children by testing cytokine expression and production in the cord blood. We were interested mainly in the cytokines involved in the development and promotion of allergy and influencing the production of IgE. Significant differences between children of HM and AM were found already at the time of birth.

MATERIAL AND METHODS

Subjects. HM and AM with physiological pregnancy and children delivered physiologically in full term at the *Institute for the Care of Mother and Child* from October 2005 to April 2006 were included. Diagnostics of allergy in mothers was based on clinical manifestation of allergy persisting for >2 years (allergy against respiratory and food allergens manifested by various individual combinations of hay fever, conjunctivitis, bronchitis, asthma, eczema, *etc*.), monitoring by an allergologist, positive skin prick tests or positive specific IgE and anti-allergic treatment before pregnancy. Father's allergy, known as having smaller impact on children´s allergy development (Tadaki *et al.* 2009), was not taken into consideration. The study

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was approved by the *Ethical Committee of the Ministry of Public Health* and was carried out with the informed consent of the mothers.

Cord blood from children of HM and AM was collected immediately after delivery with puncture of umbilical vein after careful cleaning of the cord to avoid maternal blood contamination. For RNA isolation, 2.5 mL blood was placed into Paxgene Blood RNA Tubes (*PreAnalytiX*) containing RNA stabilization solution preventing RNA degradation. Choice of 10 cord blood samples of children of both HM and AM for RNA isolation was at random, there were no selection criteria. Cord blood serum was obtained for cytokine detection.

RNA isolation. Whole cord blood RNA was isolated from CBC using Paxgene Blood RNA kit (*PreAnalytiX*) according to the manufacturer's instructions. RNA integrity was determined by gel electrophoresis in 1.5 % agarose gel stained with ethidium bromide. The purity of the RNA was assessed by the ratio of absorbance A_{260} and A_{280}. The purity was in the range 1.8–2.1. The total RNA concentration was estimated spectrophotometrically (A_{260}) assuming that 44 μg of RNA per mL equals one absorbance unit. RNA was stored in aliquots at –20 °C until used for reverse transcription.

Quantitative RT PCR. Isolated mRNA was converted to cDNA using reverse transcription reagents (*Applied Biosystems*) according to the manufacturer's instructions.

Every 25 μL reaction mix for RT PCR was made with 12.5 μL TaqMan Universal PCR master mix (*Applied Biosystems*), 100 ng of cDNA diluted in 5 μL RNAase free water, 6.25 μL RNAase free water and 1.25 μL specific TaqMan probes for mentioned genes (all *Applied Biosystems*). Eukaryotic 18S rRNA (4319413E; *Applied Biosystems*) was used as an endogenous control (housekeeping gene). Each reaction was run in duplicate.

PCR reaction was run on the 7300 RT PCR system (*Applied Biosystems*) using standard conditions. Cycling parameters were: initial denaturation (10 min, 95 °C), 40 cycles consisting of denaturation (15 s, 95 °C) and annealing–extension (1 min, 60 °C). Fluorescence was acquired after each extension step. The efficiency of reactions was very similar to that of endogenous control. A no-template control contained water instead of cDNA. Expression of all genes was normalized to cDNA of endogenous control loading for each sample and used as an internal standard.

The relative quantity of mRNA was given as $2^{-\Delta\Delta ct}$, $\Delta\Delta ct$ being calculated as follows:

$$\Delta\Delta ct = \Delta ct_a - \Delta ct_h$$
$$\Delta ct = ct_{gi} - ct_{hg}$$

where Δct_a is allergic individual, Δct_h healthy individual, calibrator, ct_{gi} gene of interest, and ct_{hg} housekeeping gene (endogenous control).

The threshold cycle (ct) is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. mRNA from cord blood from 10 children of HM was used as calibrator (its value was set = 1). The gene expressions in CBC from 10 children of AM were expressed as relative values referred to the value of the calibrator (evaluated by SDS Software; *Applied Biosystems*).

ELISA. IL-2, IL-4, IL-8, IL-10, IL-13, INF-γ, TNF-α, IL-1β, TGF-β_1 and EGF of 54 children of HM and 55 children of AM were quantified by ELISA using primary antibody–detection antibody pairs from *R&D Systems*. Analysis was carried out according to the manufacturer's recommendation. The assays were optimized by testing various dilutions of tested samples and antibodies. Samples were assayed in duplicates and the CV differs; lover CV (typically 0–0.12 %) is in assays with high concentration of cytokines detected (TGF-β, IFN-γ, IL-13) and CV is higher (0–0.26 %) in assays with lower cytokine concentration (IL-4, IL-8, IL-10, EGF, TNF-α). Detection limit for IL-4, IL-8, EGF, IFN-γ and TNF-α was 10 pg/mL, for IL-2, IL-10, IL-13 and TGF-β 30 pg/mL and for IL-1β 250 pg/mL; cytokine values were read from calibration curves. Results are expressed as medians and 25–75 % percentiles. All cytokine concentrations (Table I) are appended with numbers of samples in which concentration was upper detection limit.

Statistics. Comparison of the gene expression of cytokines and cytokine concentrations of the two groups (children of HM and of AM) was examined by non-parametric Mann–Whitney test, with $p \leq 0.05$ being considered significant.

RESULTS

Cytokine expression. For the comparison of cytokine expression in non-stimulated CBC of newborns of HM and AM tested by quantitative RT PCR *see* Fig. 1. Significant differences were detected in the expression of the majority of cytokines tested – expression of IL-2, IL-4, IL-8, IFN-γ, IL-1β, TNF-α and

TGF-β being significantly lower and that of IL-10, IL-13 and EGF higher at children of AM. IL-13 and IL-10 increase is quite evident in allergic group but non-significantly because of large individual variability.

Table I. Cytokine concentrations in cord blood sera from children of HM and AM (ELISA, pg/mL)

Cytokine[a]	HM			AM			*p*
	median	25–75 %	*n*[b]	median	25–75 %	*n*[b]	
IL-4	221	169–367	23	248	172–545	36	0.6635
IL-8	580	412–805	43	649	436–1 202	43	0.3206
IL-10	306	210–464	20	321	197–448	43	0.8943
IL-13	943	289–2 609	29	2 011	556–3 521	43	0.1666
INF-γ	1086	614–2 178	20	486	195–854	22	0.0355
TGF-β	70 008	39 911–103 040	42	42 356	24 512–88 889	54	0.1358
EGF	1498	743–2 539	40	1 304	701–1 945	44	0.0312

[a]Concentration of IL-1β, IL-2 and TNF-α were below the detection limit.
[b]Number of samples in which concentration was above the detection limit (total number was 54 in healthy group and 55 in allergic group).

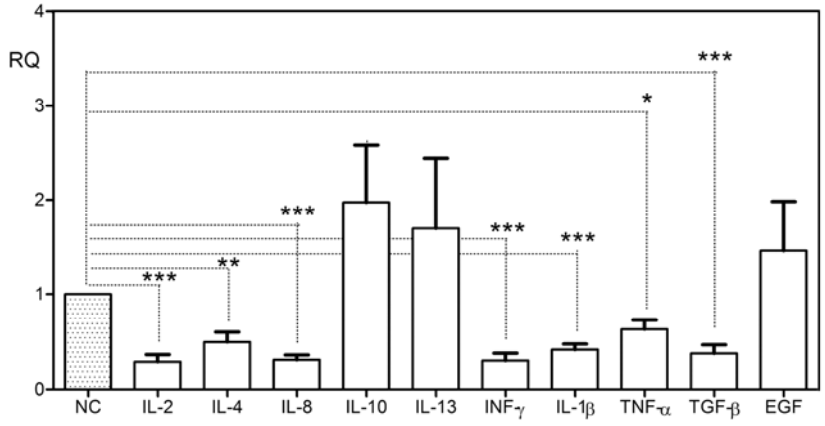

Fig. 1. Gene expression of cytokines in CBC from children of healthy and allergic mothers measured by quantitative RT PCR; means of relative values (relative quantification, RQ) of cytokine mRNA determined in CBC from 10 children of allergic mothers; $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$; NC – normal control (mRNA sample of 10 children of HM corresponding to RQ = 1).

Cytokine concentration. For cytokine concentrations detected by ELISA *see* Table I. These results are not exactly comparable with the gene expression because they reflect the cytokine production by various cells and tissues, not only production by blood cells. As concerns IL-10, IL-13, IFN-γ and TGF-β, the trend of differences between their levels in both groups corresponds to that revealed by testing cytokine expression in CBC but, except for IFN-γ, differences are not significant. An inverse relationship between cytokines of children of HM and AM was in IL-4, IL-8 and EGF. When comparing EGF by RT PCR, no significant differences were proved, while its serum levels were significantly lower in children of AM. The slight increase in IL-4 and IL-8 concentration in children of AM was not significant. IL-2, IL-1β and TNF-α values were on the border of detection sensitivity in serum.

DISCUSSION

Many authors have already dealt with cytokines in cord blood in relation to allergy but they focused mainly on IFN-γ, IL-4 and IL-13 (Chung *et al.* 2007; Schaub *et al.* 2005; Silberer *et al.* 2008) though many different cytokines are involved in allergy development and other ones have anti-allergic effect. The cytokines tested by us are supposed to have some relationship to the induction and regulation of allergic reaction: T_H1 (IL-2, IFN-γ) and T_H2 (IL-4, IL-13) cytokines which inhibit or support allergy development, respectively (Chung *et al.* 2007), IL-10 and TGF-β promoting isotype switch towards IgA (Fayette *et al.* 1997) which

is believed to act against allergy development; IL-4 and IL-13 being important for IgE production (Fayette *et al*. 2007; Graves *et al*. 2000; Tanaka *et al.* 2001); IL-10 and TGF-β mediating the establishment of peripheral tolerance hampering the rise of allergy (Jutel *et al.* 2003); EGF and TGF-β as growth and differentiation factors of great importance for intestine maturation (McGee *et al.* 1992; Nair *et al.* 2008), the failure of which can cause increased intestinal permeability leading to easier access of allergens to the immune system resulting in allergy induction; IL-1β, IL-8 and TNF-α act as inflammatory cytokines (Mohammed *et al.* 2007).

Cytokine expression detected by RT PCR revealed significant differences between CBC from children of HM and AM; our results are in accordance with the known increased risk of allergy manifestation in children of AM. Allergic phenotype is evident already on the level of CBC. We observed lower expression of IFN-γ in CBML (as we and many other authors describe on protein level in cord blood; Contreras *et al.* 2003; Kondo *et al.* 1998; Prescott *et al.* 2003) and higher expression of IL-13 and IL-10 – that points to an increased propensity of newborns of AM to T_H2 response. High expression of IL-13 is pro-allergic and was described as a marker of a high risk of future allergy (Ohshima *et al.* 2002). Increased gene expression of IL-13 in CBML of children of AM after *in vitro* stimulation in comparison to healthy ones was also observed by other authors (*e.g*., van der Veden *et al.* 2001). However, gene expression of IL-4 in CBML of children of HM was significantly higher in comparison with allergic group. This discrepancy was also described by Prescott *et al.* (1998). The tendency to the increased concentration of IL-4 in allergic group is evident only on serum level, which can point to the participation of additional sources of IL-4 influencing serum level of this pro-allergic cytokine. Therefore IL-13 expression and production seems to correlate better with increased propensity of allergy than that of IL-4. Decreased expression and production of TGF-β in these children of allergic group could contribute to the increased risk of allergy development by limiting the induction of peripheral tolerance against allergens, and by impaired maturation of the intestine and possibly by lowering IgA production.

The cytokine expression by CBC is not fully reflected by cytokine concentration in serum. This is understandable because cord serum cytokines originate not only from fetal leukocytes and could also be transferred to the newborn from the mother *via* placenta or by engulfing amniotic fluid whose constituents are partially of maternal and partially of fetal origin. Thus serum cytokines reflect summation of both intrinsic and extrinsic factors characterizing newborn's immunological milieu. In the majority of cytokines tested, the differences between children of HM and AM are similar on mRNA and serum level but serum differences are less significant. A different relationship between both groups on mRNA and serum level is only in the case of IL-4, IL-8 and EGF. mRNA for IL-8 in CBC is lower in children of AM but there is no huge difference on serum level pointing to other sources of IL-8 than leukocytes. There are no significant differences in EGF expression in CBC but there is a significantly higher concentration of EGF in healthy group on serum level. This is quite in keeping with the fact that leukocytes are not the main source of EGF. Decreased total production of EGF in allergic group reflected in decreased serum level could support the induction of allergy by its negative impact on mucosal membrane maturation. Because the differences between cord blood serum cytokines of children of HM and AM are less significant than differences detected in CBC, it may be concluded that the maternal impact on the development of offspring's allergy is asserted mainly on genetic level, the phenotypic products of maternal organism being mostly less important. Differences in gene expression of CBC cytokines and in serum levels of EGF could potentially serve for assessment of future allergy risk. Health condition (allergy development) of tested children will be followed prospectively in the effort to evaluate the prognostic value of data mentioned *above*.

Allergic phenotype pointing to the bias to T_H2 response (decreased gene expression of T_H1 cytokines IL-2, IFN-γ and increased expression of IL-13 in CBC of newborns of AM) and to impaired intestine maturation (decreased concentration of EGF in cord blood sera of children of AM) were apparent already on the level of cord blood and could serve as possible predictive sign of future allergy development.

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Cytokine expression in the colostral cells of healthy and allergic mothers

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Abstract There is no doubt about the beneficial effect of breastfeeding on the newborn's immune system. It is not fully elucidated what the differences are between the colostrum/milk of healthy and allergic mothers and how beneficial breastfeeding by an allergic mother is. The gene expression of selected cytokines was tested in cells isolated from colostra of healthy and allergic mothers using quantitative real-time PCR. Allergic phenotype was evident in colostral cells of allergic mothers: gene expressions of IL-4, IL-13 and EGF were increased and those of IFN-gamma decreased in comparison with colostral cells of healthy mothers. The allergic phenotype of the colostral cells of allergic mothers supporting the bias to a Th2 type response was found. It remains a question if a small number of these cells could influence the immature newborn immune system.

Introduction

There is no doubt about the usefulness of breastfeeding. Maternal milk includes not only an optimal nutrient composition but also many immunologically active components, with colostrum (early milk) being richer in these constituents than mature milk. Colostrum includes factors of both humoral and cellular immunity—cytokines, antibodies, various types of leukocytes, typically 40–50 % macrophages, 40–50 % polymorphonuclear neutrophils and 5–10 % lymphocytes, as well as a low number of epithelial cells (Wirt et al. 1992). Up to now, colostrum/milk humoral immunologic factors have been investigated by many authors (e.g. Islam et al. 2006; Zizka et al. 2007). The differences in the concentrations of cytokines and other humoral factors in the milk of healthy and allergic mothers have been described (e.g. Zizka et al. 2007); however, a detailed comparison of milk cells is lacking. The survival of colostral cells in the intestines of offspring and their transfer to the blood circulation were detected in animal models, and even indirectly also in humans (Hanson 2000; Reber et al. 2008; Zhou et al. 2000). The newborn organism is immature both in antibody formation and cellular immunity. This lack of antibodies is compensated by maternal immunoglobulin G, transferred transplacentally and by the colostral/milk IgA supply. Colostral lymphocytes are represented mainly by activated CD45RO+ cells (Bertotto et al. 1997). The possible effect of these maternal cells on the newborn immune system remains to be elucidated. Children of allergic mothers are at an increased risk of allergy development. The role of the genetic background is further supported by the close coexistence of child and mother during gestation and in early postnatal life mainly when a child is breastfed. Therefore, the immunological characteristics of the milk of allergic mothers are of interest. In the present study, we tested the possible differences in cytokine gene expression in the colostral cells of healthy and allergic mothers.

Materials and methods

Subjects

Mothers were divided into two groups, according to their allergy status. The diagnostics of allergy in mothers was

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based on the clinical manifestation of allergy persisting for longer than 24 months (allergy to the respiratory and food allergens; manifested with various individual combinations of hay fever, conjunctivitis, bronchitis, asthma, eczema, etc.), monitoring by an allergologist, positive skin prick tests or positive specific IgE antibodies and anti-allergic treatment before pregnancy. Detailed anamnestic data of mothers are given in Table 1. "Allergic mothers" were otherwise healthy. For data analysis were included 9 healthy mothers and 11 allergic mothers. Though a larger number of mothers were tested, not all samples either contained sufficient RNA concentration for analysis of all cytokines or passed strict criteria for RNA purity and quality ($A_{260/230}$, $A_{260/280}$). 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 the written informed consent of the mothers.

Colostrum

Colostrum from healthy and allergic mothers was obtained at day 3 after delivery. Colostrum was diluted 1:1 in minimal essential medium with 10 % fetal calf serum, and cells were obtained after centrifugation (500×*g*) and three washings with the same medium.

RNA isolation

Total intracellular RNA was isolated by an RNeasy Minikit (Qiagen) according to manufacturer instructions. RNA integrity was determined by gel electrophoresis in 1.5 % agarose gel stained with ethidium bromide. The purity of the RNA was assessed by the ratio of absorbance at 260 and 280 nm. RNA purity ranged from 1.9 to 2.3. The total RNA concentration was estimated by spectrophotometric measurements at 260 nm, assuming that 44 μg/mL RNA corresponds to one absorbance unit. RNA was stored in aliquotsat −20 °C until used for reverse transcription.

Quantitative real-time polymerase chain reaction

Isolated mRNA was converted to cDNA using reverse transcription reagents (Applied Biosystems), according to manufacturer instructions. A reaction mix for real-time PCR was made with TaqMan Universal PCR master mix, RNase free water and Assays on Demand gene expression products for interleukins (IL) IL-2, IL-4, IL-8, IL-10, IL-13, interferon gamma (IFN-gamma), transforming growth factor 1 (TGF-beta1) and epidermal growth factor (EGF), all Applied Biosystems. Eukaryotic 18S ribosomal RNA (Applied Biosystems) was used as an endogenous control. All reactions were run in duplicate. The efficiency of reactions for all assays was very similar to the efficiency of the endogenous control.

PCR reactions were carried out on the 7300 real-time PCR system (Applied Biosystems) using standard conditions as described previously (Hrdy et al. 2010). Gene expression of cytokines in the colostral cells of 11 allergic mothers was expressed relatively to the mean of the gene expression of cytokines in the colostral cells of nine healthy mothers, using GenEx software (MultiD).

Statistics

Differences between groups were evaluated using the non-parametric Mann–Whitney test, due to data not being normally distributed and because of the low number of individuals tested. Statistical significance was set at $p \leq 0.05$.

Results

Cytokine gene expression in colostral cells of 9 healthy and 11 allergic mothers was compared by quantitative real-time polymerase chain reaction. Increased levels of expression of IL-4, IL-13 and EGF and a decreased level of IFN-gamma were detected in the colostral cells of allergic mothers in comparison with those of healthy mothers (Fig. 1.). Differences in the expression of IL-2, IL-8 and regulatory cytokines IL-10 and TGF-beta were negligible.

Although the differences detected are not significant (IL-2, $p=0.37$; IL-4, $p=0.15$; IL-8, $p=0.42$; IL-10, $p=0.47$; IL-13, $p=0.18$; IFN-gamma, $p=0.18$; TGF-beta, $p=0.32$) with the exception of a border significance of difference in EGF expression ($p=0.04$), a tendency is quite evident, especially in the case of IL-4, IL-13, IFN-gamma and EGF. The low significance is mainly due to high individual variability. The expression of Th2 cytokines supporting allergy development is increased in the colostral cells of allergic mothers. Because of a low number of mRNA copies of IL-4, this cytokine was detected in only three of nine samples in the healthy group and 6 of 11 in the allergic group (Table 2); therefore, its very low expression does not allow a reliable comparison. However, the increased expression of the other Th2 cytokine tested, IL-13, is relatively convincing. Furthermore, proportional representation of individual cell types was tested in our colostrum samples, and the result corresponded with available published data. No substantial differences were found in the ratio of different types of leukocytes (macrophages, NK, CD4 and CD8 T lymphocytes) estimated by flow cytometry between healthy and allergic mothers (data not shown).

Discussion

To the best of our knowledge, there have been only three studies evaluating the gene expression of cytokines in

Table 1 Detailed anamnestic characteristics of mothers and their children

Mothers	Age of mother	Smoker	Health condition (diagnosis) excepting allergy	Allergy	Medication	Number of pregnancies	Abortions	Course of pregnancy	Course of delivery	Sex of child	Body mass (g)	Length (cm)
A1	37	No		Acylpyrin, beta-lactam antibiotics		2nd	0		Cesaeran section after delivery induction	Female	3,470	51
A2	27	No		Pollen, dust, food allergens		1st	0		Forceps delivery after induction	Male	3,960	50
A3	29	Yes		Metals		1st	1×		Cesaeran section	Male	4,020	52
A4	34	No		Metals		1st	0		Spontaneous	Female	2,900	48
A5	32	No		Pollen, dust		1st	0		Cesaeran section	Female	2,540	44
A6	29	No		Asthma bronchiale—pollen, grass, dust, mites	Symbicort, Zyrtec	1st	0		Cesaeran section	Male	3,380	50
A7	35	No	*Streptococcus* group B+	Ajatin, beta-lactam antibiotics		1st	0		Cesaeran section	Female	3,780	48
A8	29	No		Atopic dermatitis		1st	0		Cesaeran section	Male	3,710	51
A9	30	No		Dust, pollen, mites		1st	0		Cesaeran section	Female	3,620	50
A10	30	No		Pollen, dust		1st	0		Cesaeran section	Male	3,210	48
A11	32	No	*Streptococcus* group B+	Asthma bronchiale—pollen, mould	Zyrtec	1st	0		Cesaeran section	Male	3,550	50
H1	32	No		None		1st	0		Cesaeran section	Female	3,490	40
H2	39	No		None		1st	0	Pregnancy diabetes mellitus	Cesaeran section	Male	2,600	48
H3	27	No		None		1st	0		Cesaeran section	Male	3,630	51
H4	38	No	*Streptococcus* group B+, vaginal mycosis	None		1st	0		Cesaeran section	Male	3,490	50
H5	30	No	Recurrent colpitis	None		1st	0		Cesaeran section	Male	3,830	51
H6	31	No		None		2nd	1×	Pregnancy diabetes mellitus	Cesaeran section	Male	3,340	50
H7	38	No		None		2nd	1×	Pregnancy diabetes mellitus	Spontaneous	Male	3,420	51
H8	28	No		None		1st	0		Spontaneous	Female	3,030	50
H9	33	No		None		3rd	1×	Pregnancy diabetes mellitus	Cesaeran section	Female	3,450	48

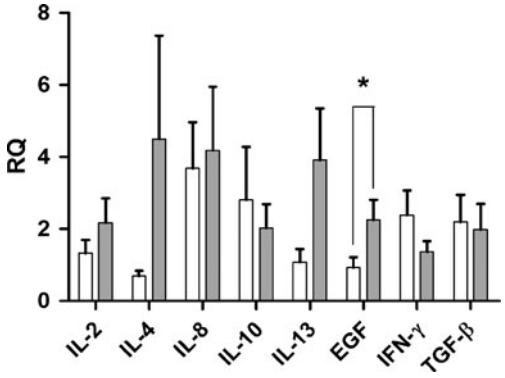

Fig. 1 Gene expression of cytokines in colostral cells of healthy (*open columns*) and allergic mothers (*filled columns*). *EGF* epidermal growth factor, *TGF* transforming growth factor, *RQ* relative quantification

colostral cells, and unfortunately, none related to the allergy status of the mother (Srivastava et al. 1996; Hashira et al. 2002; Nagatomo et al. 2004). The present study is the first report comparing the gene expression of cytokines between colostral cells of healthy and allergic mothers. The gene expression of the following cytokines was chosen: IL-2 and IFN-gamma, as representatives of Th1 cytokines; IL-4 and IL-13, as representatives of Th2 cytokines; IL-10 and TGF-beta, as regulatory cytokines; IL-8 as an inflammatory cytokine and EGF and TGF-beta, as mediators important for the development and differentiation of the intestinal epithelium, whose condition influences the permeability of the mucosal barrier for antigens and possible allergens. Increased expression of IL-4 and IL-13 and decreased expression of IFN-gamma point to the allergic phenotype of the colostral cells of allergic mothers. Increased expression of EGF in the allergic group, although being significant, is not probably of practical importance because the colostral cells are only minority producers of this growth factor.

Table 2 Individual levels of gene expression of cytokines in colostral cells of allergic and healthy mothers; values are expressed by relative quantification

Mother	IL-2	IL-4	IL-8	IL-10	IL-13	EGF	IFN-γ	TGF-β
Allergic group								
A1	0.80	0.59	18.85	1.02	13.37	1.14	0.17	0.83
A2	3.09	18.61	9.45	1.68	5.60	0.37	1.42	7.04
A3	7.60	2.29	2.71	1.72	0.09	0.70	2.78	1.31
A4	4.03	0.45	1.23	6.61	0.80	4.07	0.70	0.59
A5	0.66	1.20	0.39	0.28	2.99	4.63	2.20	3.96
A6	0.70	3.80	0.04	1.55	2.63	3.16	1.50	0.52
A7	0.62		0.15	0.18	0.51	0.19	0.25	0.65
A8	0.97		7.28	3.64	0.12	0.48	0.67	0.28
A9	1.13		0.42	0.22	4.62	4.84	3.11	1.10
A10	3.87		5.26	5.15	12.13	3.79	0.98	5.32
A11	0.38		0.25	0.18	0.19	1.29	1.21	0.16
Healthy group								
H1	0.32	0.45	1.02	0.32	0.49	0.34	1.26	0.35
H2	0.70	0.73	10.98	12.60	1.57	0.32	1.21	4.75
H3	1.84	0.92	6.86	3.04	3.13	0.10	4.07	4.64
H4	0.65		0.15	0.22	0.27	2.13	0.17	0.19
H5	4.00		1.15	0.29	0.20	1.02	0.34	0.20
H6	0.95		6.13	7.68	0.25	0.25	5.95	3.54
H7	1.53		5.52	0.56	0.48	0.36	4.27	0.46
H8	0.82		1.27	0.23	2.64	2.58	0.92	5.27
H9	1.09		0.07	0.28	0.64	0.91	3.25	0.32

In our previous work (Zizka et al. 2007), cytokine concentrations in colostra were evaluated by ELISA. On a protein level, IL-4 and IL-13 showed similar trends as the gene expression in colostral cells. IL-2 protein in colostrum was under the level of detection in both groups of mothers; IL-8 and IFN-gamma were not changed in allergic mothers; IL-10 was significantly increased, and TGF-beta and EGF were significantly decreased in the colostra of the allergic group. The results of different research groups' testing of cytokine concentrations in the colostrum/milk of healthy and allergic mothers are very heterogeneous, with large individual variability (Zizka et al. 2007; Hashira et al. 2002; Rigotti et al. 2006; Peroni et al. 2010). However, it is not possible to compare cytokine gene expression by colostral cells with cytokine concentration in colostrum because colostral cells are not the only source of colostral cytokines.

It is necessary to take into account that a certain portion of colostral cytokines could be transferred through the intestinal wall at the very beginning of postnatal life and could influence the early development of the immune system. From this point of view, the regulatory cytokines TGF-beta and IL-10 can be of importance (Rigotti et al. 2006; Peroni et al. 2010). As mentioned above, the transfer of maternal colostral cells to the newborn's circulation has been described in several mammal species, and Reber et al. (2008) proved the positive effect of these cells on the postnatal development of leukocytes in the early postnatal period of calves. Therefore, it is necessary to keep in mind a possible effect of colostral cells having the allergic phenotype transferred from the allergic mother by breastfeeding. Nevertheless, it is difficult to distinguish between the effect of transferred colostral cells and the action of soluble colostral cytokines.

In conclusion, we are able to show in the present work the increased gene expression of Th2 cytokines (IL-4 and IL-13) and decreased expression of IFN-gamma in the colostral cells of allergic mothers, in comparison with those of healthy ones. Our results point to an allergic phenotype of colostral cells of allergic mothers with a bias to a Th2-type response. It remains a question if the small number of these cells could influence the immature newborn immune system.

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Title page

Differing gene expression of subunits of the IL-12 family cytokines in mDCs derived *in vitro* from the cord blood of children of healthy and allergic mothers

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Abstract

Background

The incidence of allergic diseases is steadily increasing; arising an urgent need to clarify the immunologic processes, which occur early in life and signal an increased risk of possible future allergy development. The ratio and maturation state of DCs together with the cytokine environment are important in directing and modulating immune responses.

Methods

The maturation state of cord blood derived mDCs was estimated by flow cytometry, according to the surface expression of the CD83 activation marker.The capacity of mDCs of children of healthy and allergic mothers to express genes for subunits of IL-12 family cytokines was monitored using real-time PCR and protein secretion in culture supernatants by ELISA.

Results

The increased surface expression of CD83 activation marker on mDCs of children of allergic mothers was not significantly different from those on mDCs of children of healthy mothers, but a tendency to an increased proportion of CD83 positive mDCs after LPS-stimulation in allergic group was obvious. Significant differences were detected only in gene expression. Significantly higher gene expression of subunits of IL-12 family members was observed in mDCs of children of allergic mothers, in comparison with children of healthy mothers. The differences were evident mainly after LPS stimulation of mDC with the exception of p28 whose expression is significantly higher in allergic group even without stimulation. Proteins encoded by the genes under study were detected in culture supernatants but the differences between allergic and healthy group were not significant.

Conclusions

The observation of both higher IL-12 family gene expression and an increased presence of cell surface activation markers on mDCs of children of allergic mothers, indicates a higher reactivity of these cells, which could be reflected by an increased readiness of the immune system to allergic sensitization, later leading to allergy development.

Key words: IL-12 family, cytokine, gene expression, allergy, cord blood, dendritic cell

1. Introduction

Although APC immaturity is characteristic for the neonatal period [1], it has been suggested some infants may have a more significant and prolonged functional impairment of APC function, leading to a defective Th1 cell response, thus predisposing the child to future allergy development [2]. Prescott hypothesizes that neonatal maturational deficiency is more pronounced in individuals who go on to develop atopy but differences in IL-12 are not significant [3]. Newborns with atopic heredity show a more delayed increase in IFN-gamma responses in the early postnatal period, with the tendency of IFN-gamma to remain significantly reduced relative to low risk infants [4-8]. An impaired Th1 neonatal immune response, characterized by lower production of IL-12 and IFN-gamma, is well known. However, it remains unresolved whether the lower IFN-gamma responses are due to defective neonatal APC function. The characterization of newborn mDCs reactivity is the topic of present study.

In our former work concerning the immunologic characteristics of children with an increased risk of allergy a decreased gene expression of IFN-gamma in cord blood mononuclear cells and its decreased level in cord blood [9] were detected and increased proliferation activity, both spontaneous and stimulated, was proven with cord blood lymphocytes of children of allergic mothers [10]. The importance of dendritic cells (DCs) for T cell function directed our interest to the possible differences between mDCs derived from the cord blood of newborns of either allergic or healthy mothers. Gene and protein expression of IL-12 family cytokines and surface expression of the activation marker CD83 were monitored.

In humans, two main DC populations are present: mDC (DC1) secreting IL-12, IL-23, and IL-27 and being essential for priming naïve T cells and for their differentiation towards Th1 response, thus exerting an anti-allergic effect; and pDC (DC2) with the capacity for Th2 promotion [11;12]. The IL-12 family is represented by heterodimeric cytokines formed by alpha (p35, p19, p28) and beta (p40, EBI-3) chains. IL-12 consists of p35 and p40; IL-23 is assembled of p19 and p40; IL-27 compounds of p28 (IL-30) and EBI-3; and IL-35 comprises p35 and EBI-3, as indicated in tab.1.

Co-expression of the alpha and beta chains is required for the secretion of a bioactive cytokine. Production of each of the four heterodimeric proteins is limited by

the expression of the alpha chains, with beta chains being produced in an abundance. IL-12 family cytokines are involved in many immunological processes and, together with DC maturation stage and mDC/pDC ratio, can prevent or promote allergy development. IL-12, IL-23, and IL-27 were initially described as pro-inflammatory cytokines, promoting T-cell proliferation and cytokine production. IL-12 is known for its capability of Th1 activation and maintenance of Th1 response, while blocking Th2 [13]. IL-23 is involved in Th1 activation and the induction of IFN-gamma production, Th17 polarization, and proliferation. IL-27 promotes Th1 cell proliferation by blocking Th17 [13;14]. Like IL-12, IL-27 can directly inhibit the secretion of IL-4 and antagonize IL-2 production, hence limiting Th2 cell differentiation and promoting T cell proliferation and IFN-gamma production by naïve T cells. Both IL-12 and IL-27 are capable of inducing T-bet, IFN-gamma, and IL-12Rbeta2 expression in naïve T cells [13;15]. Besides this Th1 promoting function, a marginal effect of IL-27 on naïve B cell switch to IgE production was described in human [16;17]. Although IL-35 belongs to the IL-12 family, it serves a regulatory function by inhibiting T cell proliferation. IL-12, IL-23, and IL-27 are all primarily produced by antigen-presenting cells; but in contrast, IL-35 is mainly produced by regulatory T cells [18].

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

2. Material and methods

2.1. Subjects

Children were divided into two groups according to maternal allergy status. Healthy and allergic mothers with a physiological pregnancy and children delivered physiologically (vaginally) in full term were included in the study. Diagnostics of allergy in mothers was based on the clinical manifestation of an allergy persisting for longer than 24 months (allergy against respiratory and food allergens manifested 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. 58 children of allergic mothers and 52 children of healthy mothers were comprised in the study. The study was approved by the Ethical Committee of the Ministry of Public Health, and was carried out with the informed consent of the mothers.

2.2. Cord blood

Cord blood from children of both healthy and allergic mothers was collected immediately after delivery, with the usual procedure including careful cleaning of the cord and the puncture of the umbilical vein to avoid maternal contamination. 10-30 ml of cord blood was collected in sterile heparinized tubes (10 U heparin/ml).

2.3. Generation and maturation of mDCs

CBMC were acquired by gradient centrifugation using Ficoll-Pague (Amersham Biosciences), according to the manufacturer's instructions. Up to 6 x 10^6 CBMC in 15 ml of MEM (Sigma) were incubated for 2 h in 75 cm^2 plastic culture flasks (Nunc). After removal of the nonadherent fraction of CBMC by washing with MEM (Sigma); the adherent fraction was incubated in 15 ml of RMPI 1640 (Cambrex), supplemented with L-glutamin (2mM, Sevapharma), HEPES (2mM, Sigma), gentamicin (40mg/l), 10% FBS (Cambrex), rIL-4 (20 ng/ml, Peprotech), and rGM-CSF (500 U/ml, Leucomax) at 37°C in a 5% CO_2 atmosphere for 5 days. The purity and activation state of generated mDCs were examined by flow cytometry (CD11c+CD14- cells were evaluated as mDCs). The minimal yield and purity of cells used for further analyses was 2-4 x 10^6 mDCs from a single culture flask, (purity in the range of 85-95%) with up to 10% matured mDC (CD83+). The maturation of mDCs was induced by their cultivation (1 x 10^6 cells/ml in 24-well plates) with LPS (1µg/ml, *Escherichia coli*, Sigma) for 24 h.

2.4. FACS analysis

DCs generated *in vitro* from cord blood were subjected to flow analysis using a BD FACS Canto II in BD FACS Diva 6.1.2. (Becton Dickinson) and the data were analyzed using FlowJo 7.2.2. (Tree Star). Cells were incubated with APC conjugated CD11c mAbs (Miltenyi Biotech), PerCP conjugated CD14 mAbs, and PE conjugated

CD83 mAbs (both Becton Dickinson) for 20 min, then washed three-time with a FBS staining buffer (Becton Dickinson), and immediately analyzed. The maturation state of mDCs was checked by the fluorescence intensity of maturation marker CD83 on CD11c+ CD14-cells.

2.5. RNA isolation

Total intracellular mDCs' RNA was isolated with the RNeasy Minikit (Quiagen), according to the manufacturer's instructions. RNA integrity was determined by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The purity of RNA was assessed by the ratio of absorbance at 260 nm and 280 nm, with purity in the range of 1.9 to 2.3. The total RNA concentration was estimated by spectrophotometric measurement at 260 nm, assuming that 44 μg of RNA per milliliter equals one absorbance unit. RNA was stored in aliquots at -20 °C until used for reverse transcription.

2.6. Real-time PCR

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

PCR reactions were run on the 7300 real-time PCR system (Applied Biosystems) using standard conditions. A no-template control contained water instead of cDNA. The total amount of cDNA in the reaction was 100 ng. Expression of all genes was normalized to the mRNA loading for each sample and used as an internal

standard (endogenous control). The quantity of mRNA (relative quantification, RQ) was given as $2^{-\Delta\Delta ct}$. ΔΔct was calculated as follows: ΔΔct = Δct (mDCs of children of allergic mothers) - Δct (mDCs of children of healthy mothers). Δct = ct (concrete IL-12 family subunit) - ct (endogenous control/cyclophilin A). The ct value is the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. The gene expression of subunits of the IL-12 family cytokines in mDC of 12 children of allergic mothers was expressed relative to the mean of gene expression of the subunit of the IL-12 family in mDC of 12 children of healthy mothers, using GenEx software (MultiD).

2.7. Determination of *in vitro* secreted cytokines by ELISA method

Concentrations of IL-12, IL-23, IL-27 and IL-35 in cell culture supernatants after 3-day cultivation of mDC (nonstimulated or LPS-stimulated) were quantified by ELISA on high-adsorption polystyrene microtitration plates (NUNC) using the following reagents and producer recommendations: IL-12 – primary antibody (MAB 611), secondary antibody (BAF 219) and standard by R&D (detection limit 15 pg/ml); IL-23 – Duoset BMS2023/2MST by Bender MedSystem (detection limit 80 pg/ml); IL-27 – Duoset DY2526 by R&D (detection limit 130 pg/ml); IL-35 – Detection kit E92008Hu by Uscn, Life Science Inc. (detection limit 15 pg/ml). The results were read from calibration curves in pg/ml.

2.8. Statistics

Differences between groups were evaluated using the paired and unpaired Student's t-test for data normally distributed (comparing stimulatory indices); otherwise the non-parametric Mann-Whitney test was utilized (comparing MFI of activation markers and gene expression of subunits of cytokines of IL-12 family). Statistical significance was set at $p \leq 0.05$. For statistical evaluation of ELISA results, the values under the detection level were given as half the detection limit.

3. Results

mDC generated by cultivation of cord blood mononuclear cells were supposed to be mostly in non-activated state and we could not expect their intensive cytokine gene expression. Therefore, mDC obtained were *in vitro* stimulated with LPS and substantial

gene expression after cell activation made it possible to see better the differences between healthy and allergic group.

3.1. Activation/maturation state of mDCs

The maturation state was evaluated in both non-stimulated and LPS-stimulated cord blood mDCs in all tested children (allergic and healthy group 58 and 52 children, respectively). CD83 surface expression, measured by flow cytometry, was used as an activation marker. Because of large individual variability, the differences between nonstimulated mDCs of children of healthy and allergic mothers did not reach statistical significance - neither in cell proportions nor in MFI. However, a higher proportion of CD83+ cells and higher MFI in LPS-stimulated cultures of children of allergic mothers, in comparison with children of healthy mothers, was quite evident and significant, and pointed to a more intensive response to LPS in the allergic group – Fig. 1a - d.

3.2. Evaluation of gene expression of the IL-12 family

Gene expression was tested in mDC derived from cord blood mononuclear cells of 12 newborns of allergic mothers and 12 newborns of healthy mothers. Results obtained by expression analysis of IL-12 family subunits in non-stimulated mDCs revealed the increased expression of p19, p28, p35, and p40 in infants of allergic mothers in comparison with that of healthy mothers; however, only the difference in p28 was significant. An adverse trend was detected in EBI-3 expression. In contrast to non-stimulated mDCs, we discovered higher gene expression of all IL-12 family subunits after LPS-stimulation, with a significantly higher increase in p28, p35, p40, and EBI-3 expression in mDCs of children of allergic mothers, as compared to mDCs of children of healthy mothers. Only a hint of increase in p19 gene expression was seen in comparison to non-stimulated mDCs of children of both healthy and allergic mothers. – Fig. 2. The response to LPS stimulation is evidently higher in allergic group. The obvious expression of all alpha chains (p35, p19 and p28) represents the prerequisite for IL-12, IL-23, IL-27 and IL-35 formation in neonatal period of life.

3.3. Cytokine secretion *in vitro*

The quantity of individual cytokines secreted *in vitro* after a 3-day cultivation was measured immunoenzymaticaly using antibodies against specific epitopes of the

heterodimeric molecules under study. Non-stimulated mDC secreted measurable amount of cytokines only in the case of IL-12 and IL-27; IL-23 and IL-35 were under the detection limit. The production of all cytokines was substantially increased after stimulation with LPS, as was gene expression but the differences between allergic and healthy group were not significant while gene expression of all subunits tested except p19 was significantly higher in allergic group after LPS stimulation – Fig. 3. IL-35 is known as suppressive cytokine produced by Tregs. Here we show at first time its intensive synthesis also by stimulated mDC.

DISCUSSION

The study was addressed to evaluate mDC reactivity in children of healthy and allergic mothers, in the effort to elucidate the background of the increased risk of allergy development in children of allergic mothers. Paternal allergy was not taken in consideration in this study because maternal impact on future allergy development of offspring is much stronger due to the 9-month contact of fetus with maternal immune system [2;19;20]. In our former experiments, we proved higher proliferation activity of cord blood lymphocytes [10] in newborns of allergic mothers in comparison with children of healthy mothers. It called our attention to the possibly increased reactogenicity of the immune system of children with increased allergy risk, which could cause easier stimulation after the encounter with potential allergens. Because DC as antigen presenting cells decide about the character of oncoming immune response, we tried to follow the activation properties of mDC generated from cord blood mononuclear leukocytes. Previous studies focused mainly on comparing IL-12 gene expression of both p40 and p35 subunits in healthy adults and newborns; with the conclusion that expression of IL-12 is lower in children than in adults [3;21-24]. Differences in IL-12 gene and mainly protein expression between children with positive or negative family history of allergy were tested with contradictory results [3;19;20;25]. To the best of our knowledge, there is currently no information available about the gene expression of all of the so far described members of the IL-12 family in the mDCs of high risk newborns (infants of allergic mothers) or low risk ones (children of healthy mothers).

The reactivity of mDC derived from cord blood after cultivation of mononuclear cells with IL-4 and GM-CSF was assessed by testing activation marker surface expression, by measuring gene expression of all subunits of heterodimeric IL-12 family and by detection of IL-12 family cytokine synthesis *in vitro*. The absolute majority of mDC obtained were non-mature (non-activated), therefore all their functional properties detected were of very low, often hardly detectable, level and it was difficult or impossible to compare these data between allergic and healthy group. However, after *in vitro* stimulation of mDC with LPS, it was possible to follow and compare the reaction promptness of these cells. We have found increased reactivity of mDC of children of allergic mothers in all variables followed but substantial significance was reached only in differences in gene expression. The gene expression of IL-12 family subunits was reflected by *in vitro* synthesis of corresponding cytokines but differences between allergic and healthy group were not evident on protein level. It is possible to imagine that the potency of increased immunological reactivity of high allergy risk newborns is evident on the gene level but this potency is not yet exploited. Probably differences in secretion are influenced by further additional factors, mainly environmental factors applied only after birth.

We followed IL-12 family cytokines as important effectors of mDC, which are responsible for the bias of Th cells to anti-allergic Th1 population (IL-12, IL-23, IL-27). The next member of this family – IL-35 - is a suppressive cytokine which could damp inappropriate allergic responses. Therefore we speculated that the formation of these cytokines could be defective in allergy-prone newborns. However, the opposite is true. The gene expression of both IL-12 subunits is very well pronounced in newborn mDC even if IL-12 secretion is not very intensive. It was suggested IL-23 or IL-27 could substitute the insufficient production of IL-12 in small children [17;26]. Really, we were able to prove very convincing both gene and protein expression in the case of IL-27 , however, the formation of IL-23 in perinatal period seems to be not so very important, mainly on basis of modest expression of p19. We are looking for a reasonable explanation of increased IL-12 family gene expression in allergic group. It is broadly accepted that children of allergic mothers are at increased risk of future allergy development. However, the potential allergy is not yet manifested at birth. An increased support of Th1 cells and potentiation of suppressory mechanisms (IL-35) by IL-12

family cytokines in high risk newborns could be supposed to serve as a compensatory mechanism in genetically disadvantaged children, which can keep the equilibrium of the immune system for a certain time but can be insufficient later in life when additional environmental factors influence the predisposed organism. Increased reactivity of various cells of immune system can then facilitate allergy outbreak after an encounter with allergens under appropriate conditions.

It is possible to conclude that certain phenotypic differences pointing to increased reactivity of immune system are evident in children with increased risk of allergy already at birth. However, the allergy is such a multifactorial process [20] that it is not possible to suppose the change in one immunologic characteristic can serve as a predictive marker for future allergy development.

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Tab. 1

Subunits of heterodimeric cytokines of IL-12 family

	alpha chain	beta chain
IL-12	p35	p40
IL-23	p19	p40
IL-27	p28	EBI-3
IL-35	p35	EBI-3

Figure Legends

Fig. 1

Surface expression of the activation marker CD83 on the mDC derived from cord blood. Flow cytometry, one typical example. a – mDC of a child of healthy mother, b – mDC of a child of allergic mother

Full histogram represents MFI (median fluorescence intensity) of CD83 in non-stimulated mDC

Empty histogram represents MFI (median fluorescence intensity) of CD83 in LPS stimulated mDC

Fig.2

Gene expression of IL-12 cytokine family subunits in mDC derived from cord blood detected RQ-PCR. Results expressed as relative quantification (RQ).

HC gene expression in no-nstimulated mDCs of children of healthy mothers

AC gene expression in nonstimulated mDCs of children of allergic mothers

HLPS gene expression in lipopolysacharide stimulated mDCs of children of healthy mothers

ALPS gene expression in lipopolysacharide stimulated mDCs of children of allergic mothers, RQ relative quantification,

Fig. 2a p19

Fig. 2b p28

Fig. 2c p35

Fig. 2d p40

Fig. 2e EBI-3 (Ebstein-Barr virus induced gene 3)

* $p \leq 0.05$

** $p \leq 0.01$

** $p \leq 0.001$

Fig. 3

Protein expression of IL-12 family cytokines by mDC derived from cord blood detected by ELISA in cell culture supernatants after 3 day cultivation
IL-12 family cytokines secreted by mDC in vitro after 3 day cultivation detected by ELISA

HC cytokine concentration in cell culture supernatant of no-nstimulated mDCs of children of healthy mothers

AC cytokine concentration in cell culture supernatant of nonstimulated mDCs of children of allergic mothers

HLPS cytokine concentration in cell culture supernatant of lipopolysacharide stimulated mDCs of children of healthy mothers

ALPS cytokine concentration in cell culture supernatant of lipopolysacharide stimulated mDCs of children of allergic mothers, RQ relative quantification,

Fig. 3a IL-12

Fig. 3b IL-23

Fig. 3c IL-27

Fig. 3d IL-35

** $p \leq 0.01$

** $p \leq 0.001$

Fig.1a

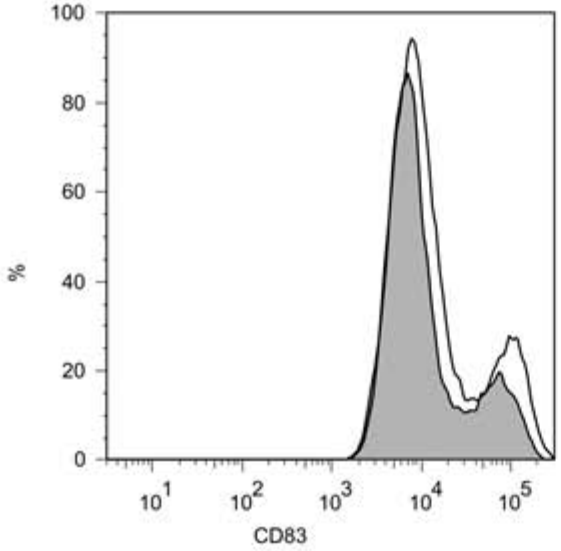


Fig.1b

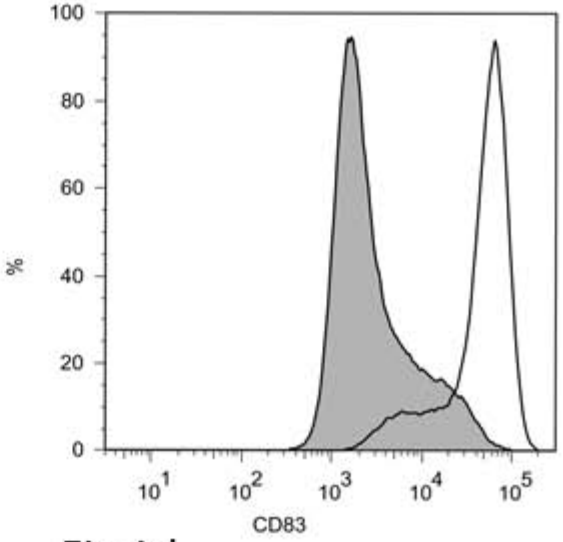


Fig.1c

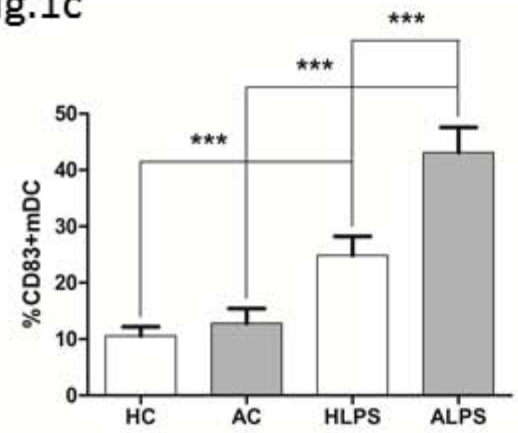


Fig.1d

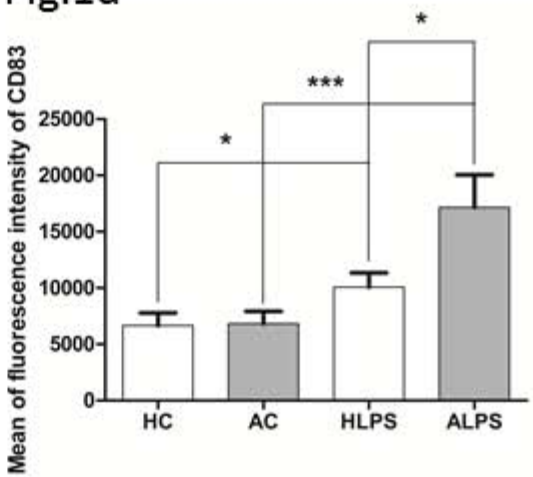
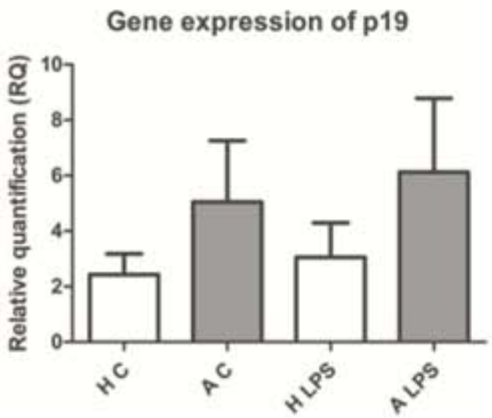


Fig.2a



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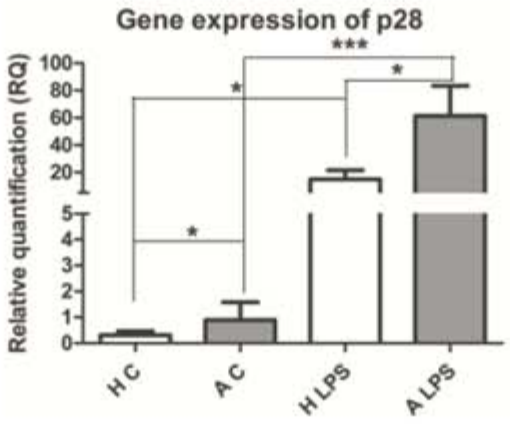

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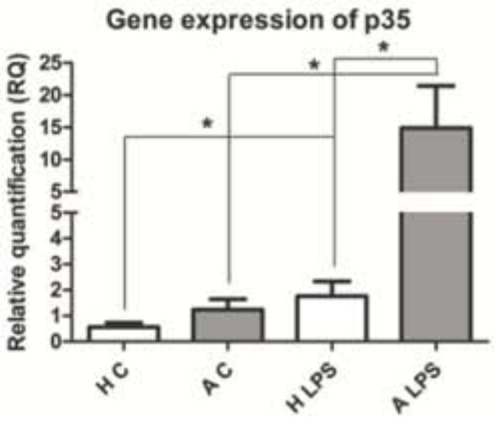

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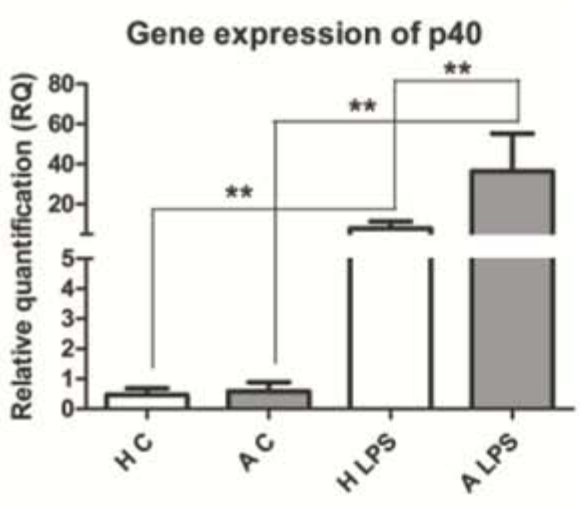

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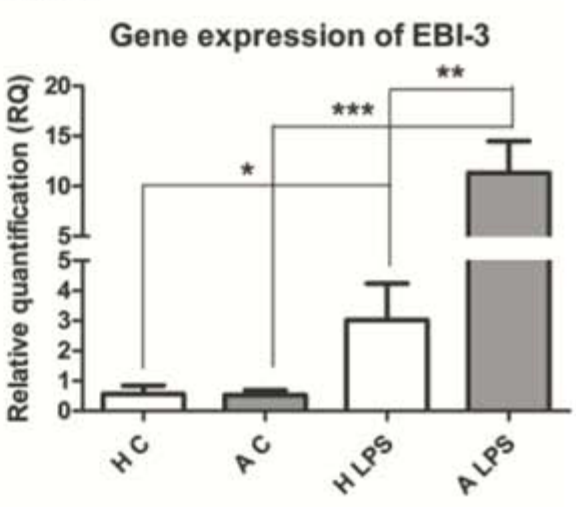


Fig.3a

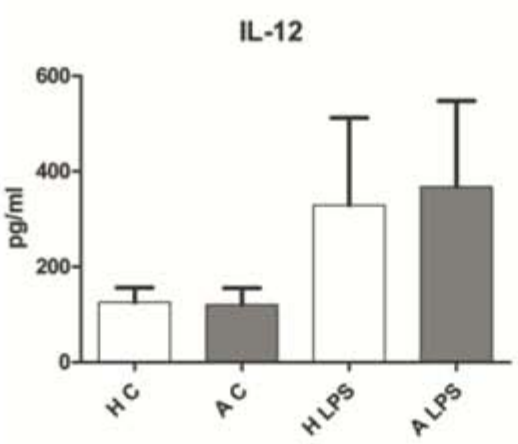


Fig.3b

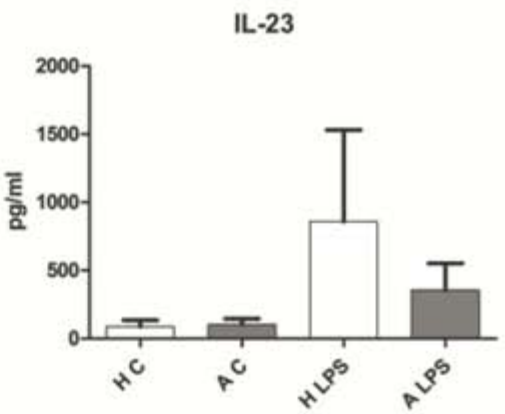


Fig.3c

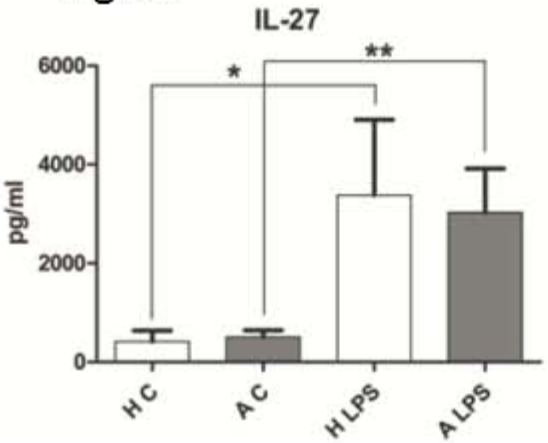
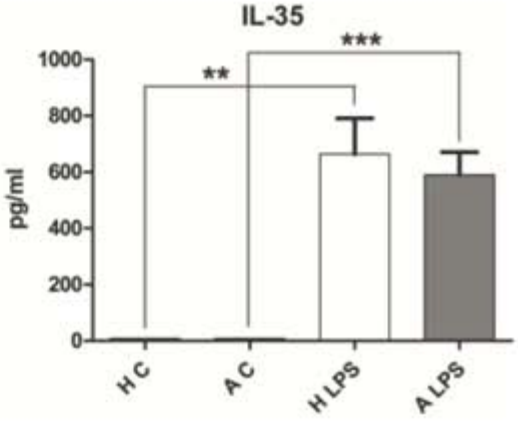


Fig.3d



Title page

Title: Differences in immunological characteristics of Tregs in cord blood of children of healthy and allergic mothers

Short Title: Tregs in cord blood of children of healthy and allergic mothers

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Abstract

Allergy is one of the most common diseases with constantly increasing incidence. Identification of prognostic markers pointing to increased risk of allergy development is of importance. Cord blood represents a suitable source of cells for searching for such prognostic markers.

In our previous work, we described increased reactivity of cord blood cells of newborns of allergic mothers in comparison to newborns of healthy mothers. That raised the question whether it was not due to the impaired function of regulatory T cells (Tregs) in high risk children. Therefore, the proportion and functional properties of Tregs in cord blood of children of healthy and allergic mothers were estimated by flow cytometry.

Proportion of Tregs (CD4+CD25^{high}CD127^{low}FoxP3+) in cord blood of children of allergic mothers tends to be higher while, in contrast, the median of fluorescence intensity of FoxP3 was significantly increased in the healthy group. Intracellular presence of regulatory cytokines IL-10 and TGF-beta was also higher in Tregs of children of healthy mothers.

Although we detected increased proportion of Tregs in cord blood of children of allergic mothers, functional indicators of those Tregs were lower in comparison to healthy group. We can conclude that impaired function of Tregs in cord blood of children of allergic mothers could be partially compensated by their increased number. Insufficient function of Tregs could facilitate allergen sensitization in high risk individuals after future allergen encounter.

Key words: Tregs, allergy, cord blood, FoxP3, regulatory cytokines

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 infants. There is a bias to Th2 immune responses in prenatal period preventing undesirable interactions with antigenically different maternal organism [1]. The establishment of a new immunological balance proceeds postnatally after the encounter 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 increased risk of allergisation. The finding of such prognostic marker would make possible introduction of preventive measures hampering allergy development or at least lowering its clinical outcomes. There are many papers dealing with this topic and analysing cord blood for this purpose but, as concluded by Prescott [3], allergy status of the mother has so far been the only reliable marker in this respect.

Several studies tried to correlate the proportion of Tregs with the clinical symptoms of allergy in adults; unfortunately, contradictory results have as yet been obtained [4-9]. Flow cytometry is usually used in these studies. Conflicting results can be mainly due to various gating strategies used, different Treg markers tested and also different ethnic groups examined. All these inconsistences together with the low number of individuals included in some studies [10;11] led to ambiguous conclusions. Studies concerning cord blood Tregs and allergy are quite scarce [11;12]. It is possible to suppose some functional insufficiency of Tregs could precede allergy development. We tested this hypothesis by analyzing and comparing Tregs in cord blood of high risk newborns (children of allergic mothers) and low risk newborns (children of healthy mothers). Using flow cytometry, we compared the proportion of Tregs (% of Tregs in

CD4+ population) and their functional properties (median of fluorescence intensity of FoxP3, IL-10 and TGF-beta).

Materials and Methods

Subjects

Healthy and allergic mothers with physiological pregnancy and children delivered physiologically (vaginaly) in full term at the Institute for the Care of Mother and Child in Prague, Czech Republic were included in the study. Diagnostics of allergy in mothers was based on clinical manifestation of allergy persisting for longer than 24 months (allergy against respiratory and food allergens manifested by various individual combinations of hay fever, conjunctivitis, bronchitis, asthma, eczema, etc.), monitoring by an 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 the Institute for the Care of Mother and Child (Prague, Czech Republic) and was carried out with the written informed consent of the mothers.

153 children participated in our study. Newborns were divided into two groups according to their mothers´ allergy status: 77 children of healthy mothers (non-allergic) and 76 children of allergic mothers. Detailed description of mothers with different types of allergy involved in our study is summarized in Tab.1.

Cord blood sampling

Typically, 10 - 20 ml of cord blood of children was collected in sterile heparinized tubes for cell analysis (Tregs). A questionnaire inquiring about the allergy status of the mother was completed during the stay at The Institute for the Care of Mother and Child.

Tregs ratio, FoxP3 staining

The proportion of Tregs was estimated in cord blood samples immediately after delivery. The whole cord blood was stained for Treg cell surface markers using the following antibodies: CD4 FITC, cat. no. 555346, CD25 PE-Cy7, cat. no. 557741, CD127 Alexa 647, cat. no. 558598, all Becton Dickinson. After lysing erythrocytes, the

permabilized/fixed cord blood samples were stained for gold standard marker for identifying Tregs by anti-human FoxP3 antibody, cat. no. 12-4776-41A (clone PCH101) using Human Regulatory T Cell Whole Blood Staining Kit (cat. no. 88-8996-40), both eBioscience, according to manufacturer's recommendations. Lymphocyte gate was set based on FCS and SSC characteristics with doublets exclusion (FCS-A x FCS-H) then CD4+ population was gated in lymphocyte gate. Approximately 500 000 events per sample were acquired for proper statistical evaluation of Tregs functional parameters. Tregs were analyzed in CD4 gate as an intercept of three subpopulations of CD4+ lymphocytes using CD25, CD127 and FoxP3 markers (CD25 x CD127, CD25 x FoxP3, CD127 x FoxP3). Detailed gating strategy for the estimation of Treg ratio is shown in Fig.1. Results are expressed as Treg ratio and MFI (median of fluorescence intensity).

IL-10 and TGF-beta in Tregs

Regulatory cytokines were detected in Tregs after erythrocyte lysis. After cell surface staining of CD4, CD25, CD127 (using antibodies indicated above), intracellular staining of cytokines IL-10 (IL-10 PE, cat. no. 506804) and TGF-beta (anti-human LAP TGF-beta1 PerCP-Cy5.5, cat. no. 341803) was performed using Fixation Buffer, cat. no. 420801 and Permeabilization Wash Buffer, cat. no. 421002 (BioLegend) according to manufacturer's recommendations.

Data analysis and statistics

Flow cytometry data were acquired on BD FACS Canto II instrument using BD FACS Diva 6.1.2. software (Becton Dickinson). FlowJo 7.2.2. (TreeStar) was exploited for data evaluation. Differences between groups were compared by the unpaired Student's t-test for data normally distributed (Treg ratio, MFI of FoxP3); otherwise the non-parametric Mann-Whitney test was utilized (comparing proportion of IL-10+ Tregs and TGF-beta+ Tregs). Statistical and graphical analysis was performed in GrapPad Prism (GraphPad Software). Statistical significance was set at $p \leq 0.05$.

Results

The immunological characteristics of Tregs in cord blood of high risk children (children of allergic mothers) and low risk children (children of healthy mothers) were compared. The proportion of Tregs was evaluated. To elucidate possible differences in

functional properties of Tregs, MFI of FoxP3 and intracellular regulatory cytokines IL-10 and TGF-beta were tested. Differences in Treg proportions and their functional properties were found between the groups.

Treg ratio

Using our gating strategy – Fig. 1 and antibodies against CD4, CD25, CD127 and FoxP3, we did not find significant differences in the proportion of Tregs in cord blood of children of healthy and allergic mothers although the trend to increased number of Tregs in CD4+ lymphocyte population from the allergic group was quite obvious ($p = 0.07$), Fig. 2a. Significantly increased proportion of Tregs in cord blood of children of allergic mothers was observed when Tregs were considered only as CD4+CD25+, Fig. 2b. Different outcome of different gating strategies used can explain some discrepancies among the results of different research groups.

Median fluorescence intensity (MFI) of FoxP3

Transcription factor FoxP3 is supposed to be a master marker for identifying Tregs [13] (as CD25 can be expressed on other activated CD4+ T lymphocytes and CD127 is present on various cell types). The values of MFI of FoxP3 in cord blood of children of allergic mothers followed an opposite trend to the proportion of Tregs. A significantly higher MFI of FoxP3 in cord blood Tregs of children of healthy mothers was detected in comparison to children of allergic mothers, Fig. 3.

Intracellular regulatory cytokines IL-10 and TGF-beta

To evaluate the possible differences in functional characteristics of Tregs, the presence of regulatory cytokines IL-10 and TGF-beta was estimated by intracellular staining. A significantly increased number of IL-10+ Tregs in cord blood of children of healthy mothers was detected in comparison to children of allergic mothers, Fig. 4. Similarly, a significantly increased proportion of TGF-beta+ Tregs in cord blood of children of healthy mothers is documented in Fig. 5.

Discussion

The importance of Tregs in immune regulations consists mainly in their role in induction of peripheral tolerance against autoantigens and harmless food and environmental antigens [14]. An insufficiency of Tregs can result in autoimmunity and allergy development [15-18]. We followed the status of newborn Tregs as a possible prognostic marker for future allergy manifestation. It is possible to assume that changes

of the immune regulation in allergy prone infants can be evident earlier than clinical signs of allergy.

We found differences in immune characteristics of Tregs in cord blood of children of allergic mothers in comparison to children of healthy mothers. Tregs were assessed on the basis of their cell surface markers (CD4, $CD25^{high}$ and $CD127^{low}$), typical transcription factor FoxP3 and intracellular regulatory cytokines IL-10 and TGF-beta. Lower presence of regulatory cytokines together with decreased MFI of FoxP3 in Tregs in cord blood of children of allergic mothers points to lower functional efficiency of these cells [19;20]. Impaired function of Tregs in the cord blood of children of allergic mothers could be partially compensated by an increased number of Tregs in comparison with the healthy group.

We documented an increased proportion of CD4+ $CD25^{high}$ $CD127^{low}$ FoxP3+ Tregs in children of allergic mothers. As indicated by Steinborn [20], FoxP3 is an important marker of regulatory cells reflecting their suppressor potency. When Tregs were detected only as CD4+ $CD25^{+}$ cells their number was still higher. It is necessary to keep in mind that the above phenotype is characteristic not only for Tregs but also for various subpopulations of activated T cells [21]. Increased proportion of CD4+$CD25^{+}$ subpopulation in cord blood of children of allergic mothers is in concordance with our previous observation of increased proliferation activity of both *in vitro* stimulated and non-stimulated cord blood cells of newborns of allergic mothers [22]. Discrimination between regulatory and activated T cells could be done on the bases of recently described inverse correlation between CD127 and FoxP3 expression [23;24].

Regulatory cytokines IL-10 and TGF-beta are important effectors of Tregs [2;25;26]. Increased secretion of IL-10 (detected by ELISA) correlated with increased Tregs markers after stimulation of cord blood cells of children of healthy mothers as reported by Schaub [27]. To our best knowledge, we are the first to report on the differences in the presence of intracellular IL-10 and TGF-beta between Tregs of children of healthy and allergic mothers. Lower proportion of Tregs producing IL-10 and TGF-beta in cord blood of children of allergic mothers (Fig. 4, 5) can signalize a decreased predisposition to limiting the aberrant immune reaction to allergens in the

future and can partially explain the increased proliferation activity of cord blood lymphocytes of children of allergic mothers mentioned above.

Regulatory T cells are a very heterogeneous population of cells and many methodological problems arise in the course of their study. Different gating strategies used for quantification of Tregs (CD4+CD25+[28], CD4+CD25^{high}[29], CD4+CD25^{high}CD127^{low}[11], CD4+CD25^{high}FoxP3+[30], CD4+CD25^{high}CD127^{low}FoxP3+[31] or the gating suggested by us based on the intercept of three different gates on CD4 subpopulation as indicated in Fig.1) can give quite different results leading to controversial conclusions. Furthermore, using different clones of FoxP3 antibodies could lead to different values of Tregs ratio [32;33]. Using different clones of FoxP3 antibodies enables the detection of different Treg subpopulations. In our early experimental setting, we used two antibody clones (PCH101 – eBioscience, 259D/C7 – Becton Dickinson) with appropriate buffers. Increased proportion of FoxP3+ Tregs was detected using PCH101, which was then used for further experiments. However, it is necessary to realize that the amount of Tregs alone is not decisive for effective suppression function [34]. 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]. Another obstacle can be caused by cell isolation. Many studies analyze Tregs in peripheral blood after Ficoll-Paque separation. We compared detection of Tregs in the whole blood and in the population of isolated cord blood mononuclear cells (CBMC) – the results were similar but the analyses obtained with the whole blood were more convincing and consistent and less time consuming (data not shown).

We acknowledge some limitations of our study, namely heterogeneity of allergies of mothers, but differentiation of the children into subgroups according to different kinds of maternal allergy decreased the power of statistical analyses. Individual types of maternal allergies are listed in Tab. 1.

Tregs are supposed to play an important role in immune regulations even during intrauterine life [36]. Increased number of Tregs in this period can be partially responsible for decreased neonatal immune responses. The function of Tregs is critical in early postnatal period when the tuning of immature immune system takes place. The

impairment of Tregs could be the underlying mechanism contributing to easier allergy development in predisposed children.

Our proof of decreased functionality of Tregs in cord blood of children of allergic mothers is fully in compliance with the work of Prescott [37], who tested immune function of neonatal CD4+CD25+CD127 $^{low/-}$ Tregs. However, both Prescott [37] and Schaub [29] did not find significant differences in transcription factor FoxP3 between high and low risk infants whereas other studies pointed to decreased function of Tregs based on the lower presence of FoxP3 (MFI) [12]. This could be explained either by low number of individuals included [11] or by different method used for quantification of FoxP3. qPCR was often used for detection of FoxP3 gene expression [11;29]. On the other hand, we exploited flow cytometry for FoxP3 protein detection. Schaub [29] suggests that mRNA level of FoxP3 in Tregs is not differently regulated in dependence on maternal atopy. Nevertheless, the same group observed quantitatively and qualitatively increased Tregs in cord blood of children of farming mothers whose children were supposed to be low risk individuals for allergy development [36].

It is believed that lower exposure to non-pathogenic microbes together with reduced T-regulatory function early in the life could lead to Th1/Th2 imbalance increasing the risk of allergy development [38]. Relationship between immune function of cord blood Tregs and allergy development requires further detailed studies.

In conclusion, our study points to decreased immunological capacity of Tregs in cord blood of children of allergic mothers in comparison to healthy ones. Insufficient function of Tregs can facilitate allergy induction in predisposed children. Long-term monitoring of children in risk is necessary for assessing the significance of prognostic value of Tregs insufficiency at birth for future allergy development.

Acknowledgements

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Figure Legends

Fig. 1
Gating strategy of proportion of Tregs in cord blood of children of healthy and allergic mothers.
Expression of the proportion of Tregs in CD4+ lymphocyte gate was considered as an intercept of three gates based on the combination of staining cell surface markers (CD4, CD25, CD127) and intracellular staining of transcription factor FoxP3.

Fig. 2
Proportion of Tregs in cord blood.
2a Four colour flow cytometry analysis (intercept of CD4+CD25^{high}CD127^{low} and CD4+CD25^{high}FoxP3+ and CD4+CD127^{low}FoxP3+).
2b Two colour flow cytometry analysis (CD4+CD25+).

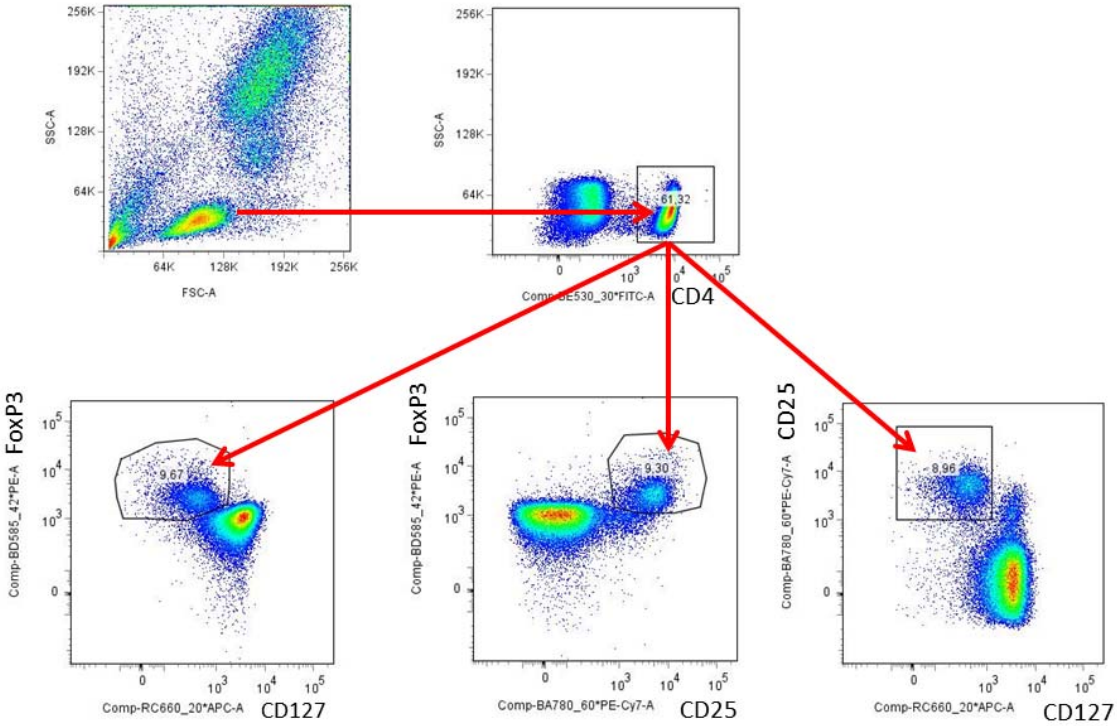
H - proportion of Tregs in CD4+ lymphocytes in cord blood of children of healthy mothers (mean of 77 tested cord blood samples)
A - proportion of Tregs in CD4+ lymphocytes in cord blood of children of allergic mothers (mean of 76 tested cord blood samples)
* $p \leq 0.05$

Fig. 3
MFI of FoxP3 in CD4+CD25^{high}CD127^{low} Tregs.
H - MFI of FoxP3 in Tregs from cord blood of children of healthy mothers (mean of 77 tested cord blood samples)
A - MFI of FoxP3 in Tregs from cord blood of children of allergic mothers (mean of 76 tested cord blood samples)
* $p \leq 0.05$

Fig. 4
Intracellular presence of IL-10 in Tregs.
% IL-10 positive Tregs (CD4+CD25^{high}CD127^{low}).
H - % IL-10+ Tregs in cord blood of children of healthy mothers (mean of 77 tested cord blood samples)
A - % IL-10+ Tregs in cord blood of children of allergic mothers (mean of 76 tested cord blood samples)
** $p \leq 0.001$

Fig. 5
Intracellular presence of TGF-beta in Tregs.
% TGF-beta positive Tregs (CD4+CD25^{high}CD127^{low}).
H - % TGF-beta+ Tregs in cord blood of children of healthy mothers (mean of 77 tested cord blood samples)
A - % TGF-beta+ Tregs in cord blood of children of allergic mothers (mean of 76 tested cord blood samples)
* $p \leq 0.05$

Fig. 1


SSC-A
FSC-A
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CD4
FoxP3
9.67
CD127
FoxP3
9.30
CD25
CD25
8.96
CD127

Fig. 2a

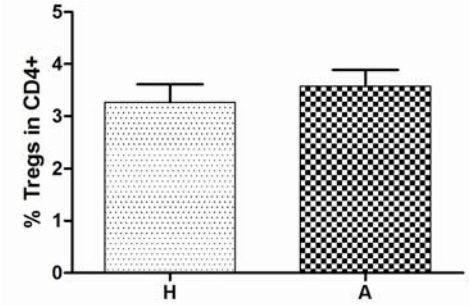


Fig. 2b

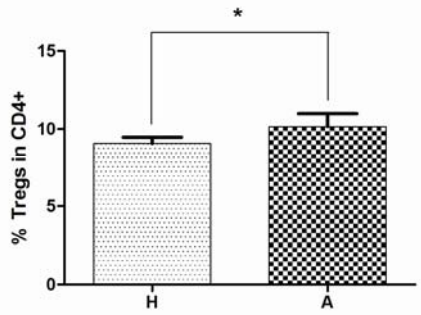


Fig. 3

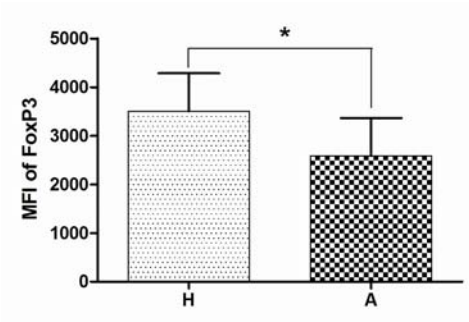


Fig. 4

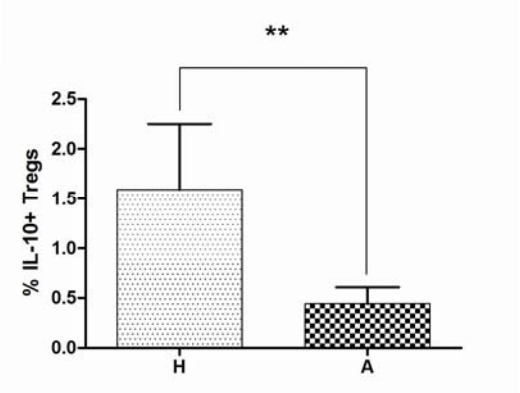
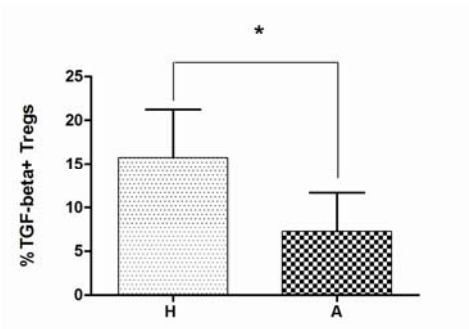


Fig. 5



Tab. 1

Detailed characteristics of mothers participating in this study.

allergy status	number	median of age	min	max	pollen	mites	dust	cat dander	food	metal	medicaments	insects	eczema	others
allergic mothers	76	31	24	37	27	9	14	2	8	2	35	6	7	18
non-allergic mothers	77	32	20	39										

Title Page

Title: The Effect of the Probiotic Vaccine Colinfant New Born on Regulatory T-cells in Six Year Old Children

Running Title: ***Effect of the probiotic vaccine Colinfant on Tregs***

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Abstract

Allergies belong to the most common diseases with steadily increasing incidence. Probiotics are believed to prevent or reduce allergy development. Nevertheless, the mechanism of their beneficial effect is still poorly understood. Immune characteristics of regulatory T cells (Tregs) in peripheral blood of probiotic colonized and non-colonized, 6 – 7 year old children of allergic mothers; and non-colonized children of healthy mothers were compared. Children were colonized within 3 days after birth and followed longitudinally. Proportion and functional properties of Tregs were estimated by flow cytometry in relation to the children's allergy status. The proportion of Tregs in the peripheral blood of children suffering from allergy tends to be higher; but on the contrary, MFI of FoxP3 was significantly decreased in the allergic group. Intracellular presence of regulatory cytokines IL-10 and TGF-beta was also lower in allergic children. Probiotic colonized children have considerably increased immune function of Tregs, both MFI of FoxP3 and intracellular presence of regulatory cytokines, in comparison to non-colonized children. Probiotic colonization by Colinfant New Born (*E. coli* O83:K24:H31) decreases allergy incidence in high-risk children. The beneficial effect of probiotics on newborn immature immune system could be, at least partially, explained by the modulating immune function of Tregs. Although we detected increased proportion of Tregs in the peripheral blood of allergic children, their functional properties were decreased in comparison with the Tregs of healthy children. It is possible to suppose that increased proportion of Tregs in allergic children reflects an effort to compensate the impaired function of these cells.

Key words: probiotic, allergy, Tregs, cytokines, Colinfant New Born, flow cytometry, FoxP3, Escherichia coli O83:K24:H31

Introduction

Allergic diseases affect a large proportion of our population, and their incidence is steadily increasing with a large health and socio-economic impact [(1)]. Preventive measures would be highly desirable, most importantly in high-risk children. Proper knowledge of the causes and pathogenesis of these ailments is the first prerequisite for their successful prevention and treatment. The causations are multi-factorial, comprising both environmental and endogenous factors. Among endogenous factors, genetic predisposition plays a pronounced role, and maternal allergy represents the most important risk factor for the child. Clinical manifestation of allergy is evoked by the pathologically altered regulation of the immune system [(2)]. In a very simplified way, it is possible to say that Th2 bias and impaired function of Tregs are mainly responsible for pro-allergic tuning of the organism. The major and decisive shaping of immunological reactivity takes place in early ontogeny – in the perinatal period. It is well known that this development is strongly influenced by microorganisms colonizing infants after birth [(3)]. Many microbes, mainly bacteria, support the development of the anti-allergic Th1 responses. In recent years, there have been many efforts to influence allergy development by the use of probiotics [(4)]. Because of the decisive role of perinatal tuning of the immune system, it is necessary to apply this preventive probiotic treatment early in life.

We have several years experience with the newborn probiotic Colinfant New Born (*Escherichia coli*, O83:K24:H31) [(5-7)]. When used in premature newborns, it decreased the incidence of postnatal infections and the future development of allergy [(8-10)]. In recent years, full-term healthy children of allergic mothers were colonized, and their allergy status has been followed already for several years (5,11). Here, we present our findings concerning the characteristics of Tregs in children colonized with Colinfant New Born at birth, 6 – 7 years ago, in the context with later allergy manifestation.

Materials and Methods

Subjects

Healthy and allergic mothers with a physiological pregnancy and children delivered physiologically (vaginally) at full term were included in the study. Diagnosis of allergy in mothers was based on the clinical manifestation of an 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.

After birth, children were divided into the following three groups (Table I): group 1 – newborns of healthy mothers (28 children), group 2 – newborns of allergic mothers (42 children), group 3 – newborns of allergic mothers, colonized with the newborn probiotic vaccine Colinfant New Born (51 children). First dose of probiotic vaccine containing $0.8x10^9$ live *Escherichia coli (E. coli*, O83:K24:H31) was applied perorally within 72 hours after delivery. Following the next 11 days, one further dose of was administered every day. Colonized children's stool was checked for the presence of the probiotic strain *E. coli* O83:K24:H31. In the case of antibiotic treatment in the group of colonized children, a new vaccine dose was administrated to guarantee the consistent *E. coli* colonization.

Children were followed longitudinally for 6 – 7 years (4 times during the first year of life, once a year thereafter). At the age of 6 – 7 years, children of each group were further divided according to their allergy status and indicated as healthy (H) or allergic (A) – the resulting 6 groups are designated as follows: 1H, 1A - non-colonized children of healthy mothers; 2H, 2A - non-colonized children of allergic mothers; and 3H, 3A - colonized children of allergic mothers.

The allergy status of children was based on maternal reports, supported by a positive skin prick test and/or increased levels of allergen specific IgE antibodies in the children's serum. Combination of different types of allergies at children suffering from allergy is summarized in Table II. 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 the written informed consent of the mothers.

Peripheral blood sampling

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

Tregs ratio

The proportion of Tregs was estimated in the whole peripheral blood within 1 – 2 hours after sample taking. Tregs were stained for cell surface markers using the

following antibodies: CD4 FITC, cat. no. 555346; CD25 PE-Cy7, cat. no. 557741; and CD127 Alexa 647, cat. no. 558598 (Becton Dickinson). After cell surface staining followed by erythrocytes lysis, the cells were permeabilized, fixed and then stained for the transcription marker of Tregs by anti-human FoxP3 antibody, cat. no. 12-4776-41A, using a human regulatory T cell whole blood staining kit (cat. no. 88-8996-40) (eBioscience), according to manufacturer recommendations. The Treg proportion was considered as an intercept of three different gates of the $CD4^+$ T lymphocyte population based on Treg markers CD25, CD127, and FoxP3 expression ($CD25^{high}CD127^{low}$, $CD25^{high}FoxP3^+$, $CD127^{low}FoxP3^+$). Gating strategy using a combination of staining of cell surface markers (CD4, CD25, CD127) and intracellular staining of transcription factor FoxP3 is indicated in Figure 1.

IL-10 and TGF-beta in Tregs

Regulatory cytokines were detected in Tregs using intracellular staining. Staining of both cytokines IL-10 (IL-10 PE, cat. no. 506804) and TGF-beta (LAP TGF-beta1 PerCP-Cy5.5, cat. no. 341803) was performed using a Fixation buffer, cat. no. 420801 and Permeabilization Wash Buffer, cat. no. 421002, all purchased from BioLegend. The complete intracellular staining was performed according to manufacturer recommendations. Results are expressed as a percentage of IL-10 or TGF-beta positive Tregs from all Tregs ($CD4^+CD25^{high}CD127^{low}$).

Data analysis and statistics

Flow cytometry data were acquired on a BD FACS Canto II instrument using BD FACS Diva 6.1.2 software (Becton Dickinson). FlowJo 7.2.2. (Tree Star) was exploited for data evaluation. Differences between groups were compared by the unpaired Student's t-test for data normally distributed (Tregs ratio, MFI of FoxP3); otherwise the non-parametric Mann-Whitney test was utilized (comparing the proportion of IL-10+ Tregs and TGF-beta+ Tregs). Statistical significance was set at $p \leq 0.05$. Bonferroni correction was used to adjust for multiple comparisons. Statistical and graphical analysis was performed using SAS (version 9.2.) and GraphPad Prism, respectively. Results are shown as individual values and mean in dot plot graphs.

Results

Peripheral blood Tregs were characterized in all followed groups of the 6 – 7 year old children. Table 1 summarizes the descriptive characteristics of the three

primary groups of children, including their allergy incidence. As indicated, probiotic administration decreased allergy development in high-risk children (children of allergic mothers) to the level comparable with low risk children (children of healthy mothers). To elucidate the beneficial effect of probiotics on the immature neonatal immune system, the proportion and immunologic characteristics (MFI of FoxP3, presence of cytokines with regulatory function: IL-10 and TGF-beta) of Tregs were evaluated. The differences in Treg proportions and their functional properties were found among our studied groups and the effect of colonization by Colinfant New Born on Tregs was proved.

Treg ratio

Significant differences in Treg numbers among groups 1, 2, and 3 were not proved; but a tendency to a higher proportion of Tregs in children of allergic mothers (groups 2 and 3) was evident, Fig. 2a. Nevertheless, after sorting the children according to their allergy status, in all 3 groups allergic children had increased numbers of Tregs in comparison with the healthy children – the difference in group 2 (non-colonized children of allergic mothers) being significant; Figure 2b.

The Tregs ratio between allergic and non-allergic children, regardless of their mother's allergy status, clearly indicated an increased number of Tregs in children suffering from allergy; Figure 2c.

Median of fluorescence intensity (MFI) of FoxP3

The decreased function of Tregs is believed to be one of the mechanisms supporting hyper-sensitization to allergies. We have found an increased proportion of Tregs in allergic children, but it does not mean these cells are fully functionally competent. Therefore, we measured not only the number of Tregs but also the MFI of transcription factor FoxP3, which is thought to be a master marker for identifying Tregs (as CD25 can be expressed on all activated CD4+ T lymphocytes, and CD127 is present on various cells).

The values of MFI of FoxP3 were similar in all 3 groups of children with the non-significantly lower values detected in non-colonized children of allergic mothers (group 2) when compared with groups 1 and 3, as shown in Figure 3a, which is in accordance with the highest allergy incidence in this group. After the differentiation of children into subgroups based on their allergy status, we can see a significantly decreased MFI of FoxP3 in non-colonized allergic children of healthy mothers (1A) and non-colonized allergic children of allergic mothers (2A), in comparison to non-allergic

children of healthy mothers (1H) and non-allergic children of allergic mothers (2H), respectively; Figure 3b. Also the MFI of FoxP3 in colonized children suffering from allergy (3A) is lower in comparison to healthy children of this group (3H), but it does not reached statistical significance. When comparing MFI of FoxP3 in peripheral blood of all children followed only according to their allergy status, the MFI of FoxP3 is significantly decreased in allergic children in comparison with the healthy children; Figure 3c. It is possible to say that the proportion of Tregs in the peripheral blood in of healthy and allergic children is in an inverse trend to MFI of FoxP3.

Proportion of IL-10 and TGF-beta positive Tregs

The further functional characteristic of Tregs is the production of suppressive cytokines IL-10 and TGF-beta (12,13). We identified and utilized these cytokines as further functional markers and detected them in Tregs using intracellular staining.

There were no significant differences in the numbers of IL-10 producing cells among the children of the three basic groups; even if, according to our expectations, the highest numbers of IL-10+ Tregs were in children of healthy mothers; and on the contrary, high-risk children of allergic mothers exert lower numbers. The colonization of high-risk children leads more or less to normalization of IL-10+ Tregs count; see Figure 4a. When the three groups of children mentioned above were divided into subgroups according to their allergy status, the depression of IL-10+ Tregs in allergic children is evident; and even significantly in untreated high-risk group (2H versus 2A); Figure 4b. Similarly, after dividing all children into only two groups – allergic and healthy – the number of IL-10+ Tregs was again significantly lower in the allergic group; Figure 4c.

Testing intracellular TGF-beta in Tregs provided only insignificant differences among the groups of children, yet in the same sense as in the case of IL-10; Figures 5a, 5b, 5c.

Discussion

There is a substantial increase of allergic diseases mainly in western countries in the last decades. Therefore, searching not only for an efficient treatment of allergy but also for its prevention and prognostics is an important challenge.

In our previous work, the convincing preventive effect of the newborn probiotic Colinfant New Born (*E. coli,* O83:K24:H31; Dyntec) was reported (5,14). The

development of the allergy in high-risk children of allergic mothers was limited when newborns were colonized by Colinfant New Born within 3 days after birth.

The mechanism of the probiotic effect is not fully understood, but it is supposed that the function of Tregs could be influenced, among others. Therefore, we attempted to characterize the differences in Tregs in children 6 – 7 year old, who were or were not colonized early after birth with Colinfant New Born, and who developed or did not develop the allergy later. We have found differences in Tregs dependent both on colonization and on later allergy status.

In this study, the immune characteristics of Tregs in the peripheral blood of 121 children, six to seven years old colonized or non-colonized were compared. Functional characteristics of Tregs (judged according to the MFI of FoxP3 and the intracellular presence of regulatory cytokines IL-10 and TGF-beta) in colonized children of allergic mothers (group 3) were increased in comparison to non-colonized children of allergic mothers (group 2). Functional characteristics of Tregs of colonized children of allergic mothers (group 3) were comparable with non-colonized children of healthy mothers (group 1); Figures 2a, 3a, 4a. Functional characteristics of Tregs of children suffering from allergy in all groups were substantially decreased in comparison to non-allergic children; Figures 2b, 3b, 4b. The increased proportion of Tregs in allergic children could be interpreted as an effort to compensate, at least partially, for the Tregs insufficient function. It is evident that not only numbers of Tregs should be followed, but also their functional characteristics.

As our results show, probiotic colonization leads to an increased immune function of Tregs, judged according to the intracellular presence of cytokines with regulatory properties and an increased MFI of transcription factor FoxP3. FoxP3 is responsible for regulatory Tregs phenotype, and the presence of FoxP3 is positively correlated with the suppressive function of Tregs [15-17]. Therefore an increased MFI of FoxP3 in colonized children of allergic mothers indicates a boost effect of the probiotic on the functional properties of Tregs. In the current study we did not have sufficient number of cells to perform *in vitro* suppressive study to test the suppressive effect of Tregs ($CD4^+CD25^{high}CD127^{low}$) on $CD4^+CD25^-$ cells due to the obvious ethical reasons. A beneficial effect of probiotics on Tregs potency was detected *in vitro* (18,19), in animal models [20-22] and several clinical trials (23,24). On the other hand, our results are partially in conflict with the work of Taylor et al. (25,25,26), who claim they failed to prove significant effect of probiotic colonization on the decrease of atopic dermatitis in

6-month-old children. However, they were tracking FoxP3 at the age of 6 months after birth relative to the mRNA level. In addition to this, the discrepancy can be caused by different probiotic bacteria used, as there are large differences not only among different probiotic bacteria, but also among their individual strains. Thus, some authors suggest using the combination of several probiotic bacteria to achieve the highest immunomodulatory effect [27-29]. Furthermore, the timing of probiotic administration seems to be critical, as presented by Anderson et al [30].

We were able to prove impaired function of Tregs in children suffering from allergy (regardless of maternal allergy status) on two levels: MFI of FoxP3 and proportion of regulatory cytokines. Our observation is in agreement with the study by Ling et all [31], describing the less effective inhibition of allergen driven responses by Tregs in allergic patients, where regulatory cytokines released by Tregs play important role in regulation of immune responses. In our current study, we detected the decreased intracellular presence of IL-10 and TGF-beta in Tregs of allergic children, regardless of the allergic status of their mothers. Although the difference in TGF-beta was not significant, it was in the same sense as the difference in IL-10. These regulatory cytokines play an important role in preventing allergy development (12,32,33), and our results are in concordance with those previous observations. Surprisingly, even Tregs in the peripheral blood of colonized children suffering from allergy (group 3A) show increased immune function in comparison to Tregs in the peripheral blood of allergic non-colonized children of allergic mothers (group 2A); yet still lower than Tregs in allergic children of healthy mothers (group 1A); Figures 2a, 3a, 4a. Taken together, early probiotic administration (at least in the case of Colinfant New Born *E. coli*) seems to be a positive solution for minimizing allergy development in predisposed / high-risk children.

Regulatory T cells (Tregs) are a very heterogeneous population of cells, and many methodological problems arise in the course of their study. Different gating strategies used for quantification of Tregs ($CD4+CD25+$[34], $CD4+CD25^{high}$[16], $CD4+CD25^{high}CD127^{low}$[35], $CD4+CD25^{high}FoxP3+$[36], $CD4+CD25^{high}CD127^{low}FoxP3+$[37], or the gating suggested by us based on the intercept of three different gates on CD4 subpopulation using combination of cell surface markers (CD4, CD25, CD127) and intracellular transcription marker FoxP3, as indicated in Figure E1 in the Online Repository) can give quite different results leading to controversial conclusions. Furthermore, using different clones of FoxP3 antibodies

could lead to different values of Tregs ratio (38,39), and to the detection of different Treg subpopulations. In our early experimental setting, we used two antibody clones (PCH101, eBioscience; and 259D/C7, Becton Dickinson) with appropriate buffers (data not shown). Higher values in both the proportions of FoxP3+ Tregs and MFI of FoxP3 were detected using PCH101, which was then used for further experiments. However, it is necessary to realize that the amount of Tregs alone is not decisive for effective suppression function [(40)]. Functional analyses of Tregs are therefore, probably more informative. Further, it is necessary to keep in mind that not all lymphocytes exerting suppressor function express FoxP3 [(41)]. Another obstacle can be caused by cell isolation. Many studies analyse Tregs of peripheral blood after Ficoll-Paque separation. Yet, we compared detection of Tregs in the whole blood, and in the population of isolated blood mononuclear cells – the results being similar, but the analyses obtained with the whole blood were more convincing and consistent and less time consuming (data not shown).

Probiotics can influence the development of the immune system when applied early after the birth at the crucial time of changes and fine-tuning of the immune system. The growing popularity of probiotic use corresponds with the widespread hygienic hypothesis suggesting that increase of allergies in western countries is due to the lower microbe burden early in the life. Microbes constituting microbiota and other antigens of the outside environment are very important as stimulating and modulating agents.

The capability of probiotics to promote the immune function of Tregs could be exploited in prevention and possibly treatment of various diseases including allergy. Although there are some early reports of allergy treatment by probiotic administration, the results are neither consistent nor convincing. The early finding of prognostic markers indicating an increased risk of allergy development could enable the introduction of early preventive measures leading to a decrease of allergy incidence, or at minimum lowering the clinical significance of allergy manifestation. There is much work searching for markers signalling the increased risk of future allergy development in newborns, but as has been concluded by Prescott, so far the only reliable marker indicating increased risk of allergy development is the allergy status of the mother [(42)]. Nevertheless, the combination of several other markers (e.g. functional insufficiency of Tregs) can further support prognostic judgment.

In conclusion; early Colinfant New Born administration (within 72h after delivery) decreased allergy incidence in predisposed children later in life. The beneficial effect of Colinfant New Born on the immature newborn's immune system involves the

modulation of immune characteristics of Tregs. The capability of Colinfant New Born to influence the functional properties of Tregs could be exploited as a preventive and probably a therapeutic approach for immunotherapy of immune disorders (allergy, autoimmunity, and transplantation reaction).

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Figure Legends

Figure 1

Gating strategy for estimation of proportion of Tregs in CD4+ population in peripheral blood of children.

The intercept of three gates (CD4+CD25^{high}CD127^{low}; CD4+CD25^{high}FoxP3+; CD4+CD127^{low}FoxP3+) based on the combination of staining cell surface markers (CD4, CD25, CD127) and intracellular staining of transcription factor FoxP3 was considered as Tregs region.

Figure 2a

Proportion of Tregs in peripheral blood of six year old children.

Comparison of proportion of Tregs in peripheral blood of colonized and non-colonized children of allergic mothers and non-colonized children of healthy mothers using four colour flow cytometry analysis (intercept of CD4+CD25^{high}CD127^{low} and CD4+CD25^{high}FoxP3+ and CD4+CD127^{low}FoxP3+).

1 - children of healthy mothers

2 - children of allergic mothers

3 - Colinfant New Born colonized children of allergic mothers

Figure 2b

Proportion of Tregs in peripheral blood of six year old children.

Comparison of proportion of Tregs in CD4+ lymphocytes in peripheral blood of colonized and non-colonized children of allergic mothers and non-colonized children of healthy mothers with respect to allergy status of children using four colour flow cytometry analysis (intercept of CD4+CD25^{high}CD127^{low} and CD4+CD25^{high}FoxP3+ and CD4+CD127^{low}FoxP3+).

1H - healthy children of healthy mothers
1A - allergic children of healthy mothers
2H - healthy children of allergic mothers
2A - allergic children of allergic mothers
3H - healthy Colinfant New Born colonized children of allergic mothers
3A - allergic Colinfant New Born colonized children of allergic mothers

Figure 2c

Proportion of Tregs in peripheral blood of six year old children.

Comparison of proportion of Tregs in CD4+ lymphocytes in peripheral blood of non-allergic and allergic children regardless of their mother allergy status using four colour flow cytometry analysis (intercept of $CD4+CD25^{high}CD127^{low}$ and $CD4+CD25^{high}FoxP3+$ and $CD4+CD127^{low}FoxP3+$).

H non-allergic children

A allergic children

Figure 3a

MFI of FoxP3 in Tregs of peripheral blood of six year old children.

Comparison of median of fluorescence intensity (MFI) of transcription factor FoxP3 in Tregs (gated on $CD4+CD25^{high}CD127^{low}$) of peripheral blood of probiotic colonized and non-colonized children of allergic mothers and non-colonized children of healthy mothers.

1 - children of healthy mothers

2 - children of allergic mothers

3 - Colinfant New Born colonized children of allergic mothers

Figure 3b

MFI of FoxP3 in Tregs of peripheral blood of six year old children.

Comparison of median of fluorescence intensity (MFI) of FoxP3 in Tregs (gated on $CD4+CD25^{high}CD127^{low}$) of peripheral blood of probiotic colonized and non-colonized children of allergic mothers and non-colonized children of healthy mothers according to children allergy status.

1H - healthy children of healthy mothers
1A - allergic children of healthy mothers
2H - healthy children of allergic mothers
2A - allergic children of allergic mothers
3H - healthy Colinfant New Born colonized children of allergic mothers
3A - allergic Colinfant New Born colonized children of allergic mothers

1H versus 1A $p = 0.0058$

2H versus 2A $p = 0.0043$

Figure 3c

MFI of FoxP3 in Tregs of peripheral blood of six year old children.

Comparison of median of fluorescence intensity (MFI) of FoxP3 in Tregs (gated on $CD4+CD25^{high}CD127^{low}$) of peripheral blood of healthy and allergic children regardless of their mother allergy status

H non-allergic children

A allergic children

Figure 4a

Intarcellular presence of IL-10 in Tregs in peripheral blood of six year old children.

Comparison of intracellular presence of IL-10 in Tregs in peripheral blood of colonized and non-colonized children of allergic mothers and non-colonized children of healthy mothers (% IL-10 positive Tregs in $CD4+CD25^{high}CD127^{low}$).

1 - children of healthy mothers

2 - children of allergic mothers

3 - Colinfant New Born colonized children of allergic mothers

Figure 4b

Intarcellular presence of IL-10 in Tregs in peripheral blood of six year old children.

Comparison of intracellular presence of IL-10 in Tregs in peripheral blood of colonized and non-colonized children of allergic mothers and non-colonized children of healthy mothers (% IL-10 positive Tregs in $CD4+CD25^{high}CD127^{low}$) according to children allergy status.

1H - healthy children of healthy mothers
1A - allergic children of healthy mothers
2H - healthy children of allergic mothers
2A - allergic children of allergic mothers
3H - healthy Colinfant New Born colonized children of allergic mothers
3A - allergic Colinfant New Born colonized children of allergic mothers

Figure 4c

Intarcellular presence of IL-10 in Tregs in peripheral blood of six year old children.

Comparison of intracellular presence of IL-10 in Tregs in peripheral blood of non-allergic and allergic children (% IL-10 positive Tregs in CD4+CD25^{high}CD127^{low}) according to children allergy status regardless of their mother allergy status.

H non-allergic children
A allergic children

Figure 5a

Comparison of proportion of TGF-beta positive Tregs in peripheral blood of six year old children.

Comparison of proportion of TGF-beta positive Tregs in peripheral blood of colonized and non-colonized children of allergic mothers and non-colonized children of healthy mothers (% TGF-beta positive Tregs in CD4+CD25^{high}CD127^{low}).

1 - children of healthy mothers
2 - children of allergic mothers
3 - Colinfant New Born colonized children of allergic mothers

Figure 5b

Comparison of proportion of TGF-beta positive Tregs in peripheral blood of six year old children.

Comparison of proportion of TGF-beta positive Tregs in peripheral blood of colonized and non-colonized children of allergic mothers and non-colonized children of healthy mothers according to children allergy status. (% TGF-beta positive Tregs in CD4+CD25^{high}CD127^{low})

1H - healthy children of healthy mothers
1A - allergic children of healthy mothers
2H - healthy children of allergic mothers
2A - allergic children of allergic mothers
3H - healthy Colinfant New Born colonized children of allergic mothers
3A - allergic Colinfant New Born colonized children of allergic mothers

Figure 5c

Comparison of proportion of TGF-beta positive Tregs in peripheral blood of six year old children.
Comparison of proportion of TGF-beta positive Tregs in peripheral blood of healthy and allergic children regardless of their mother allergy status. (% TGF-beta positive Tregs in CD4+CD25^{high}CD127^{low})
H non-allergic children
A allergic children

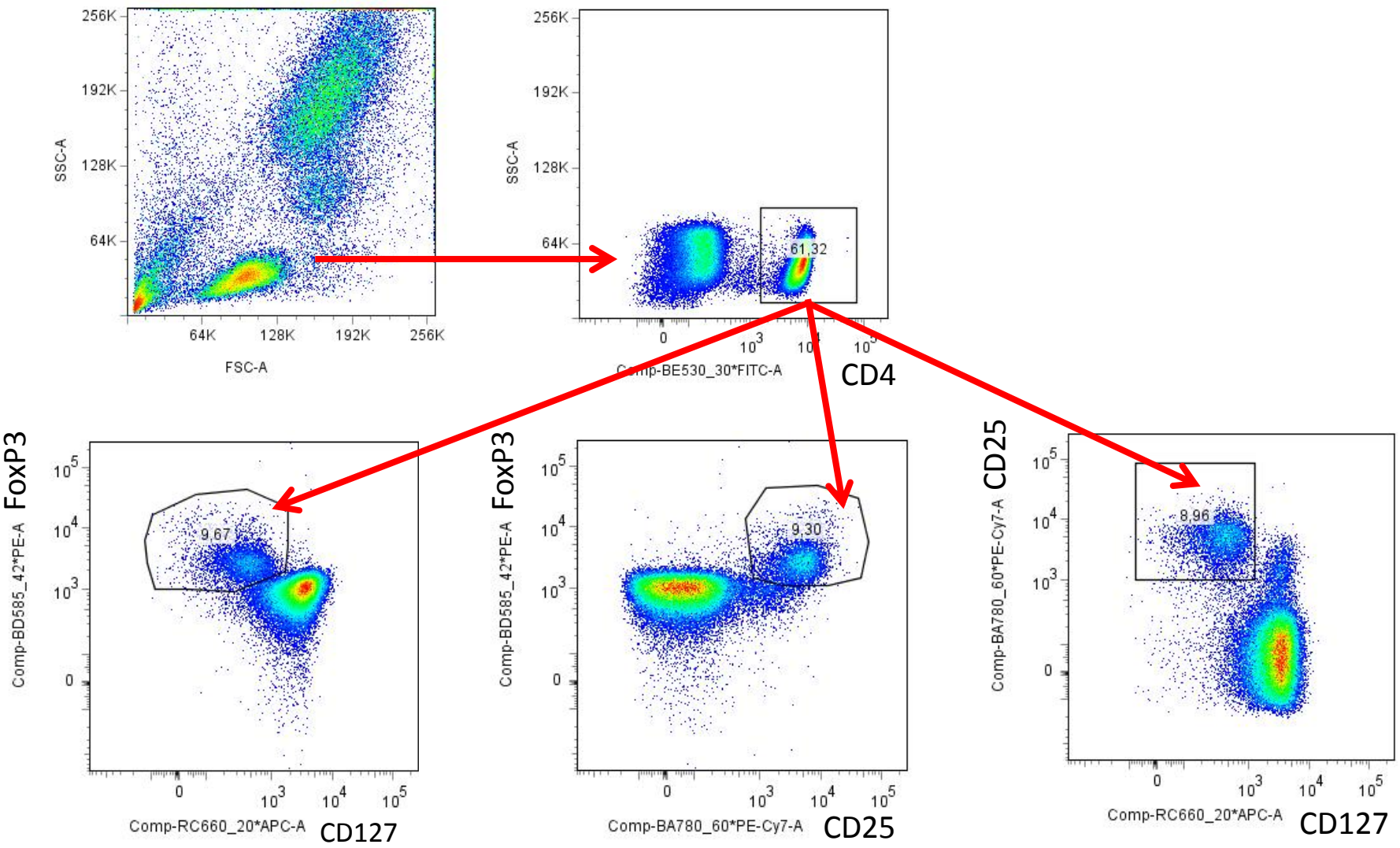
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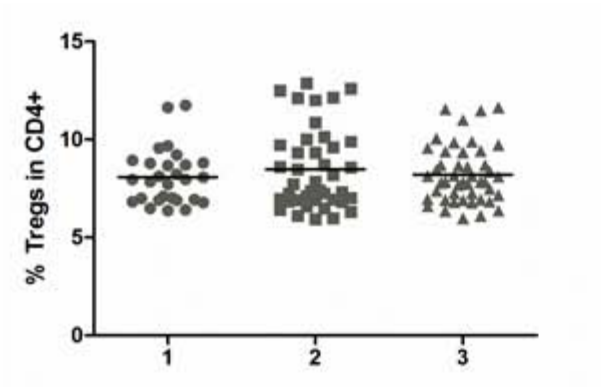
Fig. 2a



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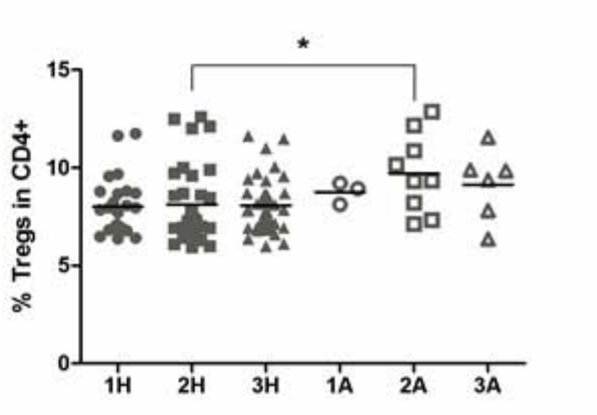

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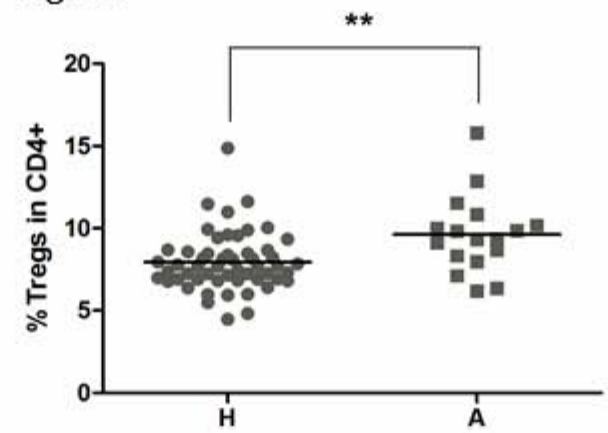

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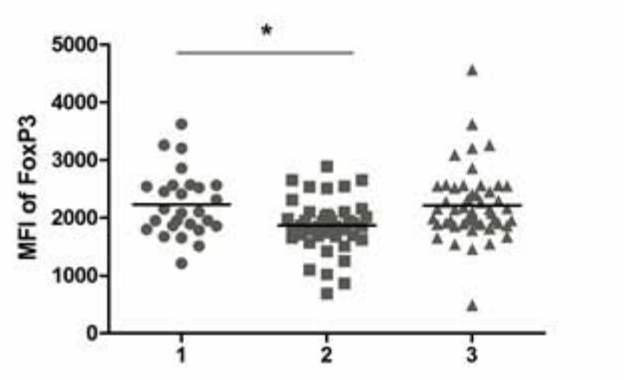

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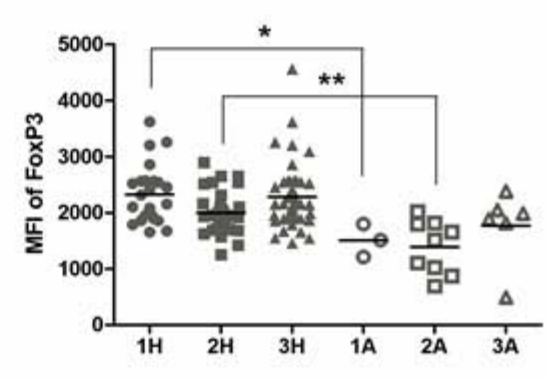

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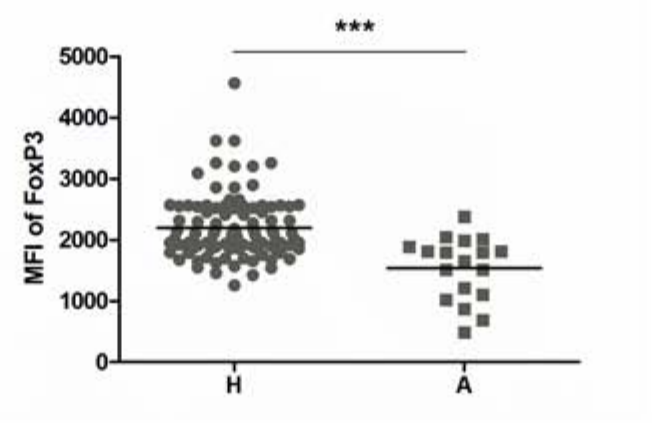

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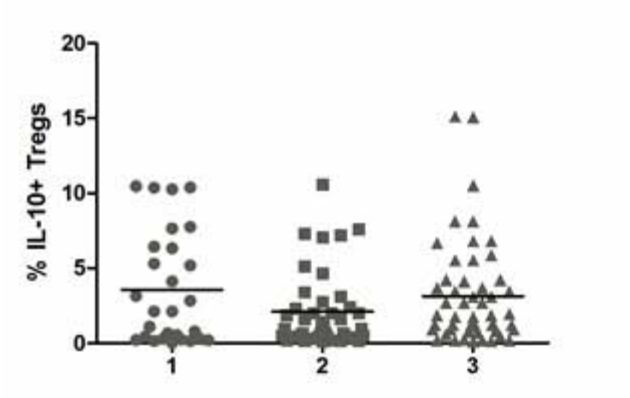

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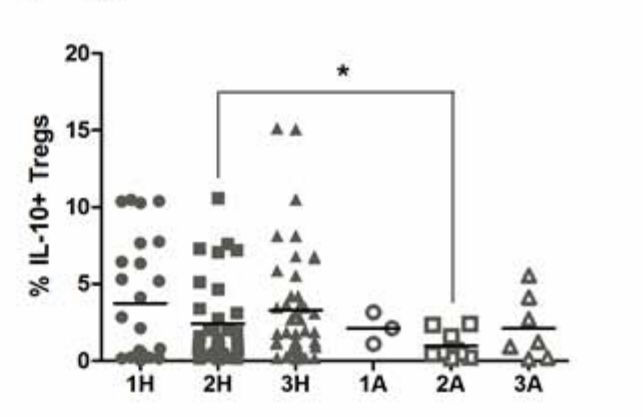

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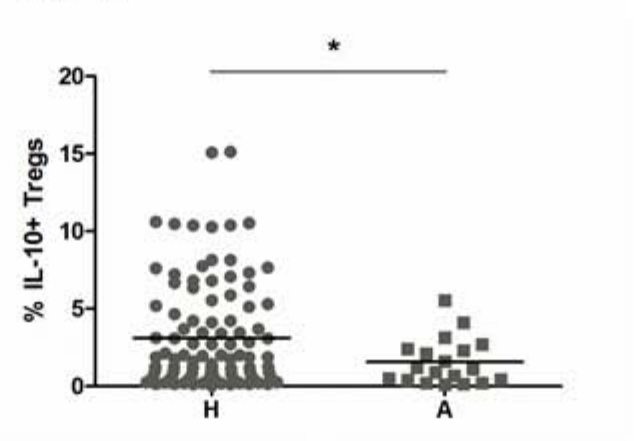

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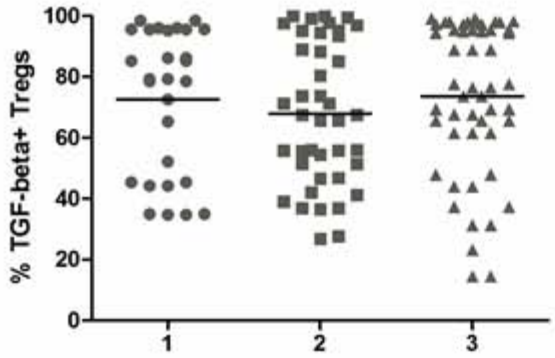

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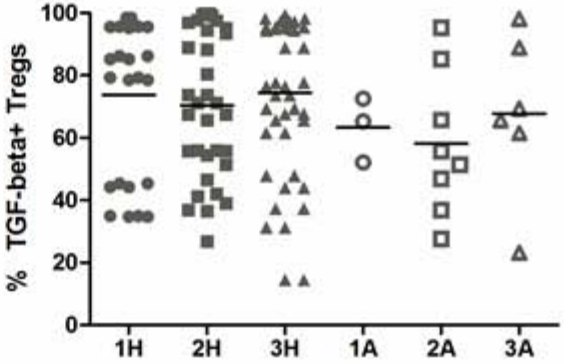

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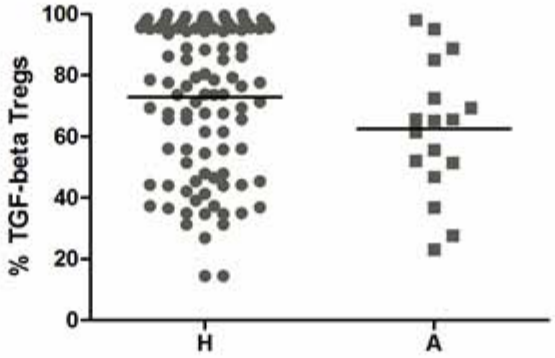

Table I

Study group characteristics

group	n	median of age	mean of age	number of allergic children	percentage of allergic children
1	28	7	6.857	3	10.714%
2	42	6	6.458	9	21.429%
3	51	6	6.321	6	11.765%

1 – children of non-allergic mothers

2 – children of allergic mothers

3 – probiotic colonized children of allergic mothers

Table II

Combination of different allergy outcomes in children suffering from allergy

group	individual	allergy outcomes (allergens)
1A	1	polinosis, bronchitis (mites, dust, grass pollen)
1A	2	polinosis (rodent, grass pollen, tree pollen)
1A	3	(dust, feathers)
2A	1	atopic eczema, bronchitis, polinosis (grass pollen, cacao, tomatoes, chocolate, dog dander)
2A	2	atopic eczema, polinosis (chocolate, diary produce, tomatoes, nuts, nectarines)
2A	3	eczema, polinosis (grass pollen, tree pollen: birch, alder, nut tree)
2A	4	coeliac disease, polinosis (grass pollen)
2A	5	bronchitis (mites)
2A	6	atopic eczema (chocolate)
2A	7	atopic eczema, coeliac disease, polinosis (mites, grass pollen, spring tree pollen)
2A	8	polinosis (mites, pollen, animal dander)
2A	9	polinosis (grass pollen)
3A	1	atopic eczema, polinosis (mites, pollen, grass)
3A	2	polinosis (grass pollen, nuts, strawberries, citrus, tomatoes)
3A	3	polinosis (grass pollen)
3A	4	atopic eczema, polinosis (grass pollen)
3A	5	atopic eczema, polinosis (birch pollen, mould, peanut, egg white, cat dander)
3A	6	atopic eczema, polinosis (pollen, mould, dander, poppy seed, nuts, asthma)

group 1A – allergic noncolonized children of healthy mothers

group 2A – allergic noncolonized children of allergic mothers

group 3A – allergic probiotic colonized children of allergic mothers