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

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

Doctoral thesis

Supervisor: RNDr. Jiří Hrdý, Ph.D.

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Dedication

Disertační práci bych rád věnoval svým strážným andělům – své manželce, svým rodičům i prarodičům, a také všem svým vynikajícím pedagogům, kteří již nejsou mezi námi.

Abstrakt

Vzhledem k vysoké incidenci a socioekonomické i individuální zátěži představují alergická onemocnění jeden z klíčových problémů, kterým imunologie v 21. století čelí. Zejména v poznání časných mechanismů, podílejících se na rozvoji alergie, zatím existují významné mezery. Aby bylo u rizikových jedinců možné účelně a včasně přistoupit k preventivním opatřením, je třeba zavést spolehlivé prediktory vysokého rizika alergie.

Podkladem alergie je dysregulace rovnováhy mezi jednotlivými větvemi imunitní reakce, především nežádoucí převaha Th2 odpovědi. Po narození v novorozenci probíhají rozsáhlé změny polarizace imunitního systému za účelem přípravy zrající imunity k adekvátní reaktivitě vůči nově přístupným podnětům zevního prostředí. Regulační T lymfocyty (Treg) hrají klíčovou roli v jemném nastavování této rovnováhy a zodpovídají též za periferní toleranci vůči neškodným externím antigenům včetně alergenů. Na intenzivně se vyvíjející imunitní systém vykazují během časně postnatálního „okna příležitosti“ rovněž výrazný vliv externí faktory, především interakce s kolonizující mikrobiotou.

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

Potenciálně slibným přístupem pro prevenci alergie je podávání probiotických bakterií. V naší práci jsme pozorovali ve věku 8 let snížení incidence alergie u dětí alergických matek, kterým byl záhy po narození podán probiotický kmen *E. coli* O83:K24:H31 (EcO83). Toto snížení bylo pravděpodobně způsobeno normalizací produkce IL-10 a IFN-γ, k níž u kolonizovaných dětí došlo. Biologický efekt EcO83 může být zprostředkován podporou imunoregulačních mechanismů: po *in vitro* stimulaci izolovaných mononukleárních leukocytů pupečníkové krve kultivací s EcO83 jsme pozorovali zvýšenou produkci IL-10 a IFN-γ. Kromě toho kultivace s EcO83 indukovala zvýšení produkce IL-10 v *in vitro* generovaných DC, a takto stimulované DC indukovaly v kokultivaci s CD4$^+$ T lymfocyty vyšší procento IL-10$^+$ T buněk.

Klíčová slova: Treg, alergie, pupečníková krev, regulace imunity, cytokiny, probiotika, *E. coli* O83:K24:H31

Abstract

Due to high incidence, medical and socioeconomic burden and impact on individual quality of life and productivity, allergic disorders are a crucial issue for 21st century immunology. Much still remains to be elucidated, particularly regarding the very early processes in allergy development. In order to introduce timely, effective preventive measures, novel, more reliable predictive factors of allergy risk also need to be established.

Dysregulation of proper balance between the branches of immune response, particularly unwarranted dominance of Th2, is the underlying cause of allergy. After birth, new immune balance needs to be established to prepare the neonate for adequate reactivity towards newly encountered environmental stimuli. Regulatory T cells (Treg) play a central role in finely setting this balance and inducing tolerance towards harmless environmental antigens, including allergens. Interactions with external factors, most importantly microbiota, modulate this process during the early postnatal "window of opportunity."

Analysis of cord blood Treg of children of allergic mothers uncovered decreased presence of function-associated surface markers and lower production of IL-10. Furthermore, decreased proportion of Helios^{-} induced Treg was observed in children with higher risk of allergy. Together, these findings hint at delayed functional maturation of Treg in the high-risk group, consistent with observation of increased dendritic cell (DC) reactivity of these children.

Supplementation with probiotic bacteria is considered a potentially promising approach for allergy prevention. In our studies, we show that early postnatal colonisation with probiotic *E. coli* strain O83:K24:H31 (EcO83) is able to reduce allergy incidence in colonised children of allergic mothers, likely owing to normalisation of IL-10 and IFN-γ production in the colonised children. This effect may be due to promotion of regulatory responses by EcO83 administration to the neonate. Upon *in vitro* stimulation with EcO83, we observed increase in production of IL-10 and IFN-γ by cord blood mononuclear cells, higher ability of DC to produce IL-10 and higher induction of IL-10^{+} CD4^{+} T cells in coculture with the stimulated DC.

Keywords: Treg, allergy, cord blood, immune regulation, cytokines, probiotics, *E. coli* O83:K24:H31

Contents

Abbreviations

ACK	ammonium-chloride-potassium
AD	atopic dermatitis
AP-1	activator protein 1
APC	antigen-presenting cell
ATP	adenosine triphosphate
BCL6	B-cell lymphoma 6 protein
Breg	regulatory B cells
CBMC	cord blood mononuclear cells
CD	cluster of differentiation
CFSE	carboxyfluorescein succinimidyl ester
CNS	conserved noncoding sequence
CREB	cAMP response element-binding protein
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAG	diacylglycerol
DC	dendritic cells
EcN	*Escherichia coli* strain Nissle 1917
EcO83	*Escherichia coli* strain O83:K24:H31
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FAO	United Nations Food and Agricultural Organization
FasL	Fas ligand
FcεRI	high-affinity IgE Fc receptor
FoxP3	forkhead box P3
GALT	gut-associated lymphoid tissue
GATA3	GATA binding protein 3
GF	germ-free
GITR	glucocorticoid-induced TNFR-related protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HLA	human leukocyte antigen
Id3	inhibitor of DNA binding 3
IDO	indolamine 2,3-dioxygenase
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cells
ILCreg	regulatory innate lymphoid cells
IP3	inositol 1,4,5-trisphosphate

IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked
IRF	interferon regulatory factor
iTreg	induced regulatory T cells
LAG-3	lymphocytes activation gene-3
LPS	lipopolysaccharide
M2	alternatively activated macrophages
MACS	magnetic-activated cell sorting
MAP-K	mitogen-activated protein kinase
MDSC	myeloid derived suppressor cells
MFI	median fluorescence intensity
MHC	major histocompatibility complex
moDC	monocyte-derived dendritic cells
NCDs	non-communicable diseases
NF/AT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer cells
NO	nitric oxide
nTreg	natural regulatory T cells
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
rh	recombinant human
RORγt	retinoic acid receptor-related orphan receptor gamma
ROS	reactive oxygen species
rt-qPCR	quantitative real-time polymerase chain reaction
SCFA	short-chain fatty acid
SIT	allergen-specific immunotherapy
SPF	specific-pathogen-free
STAT5	signal transducer and activator of transcription
T-bet	T-box transcription factor 21
Tconv	conventional T cells
TCR	T cell receptor
TGF-β	transforming growth factor beta
Th	helper T cells
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
Tr1	type 1 regulatory T cells
Treg	regulatory T cells
TSDR	Treg specific demethylated region
TSLP	thymic stromal lymphopoietin
WHO	World Health Organisation

1. Introduction

a. Allergy

Allergy represents a heterogeneous group of disorders, characterised by unwarranted and/or dysregulated immune response toward harmless external antigens, i.e. allergens. As is common for multifactorial disorders, genetic predisposition and environmental factors contribute comparably to allergy development. The many ways in which physiological tolerance of innocuous antigens can be broken cause a scale of diseases of varying localisation, severity and symptoms. Taken together as a group, allergic diseases are characterised by high incidence and prevalence, which are still rising worldwide. Because of the often-lifelong character, potentially severe or even fatal complications and overall effect on quality of life of the sufferers, allergy poses globally high socio-economic burden. While there are many options for symptomatic treatment, causal therapy of allergic diseases and especially effective predictive and preventive measures remain limited. Research into the underlying causes and early processes involved in sensitization and allergy development therefore continues to be among the key areas of interest of both clinical and experimental immunology.

Hypersensitivity diseases were broadly categorized into four types by Philip Gell and Robin Coombs in 1963, distinguished by the major effective mechanisms employed:

Type I (immediate hypersensitivity) is characterised by the formation of allergen-specific IgE antibodies and engaging effector cells expressing high-affinity Fcε receptors (FcεRI), most importantly mast cells. As this includes the most common forms of allergy (e.g. respiratory, food and medication allergies), the following sections on allergy will deal with this type in particular.

Type II (the cytotoxic type) hypersensitivity is driven by IgM or IgG antibodies binding to cell membrane antigens, leading to complement activation and/or attack by Fc receptor expressing effector cells such as natural killer (NK) cells and phagocytic cells. This hypersensitivity type includes some autoimmune diseases such as autoimmune haemolytic anaemia, haemolytic disease of the newborn and some forms of drug-induced thrombocytopaenia.

Type III (immunocomplex-driven), where pathology is caused by formation of antigen-antibody complexes (i.e. immunocomplexes) and subsequent inflammation caused mainly by complement activation. Typical examples might include serum sickness, small vessel vasculitis and some forms of glomerulonephritis.

Type IV (delayed-type hypersensitivity) is a cell-based immunopathology, with T cell and myeloid cell activation playing the major role. This form of hypersensitivity most commonly manifests as contact dermatitis.

It is also necessary to distinguish between atopy, a genetic predisposition to type I hypersensitivity reactions, and allergy itself, i.e. actual disease caused by unwanted immune reaction, which is due to this predisposition. Allergy itself essentially consists of two phases. In the first phase, immune system is sensitized, i.e. physiologic tolerance is broken and an allergen-specific immune response is induced, with effector mechanisms and immunological memory forming. When the immune system is later exposed to the cognate allergen, these memory cells and effector mechanisms swiftly cause secondary immune, i.e. allergic, reaction.

In type I hypersensitivity, exposure to an allergen leads to cross-linking of high affinity FcεRI receptors with bound IgE specific for the allergen. A signalization cascade

is then initiated by Src-family tyrosine kinase Lyn and engages the various mast cell effector functions via the second messengers including inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) activating the mitogen-activated protein kinase (MAP-K) pathway and spiking cellular calcium level. In the first phase of activation, the cell degranulates, i.e. releases the contents of preformed secretion granules in a process of Ca^{2+}-dependent fusion of the granules with cytoplasmic membrane. Secretion granules contain strongly bioactive compounds such as histamine, heparin, proteases and chemotactic factors, which are physiologically employed very rapidly in immune reaction against extracellular parasites. At the same time, the activated mast cell initiates de-novo production of bioactive mediators, including cytokines (e.g. interleukins [IL] IL-3, IL-4, IL-5, IL-13, IL-33, granulocyte macrophage colony stimulating factor [GM-CSF], tumor necrosis factor α [TNF-α]) and chemokines (CCL3) as well as lipid mediators synthesised upon phospholipase A2 activation (e.g. prostaglandins, leukotrienes, platelet activation factor etc.). These inflammatory compounds constitute the secondary phase of mast cell activation and play a critical role in attraction and activation of other immune cells including eosinophilic, neutrophilic and basophilic granulocytes, T cells and B cells. These cells then contribute to long-term tissue damage, establish local pro-allergic milieu and maintain inflammation by releasing various tissue-damaging mediators (granulocytes), large amounts of inflammatory cytokines (T cells) and allergen-specific IgE (B cells).

Histamine is the primary mast cell effector molecule and a strongly bioactive compound. It forms about 10% of the preformed granules and binds to a variety of receptors, increasing vascular permeability, causing smooth muscle contraction and promoting mucus secretion. This leads to the typical symptoms of allergy, such as oedema of connective tissues, airway congestion and hypersecretion, diarrhoea and bronchial

constriction. In the most severe cases, the patient can be at risk of circulatory failure due to critical drop of systemic vascular resistance, a condition known as anaphylaxis.

Pathophysiology of sensitization crucially involves dysregulation of balance among different branches of immune reaction and associated regulatory mechanisms. Type 1 allergy was historically viewed mainly as dysregulation of Th1 and Th2 response, with aberrant Th2 polarization being the chief culprit that ultimately causes production of allergen-specific IgE. As the understanding of immune regulation networks and the number of participating cells grew, it became clear that other Th populations play key roles in setting up and maintaining or disturbing immune balance, importantly including regulatory T cells (Treg). These represent the chief immunoregulatory population and will be described in greater detail in a later section, including their role in allergy. Other more recently described cell populations playing a major role in sensitization and allergy include Th subpopulations Th17, Th9, Th22, as well as innate lymphoid cells (ILC), particularly type 2 ILC (ILC2). Antigen presenting cells (APC) responsible for allergen presentation to T and B lymphocytes are most commonly dendritic cells (DC) and basophile granulocytes (Hammad et al., 2010).

The various subpopulations of helper T lymphocytes arise upon antigen stimulation under influence of specific cytokine milieu and can be distinguished by the production of typical cytokines and usually also the expression of lineage-specific transcription factors. Significant plasticity also exists among $CD4^+$ T cell subtypes, further complicating our understanding of regulatory networks. The role of the different Th populations in allergic diseases has recently been reviewed e.g. by Berker et al. (Berker et al., 2017).

Th2, the principal Th subpopulation in type 1 hypersensitivity, are induced by IL-4 and characterised by the expression of lineage-specific transcription factor GATA3. Thymic stromal lymphopoietin (TSLP) and IL-33 have likewise been implicated in Th2 induction (Ito et al., 2005; Jackson et al., 2014). Th2 produce large amounts of cytokines such as IL-4, IL-5, IL-9, IL-13, IL-25 and IL-33. IL-4 and IL-13 support B cells in antibody production and promote IgE class switch (Jutel and Akdis, 2011), arming FcεRI-expressing cells for allergen recognition, while IL-5 is chiefly responsible for eosinophil attraction and activation.

Th1 cells, characterised by the expression of transcription factor T-bet and production of interferon γ (IFN-γ) and IL-2, are induced by IL-12. Their physiological role is in supporting immune responses against intracellular pathogens. While they have historically been viewed as protective with regard to allergy due to their role in counter-regulating Th2 (Szabo et al., 2003), they can also play a pathogenic role, particularly in the chronic phases of asthma and atopic dermatitis (AD) (Trautmann et al., 2002; Chen et al., 2004).

The production of cytokine IL-17 is the hallmark of Th17, a population induced under control of IL-6 and transforming growth factor β (TGF-β) and responsible for immunity against extracellular bacteria. Their specific transcription factor is RORγt. In the context of allergy, these cells play important role especially in some forms of asthma (e.g. neutrophilic (Sorbello et al., 2015)) and AD (Chan et al., 2018; Nomura et al., 2018).

While originally IL-9 has been considered a Th2 cytokine, recently a novel population of distinct Th9 cells, originally described in mouse models, has been confirmed responsible for the majority of its production (Campos Carrascosa et al., 2017). Th9 are induced by stimulation with IL-4 and TGF-β, and transcription factors PU.1 and

interferon regulatory factor 4 (IRF4) and 1 (IRF1) play a role in their development (Campos Carrascosa et al., 2017). IL-9 produced by these cells is important for the accumulation of mast cells and goblet cell hyperplasia, contributing significantly to allergy (Sehra et al., 2015; Townsend et al., 2000). The role of Th9 has also been confirmed in human-based studies, and the cells have been implicated in allergic contact dermatitis (Liu et al., 2014) and atopic dermatitis (Ma et al., 2014).

Th22 cells are the major producers of IL-22 while being incapable of secreting IL-4, IL-17 and IFN-γ. IL-6 and TNF-α are key for Th22 induction, and aryl hydrocarbon receptor has been proposed as the transcription factor controlling IL-22 production (Ramirez et al., 2010). The role of Th22 in allergic diseases is complex and context-dependent. While they promote barrier protection and play a role in wound healing (Wolk et al., 2006), they can also play a pathological role in AD (Nograles et al., 2009) and correlate with disease severity in adults with allergic rhinitis (Tang et al., 2014).

Cells of innate immunity likewise play essential roles in allergy pathogenesis. Because these cells can swiftly react to nonspecific perturbances to barrier tissues (e.g. the release of IL-25, IL-33 and TSLP by stressed epithelial cells, release of inflammatory mediators by mast cells), they are particularly important early in the allergic inflammation. ILC2 (originally described in the gut and dubbed “nuocytes” (Barlow et al., 2012)) produce substantial amounts of Th2 cytokines such as IL-4, IL-5, IL-9 and IL-13 in the early phases of allergic reaction, and may be the crucial original provider of IL-4 for Th2 induction. ILC2 thus play a manifold role in the pathogenesis of allergy, enhancing and promoting both Th cell and B cell function (Gurram and Zhu, 2019).

The exact clinical course of allergy arises from a combination of external and intrinsic influences. The particular symptoms, dynamics and severity of the allergic

reaction depend upon the nature of allergen and any biological activities it may possess, as well as route, timing, dose and dynamics of the original exposure. Among the most important intrinsic factors is the maturity of immune system when it first encounters the allergen. Immune system maturity is determined genetically, environmentally and developmentally, and is chiefly due to the age of the patient. The typical (though not exclusive) sequence consists of atopic eczema in the first few months of life, food allergy in following months and finally respiratory allergies (allergic rhinitis) and asthma later in life and has been termed the "atopic march" (Spergel, 2010).

Th2 immune response is favoured prenatally as unchecked Th1 response could lead to unwanted reactivity against antigenically foreign maternal constituents (Sykes et al., 2012). Maternal side of the maternal-foetal interface also maintains Th2 bias over the majority of pregnancy to prevent reaction against paternal antigens in the foetus (Sykes et al., 2012; Wegmann et al., 1993). This helps explain the well-known amelioration of Th1 and Th17-type autoimmune diseases during pregnancy (Buyon, 1998) as well as the observed rise in flares of systemic lupus erythematosus, a Th2-type autoimmune disease, during pregnancy (Jara et al., 2014). Maternal organism is tightly interconnected with the nascent foetal immune system, and environmental factors affecting the mother have significant influence on the developing foetus (Herberth et al., 2011; Hinz et al., 2010; Schaub et al., 2009). Maternal and foetal immune systems exhibit a significant and well-described crosstalk, including the passage of antigens (Mold et al., 2008), antibodies (Rindsjö et al., 2010), cytokines and even cells through the placental interface. The exchange of immune and non-immune cells is bidirectional and forms the basis of the clinically relevant phenomenon of microchimerism (Wegmann et al., 1993; Lissauer et al., 2009). All the mechanisms play an important role in promotion of tolerance, including formation of foetal regulatory T-cells, as evidenced by studies in animals (Polte and

Hansen, 2008) as well as in human (Mold et al., 2008). It has been proposed that allergic diseases may be tracked to the foetal period (Jones et al., 2000).

Postnatally, the immune crosstalk between mother and child continues via breastfeeding, as maternal milk and especially colostrum serve to support and modulate the neonatal immunity via delivering large amounts of antibodies (mostly of IgA isotype in humans), cytokines and immune cells (Ciardelli et al., 2008; Järvinen et al., 2019). The immune system of a newborn is highly immature, and the immunological role of breastfeeding is critical in the first few months, until the production of immunoglobulins reaches sufficient levels in the child. IgA present in maternal milk also shapes the colonisation of neonatal gut by microbiota, exerting a highly important regulatory role and possibly influencing sensitization and oral tolerance (Orivuori et al., 2014). Thus, it is generally accepted that breastfeeding is highly beneficial for the maturation of the immune system. It remains to be confirmed whether maternal milk of allergic mothers is as beneficial as that of non-allergic mothers, however, as differences in cytokine content and other characteristics have been described between the two groups (Hrdý et al., 2012a), even though in other works by our group, no differences in biological function were observed (Žižka et al., 2007b).

Because Th2 bias is still present at birth, postnatal re-balancing of the immune system in favour of Th1 and the other immune reactions has to be performed to prepare the immune system for new needs imposed by the change in environment. This re-balancing is orchestrated by immune regulatory mechanisms (importantly including Treg) as well as exposure to external factors, such as environmental antigens and colonising microbiota (McCoy and Köller, 2015). Indeed, lower interaction of infants with microbiota and helminths and their products has been proposed as one of the reasons

of the observed increase in allergy incidence in developed countries by the so-called "hygiene hypothesis" (Strachan, 1989; Thomsen, 2015). This was postulated by Strachan in 1989 and proposes that reduced exposure to Th1-promoting microbial agents in environs with higher standards of hygiene delays the postnatal maturation of the immune system and disrupts the formation of proper balance between Th2 and the other branches of immune response, promoting allergy. Different availability of microbial stimuli as well as the particular composition of bacterial taxa may be among the factors contributing to conflicting results of studies performed in varying environments (Eriksson et al., 2010), especially when comparing cohorts collected in urban (McLoughlin et al., 2012) vs. rural settings (Schaub et al., 2008, 2009; Strömbeck et al., 2014).

Genetic and epigenetic factors are the intrinsic players of atopy, sensitization and allergy development. It is well known that children with one or both atopic parents are several times more likely to develop atopy then children from non-atopic families. Polymorphisms in numerous genes have been implicated in atopy and allergy development, including genes for cytokines (e.g. IL-4, IL-5, IL-10 and IL-13), chemokines, proteases or specific human leukocyte antigen (HLA) alleles (Ferreira et al., 2017; Ober and Yao, 2011). Different genetic background may thus account for some of the discrepancies observed between various studies, and it has been suggested that gene expression and epigenetic control may be prenatally influenced by environmental factors affecting the mother (Waterland and Michels, 2007). Epigenetic control has been described for various immune populations and mechanisms, including Th1 (White et al., 2002), Th2 (Su et al., 2008) and Treg (Lluis et al., 2014; Paparo et al., 2016).

Therapy of allergy is in most cases symptomatic, consisting of antihistamines and/or mast cell stabilizers (chromones), and allergen avoidance where possible. In more

severe cases, corticosteroids are employed either topically or systemically as a form of general immune suppression, and adrenaline is used to stabilize circulation in case of anaphylaxis. The therapy of asthma is different from other allergic diseases due to its localised nature and specific pathophysiology, and usually includes locally applied inhalatory corticosteroids and beta-blockers to control bronchial inflammation and hyper-reactivity, with systemic immunosuppressive therapy being reserved for more severe cases or non-classical forms. Generally, the only causal therapy approach in allergy treatment is allergen-specific immunotherapy (SIT) (Jutel and Akdis, 2011; Larché et al., 2006). In this therapeutic modality, increasing doses of allergen are administered over a long period of time in order to induce specific tolerance, and success has been shown to be dependent on regulatory T-cells (Radulovic et al., 2008; Syed et al., 2014), characteristic regulatory cytokines IL-10 and TGF-β (Palomares et al., 2014) and on generation of antibodies of IgG4 isotype (James et al., 2012).

Because of the high overall cost and symptomatic nature of most anti-allergic therapy, as well as the pronounced effect on quality of life and productivity of patients, there is a pressing need of effective preventive measures. Furthermore, to determine individuals who would best benefit from such preventive measures and more regular observation, a reliable way to assess individual risk of future sensitization and allergy development very early in life is necessary. Despite the extensive understanding of pathophysiology of allergic diseases, there is still a marked lack of sufficiently reliable early predictors, making further research warranted. Cord blood is a material readily and non-invasively available at birth, and as such it potentially provides valuable insight into the immune status at birth. Many groups have tried to search for predictive factors using cord blood. Among parameters studied in cord blood were levels of Th1 and Th2 cytokines (Chung et al., 2007; Hrdý et al., 2010), proliferation of and cytokine production by cord blood

cells stimulated with polyclonal stimulators, allergens or microbial agents (G^+ and G^-) (Prescott et al., 2003; Lappalainen et al., 2009; Rindsjö et al., 2009; Schaub et al., 2009), specific IgE levels (Prokešová et al., 2008; Peters et al., 2012) as well as regulatory T cell characteristics (Hrdý et al., 2012b; Meng et al., 2016; Schröder et al., 2016). However, the conclusiveness of such studies so far has been limited, and few predictors have been unambiguously accepted as sufficiently strong. Parental, especially maternal allergy status is still considered the strongest predictor (Björkander et al., 2019; Prescott et al., 2003).

b. Immune regulation

Immune system is armed with numerous remarkably powerful and destructive effector mechanisms. While this is necessary in order to contend with the multitude of external and internal threats we are constantly exposed to, it also poses a great risk to the organism itself. Therefore, we can consider immune system as a double-edged sword. Tightly balanced regulation is needed to ensure that immune reaction and the associated risk of tissue damage is limited strictly to the context and extent it is truly needed. This control is built into the very way the immune system is organised and present at every level, from self/nonself and danger recognition, innate immune cell activation and function, to antigen presentation, engagement of adaptive immunity and its effector mechanisms, and finally the entire process of negative selection of T and B cells in the central lymphoid organs. When the immune regulation fails, autoimmune diseases, allergy or other forms of immune-mediated inflammatory disorders arise.

The major goals of immune regulation in the periphery are limiting the damage to own tissues, resolution of inflammation and wound healing, retraction of the immune

response when it is no longer needed, and induction and maintenance of tolerance of self-antigens, innocuous environmental antigens and normal microbiota components. These goals are achieved via multiple ways, including spatial and functional barriers, feedback loops which give the immune reaction a self-limiting nature unless constantly stimulated, creation of tissue- and context-specific cytokine milieu that promotes tolerance and hinders reactivity unless disturbed, and finally the existence of specialised regulatory cell populations dedicated to keeping the entire system in balance. Among various such populations, undeniably the most important are the regulatory T cells, or Treg.

i. Regulatory T cells

Treg were first identified by Shimon Sakaguchi in 1995 as a population of $CD4^+CD25^+$ T cells with suppressive phenotype (Sakaguchi et al., 1995). Since then, remarkable progress has been made into describing and functionally defining these cells as well as various functionally relevant subpopulations. Of particular importance was the discovery of the transcription factor specific for Treg lineage, forkhead box protein 3 (FoxP3) in 2003 (Fontenot et al., 2003). Treg are crucial for maintaining peripheral tolerance, and a defect in their number or function plays a key role in various autoimmune and allergic diseases (Alroqi and Chatila, 2016). On the other hand, the biological significance of their suppressive and immunomodulatory properties is clearly demonstrated in tumor stroma, where infiltrating Treg create potently immunosuppressive environment that impairs immune response and favours tumor progression. Indeed, Treg are key for tumor immune evasion, and Treg infiltration correlates with poor prognosis (Chaudhary and Elkord, 2016).

While precise identification of the cells can be challenging and is sometimes inconsistent among different studies, human Treg are nowadays generally accepted to be $CD4^{+}CD25^{high}CD127^{low}$ cells with intracellular $FoxP3^{+}$ expression. Due to their high levels of CD25, the high-affinity α subunit of IL-2 receptor (IL-2Rα), another defining characteristic of Treg is their strong dependence on IL-2. In terms of function, Treg are important producers of regulatory cytokines such as IL-10, TGF-β and IL-35 and are capable of suppressing the function of most other effector cells. Some measure of plasticity exists between Treg and other Th subtypes, e.g. Th17 (Sakaguchi et al., 2013). Importantly, in humans, it seems that some of the accepted Treg markers can also be found in non-Treg cells in specific context. For instance, CD25 is expressed by activated effector T cells, and FoxP3 has likewise been described to transiently upregulate upon activation of T cells (Kmieciak et al., 2009). Thus it is necessary to take into account any activation of the immune system when evaluating Treg, and preferably to use combinations of markers and methods for the study of these cells (Nettenstrom et al., 2013). Likewise, it has been shown that very high expression (approx. the top 2%) of CD25 more reliably corresponds with Treg phenotype than simple positivity for CD25 marker (Roncador et al., 2005); hence, Treg are now usually described as $CD25^{high}$ rather than the previously used $CD25^{+}$.

Expression of FoxP3 plays a key role in development, function and stability of Treg, and median fluorescence intensity (MFI) of FoxP3 has even been proposed to correlate with suppressive function (Steinborn et al., 2010). Transgenic $FoxP3^{-/-}$ mice express the so-called “scurfy” phenotype with pronounced autoimmune and allergic symptoms (Lin et al., 2005). In humans, genetic deficiency of FoxP3 is the cause of the syndrome IPEX (immune dysregulation-polyendocrinopathy-enteropathy-X-linked), comprising severe multiorgan autoimmunity and allergic symptoms, including atopic

dermatitis, food allergy and eosinophilic inflammation (Bacchetta et al., 2006). Similar clinical course can also be found in the IPEX-like syndrome, which is caused by the disturbed epigenetic control of an otherwise intact *FOXP3* locus (Barzaghi et al., 2012). This highlights the importance of epigenetic regulation of FoxP3 expression for Treg function. Specific mechanisms of epigenetic control of Treg phenotype have been identified to maintain Treg stability, most importantly the demethylation of Treg specific demethylated region (TSDR) in *FOXP3* promoter area (Baron et al., 2007; Kitagawa et al., 2015). TSDR comprises three conserved noncoding sequences (CNS) in *FOXP3* gene, CNS1 in the promoter and CNS2 and CNS3 in the first intron (Kim and Leonard, 2007). The conserved noncoding sequences contain motives for binding various transcription factors (e.g. NF/AT, AP-1, NF-κB, STAT5, CREB etc.) and allow Treg to integrate information from cell-intrinsic as well as extrinsic signals and modulate FoxP3 expression accordingly (Zheng et al., 2010). Several CpG islands which are found within TSDR are differentially methylated between Treg and non-Treg cells (Baron et al., 2007) and likewise serve regulation purposes in FoxP3 induction. Demethylation of CNS2 of TSDR in particular correlates with stable Treg phenotype and regulatory function (Baron et al., 2007; Iizuka-Koga et al., 2017; Lee et al., 2018). Some studies claim that TSDR methylation status can be used to distinguish between *bona fide* Treg and the upregulation of FoxP3 and CD25 in activated effector T cells (Baron et al., 2007). Increased demethylation of TSDR in Treg has been described to correlate with decreased allergic sensitization (Lluis et al., 2014) and in therapeutically induced oral tolerance (Paparo et al., 2016). Methylation status of TSDR has also been evaluated in autoimmune diseases (Barzaghi et al., 2012; Shu et al., 2017) and high demethylation is considered a marker of poor prognosis in tumor diseases (Timperi et al., 2016). The role and mechanisms of

epigenetic control of *FOXP3* in Treg have been reviewed e.g. in (Iizuka-Koga et al., 2017; Morikawa and Sakaguchi, 2014).

Treg are capable of inhibiting all types of immune reaction, employing an array of both contact-dependent and distal mechanisms. These are utilised both to directly inhibit function of effector cells and on APC, subverting costimulation and leading to ineffective antigen presentation. The particular subset of mechanisms Treg use to suppress immunity and promote tolerance may vary depending on Treg subpopulation and biological context. They can selectively inhibit the expansion and activation of a broad spectrum of cell types, including naïve and effector T lymphocytes, B lymphocytes, APC, NK cells and others (Palomares et al., 2014, 2017). Regulatory T cells are the chief producers of immunosuppressive cytokines IL-10, TGF-β and IL-35 (Akdis et al., 2011) and thus may establish tolerogenic and anti-inflammatory cytokine milieu in tissues. Furthermore, Treg utilise various surface molecules for cell-to-cell suppression, including CTLA-4 (cytotoxic lymphocytes antigen 4), PD-1 (programmed death domain 1), PD-L1 (PD-1 ligand 1) and LAG-3 (lymphocytes activation gene-3). CTLA-4 and PD-1 are co-inhibitory members of CD28 family and are referred to as "immune checkpoints" for their central role in controlling autoimmune diseases (Fife and Bluestone, 2008; Watanabe and Nakajima, 2012) as well as allergy (Kumar et al., 2013; McAlees et al., 2015). Targeted inhibition of these molecules by monoclonal antibodies forms the basis of a novel anti-tumor therapy, termed checkpoint blockade (Ito et al., 2015). The high effectivity of this therapy in some types of tumor diseases, as well as the pronounced immunologic adverse effects observed (Michot et al., 2016), both testify to the biological relevance of CTLA-4 and PD-1 in Treg function. Specifically, CTLA-4 has been shown to bind CD80 and CD86 on APC more strongly than CD28 and convey inhibitory signalling to effector T cells. In direct contact of Treg with APC, CTLA-4

segregates and downregulates CD80 and CD86, hampering costimulation the APC provide to effector cells (Wing et al., 2008). PD-1 interacts with a pair of ligands, PD-L1 (constitutively expressed on B cells, DC, macrophages, mast cells, T cells and a variety of non-hematopoietic cells) and PD-L2 (inducibly expressed on DC, macrophages, and some B cells). Engagement of PD-1 differentially regulates T cell homeostasis. In effector T cells, PD-1 signalling leads to anergy and deletion through inhibition of the key metabolic Akt/mammalian target of rapamycin pathway, providing an important cell-intrinsic negative feedback mechanism, while in Treg it promotes FoxP3 expression and supports regulatory phenotype (Francisco et al., 2010). In addition, Treg utilize PD-L1 expression to trigger PD-1 signalling in DC, negatively regulating their function. Thus, PD-1:PD-L interaction represents a complicated, multifaceted inhibitory pathway that can both directly and indirectly mediate Treg suppression (Francisco et al., 2010). LAG-3 is a CD4 homolog that strongly binds to major histocompatibility complex class II (MHCII), is strongly involved in Treg function (Huang et al., 2004) and contributes to cell-contact-mediated Treg inhibition of APC (Liang et al., 2008). Expression of ectoenzymes CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5'-nucleotidase) allows Treg to degrade extracellular ATP, a potently proinflammatory danger signal, into immunosuppressive adenosine. Other routes of suppression include competition for IL-2 with effector T cells; as Treg express high levels of CD25, they have competitive advantage over other T cell populations and effectively deplete IL-2 from the environment, starving effector T cells in addition to supressing its production by other means (Pandiyan et al., 2007). Treg have also been described to be capable of directly killing target cells utilizing perforin – granzyme route, similar to CD8 cytotoxic T cells. In addition to antigen-specific inhibition of effector cells with corresponding specificity, the effect Treg exert on cytokine milieu, danger signals and antigen presentation can also

cause indirect inhibition ("bystander effect", (Jun et al., 2012)), introducing generally immunosuppressive environment in tissues. Treg can furthermore induce Treg phenotype in conventional T cells in this way, a phenomenon called infectious tolerance (Shevach and Thornton, 2014). Effector mechanisms of Treg have been comprehensively reviewed on many occasions, e.g. by (Palomares et al., 2014; Shevach, 2009). The biological importance of various molecular constituents of Treg phenotype and function is demonstrated by the Treg deficiency observed in various "tregopathies", i.e. monogenic syndromes caused by mutation in various genes important for Treg homeostasis and function, including but not limited to *FOXP3, CTLA-4, PD-1* and *IL-10* (Cepika et al., 2018).

Over 25 years of research, various subpopulations of Treg have been described, distinguished phenotypically, functionally and by the context in which they arise and operate. Conventional $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ Treg can be divided into two major populations, natural Treg (nTreg) and induced Treg (iTreg). Arising in thymus during negative selection from mildly self-reactive single positive thymocytes, nTreg possess exert strongly immunosuppressive, stable Treg phenotype and represent an important mechanism of peripheral tolerance (Shevach and Thornton, 2014). They are responsible for control of autoimmune diseases. On the other hand, iTreg arise from naïve T cells on periphery, typically upon foreign antigen recognition in specific conditions (suboptimal antigen presentation in presence of TGF-β) (Chen et al., 2003). Induced Treg are crucial for the maintenance of tolerance to harmless, mostly environmental antigens, including allergens (Jutel et al., 2003). Some authors use the term "peripheral Treg" or pTreg rather than iTreg for this population when it arises *in vivo,* reserving the term iTreg for artificially induced regulatory T cells used in *in vitro* studies or prepared for therapeutic purposes (Shevach and Thornton, 2014). Induced Treg (particularly those

induced *in vitro*) exhibit significant plasticity and may lose FoxP3 expression and Treg phenotype under inflammatory conditions. This may play a vital role in allergic or autoimmune disease pathogenesis and also complicates the use of these cells in therapy (Sakaguchi et al., 2013; Diller et al., 2016; Xin et al., 2018). As mentioned before, methylation analysis of TSDR may be used to ascertain whether these cells are stably committed to Treg lineage or not. While the two Treg populations are quite distinct functionally, they are rather difficult to distinguish in order to study the differences. Expression of Helios, an Ikaros family transcription factor (Thornton et al., 2010), and surface marker neuropilin 1 (CD304) (Weiss et al., 2012) have been proposed to be specific for nTreg (Singh et al., 2015). Doubt has since been cast on both of these markers, however (Szurek et al., 2015). Helios has been described to be upregulated in activated T cells, similar to FoxP3 itself (Akimova et al., 2011). Interestingly, it has also been shown to play a role in maintaining stable Treg function (Kim et al., 2015), and similar function has been proposed for neuropilin 1 (Chen et al., 2019; Delgoffe et al., 2013). As nTreg are commonly considered to be more firmly committed into Treg lineage then iTreg, it is possible that Helios and neuropilin 1 play a role in controlling their homeostasis. Thus, the value of Helios and neuropilin 1 as nTreg markers still remains to be conclusively determined (Thornton and Shevach, 2019). Difference in T-cell receptor (TCR) specificity repertoire has been described between $Helios^{+}$ and $Helios^{-}$ Treg, and may reflect differential function of these cell subsets (Lord et al., 2015; Thornton et al., 2019).

Using flow cytometry, various other subpopulations of Treg have been described. These subpopulations play different biological roles and occur in different context. Type 1 regulatory cells (Tr1) are usually defined as $CD4^{+}CD25^{high}CD127^{low}FoxP3^{-}$ cells which produce large amounts of IL-10 (Meiler et al., 2008a). They have been established as

important producers of IL-10 in the context of allergen-specific immunotherapy and are a major part of its success (Akdis and Akdis, 2011). A population of Th3 cells is mostly present in mucosal tissues and produces large amounts of TGF-β (Konkel and Chen, 2011; Palomares et al., 2014), setting up a strongly tolerogenic environment and playing important role in oral tolerance induction (Carrier et al., 2007). Specific subpopulations of Treg can also be identified based on the coexpression of FoxP3 with transcription factors typical for other Th lineages, including T-bet, GATA3, RORγt and BCL-6. It has been proposed that the expression of transcription factors specific for an effector cell line allows Treg to also express the corresponding chemokine receptors, thus homing them into the relevant tissues where they can specifically inhibit these cells (Campbell and Koch, 2011).

Numerous studies have demonstrated the importance of Treg in controlling allergy and inhibiting pathologic Th2 responses (Palomares et al., 2017; Xystrakis et al., 2006). They are responsible for inducing and maintaining peripheral tolerance, acting to both prevent sensitization and counteract the effector mechanisms of allergy (Palomares et al., 2014). Via IL-10 secretion, Treg can inhibit IgE secretion and promote the switch to IgG4, which is considered protective in the context of allergy (Meiler et al., 2008b). Furthermore, they prevent the immigration and degranulation of basophiles and mast cells (Gri et al., 2008; Kashyap et al., 2008) as well as the recruitment of effector T cells into inflamed tissues (Ring et al., 2006). By releasing TGF-β and inducing tolerogenic DC (Wing et al., 2008), they establish broadly tolerogenic environment in peripheral immune tissues, forming the necessary basis for oral tolerance (Palomares et al., 2012). Such environment also promotes further Treg induction from naïve T cells (Chen et al., 2003), acting as an important feedback mechanism especially in mucosal tissues (Palomares et al., 2014; Wawrzyniak et al., 2017). By regulating tissue repair, Treg can also mitigate

the damage caused by allergic inflammation (Gri et al., 2008). It is therefore not surprising that Treg are considered indispensable for allergen-specific immunotherapy (Palomares et al., 2014; Radulovic et al., 2008). Expansion of conventional Treg (Suárez-Fueyo et al., 2014) or IL-10 producing $CD4^+$ cells such as Tr1 (Akdis and Akdis, 2011); hypomethylation of *FOXP3* (Paparo et al., 2016); and functional changes in Treg (Gonzalez et al., 2017) have all been considered as markers of successful SIT. In a similar vein, expansion of bee-venom-specific Tr1 cells producing large amounts of IL-10 has been observed in beekeepers during the beekeeping season and correlated with the suppression of effector T cell functions (Meiler et al., 2008a).

Dysbalance in the numerous factors acting prenatally may already *in utero* prepare the terrain for postnatal atopy and allergy development (Jones et al., 2000). Due to their potent and finely controlled immunomodulatory capacity, Treg play a central part in the critical foetal and perinatal periods, orchestrating the nascent immune system balance, immune maturation and allergy prevention. Maternal Treg are also critical for maintenance of pregnancy, as low levels of Treg and imbalance between Th17 and Treg have been linked to increased risk of abortion and premature birth (Figueiredo and Schumacher, 2016). Major interplay between maternal and foetal immunity exists during pregnancy, and supports Treg induction (Mold et al., 2008). Shared regulation of this population is reflected in positive correlation between the population sizes of maternal and foetal Treg as well as IL-10 levels in maternal peripheral blood and cord blood (Santner-Nanan et al., 2013). Reduced Treg population in maternal peripheral blood correlates with increased maternal Th2 cytokine production and higher levels of IgE in cord blood (Hinz et al., 2010). Maternal and foetal immune system are maintained under Th2 bias and tolerogenic conditions throughout the pregnancy (Aluvihare et al., 2004; Sykes et al., 2012), and the tightly interdependent regulatory network mediates the

influence of environmental effects faced by the pregnant mother on foetal immune system. Protective factors including farm exposure (Hrusch et al., 2019; Lluis et al., 2014; Schaub et al., 2009; Stein et al., 2016) and risk factors such as air pollution (Baïz et al., 2011; Hinz et al., 2012) or maternal allergy (Fu et al., 2013; Hrdý et al., 2012b) may influence Treg levels and functional properties in the perinatal period, exerting their effect through Treg. This is not absolute, however, as for instance Rindsjö *et al.* observed no effect of maternal allergy on $FoxP3^+$ Treg and cord blood cell reactivity towards allergens. While usually, larger Treg population size confers protection (e.g. (Hinz et al., 2012)), some studies have also demonstrated increased size of Treg population in cord blood of children at higher risk of allergy development, accompanied by defective function (e.g. (Hrdý et al., 2012b; Strömbeck et al., 2014)). This may be attributable to a compensatory expansion of Treg in an effort to make up for insufficient function, as has been described in atopic dermatitis (Roesner et al., 2015) and systemic lupus erythematosus (Alexander et al., 2013).

ii. Breg and other cell types with immunoregulatory capacity

Apart from Treg, other regulatory cell populations have been described in experimental animal models as well as various human immunopathological states. Among these, regulatory B cells (Breg) are the most relevant and well-studied in the context of allergy. Breg have been described and researched in both human and mice as a heterogeneous group of B cell subpopulations of various stages of maturation and differentiation and patterns of surface markers expression that nonetheless share a common, biologically relevant regulatory capacity (Palomares et al., 2017; van de Veen et al., 2016). Their suppressive and immunoregulatory functions are due to production of

high amounts of IL-10 as well as TGF-β and IL-35 (Rosser and Mauri, 2015). Additionally, some subpopulations of Breg have been described to express a variety of regulatory surface molecules, allowing for additional contact-dependent and distal modes of suppression, possibly including induction of apoptosis by FasL (Klinker et al., 2013) and PD-L1 dependent co-inhibition (Shalapour et al., 2015). In the context of IgE-mediated allergy, IL-10 producing Breg importantly secrete allergen-specific IgG4 antibodies (van de Veen et al., 2013). This highly specialized blocking antibody isotype is commonly considered to be anti-inflammatory due to its inability to activate complement and the unique ability to exchange antigen-binding arms, forming bispecific functionally monovalent antibodies (van der Neut Kolfschoten et al., 2007). Importantly, Breg also secrete less IgE and can support tolerogenic DC maturation (Stanic et al., 2015).

IL-10- and IgG4-producing Breg play an important role in allergen tolerance, as has been demonstrated in studies of healthy beekeepers (Meiler et al., 2008a; van de Veen et al., 2013) and cat owners (Platts-Mills et al., 2001) who are exposed to high doses of the particular allergens. As such, induction of allergen-specific Breg is another essential component of successful allergen-specific immunotherapy (Palomares et al., 2017). In mice, infection with some helminths and microbes induced regulatory B cells able to limit allergic airway inflammation, possibly contributing to the mechanisms asserted by "hygiene hypothesis" (Smits et al., 2007). Furthermore, various Breg subpopulations exert suppressive function in numerous models of murine and human autoimmune diseases (van de Veen et al., 2016). For instance, their depletion by anti-CD20 monoclonal antibody B cell-depleting therapy has been observed to cause exacerbations of colitis (Goetz et al., 2007) or new onset of psoriasis (Dass et al., 2007).

Other immunoregulatory cell populations with less-well-described roles in allergy include three types of innate suppressive cells: regulatory innate lymphoid cells (ILCreg) and myeloid derived suppressor cells (MDSC) and M2 macrophages, both of myeloid origin. ILCreg are a $CD3^{-}FoxP3^{-}$ ILC subpopulation that has recently been described in both human and murine models of allergy (Morita et al., 2019) and intestinal inflammation (Wang et al., 2017). ILCreg exert their regulatory functions mainly in early mucosal regulation of inflammation by IL-10 and TGF-β (Wang et al., 2017) secretion and are a somewhat heterogeneous population that can be induced from ILC2 by retinoic acid (Morita et al., 2019). Expression of transcription factor Id3 (inhibitor of DNA binding 3), implicated also in Treg maintenance (Miyazaki et al., 2014), is required for their development (Morita et al., 2019; Wang et al., 2017). Myeloid derived suppressor cells are a heterogeneous immunosuppressive population arising from immature myeloid cells that has been studied extensively in the context of cancer (Gabrilovich and Nagaraj, 2009; Kwak et al., 2015). MDSC utilise a plethora of mechanisms to suppress T cell function, induce Treg and modulate other cell populations (e.g. macrophages and NK cells), including IL-10 production, L-arginine depletion by arginase, reactive oxygen species (ROS) release and nitric oxide (NO) synthesis by inducible NO synthase (Kolahian et al., 2016; Kwak et al., 2015). Recently, complex regulatory functions of these cells in inflammatory diseases including asthma have been observed (Kwak et al., 2015; Zhang et al., 2013) and a dual inflammatory/anti-inflammatory nature has been proposed (Kolahian et al., 2016). Complex anti-inflammatory functions have also been described in some macrophage populations, most importantly alternatively activated or M2 macrophages. M2 generally play a key role in tissue remodelling, wound healing and resolution of inflammation; in cancer, they are commonly associated with tumor stroma and contribute to immune evasion (Shapouri-Moghaddam et al., 2018). M2 macrophages

are induced by IL-4 and IL-13 and exert suppressive functions mainly by secreting IL-10 and TGF-β. They can thus recruit or induce other suppressive populations such as Treg and set up a suppressive environment in tissues (Saradna et al., 2018; Shapouri-Moghaddam et al., 2018). In the context of allergy, M2 cells are usually considered pathogenic as they suppress Th1 and support Th2 responses and promote inflammatory tissue remodelling (Lou et al., 2019; Saradna et al., 2018).

c. Microbiota and immunity

The last several decades have seen a breakthrough in our understanding of the exceedingly complex interplay of the host macroorganism and the myriad microorganisms present in microbiota that colonise its external and internal surfaces. It is now becoming increasingly evident that the immune system in particular has co-evolved in tight-knit interconnection with our microbiota and that their mutually regulated maturation is essential during ontogeny. Thus, factors such as composition, diversity, metabolic activity and spatiotemporal properties of exposure to symbiotic, commensal and pathogenic microbes alike play a key role in the induction of or protection from numerous immune-related, inflammatory disorders including infectious diseases (Belkaid and Naik, 2013; Buffie and Pamer, 2013; Kamada et al., 2013), autoimmunity (Antonini et al., 2019; Maloy and Powrie, 2011), allergy (Wesemann and Nagler, 2016), cancer (Sheflin et al., 2014), atherosclerosis (Li and Tang, 2017) and metabolic syndrome (Turnbaugh et al., 2006; Vijay-Kumar et al., 2010).

Gut represents the compartment colonised by the largest numbers of microorganisms and possesses extensive mucosal immune system; consequently, the influence exerted by gut microbiota on local as well as systemic immunity is the best

studied. The composition of colonising microbiota is nonetheless highly location-specific, individual and influenced by genetic and environmental factors, especially during establishment of colonisation in the perinatal period (Levin et al., 2016). Furthermore, while bacteria predominate, colonising microbiota includes a plethora of organisms from other taxa, such as archaea, viruses, protozoa and fungi. Many of them are difficult to study, and a vast majority of the species cannot be easily cultivated; their functional significance is only now beginning to be appreciated. This complexity sometimes makes it hard to reconcile inconclusive or contradictory results of studies regarding the biological role of individual factors or microbial agents. Nonetheless, it is clear that microbes are major players in the establishment of a healthy immune system and disturbed microbiota was linked to number of disorders, as has been reviewed e.g. by Belkaid in (Belkaid and Hand, 2014). The importance of microbiota for immune regulation, maturation of lymphoid organs and health in general was convincingly illustrated by studies in germ-free (GF) animals which exhibit widespread immune dysregulation and aberrant intestinal mucosal system organisation and function (Cahenzli et al., 2013; Herbst et al., 2011).

The notion of microorganisms as major players in the pathogenesis of non-communicable diseases (NCDs) is not entirely new. Indeed, the importance of immune modulation by microbiota is the common denominator of several hypotheses that have attempted to explain the observed rise in non-communicable disease (chiefly allergy) in the Western world over the last century. In 1989, Strachan postulated the "hygiene hypothesis," stating that the decrease in exposure to infancy and early childhood infections was behind the dysregulation of immune system leading to the development of allergy and other NCDs (Strachan, 1989). This was re-evaluated and superseded by the "old friends" hypothesis proposed by Rook in 2010, which holds that colonising

microbes, not pathogens, possess the key immunomodulatory capacity, having undergone evolution side by side with the evolving immune system from the dawn of human species (Rook, 2010). Another related hypothesis that has gained traction in the past decade is the "biodiversity" hypothesis, proposing that it is a general decrease in diversity of the microbial community, and not the positive or negative influence of individual strains, that leads to immune dysregulation (Haahtela et al., 2013). The aforementioned theories are not mutually exclusive; in fact, it is likely that a combination of all the mechanisms plays a role with varying contributions to different pathologies and in different individuals. Taken together, they suggest a concept of microbial dysbiosis, i.e. pathological microbiota, as the key culprit of dysregulation of immune system common to many NCDs with increasing prevalence. Furthermore, considering the importance of early postnatal and antenatal period for the establishment of healthy microbiota – immune system balance and the potentially lifelong influence of this early event, colonising microbes have been implicated as one of mechanisms participating in developmental origins of health and disease hypothesis (Stiemsma and Michels, 2018).

Microbiota modulates the mucosal as well as systemic immune system via a complex array of mechanisms, which under physiological conditions integrate to promote tolerogenic and regulatory immunological states. From the standpoint of allergy, the regulation of Th2 immune responses is especially important. Pathways utilised in microbiota – immune system crosstalk are manifold and include Treg induction (Atarashi et al., 2013; Josefowicz et al., 2012); tolerogenic antigen presentation introduced by modified signalling via Toll-like receptors (TLR) (Round et al., 2011); competition with pathogenic or dysbiotic microbiota (Kamada et al., 2013); induction of IgA responses in mucosal B cells; production of immune-relevant metabolites, chiefly short-chain fatty acids (SCFA) (Smith et al., 2013) and certain vitamins (LeBlanc et al., 2017); and

epigenetic modulation of mucosal immune cells (Luo et al., 2017). SCFA – acetate, propionate and most importantly butyrate – are produced by certain bacterial species (e.g. Firmicutes including *Faecalibacterium prausnitzii*, *Eubacterium rectale* and *Roseburia* spp. (Louis and Flint, 2017)), serve as a major substrate for colonic epithelium (Morrison and Preston, 2016), contribute to induction of Treg and promote regulatory environment in mucosae (Arpaia et al., 2013). This is likely at least in part achieved by epigenetic modification, as butyrate can regulate histone deacetylase activity (Davie, 2003) and mice deficient in CNS1 of TSDR region fail to create microbiota-induced Treg in mucosa and instead develop Th2 inflammation (Arpaia et al., 2013). Recently, it has been shown that not only microbiota but also food antigens can promote iTreg induction in the gastrointestinal tract, mediating oral tolerance (Kim et al., 2016). The described microbial effects are highly strain-specific, and various dysbiotic patterns have been described to associate with risk of atopy by different studies (reviewed by McCoy in (McCoy et al., 2018)). This has been described in the context of airway allergy (Arrieta et al., 2015, 2018; Fujimura et al., 2016), food allergy (Bunyavanich et al., 2016; Fazlollahi et al., 2018) as well as atopic dermatitis (Kennedy et al., 2017; Kobayashi et al., 2015).

It is commonly accepted that the perinatal period and early infancy represent the crucial timeframe during which commensal microbiota is established and the key microbial-immune crosstalk occurs. In studies on GF animals, colonisation with specific pathogen free (SPF) biota early in life, but not in adulthood, can rescue many facets of the immune system dysregulation (El Aidy et al., 2013). Correspondingly, very early perturbations of human gut microbiome by various factors have been described to associate with immune-related disorders (chiefly allergic diseases) later in life (reviewed e.g. by Gensollen in (Gensollen et al., 2016)). On the other hand, this period in life is also amenable to positive modulation of the immune system (e.g. by administration of

probiotic microbial strains) and has been termed the “window of opportunity” (Torow and Hornef, 2017). In humans, microbial colonisation continues to fluctuate for the first few years of life until stabilising and remaining mostly stable for the rest of life unless disturbed by extreme environmental changes (Palmer et al., 2007; Spor et al., 2011).

While it is still not unambiguously clear whether foetus is prenatally in contact with microbiota from the maternal organism, physiologically the first massive microbial exposure occurs during vaginal delivery with the microbiota found in the birth canal and maternal faeces (Dominguez-Bello et al., 2010; Gueimonde et al., 2006). Delivery by caesarean section is associated with a dysbiotic microbiota that can potentially predispose to various NCDs (Blustein et al., 2013; Magne et al., 2017). Breastfeeding is probably the most important factor determining microbial colonisation of the infant’s gut (Bäckhed et al., 2015). Human breast milk contains both live microbes and oligosaccharides which serve microbiota as an energy source, modulating microbial communities in the gut and promoting physiologic microbiota (Moossavi et al., 2018). In addition, breastfeeding protects the infant from infections and stimulates neonatal immunity by supplementing with immune cells and IgA (Ciardelli et al., 2008; Järvinen et al., 2019), helping maintain physiological balance between gut-resident microbiota and mucosal immune system. Other environmental factors that can influence human microbiota and immune system during the early infancy include exposure to household pets (Levin et al., 2016) or farming environment with high microbial burden (Hrusch et al., 2019; Lluis et al., 2014; Schaub et al., 2009; Stein et al., 2016); season of birth (Thysen et al., 2016); antibiotic use (Ahmadizar et al., 2018); or use of anti-acid medication (Mitre et al., 2018). Most of these factors can influence immune regulation and either serve as protective influences or lead to predisposition to atopic diseases under specific conditions. Owing to the

exceptional complexity of the systems involved the studies into the topic commonly report conflicting results, however (reviewed e.g. by Kim et al. in (Kim et al., 2019)).

i. Probiotic intervention in allergy prevention

It logically follows that beneficial health effects, up to and including allergy prevention, could be achieved by a rational modification of gut microbiota, particularly when attempted during the "window of opportunity". Most common approach to achieve this is through the administration of probiotics, which were defined as "live organisms that when administered in adequate doses confer a health benefit to the host" by United Nations Food and Agricultural Organization (FAO) and World Health Organization (WHO) in 2001. Another possible way to modulate microbiota is with prebiotics, defined as metabolic substrates (usually nondigestible by host) that promote the growth and/or activity of beneficial microorganisms, mostly in the gastrointestinal tract, and provide health benefit to the host. Lastly, synbiotics are preparations that include both probiotics and prebiotics to further increase the health benefits, and postbiotics encompass "non-viable bacterial products or metabolic byproducts from microorganisms that have biologic activity in the host" (Patel and Denning, 2013). The idea of probiotic use to improve human health is not new to modern science, as already at the beginning of 20th century Elie Metchnikoff proposed the use of bacteria in this fashion upon observing the longevity of Bulgarian peasants whose diet contained large amounts of fermented milk products. As our understanding of the importance of microbial commensals for immune system grew over the last several decades, so did the interest in the possible capacity of probiotic preparations to modify various immune disorders. Recently, the ascent of highly effective, culture-independent analytic techniques led to a boom in studies of human

commensal microbiota and its perturbations (termed dysbiosis) associated with various pathological states. This methodical breakthrough led to identification of novel probiotic species and strains associated with healthy microbiota, which were previously difficult to isolate and study. These probiotic species (e.g. *Faecalibacterium prausnitzii, Akkermansia muciniphila*) are termed next-generation probiotics and defined as "live microorganisms identified on the basis of comparative microbiota analyses that, when administered in adequate amounts, confer a health benefit on the host" (Martín and Langella, 2019). Due to their recent discovery, they lack the long-term record of safety in human use traditional probiotics possess and their safety is so far not considered proven.

The biological and clinical effects are highly dependent on factors including choice of the used probiotic species or strains, dosage of the microbes, age and mode of administration. It is crucial to decide whether the probiotic should be administered to infants, pregnant women, breastfeeding mothers or a combination of the three. Supplementation of mother during pregnancy or breastfeeding can lead to colonisation of the infant and this effect is strain-dependent (Dotterud et al., 2015). It has been shown that prenatal exposure to microbial components can influence the immune system and confer protection from allergic sensitization (Abrahamsson et al., 2015; Gomez de Agüero et al., 2016; von Mutius, 2016). In addition to possibly mediating the probiotic colonisation in the infant, supplementation of breastfeeding mothers may also modify human breast milk composition and regulatory capacity, e.g. by increasing TGF-β or IL-10 levels in colostrum and breast milk (Böttcher et al., 2008; Rautava et al., 2002). The choice of strain is equally critical, and a broad spectrum of microbes including Gramm-positive (e.g. *Lactobacilli* such as *L. rhamnosus GG*, *L. reuteri* and others; *Bifidobacteria* including *B. infantis* and *B. longum* or some *Bacilli*) and Gramm-negative

bacteria (*Escherichia coli* strains Nissle 1917 or O83:K24:H31 (Wassenaar, 2016)) as well as yeasts (e.g. *Saccharomyces boulardii, S. cerevisiae*) have been studied (Liu et al., 2018). Furthermore, combination of distinct probiotics may have stronger effect than preparations including only a single strain (Timmerman et al., 2004).

While the probiotic strains may be able to colonise the gut and exert their effects *in situ* for extended periods, this is not always the case and many probiotics need to be repeatedly applied. Colonisation resistance by the already established microbiota as well as the need to overcome adverse conditions in the stomach (low pH) and the small intestine (bile acids) are likely to blame for the strain- and individual-specific inconsistency of long-term colonisation after probiotic administration (Buffie and Pamer, 2013; Libertucci and Young, 2019; Zmora et al., 2018). The desired effects, however, do not necessarily require persistent colonisation, especially when exerted in the early infancy. Prenatal administration of probiotics can modify the microbial development in infants, e.g. *Lactobacillus rhamnosus* GG strain has been shown to promote *Bifidobacterium longum* (Lahtinen et al., 2009). Role of probiotics in immune regulation is complex and strain-specific; described effects include tolerogenic DC and Treg induction (Baba et al., 2008; Thomas and Versalovic, 2010), TLR signalling modulation (Ciorba et al., 2012); promotion of IL-10 and TGF-β secretion (Di Giacinto et al., 2005; Niers et al., 2007); increase in secretory IgA production (Corthésy et al., 2007) and production of anti-inflammatory microbial metabolites including SCFA (Peng and Biswas, 2017).

Effect of probiotic treatment on disease prevention has been studied in various contexts, and numerous meta-analyses have tried to reconcile the published data. So far, the most convincing evidence of beneficial effect has been described in the context of

necrotising enterocolitis, acute infectious diarrhoea, acute respiratory tract infections, and infant colic, as reported in the work of Liu (Liu et al., 2018). While studies have been published reporting a protective effect of specific probiotic treatments in the context of atopic eczema (Kalliomäki et al., 2007) and allergic sensitization (Allen et al., 2014; Wickens et al., 2013), as a whole, the role of probiotics in allergy prevention remains inconclusive and other studies have reported no effect on allergy or sensitization outside atopic eczema (Cuello-Garcia et al., 2015). Meta-analyses and comprehensive overview studies in this field are complicated due to the extreme heterogeneity introduced by methods, utilised probiotics strains and the particulars of their use as well as differences in environmental and genetic factors. The resulting complexity and numerous confounding factors make the comparison of results and interpretation of studies quite challenging. In general, current data seems to indicate that combination of prenatal and postnatal probiotic treatments shows the greatest protective effects against atopic eczema (Zuccotti et al., 2015) and sensitization in general (Zhang et al., 2016). While probiotic supplementation by itself has so far not been demonstrated to have clear benefit in prevention of allergic disease development in atopic patients, the possibility remains open and more well-designed and randomised, double-blind, placebo controlled studies will be needed to definitively resolve the heterogeneous data (Forsberg et al., 2016). Since no probiotic strain that would provide optimal risk-benefit ratio has been indisputably established as of yet, great care needs to be taken when considering probiotics for allergy prevention (Szajewska and Horvath, 2018). Despite the overall inconclusiveness, World Allergy Organisation stated in 2015 that probiotic use likely confers net advantages, mostly due to eczema prevention (Fiocchi et al., 2015). Of potential interest is also the possibility of using probiotic supplementation as an adjuvant in the context of oral allergen-specific immunotherapy (Loh and Tang, 2018).

a. *Escherichia coli* O83:K24:H31

The probiotic strain of *E. coli* O83:K24:H31 (EcO83) is commercially available in the Czech Republic as a registered drug for oral administration. This preparation is named “probiotic vaccine” under the name Colinfant New Born. EcO83 has been introduced into Czech paediatric practice by the efforts of Dr. Lodinová-Žádníková, who championed its use in preterm newborns for prevention of nosocomial infections and diarrhoea (Lodinová-Žádníková et al., 1991a, 1998). The strain has several desirable characteristics that make it an efficient probiotic, such as good epithelial adhesion and ability to survive in intestine long term, low pathogenic potential and absence of plasmids, and good sensitivity to broad spectra of antibiotics. A retrospective analysis of infectious and allergic anamneses of a cohort of patients who had been treated with EcO83 in early 1980s and 1990s revealed both a decrease in recurrent infections reported at the age of 10 years and a decrease in allergy both at 10 and at 20 years of age (Lodinová-Žádníková et al., 2003). Following this remarkable discovery, a still ongoing prospective study was started by our group in an effort to better describe this phenomenon and elucidate the underlying immunologic mechanisms. While the entire mode of function of this probiotic still hasn’t been conclusively explained, immune-relevant changes including an effect on Treg functional markers have been observed in supplemented children which might contribute to observed prevention of allergy development (Lodinová-Žádníková et al., 2010; Hrdý et al., 2016).

The PhD thesis is based on the following papers:

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

Černý, V., Hrdý, J., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., and Prokešová, L.

PLoS ONE (2018) 13(11): e0207998. https://doi.org/10.1371/journal.pone.0207998

IF_{2018} = 2.776

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

Černý, V., Petrásková, P., Novotná, O., Boráková, K., Prokešová, L., Kolářová, L., and Hrdý, J.

Cent. Eur. J. Immunol. (2019), accepted for publication.

IF_{2018} = 1.455

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

Hrdý, J., Vlasáková, K., Černý, V., Súkeníková, L., Novotná, O., Petrásková, P., Boráková, K., Lodinová-Žádníková, R., Kolářová, L., and Prokešová, L.

Eur. J. Immunol. (2018) 48, 2015–2030. https://doi.org/10.1002/eji.201847636

IF_{2018} = 4.695

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

Súkeníková, L., Černý, V., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., Prokešová, L., and Hrdý, J.

Immunol. Lett. (2017) 189, 82–89. https://doi.org/10.1016/j.imlet.2017.05.013

IF_{2018} = 2.552

2. Aims

2.1. First major aim was to characterise Treg cell populations involved in immune regulation during the perinatal period, which might play a role in early phases of allergy development, and to evaluate their potential usefulness in allergy prediction. In particular, we aimed to:

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

2.2. Second major aim was to describe the effect of probiotic strain *E. coli* O83:K24:H31 on cell populations in the context of allergy in order to uncover the possible mechanisms behind the ability of this probiotic strain to decrease allergy incidence in the high-risk group. In particular, our aims were to:

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

3. Results

Despite the progress made in the field of allergy research over the last decades, allergy remains a key issue of contemporary medicine. The virtually epidemic incidence of allergic diseases, high individual and systemic burden they pose, and limited options of causal therapy make the search for potential preventive measures an important area of immunological research.

While a consensus has yet to be reached regarding the usefulness of probiotic treatment for allergy prevention, the possibility remains open and further research is warranted. Considering the multifactorial nature of allergic diseases and the highly complex, strain-specific effects of probiotics, it is conceivable that particular strains could be identified that can be of benefit to individual patients or groups of patients. For such an individualised approach to be feasible, however, we need a deeper understanding of both the immunoregulatory effect of probiotics and the early events involved in immune dysregulation and allergy development. Hence, improving our knowledge of biological effects of probiotic strains that have already been described to lower allergy incidence is imperative. Furthermore, probiotic intervention is likely most effective when attempted during the perinatal period, i.e. when probiotics are administered prenatally to pregnant mothers or postnatally to neonates during the first days after birth. Identification of very early markers able to predict the risk of subsequent allergy development and the potential benefit of timely probiotic supplementation would thus be highly desirable. As a biological material readily and non-invasively available at birth, cord blood represents a promising source of such early predictive markers.

In our study, we followed the children of allergic mothers (i.e. with higher risk of future allergy development – **A**) and children of healthy mothers (i.e. with lower risk of

allergy development later in life – **H**). Firstly, we compared both proportional and functional characteristics of Treg between these two groups in order to uncover whether there is a discernible pattern of abnormality of Treg associated with allergy risk determined by maternal allergy status (Černý et al., 2018). Furthermore, in a different, older cohort of probands, children from high-risk and low-risk group were subdivided according to their own allergic status and a retrospective analysis of Treg population properties and functional characteristics were performed in order to evaluate their predictive potential (Černý et al., accepted 2019).

In order to test suppressive function of Treg in a direct suppression assay based on coculture with carboxyfluorescein succinimidyl ester (CFSE)-stained $CD4^+CD25^-$ non-Treg target cells, we needed to optimize the method for isolating $CD4^+CD25^+CD127^{low}$ Treg and target cells, i.e. conventional T cells (Tconv), from cord blood mononuclear fraction (CBMC; obtained by density gradient centrifugation using Histopaque). The first method we tested was magnetic bead-based separation using magnetic-activated cell sorting (MACS) technology by Miltenyi Biotec. First, we used $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II, human (cat.no. 130-094-775; Miltenyi Biotec, Bergisch Gladbach, Germany). While the isolation yielded sufficient numbers of Treg and Tconv for downstream experiments, purity of the obtained cells was generally low, with the Tconv fraction consistently containing ~40% non-CD4 cells (representative dot plots of isolates stained for CD4,CD25 and CD127 shown in Fig. 1A) and the Treg fraction containing only around 20% of $CD25^+CD127^{low}$ Treg cells (representative dot plots of Treg characterised by combination of cell surface markers CD4, CD25 and CD127 shown in Fig. 1B).

Figure 1. MACS CD4$^+$CD25$^+$CD127^{dim} Regulatory T Cell Isolation Kit II

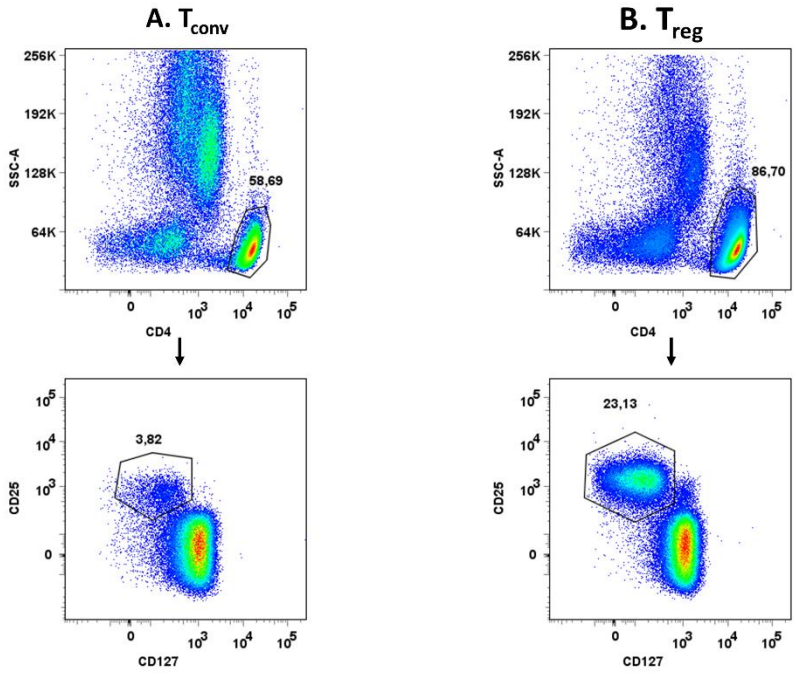

Figure 1. *Cells were isolated from cord blood mononuclear cell fraction by magnetic separation using CD4$^+$CD25$^+$CD127$^{dim/-}$ Regulatory T Cell Isolation Kit II, human from Miltenyi Biotec. To ascertain the purity of isolated cells, obtained cell suspensions were stained for surface markers CD4, CD25 and CD127. Top row, CD4+ cell population gated from all singlet cells. Bottom row, CD25$^+$CD127^{low} Treg cell population gated from CD4$^+$. A: CD4$^+$CD25$^-$ Tconv. B: CD25$^+$CD127^{low} Treg.*

We further tried to isolate Treg using a different MACS kit, CD4$^+$CD25$^+$CD45RA$^+$ Regulatory T Cell Isolation Kit, human (cat.no. 130-093-631, Miltenyi Biotec). While the purity was slightly higher, particularly in the case of Tconv fraction (representative dot plots of CD4$^+$ T cells and CD25$^+$CD127^{low} cells shown in Fig. 2A), isolated Treg still contained only 30%-40% of CD25$^+$CD127^{low} (representative dot plots of CD4, CD25 and CD127 shown in Fig. 2B).

Figure 2. MACS CD4⁺CD25⁺CD45RA⁺ Regulatory T Cell Isolation Kit

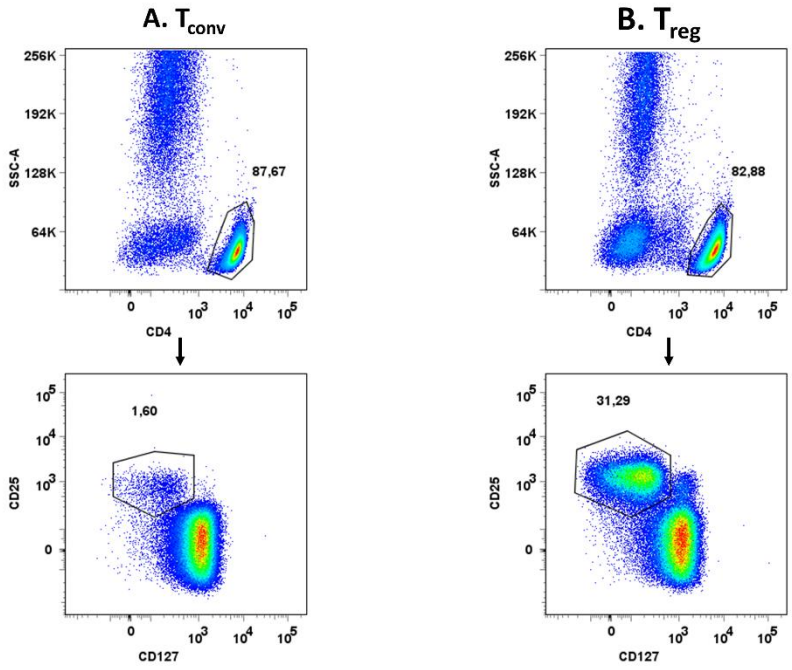

Figure 2. *Cells were isolated from cord blood mononuclear cell fraction by magnetic separation using CD4+CD25+CD45RA+ Regulatory T Cell Isolation Kit, human from Miltenyi Biotec. To ascertain the purity of isolated cells, obtained cell suspensions were stained for surface markers CD4, CD25 and CD127. Top row, CD4+ cell population gated from all singlet cells. Bottom row, $CD25^{+}CD127^{low}$ Treg cell population gated from $CD4^{+}$. A: $CD4^{+}CD25^{-}$ Tconv. B: $CD25^{+}CD127^{low}$ Treg.*

Both Miltenyi Biotec kits are optimized by the manufacturer for the use with adult peripheral blood, therefore, use with cord blood may be problematic; major differences include the presence of more resilient nucleated foetal erythrocytes in cord blood. For this reason, we decided to perform erythrocyte lysis with ammonium-chloride-potassium (ACK) lysis buffer in an attempt to improve purity of the isolated cells. Unfortunately, this did not solve the issue with Treg purity (representative dot plots of lysed and non-

lysed isolates for CD4 and CD25 shown in Fig. 3), and we abandoned Miltenyi MACS technology for other options.

Figure 3. MACS CD4⁺CD25⁺CD127^{dim} Regulatory T Cell Isolation Kit II – isolated Treg cells before and after erythrocyte lysis

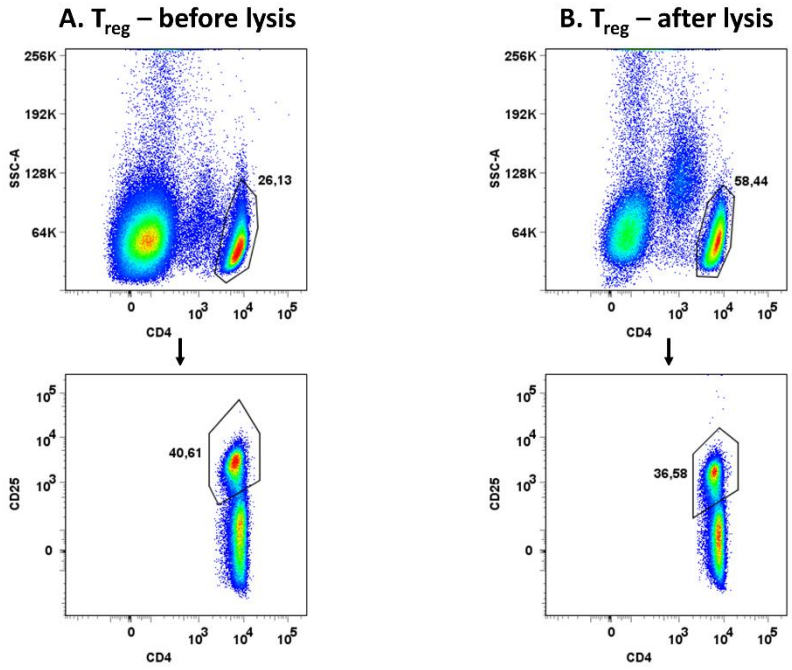

Figure 3. *Cells were isolated from cord blood mononuclear cell fraction by magnetic separation using CD4⁺CD25⁺CD127$^{dim/-}$ Regulatory T Cell Isolation Kit II, human from Miltenyi Biotec and erythrocytes lysed using ammonium-chloride-potassium lysis buffer. To ascertain the purity and viability of isolated cells, after lysis cell suspensions were washed and stained for surface markers CD4 and CD25. Top row, CD4+ cell population gated from all singlet cells. Bottom row, CD4⁺CD25⁺ Treg cell population gated from CD4⁺. A: CD4⁺CD25⁺ Treg before erythrocyte lysis. B: CD4⁺CD25⁺ Treg after erythrocyte lysis.*

Consequently, we attempted to obtain Treg by magnetic isolation with CD4+ CD25+ Treg Fab Streptamer® Isolation Kit MB, human (cat.no. 6-8000-205, IBA GmbH, Göttingen, Germany). While the quantity and purity of the obtained Tconv was acceptable, we were able to obtain only extremely few Treg of questionable purity

(representative dot plots of isolated Tconv and Treg shown in Fig. 4A and Fig. 4B, respectively).

Figure 4. CD4+ CD25+ Treg Fab Streptamer® Isolation Kit MB

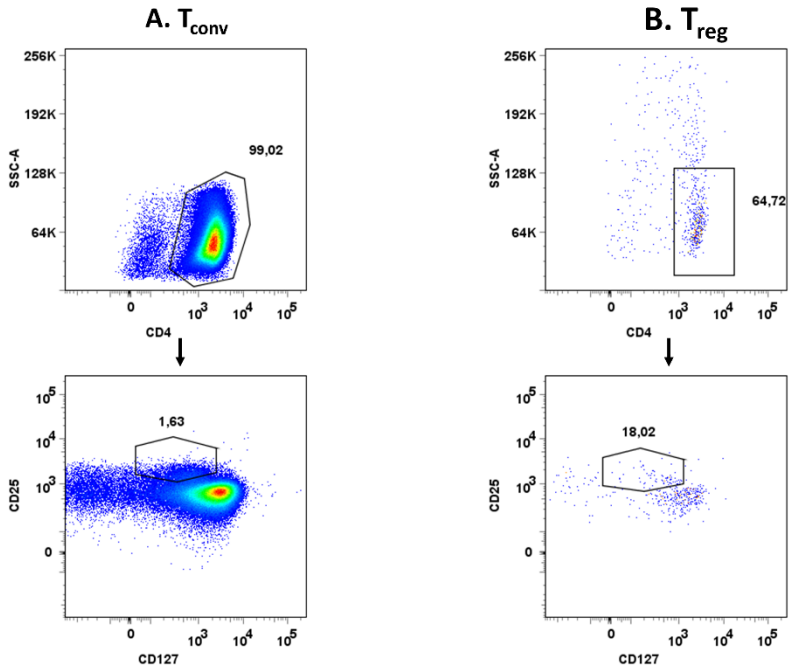

Figure 4. *Cells were isolated from cord blood mononuclear cell fraction by magnetic separation using CD4+ CD25+ Treg Fab Streptamer® Isolation Kit MB, human from IBA. To ascertain the purity of isolated cells, obtained cell suspensions were stained for surface markers CD4, CD25 and CD127. Top row, CD4+ cell population gated from all singlet cells. Bottom row, $CD25^{+}CD127^{low}$ Treg cell population gated from $CD4^{+}$. A: $CD4^{+}CD25^{-}$ Tconv. B: $CD25^{+}CD127^{low}$ Treg.*

Next, we proceeded to attempt fluorescence-activated cell sorting (FACS) of $CD4^{+}CD25^{+}$ Treg and $CD4^{+}CD25^{-}$ Tconv using BD FACS Aria sorter. Although both Tconv and Treg yields and purity were acceptable after sorting (representative dot plots of sorted Tconv and Treg shown in Fig. 5A and Fig. 5B, respectively), control staining of the Treg fraction 24 hours after sorting uncovered poor survival and loss of $CD4^{+}CD25^{+}$

phenotype of Treg, rendering the yielded cells unsuitable for cultivation assays performed over several days (Fig. 5C).

Figure 5. FACS sorting (BD FACS Aria™)

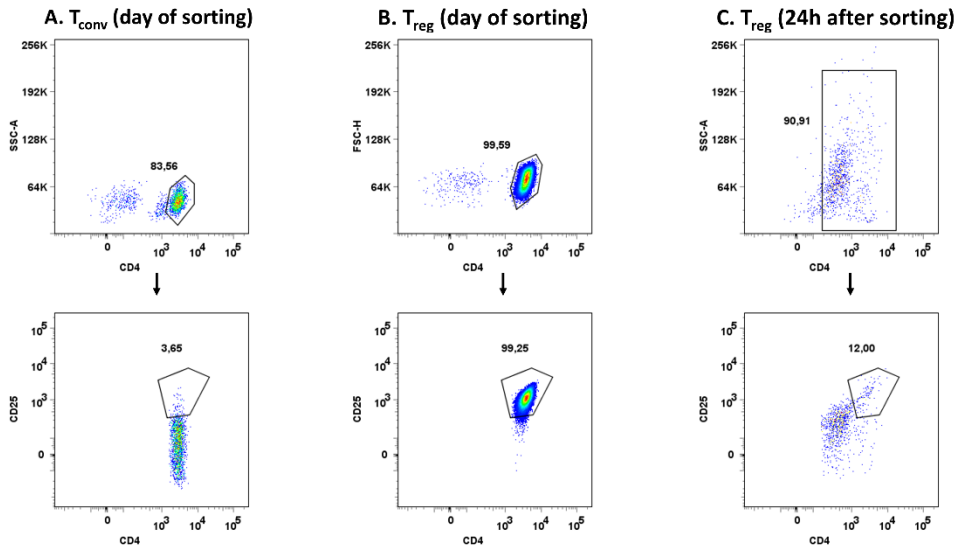

Figure 5. *$CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ Tconv were obtained from cord blood mononuclear cell fraction by FACS sorting on BD FACS Aria sorter. To ascertain the purity of sorted cells, obtained cell suspensions were stained for surface markers CD4 and CD25. To assess Treg stability and viability after sorting, Treg were stained for surface markers CD4 and CD25 24 hours after sorting. Top row, CD4+ cell population gated from all singlet cells. Bottom row, $CD4^+CD25^+$ Treg cell population gated from $CD4^+$. A: $CD4^+CD25^-$ Tconv on day of sorting. B: $CD25^+CD127^{low}$ Treg on day of sorting. C: $CD25^+CD127^{low}$ Treg 24h after sorting.*

Lastly, we performed Treg separation from CBMC using EasySep™ Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (cat.no. 18063, STEMCELL Technologies, Vancouver, Canada). Treg isolation using this kit yielded high numbers of pure $CD4^+CD25^-$ Tconv for use as target cells (>90%, representative dot plots shown in Fig. 6A), as well as sufficient numbers of Treg of high purity (>90% of

$CD4^+CD25^+CD127^{low}$, representative dot plots shown in Fig. 6B). We proceeded to use this kit for subsequent coculture-based assays.

Figure 6. EasySep™ Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit

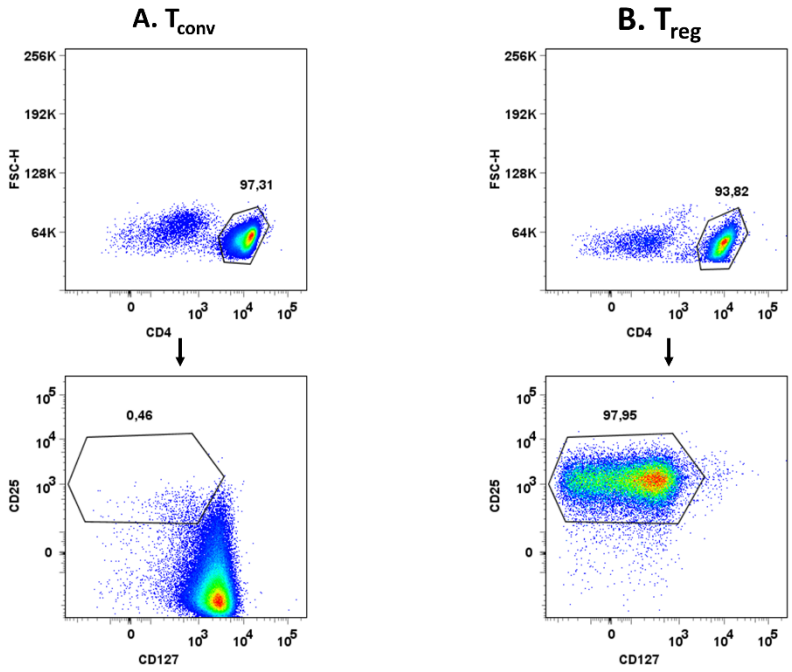

Figure 6. *Cells were isolated from cord blood mononuclear cell fraction by magnetic separation using EasySep™ Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit from STEMCELL Technologies. To confirm purity, obtained cells were stained for surface markers CD4, CD25 and CD127. Top row, CD4+ cell population gated from all singlet cells. Bottom row, $CD25^+CD127^{low}$ Treg cell population gated from $CD4^+$. A: $CD4^+CD25^-$ Tconv. B: $CD25^+CD127^{low}$ Treg*

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

Černý, V., Hrdý, J., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., Prokešová, L.

PLoS ONE (2018) 13(11): e0207998. https://doi.org/10.1371/journal.pone.0207998

(Attachment 1)

The goal of this work was to evaluate whether any difference could be found in the population and phenotypic characteristics of Treg in cord blood of children of allergic mothers and cord blood of children of healthy mothers. The hypothesis, based on observations our group made in previous studies (Hrdý et al., 2010, 2012b, 2014; Žižka et al., 2007a), was that differences in functional characteristics of CBMC, myeloid DC and cytokine expression in CBMC could be mediated by abnormal proportion and/or functional deficiency/dysregulation of Treg in cord blood of the high-risk group. Since iTreg arise in the periphery upon contact with harmless environmental antigens and play an important role in allergy control, we also compared the proportions of iTreg and nTreg among cord blood Treg between the two groups to ascertain if iTreg deficiency in children of allergic mothers could contribute to the higher risk of allergy development. Antibodies against Helios, a transcription factor found in natural but not induced Treg, were added to distinguish the two subpopulations.

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

Proportions of Treg (defined as $CD4^+CD25^+CD127^{low}FoxP3^+$ among $CD4^+$ T cells), nTreg (identified as $CD4^+CD25^+CD127^{low}FoxP3^+$ $Helios^+$ cells among Treg) and iTreg (identified as $CD4^+CD25^+CD127^{low}FoxP3^+$ $Helios^-$ cells among Treg) were determined by flow cytometry. Somewhat surprisingly, percentage of Treg in $CD4^+$ cells was significantly higher in children of allergic mothers than in the low-risk group. The children of healthy mothers had significantly higher proportion of $Helios^-$ iTreg among Treg, and conversely, children of allergic mothers had significantly higher proportion of $Helios^+$ nTreg among cord blood Treg. These findings might hint at a delayed maturation

and formation of iTreg in the high-risk group, which could potentially contribute to the increased risk of allergy observed.

Determination of selected phenotypic characteristics associated with Treg regulatory function

Surface expression of CTLA-4, PD-1 and GITR (markers of regulatory function) and intracellular presence of chief regulatory cytokines (IL-10, TGF-β) were measured in Treg (defined as $CD4^+CD25^+CD127^{low}$ cells) by flow cytometry in order to indirectly assess and compare the functional capabilities of these cells between the high-risk and low-risk groups. Significantly higher proportion of PD-1^+ Treg was observed in cord blood of children of healthy mothers, with a similar albeit non-significant trend discernible for CTLA-4^+ Treg. Furthermore, children of healthy mothers had significantly higher percentage of IL-10^+ Treg in cord blood than children of allergic mother, with an observable similar but non-significant trend for TGF-β. Taken together, markers associated with Treg function are observably lower in the high-risk group, potentially hinting at a compromised function of these cells.

Assessment of Treg regulatory function

To confirm the findings suggesting lower functional capacity of Treg in cord blood of children of allergic mothers in a more direct fashion, we performed a functional assay based on cocultivation of Treg isolated from cord blood (using EasySep magnetic separation) with CFSE-stained non-Treg ($CD4^+CD25^-$) conventional T cells. Although the low number of samples we were able to include did not allow for proper statistical analysis, we observed lower ability of cord blood Treg of children of allergic mothers to inhibit proliferation of conventional T cells, hinting at defective suppressive function of Treg in the high-risk group. Indeed, stimulated cells from children of healthy mothers

produced significantly more IL-10 than stimulated cells from children of allergic mothers in these cocultivation assays, with maternal allergy status exerting greater effect on IL-10 concentration in supernatant than presence and number of cocultured Treg (Figure 7).

Figure 7. Concentration of IL-10 in cell culture supernatant from Treg:Tconv coculture

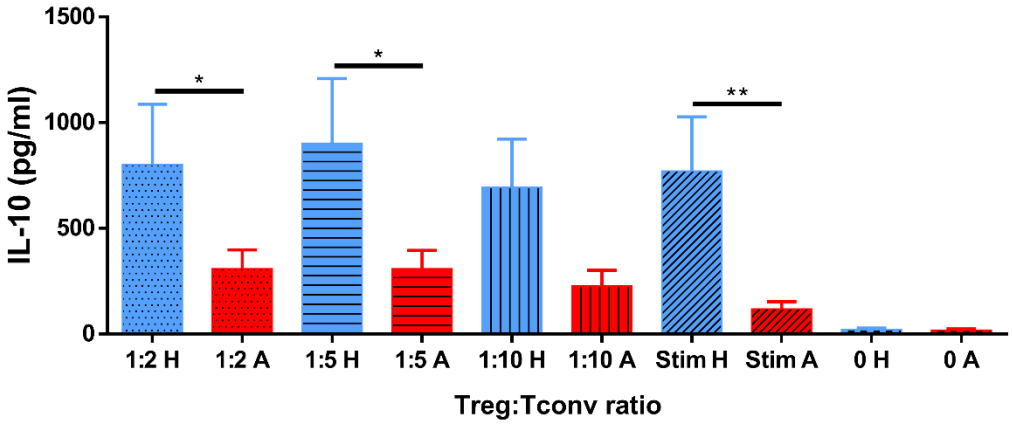

***Figure** 7. $CD4^+CD25^+CD127^{low}$ Treg and $CD4^+CD25^-$ conventional T cells (Tconv) were isolated from cord blood mononuclear cell fraction by magnetic separation using EasySep™ Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit from STEMCELL Technologies. Tconv were stained with carboxyfluorescein succinimidyl ester (CFSE) and plated into 48-well plates with Treg at different Treg:Tconv ratios. Recombinant human IL-2, functional grade human anti-CD3 and functional grade human anti-CD28 were used to stimulate cells. After 72 hours of culture, supernatant was collected and concentration of IL-10 was determined by ELISA (R&D System.) A – children of allergic mothers (n=14). H – children of healthy mothers (n=11). Stim, stimulated Tconv. 0, unstimulated T conv. 1:2, 1:5, 1:10 – various ratios of Treg:Tconv in the culture.*

Lastly, we quantified IL-10 and TGF-β levels in cord blood plasma by enzyme-linked immunosorbent assay (ELISA) to see if the trend of compromised regulatory capacity was observable on the systemic level. Levels of IL-10 and TGF-β were significantly lower

in cord blood plasma of children of allergic mothers, further hinting at the dysregulated pattern of immune regulation in the high-risk group.

Together, results of the study exposed impaired regulatory capabilities of cord blood Treg of children of allergic mothers that may play a biologically significant role in the higher risk of allergy described in literature. Another interesting finding is that children from the high-risk group have lower proportion of iTreg among their Treg. We hypothesise that our data reflect dysregulated development of Treg in children of allergic mothers, potentially due to delayed maturation of their immune system. Such defect in regulatory mechanisms is consistent with our previous observations of higher reactivity of immune system of children from the high-risk group to a variety of stimuli. The counterintuitively higher percentage of total Treg among $CD4^+$ T cells in the high-risk group may be attributed to attempts to compensate for their functional insufficiency by expanding the population.

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

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In order to plausibly evaluate the potential of cord blood Treg characteristics for allergy prediction, a retrospective analysis in the context of actual development allergy is required. In this follow-up study, we compared cord blood Treg population properties and phenotypic characteristics, measured at the time of birth (Hrdý et al., 2012b), among

children divided into two groups according to their allergic status at the age of 6 – 10 years. Healthy and allergic children were further subdivided into four groups with different risk of allergy predicted at birth according to maternal allergy (the 4 subgroups being healthy children of healthy and allergic mothers – H/H and H/A, respectively, and allergic children of healthy and allergic mothers – A/H and A/A, respectively).

Proportion characteristics of cord blood Treg in allergic and healthy children

When Treg were identified as $CD4^+CD25^+CD127^{low}$ cells, no differences in percentage from $CD4^+$ T cells was observed among neither the two basic groups nor the four subgroups. When $CD4^+CD25^+$ T cells (including Treg but potentially also activated T cells) were compared, significantly higher percentage of these cells was found in the group of healthy children. Upon subdivision according to maternal allergy status, the group of healthy children of healthy mothers, i.e. healthy children from the low-risk group, showed a higher percentage of these cells than both healthy children of allergic mothers and allergic children of healthy mothers. Notably, in children of allergic mothers, no difference of $CD4^+CD25^+$ T cells was observed regardless of the children's allergy. This may point to a different composition and/or biological role of this population between the high-risk and the low-risk groups.

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

The original study included MFI of FoxP3 as a marker associated with Treg function, in addition to intracellular presence of regulatory cytokines IL-10 and TGF-β. Upon division according to the children's allergic status (and subdivision according to maternal allergy), no difference was observed in FoxP3 MFI. While IL-10^+ and TGF-β^+ Treg showed only nonsignificant tendency towards higher proportions in healthy children,

upon subdivision according to maternal allergy status, significantly higher proportion of IL-10^+ Treg was uncovered in healthy children of healthy mothers and conversely, a significantly lower proportion of TGF-β^+ cells was discernible in allergic children of allergic mothers.

Concentration of cytokines with regulatory functions in plasma of cord blood

Cord blood serum concentrations of IL-10 and TGF-β were also determined in the original study (Hrdý et al., 2012b). In the follow-up, levels of cytokines were not different between allergic and non-allergic children. After division according to maternal allergy status, healthy children of healthy mothers exhibited significantly higher serum concentration of IL-10 than allergic children of allergic mothers, as well as significantly higher levels of TGF-β than both groups of children of allergic mothers.

The results of this study further support the notion that analyses of functional aspects of Treg will likely have greater predictive value than simple determination of population proportions. In addition, the differences observed between the original study and the follow-up serve to stress the importance of maintaining sufficient cohort sizes, as limiting the analysis to a selection from a larger cohort may strongly influence the results, contributing to discrepancies observed between numerous published studies.

In another study on a different group of children (Hrdý et al., 2018), some newborns from the high-risk group were randomly chosen for supplementation with probiotic EcO83 (colonised children of allergic mothers, **C A**) and their allergic status was observed and compared with non-colonised children of allergic mothers (**NC A**) and non-

colonised children of non-allergic mothers (**NC H**) at the age of 8 years. Additionally, these groups were further divided according to the allergy status of the children, into allergic (A C A; A NC A or A NC H) and non-allergic (H C A; H NC A or H NC H) subgroups in an attempt to describe the effect of probiotic treatment on immune regulation in the context of allergy. In an effort to uncover the possible modes of action of EcO83 supplementation, we evaluated the effect of *in vitro* stimulation by EcO83 on CBMC and *in vitro* generated myeloid DC (i.e. monocyte-derived DC, moDC).

All the studies were made possible thanks to a long-term collaboration with the Institute for the Care of Mother and Child, where the children were delivered, samples of cord blood obtained, probiotic supplementation of the colonised group realised and subsequent monitoring of the children by paediatricians and allergists took place.

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

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Supplementation by suitable probiotic strains is a potentially useful approach to prevent or mitigate allergy development. A decrease in allergy incidence in high-risk children of allergic mothers by colonisation with *E. coli* O83:K24:H31 has been retrospectively observed (Lodinová-Žádníková et al., 2003), warranting further prospective studies to better describe the phenomenon and uncover the mechanisms involved. The present study reports lower allergy incidence at the age of 8 years in infants

of allergic mothers supplemented early postnatally with EcO83. To help elucidate the possible ways in which this protective influence may be enacted, a selection of immunological characteristics including Treg population proportions and regulatory cytokine profiles at the age of 8 years were followed. In addition, effect of *in vitro* stimulation of CBMC with EcO83 was tested.

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

Effect of EcO83 supplementation on allergy incidence, IgE sensitization and regulation

Colonised children of allergic mothers had lower incidence of allergy than non-supplemented children of allergic mothers, effectively reaching incidence observed in low-risk group of children of healthy mothers. Despite this, colonisation had little effect on serum levels of specific IgE against separate mixtures of food or respiratory allergens, nor on levels of IgG4 against the same allergens. Allergic children had significantly higher titres of respiratory allergen-specific IgE than healthy children, both when considered in total and within each of the three basic groups. Surprisingly, allergic colonised children of allergic mothers had higher titres of these IgE than allergic non-colonised children of allergic mothers.

No difference of total Treg population (percentage of $CD4^+CD25^{high}FoxP3^+$ cells from $CD4^+$ T cells, measured using flow cytometry) was observed among the three basic groups or the six subgroups. However, healthy children had significantly higher

proportion of Helios$^-$ iTreg (and, correspondingly, lower proportion of Helios$^+$ nTreg), regardless of maternal allergy status. This increase in iTreg was driven by their higher percentage in the group of colonised children of allergic mothers, and particularly those colonised children who did not proceed to develop allergy (H C A group). The upregulation of iTreg was not observed in colonised children who developed allergy (A C A), thus an inability to induce Treg generation upon probiotic supplementation might be one potential cause of probiotic intervention failure.

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

Cytokine production represents a crucial pathway of regulation, setting the organism up both locally and systemically to either develop allergy or be protected. Thus, we expected the broadly preventative effect of EcO83 colonisation to influence plasma levels of relevant cytokines, i.e. IFN-γ, IL-4 or IL-10. Indeed, quantification of plasma IFN-γ and IL-10 by ELISA revealed significantly higher levels of both cytokines in children of healthy mothers and importantly, in colonised children of allergic mothers as opposed to non-colonised children of allergic mothers. After subdivision according to the children's allergic status, it became clear that this difference is mostly due to higher levels in healthy colonised children of allergic mothers and children of healthy mothers. Notably, healthy non-colonised children of allergic mothers had lower levels of IFN-γ and IL-10 than the other two healthy groups, hinting at less developed regulatory mechanisms preventing aberrant Th2 immune response activation in the untreated high-risk group even in absence of overt disease. On the other hand, colonisation with EcO83 did not influence plasma levels of the chief Th2 cytokine IL-4, as both groups of children of allergic mothers had significantly higher IL-4 levels than children of healthy mothers. This pattern held true

after subdivision according to the children's allergy, although non-allergic children in each basic group had lower IL-4 levels than allergic children from the same group. To more directly assess the effect of EcO83 supplementation on functional capacity of Treg in 8-year-old children, we performed intracellular staining of IL-10 in $CD4^+CD25^{high}CD127^{low}$ Treg. Furthermore, as IL-10 producing Tr1 cells are functionally important for the success of SIT and are described as a $CD4^+$ population distinct from conventional $CD25^{high}CD127^{low}$ Treg, we also analysed the intracellular presence of IL-10 in $CD4^+$ cells after exclusion of $CD25^{high}CD127^{low}$ Treg. We observed a significantly higher proportion of IL-10^+ Treg in the blood of children of healthy mothers and, importantly, also in the blood of children of allergic mothers colonised by EcO83 than in samples from non-colonised children of allergic mothers. Upon subdivision according to the allergy status, both healthy children of healthy mothers and healthy colonised children of allergic mothers had significantly more IL-10^+ Treg than healthy non-colonised children of allergic mothers; both groups likewise had significantly more IL-10^+ Treg than allergic children from the respective basic groups. Intracellular presence of IL-10 in non-Treg $CD4^+$ T cells showed a similar pattern, with children of healthy mothers and EcO83 colonised children of allergic mothers both having significantly higher percentage of IL-10^+ $CD4^+$ non-Treg cells than non-supplemented children from the high-risk group. Additionally, significantly larger proportions of these cells were present in samples of both healthy children of healthy mothers and healthy colonised children of allergic mothers in comparison with healthy non-colonised children of allergic mothers, and the healthy colonised children also had significantly higher percentage of IL-10^+ CD4 T cells than allergic non-colonised children of healthy mothers. Collectively, the observed patterns of cytokine production are consistent with the known preventive effect of EcO83

supplementation and might represent a possible immunoregulatory mechanism underlying this phenomenon.

Biological effects of stimulation of CBMC by EcO83

In order to elucidate the possible mechanisms involved in EcO83 probiotic function, we investigated the effect of *in vitro* stimulation by the strain on cytokine production by mononuclear cells isolated from cord blood of children of allergic and of healthy mothers. Stimulation by lipopolysaccharide (LPS) and *E. coli* Nissle 1917 (EcN), another probiotic strain of *E. coli*, was used for comparison. Production of relevant cytokines (IFN-γ, IL-4 and IL-10) was assessed both by analysing gene expression by quantitative real-time PCR (rt-qPCR) and by detection of these cytokines in cell culture supernatants. No changes in gene expression of IL-4 were present, regardless of the stimulation or of maternal allergy status. Gene expression of IFN-γ was significantly lower in unstimulated controls than in CBMC stimulated by EcO83, EcN and LPS; the difference remained significant even after subdividing the subjects according to maternal allergy status, with the exception of stimulation of CBMC isolated from cord blood of children of allergic mothers by EcN. Gene expression of the regulatory cytokine IL-10 determined by rt-qPCR was significantly increased after culture with all three stimulants; importantly, stimulation of CBMC by EcO83 led to significantly higher upregulation of IL-10 expression than stimulation with LPS and EcN. This pattern was also found in the subgroup of children of healthy mothers, while in children of allergic mothers, only CBMC cocultured with EcO83 and LPS had higher IL-10 gene expression in comparison with unstimulated cells.

Supernatant levels of IL-4 showed no significant changes after stimulation with EcO83, LPS and EcN; however, after subdivision according to the maternal allergy, children of allergic mothers produced significantly higher levels of IL-4 than children of

healthy mothers, regardless of the stimulation used or lack thereof. CBMC cocultured with LPS and EcO83 produced significantly higher amounts of IFN-γ into the supernatant than unstimulated cells or cells stimulated with EcN. EcO83 stimulated CBMC of children of allergic mothers also produced higher amounts of IFN-γ than unstimulated CBMC of the same subgroup. In a similar fashion, supernatants of CBMC cocultured with LPS contained significantly more IL-10, and stimulation of CBMC by EcO83 induced significantly more IL-10 than all the other conditions. After division of CBMC according to the mothers' allergic status, significantly increased IL-10 levels were detected after all three modes of stimulation in CBMC of children of both healthy and allergic mothers. Moreover, CBMC of children of allergic mothers stimulated by EcO83 produced significantly more IL-10 than corresponding cells stimulated by LPS or EcN.

The results of this study further corroborate the potential of probiotic *E. coli* strain O83:K24:H31 for allergy prevention, an effect that persists from birth into the age of 8 years. Furthermore, higher proportion of iTreg in the colonised group supports the hypothesis that probiotic supplementation with EcO83 promotes tolerogenic environment necessary for mitigation of allergy development in the high-risk group. While colonisation had no effect on IL-4 production, colonised children of allergic mothers exhibited significantly larger proportion of IL-10$^+$ Treg and non-Treg CD4$^+$ T cells and higher levels of IFN-γ and IL-10 in serum, reaching levels found in the low-risk group. Increased production of IFN-γ and IL-10, observed on both mRNA and protein level upon *in vitro* stimulation of CBMC with EcO83, could help account for the long-term beneficial effects described in the study.

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

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Dendritic cells occupy a central position in immune regulatory networks as the cell population responsible for antigen presentation, T cell activation control and deciding which of the different immune response branches to employ. Due to the key role of DC in shaping immune milieu and informing T cell subpopulation development from early age, proper function of these cells is crucial for oral tolerance and dysregulation can promote allergy development. Thus, we decided to compare functional properties and responsiveness to EcO83 stimulation of myeloid DC generated *in vitro* from cord blood of children of allergic and of healthy mothers.

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

moDC were generated from adherent fraction of CBMC by 6-day culture with recombinant human (rh) IL-4 and rhGM-CSF, then stimulated with EcO83 or LPS for 24 hours before expression of activation and functional markers was evaluated. Flow cytometry was used to quantify surface expression of CD40, CD80, CD83, CD86 and MHCII on $CD11c^+$ moDC. While most activation markers exhibited no difference according to maternal allergy status nor any change upon stimulation, surface expression of CD83 was significantly upregulated after culture with EcO83 and LPS, both in moDC from children of healthy and from children of allergic mothers. Furthermore, moDC from

allergic mothers had significantly higher CD83 than moDC from healthy mothers, regardless of stimulation, implying higher reactivity in the high-risk group. Gene expression of indolamine 2,3-dioxygenase (IDO) and IL-10, important regulatory molecules, was evaluated by rt-qPCR after 5 hours of stimulation with EcO83. Unstimulated moDC derived from CBMC of healthy mothers had significantly higher gene expression of both IDO and IL-10 than unstimulated moDC from CBMC of children of allergic mothers. After stimulation with EcO83, gene expression of IDO was significantly upregulated in both low-risk and high-risk group, reaching comparable levels. While the expression of IL-10 likewise increased in both stimulated groups compared to the respective unstimulated moDC, the upregulation was significantly more pronounced in moDC derived from CBMC of children of healthy mothers, possibly indicating impaired regulatory function in the high-risk group of children of allergic mothers. Levels of IL-10, TNF-α and IL-6 in cell culture supernatant were then quantified by ELISA. Production of inflammatory cytokines TNF-α and IL-6 was increased after stimulation with EcO83 and LPS to a comparable degree, with no difference in scale of the upregulation between moDC from CBMC of children of allergic and healthy mothers. While IL-10 was likewise significantly upregulated upon culture with LPS and EcO83 in moDC from children of both healthy and allergic mothers, the increase was significantly higher when moDC from the low-risk group were stimulated with EcO83, compared both with stimulation by LPS and with stimulation of moDC from allergic mothers by EcO83. Taken together, the data imply that moDC derived from children of allergic mothers show higher reactivity and activate upon microbial stimulation more strongly, but show signs of regulatory deficiency, including lower IL-10 production and lower IDO expression.

Induction of Th responses by probiotic primed moDC

The ability of moDC stimulated by EcO83 to induce various types of Th response was evaluated by flow cytometric analysis of intracellular presence of the respective characteristic cytokines and transcription factors in T cells cocultured with the moDC. No stimulation-dependent differences were observed for markers of Th1 (IFN-γ; T-bet) or Th2 axis (IL-4, IL-13; GATA3); however, proportion of $CD4^+$ T cells positive for GATA3 as well as for intracellular IFN-γ and IL-4 was significantly higher upon coculture with moDC derived from CBMC of children of allergic mothers compared with moDC derived from CBMC of children from the low-risk group. After coculture with non-stimulated, but not EcO83-stimulated, moDC from the high-risk group, significantly higher percentage of IL-13^+ $CD4^+$ T cells was also observed. Intracellular staining for cytokines and transcription factors characteristic for Treg (IL-10; FoxP3) and Th17 cells (IL-17A, IL-22; RORγt) was performed. No difference in the percentage of $FoxP3^+$ Treg was observed upon coculture with moDC, regardless of maternal allergy status or microbial stimulation; however, moDC stimulated with EcO83 were able to induce significantly larger proportion of IL-10^+ $CD4^+$ T cells than non-stimulated moDC. Surprisingly, significantly higher percentage of IL-10^+ cells was also present after $CD4^+$ T cell coculture with moDC derived from CBMC of children of allergic mothers compared with moDC from CBMC of children of healthy mothers. In addition, we observed significantly lower percentage of both IL-$17A^+$ and RORγt^+ cells in $CD4^+$ T cells cocultured with non-stimulated moDC from children of healthy mothers, compared with EcO83 stimulated moDC from the low-risk group as well as with non-stimulated moDC derived from CBMC obtained from children of allergic mothers. Proportions of IL-22^+ cells as well as $IL10^+IL17^+$ double positive cells were also higher in $CD4^+$ T cells

cocultured with non-stimulated moDC of high-risk children compared to the low-risk group, but no influence of EcO83 stimulation was found.

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

4. Discussion

In the field of allergy research, identification of early risk predictors and prevention strategies are currently two of the chief aims. To tackle these challenges conclusively, we need both to gain more insight into the early processes shaping development of allergic diseases and to improve our understanding of the mechanisms by which probiotic bacteria can promote immune homeostasis. In our work, we contributed to this area of study by comparing the role of Treg and their subpopulations in cord blood of children with higher and lower expected risk of allergy development, as well as describing the effect of exposure to probiotic *E. coli* strain EcO83 on relevant immune populations both *in vivo* and *in vitro*.

Somewhat surprisingly, in cord blood of children of allergic mothers we observed larger total proportion of Treg in $CD4^+$ T cells. Nevertheless, this group had fewer $Helios^-$ induced Treg and more $Helios^+$ Treg in cord blood than children of healthy mothers. In addition, smaller proportions of PD-1^+ and IL-10^+ Treg cells were present in cord blood of high-risk children, with similar trends noticeable for CTLA-4^+ and TGF-β^+ Treg. These observations are consistent with the hypothesis that impaired or immature functional capacity of Treg in the high-risk group might contribute to allergy development; the increase of total Treg in CD4+ T cell population might then result from compensatory upregulation in an attempt to correct the deficiency. Induced Treg arise in peripheral lymphoid tissues after exposure to environmental factors and are crucial in allergy control and maintenance of tolerance towards harmless antigens. Higher proportion of iTreg may result from more efficient induction of these cells and thus reflect more matured immune system of the low-risk children at birth, contributing to control of allergy development.

Analysing a different cohort of older children, we were able to compare the actual allergy development with subpopulation proportions and functional properties of cord blood Treg originally described by our group in 2012 (Hrdý et al., 2012b). Main findings include higher proportion of IL-10$^+$ Treg in healthy children of healthy mothers and lower TGF-β in allergic children of allergic mothers, reflecting the role of these cytokines as key immune regulators responsible for tolerance induction and maintenance and supporting their prognostic value. Importantly, we also observed increased proportion of CD4$^+$CD25$^+$ cells in peripheral blood of healthy children of healthy mothers, but no difference in children of allergic mothers at the age of 6 – 10 years (Černý et al., 2019). As CD4$^+$CD25$^+$ cells include both Treg and activated conventional T cells (Kmieciak et al., 2009), this observation may point to a differential composition or functional role of this cell population between the high-risk and low-risk groups at birth. Children of allergic mothers have been described to be overall more prone to immune activation (Hrdý et al., 2014; Žižka et al., 2007b), thus CD4$^+$CD25$^+$ cells might include higher ratio of activated non-Treg cells to Treg in this group, making the role of these cells in allergy development harder to evaluate. On the other hand, fewer activated Tconv and more Treg among CD4$^+$CD25$^+$ cells could lead to a clearer association of this cell population with better prognosis in the low-risk group.

Interestingly, we also observed higher expression of activation marker CD83 on DC generated from CBMC of children of allergic mothers compared to children of healthy mothers, coupled with lower expression of regulation-associated genes (IDO, IL-10) as well as greater reactivity of these cells to stimulation by EcO83. This suggests that DC of the high-risk neonates are more prone to immune activation and less tolerogenic, a finding consistent with our previous observations of overall higher immune reactivity in children of allergic mothers (Hrdý et al., 2014; Žižka et al., 2007b). This is

in line with the hypothesis that a general insufficiency of immune regulatory mechanisms such as Treg and tolerogenic DC contributes to the increased risk of allergy.

Due to the central position of Treg in immune regulation from the perinatal period onward, various groups have already attempted to elucidate the role of these cells in influencing the risk of allergy development. The results have so far been hardly consistent. While some groups report increased Treg proportions in cord blood of children with higher risk of allergy (Hrdý et al., 2012b; McLoughlin et al., 2012; Strömbeck et al., 2014), similar to the results presented in this thesis, in other studies the opposite trend was observed (Hinz et al., 2012; Meng et al., 2016) or no differences were described (Fu et al., 2013). Various factors can be held responsible for this inconclusiveness. Firstly, as both genetic background and environment combine to determine allergy development, it stands to reason that data gathered within the context of different populations will vary. This is particularly important to bear in mind when comparing studies carried out in urban (McLoughlin et al., 2012) vs. rural or farming environment (Strömbeck et al., 2014): both environmental pollution (Baïz et al., 2011; Hinz et al., 2012) and exposure to microbial agents (Lluis et al., 2014; Schaub et al., 2009) are crucial factors in allergy development and vary wildly between the two environmental types (rural vs. urban).

Another important point to consider is the incongruity in Treg identification among the different studies. While $CD4^{+}CD25^{+/high}CD127^{low}$ $FoxP3^{+}$ phenotype is nowadays generally accepted to define human Treg cells, this was not always the case and heterogeneity stemming from older studies still influences contemporary interpretations. Gating strategy and the choice of markers therefore need to be carefully considered when planning or interpreting studies of Treg (Hrdý et al., 2012b). Considering Treg as $CD4^{+}CD25^{+}$ cells is especially problematic, since CD25 is

upregulated in effector T cells (Kmieciak et al., 2009) and thus such Treg will invariably be contaminated with activated non-Treg cells. In fact, Treg are better identified as $CD25^{high}$ cells, as the very highest CD25 expression (approximately the top 2%) correlates better with the regulatory characteristics than simple $CD25^{+}$ positivity (Roncador et al., 2005). Furthermore, transient FoxP3 upregulation has also been described in activated Tconv (Allan et al., 2007; Kmieciak et al., 2009). While few activated T cells should be expected in cord blood due to the immaturity of the neonate's immune system, we have previously observed more pronounced tendency towards immune activation in cord blood cells of children of allergic mothers (Hrdý et al., 2014; Žižka et al., 2007b). Therefore, cells described as Treg might in fact contain larger proportion of contaminating recently activated non-Treg $CD4^{+}$ T cells in the high-risk group. Such inclusion of activated T cells could provide an alternative explanation for the higher proportion of Treg in cord blood of children of allergic mothers described in this thesis.

Methodical differences among individual studies can also introduce significant discrepancy. Specifically, the choice of clone of monoclonal antibodies as well as appropriate fixation/permeabilization buffers used for FoxP3 staining are of paramount importance. Using different clones can provide different estimates of Treg proportions or identify Treg subpopulations with different effectiveness (Law et al., 2009; Presicce et al., 2010). In our studies, we used clones PCH101 (in combination with human regulatory T cell whole blood staining kit, cat. no. 88–8996-40, manufactured by eBioscience; San Diego, CA, USA) and 3G3 (in combination with TregFlowEx kit, manufactured by Exbio, plc; Vestec, Czech Republic), which in our experimental setting using cord and peripheral blood collected into heparinised tubes provided the clearest staining of FoxP3 (Hrdý et al., 2012b, 2018).

In the last decade, epigenetic control of TSDR locus has been established as a crucial regulator of Treg phenotype, stability and function (Baron et al., 2007; Iizuka-Koga et al., 2017; Morikawa and Sakaguchi, 2014). Demethylation of conserved sequences in *FOXP3* promoter area is necessary for stable, *bona fide* Treg cells; therefore, the analysis of TSDR demethylation is currently being proposed as a useful marker for identification of lineage-determined FoxP3$^+$ Treg. Such approach could remove the uncertainty stemming from Treg contamination by activated Tconv with transient FoxP3 upregulation and thus be used to resolve some of the discrepancies among the conflicting studies.

Among Treg subpopulations, iTreg are expected to be particularly important for oral tolerance and allergy control due to the fact that they arise upon harmless antigen recognition in periphery. So far, no universally reliable and indisputable markers have been proposed for iTreg identification. Expression of Ikaros-family transcription factor Helios has been proposed as a marker of thymus-derived nTreg (Thornton et al., 2010), with peripherally generated iTreg being a Helios$^-$ fraction of FoxP3$^+$ Treg. The suitability of Helios as an exclusive and indispensable marker of nTreg has been questioned by studies in mice (Gottschalk et al., 2012; Szurek et al., 2015) as well as in humans (Akimova et al., 2011; Himmel et al., 2013), though the issue is yet to be resolved (Thornton and Shevach, 2019). Alternative explanations of Helios biological role have been proposed, including its upregulation in T cells upon activation, similarly to FoxP3 (Akimova et al., 2011). In such case, the observed higher proportion of Helios$^+$ Treg in the high-risk group might reflect generally higher tendency toward immune activation and inflammation, including allergy. This would be consistent with our observations of higher reactivity in cord blood of children of allergic mothers, supporting our hypothesis of impaired regulatory capacity in this high-risk group. An alternative role has been

proposed for Helios, namely involvement in Treg development and acquisition of stable regulatory phenotype (Kim et al., 2015). Thus, upregulation of Helios might in fact be a sign of the compensatory expansion of Treg in an attempt to make up for their insufficient regulatory function. Another marker potentially proposed as specific for nTreg is neuropilin 1 (Singh et al., 2015), a surface molecule which has likewise been implicated in Treg stability maintenance (Chen et al., 2019; Delgoffe et al., 2013) and as such may play similar role as Helios. Recently, blockage of neuropilin 1 by antagonistic monoclonal antibody was shown to inhibit suppressive function of intratumoral Treg, highlighting its importance in Treg function (Jung et al., 2019). In our studies, we observed higher MFI of neuropilin in Helios$^+$ cells than Helios$^-$, indicating common regulation or functional role of the two markers. However, most studies regarding neuropilin 1 in Treg have been performed in mice (Chen et al., 2019; Singh et al., 2015; Weiss et al., 2012) or in the context of lymph nodes (Battaglia et al., 2008), so the suitability of this marker for study of circulatory Treg in humans needs to be confirmed. A possible method of distinguishing nTreg and iTreg consists in determining their TCR specificity, as this has been described to differ substantially between the two subpopulations (Lord et al., 2015); however, this method would be far too challenging for convenient use in routine prediction in human patients. Nevertheless, results obtained from studies determining TCR specificity of Helios$^+$ and Helios$^-$ cells give valuable insight into the differences of the subpopulations of FoxP3$^+$ Treg and could decisively resolve the validity of using this transcription factor as a marker of nTreg (Thornton et al., 2019).

Considering all the issues mentioned above, it follows that analysis of characteristics associated with Treg function will give substantially more reliable information than simple enumeration of these cells or their subpopulations. Treg function

can be assessed indirectly by estimating presence of relevant surface markers with immunoregulatory functions such as CTLA-4 and PD-1 or regulatory cytokines such as IL-10, IL-35 and TGF-β, or directly using assays based on coculture with CFSE-stained or radioactive ^{3}H-thymidine labelled target cells.

CTLA-4 and PD-1, together dubbed the immune checkpoints, are the most important co-inhibitor molecules involved in Treg function (Chen, 2004) and play an indispensable role in contact-dependent suppression mediated by Treg, as evidenced by various autoimmune and lymphoproliferative syndromes associated with deficiency of these molecules (Cepika et al., 2018). CTLA-4 has been implicated in food allergy (Kumar et al., 2013), as well as in regulating the Th1/Th2 crosstalk in allergy and asthma (Munthe-Kaas et al., 2004). PD-1 has been shown to control asthma in animal models (McAlees et al., 2015; McGee et al., 2010). The importance of the immune checkpoints for immune control is evidenced by the clinical success of "checkpoint blockade", i.e. therapeutic blocking antibodies against these molecules, in therapy of certain types of tumors (Ito et al., 2015); just as importantly, severe immune-mediated adverse effects have been observed upon use of checkpoint blockade therapy (Michot et al., 2016). Tendency toward lower expression of CTLA-4 and PD-1 in cord blood Treg of children of allergic mothers further points toward the lower functional capacity of these cells.

IL-10 and TGF-β are the chief immunoregulatory cytokines, and their importance for allergy control is universally accepted (Akdis et al., 2011). Importantly, IL-10 production by Treg and FoxP3^{-} CD4^{+} Tr1 cells is indispensable for the success of SIT, the sole causal therapy of allergy (Akdis and Akdis, 2015; Gonzalez et al., 2017), highlighting IL-10 as major suppressor of unwanted reactivity. TGF-β is responsible for maintenance of mucosal homeostasis and generation of iTreg, and thus plays a key part

in oral tolerance (Chen et al., 2003; Palomares et al., 2012). The lower production of IL-10 and TGF-β by Treg from the high-risk children which we have observed in the current study are likely an important factor for allergy risk, in line with the accepted consensus (Palomares et al., 2014).

Decrease in intracellular and surface function-associated markers likely reflects impaired Treg functional capacity. To decisively confirm this, however, direct functional assays based on Treg coculture with labelled target cells need to be performed. So far, directly measured decrease of Treg suppressive function has been described e.g. in children of allergic mothers as opposed to children of healthy mothers (Meng et al., 2016) or in non-farming mothers compared to farming mothers (Yu et al., 2018). In the thesis, we include some preliminary results hinting at impaired suppression of CFSE-stained Tconv by Treg isolated from CBMC of children of allergic mothers (Černý et al., 2018), although further studies need to be performed to conclusively confirm this phenomenon.

Taken together, our observations (decreased expression of PD-1, lower production of IL-10, impaired suppressive ability estimated by a coculture-based assay) unveil compromised Treg function in children of allergic mothers. This defect in suppressive activity of Treg likely explains our previous observations of exaggerated immune reactivity in the high-risk group – increased CBMC proliferation upon polyclonal stimulation (Žižka et al., 2007b) and higher DC reactivity to stimulation (Hrdý et al., 2014; Súkeníková et al., 2017) – and may be a major cause of the higher risk of allergy development in this group.

A particularly important approach also consists in correlating the putative predictors with actual allergy development at a later age (Björkander et al., 2019; Prescott et al., 2003). We performed a follow-up study on a cohort described previously (Hrdý et

al., 2012b) to assess the usefulness of Treg for allergy prediction. Unfortunately, the follow-up was complicated by significant dropout, which reduced statistical power and limited our ability to conclusively confirm or deny the predictive potential of Treg proportions. Production of IL-10 and TGF-β proved more robust, further supporting the notion that functional properties are more strongly associated with future allergy development and therefore more suitable to be considered for predictive role. We also noted that maintaining sufficient cohort sizes is of supreme importance for comparability of experiments, as we were able to confirm most but not all observations from the original study when we reanalysed the data according to maternal allergy status in the limited selection we had available in the follow-up.

Preventative approaches based on selectively influencing microbiota in order to modulate host immunity have been at the forefront of allergy research for decades. Supplementation with selected strains of probiotic bacteria in particular represents a promising possibility of easy intervention, but much still remains to be elucidated before the final verdict regarding the optimal strains and mode of such supplementation can be reached. In our study, we describe the effects of early postnatal administration of probiotic *E. coli* strain O83:K24:H31 in a prospectively followed cohort of colonised children of allergic mothers, compared with non-supplemented children of allergic mothers and non-supplemented children of healthy mothers. The children have been observed at selected time points of life, with the current report describing allergy status and relevant immune characteristics at the age of 8 years. Consistently with our previous reports (Hrdý et al., 2016; Lodinová-Žádníková et al., 2010), in the supplemented group of high-risk children we observed lower incidence of allergic diseases, comparable with the incidence in the low-risk group. This was likely at least in part due to the effect on Treg: colonised children had more induced Treg than both other groups, an effect driven by a particularly high

number of these cells in healthy as opposed to allergic colonised children. In addition, supplementation of high-risk children with EcO83 increased the production of IL-10, evaluated both as intracellular presence of IL-10 in Treg and FoxP3$^-$ CD4$^+$ cells and as levels in plasma at the age of eight years. This was evident especially in those members of the group who did not develop allergy, and the cytokine production reached the levels seen in children belonging to the low-risk group, with an equally strong upregulation observed for IFN-γ. Thus, EcO83 intervention effectively normalised the production of the main regulatory cytokine IL-10 and IFN-γ, a chief effector in Th1 response, which is likewise seen as important regulator in the context of Th2-mediated allergy. On the other hand, no effect of colonisation was seen on production of IL-4, as both supplemented and non-supplemented children of allergic mothers alike had higher IL-4 production than children of healthy mothers. Therefore, the decrease in allergy incidence likely depends on Th1- and Treg-mediated suppression of excessive Th2 responses rather than preventing Th2 induction in the first place.

We also performed studies to determine the immediate effect EcO83 can have on cells isolated from cord blood, which conveniently reflect the state of neonatal immune system during the first days after birth when supplementation with EcO83 occurs. In one study, we observed increased expression of IL-10 and IFN-γ in CBMC, both on mRNA and on protein level, demonstrating the ability of our probiotic strain to promote Treg and Th1 response in the context of perinatal, immature immune system. Furthermore, we tested the effect of EcO83 stimulation on moDC generated *in vitro* from CBMC in order to establish if promoting effective antigen presentation needed for T cell subpopulation rebalancing in the context of novel stimuli after birth might play a role in EcO83 probiotic effect. EcO83 was able to induce upregulation of CD83, gene expression of IL-10 and IDO as well as release of IL-10, IL-6 and TNF-α from the DC. This evidences a complex

stimulatory effect of EcO83 on model DC. Importantly, DC generated from CBMC isolated from children of healthy mothers upregulated IL-10 production to a significantly greater degree, implying higher tolerogenic capacity in the low-risk group. This is supported by the fact that EcO83 stimulated DC of the low-risk group, but not of the high-risk group, were able to induce more IL-10^+ $CD4^+$ T cells upon coculture, illustrating the more robust immune regulation present in the low-risk children.

Probiotic intervention for allergy prevention is a highly complicated concept, and numerous aspects need to be taken into account if it is to be considered. Firstly, biological effects of probiotic supplementation are highly strain specific, leading to different outcomes via different routes of function. In our study, we demonstrate that the key effect of EcO83 administration in the context of allergy is likely its ability to promote IL-10 production by Treg and Tr1 as well as Th1 response. Induction of Treg by various probiotic bacteria, including *Clostridia*, *Bifidobacteria* and *Lactobacilli* strains, has been described by many studies performed in mice (Atarashi et al., 2013; Kim et al., 2014; Kwon et al., 2010) as well as studies using human cells (Eslami et al., 2016; Qiu et al., 2013; Smits et al., 2005). Furthermore, the effect of EcO83 is apparently mediated through the priming of neonatal DC toward Treg induction, similar to the reports of other groups (Niers et al., 2007; Smits et al., 2005).

Since probiotic are by definition live bacteria, an important aspect of any probiotic strain is its safety for administration, particularly when considering perinatal treatment. Since EcO83 does not express potential major factors of virulence such as plasmids (Lodinová-Žádníková et al., 1991b) or enterotoxin (Lodinová-Žádníková et al., 1998) and possesses sufficient sensitivity to a broad variety of antibiotics, it has been deemed safe enough for registering as a clinical preparation in the Czech Republic (Lodinová-

Žádníková et al., 1998). Sequencing analysis of EcO83-specific genome regions uncovered the presence of several elements traditionally linked to virulence, including a siderophore system, adhesive fimbriae and several metabolic pathways (Hejnová et al., 2006). As it was previously shown that various factors originally considered pathogenic may in fact be present in commensal *E. coli* strains (Dobrindt et al., 2003), the authors concluded that the genetic information observed in EcO83 in fact encodes factors increasing fitness, adaptability and colonising capacity of the probiotic strain (Hejnová et al., 2006). Other probiotic preparations of *E. coli* commonly used in clinical practice include Mutaflor containing the probiotic strain *E. coli* Nissle 1917 (Jacobi and Malfertheiner, 2011) and Symbioflor 2, a mixture of six probiotic *E. coli* strains (Beimfohr, 2016). Collectively, it is possible to conclude that probiotic strains of *E. coli* currently on the market are sufficiently safe for use in human medicine (Wassenaar, 2016). Finally, it needs to be pointed out that particular care needs to be taken in the case of immunocompromised patients, where any exposure to live bacteria may pose a severe danger. Nevertheless, the long account of clinical safety in infants, including pre-term neonates, coupled with the good antibiotic sensitivity described for EcO83 make it particularly suitable for use in newborns (Lodinová-Žádníková et al., 1991a, 2003).

Closely related to safety is the ability of a probiotic strain to maintain long-term colonisation. EcO83 has been shown to colonise full-term neonates for 12 – 16 weeks (Lodinová-Žádníková et al., 1998) and has even been isolated from stool of colonised subjects after several years (Lodinová-Žádníková et al., 2010). This ability to colonise the supplemented individuals for long time periods is due to good epithelial adhesion of EcO83, as well as the selective advantage conferred by the presence of the aforementioned fitness factors (Hejnová et al., 2006). Thanks to its high antibiotic

sensitivity, however, EcO83 can only persist until the colonised individual is treated with antibiotics.

Although the biological effects of EcO83 treatment on the immune system can be seen even after 8 years (Hrdý et al., 2018) and clinical effect has been observed even two decades after supplementation (Lodinová-Žádníková et al., 2003), this is not necessarily due to persistent colonisation. Indeed, many of the important regulatory effects of microbiota are imprinted on the immune system during the early perinatal 'window of opportunity' and carried on as the immune homeostasis stabilises, exerting potentially lifelong impact regardless of whether long-term colonisation took place (Laursen et al., 2017; Torow and Hornef, 2017). Because of the limited duration of this highly susceptible window of opportunity, the timing of probiotic administration represents a crucial consideration. According to recent reports, combined prenatal and postnatal administration of probiotics (i.e. administration both to mothers before birth and to neonates shortly after birth) shows the most promise for allergic disease prevention, particularly in atopic dermatitis (Fiocchi et al., 2015; Zuccotti et al., 2015). Upon combined pre- and postnatal supplementation, the newborn will likely first be exposed to minor amounts of the probiotic bacteria via placenta, with major exposure occurring during passage through birth canal (in spontaneous delivery) or from maternal skin and via breastfeeding in the case of caesarean section (Dominguez-Bello et al., 2010). Subsequently, boosting by direct administration of the probiotic preparation to the newborn will ensure sufficient strength of the beneficial effect during the critical early postnatal period.

Lastly, it is also necessary to consider the application pathway of probiotics. Most often, administration is done orally and therefore the effect will be mediated through gut

microbiota and immune modulation in gut associated lymphoid tissue (GALT), regardless of the expected route of allergen exposure. Oral route of application presents some challenges to the probiotic bacteria, namely passage through the adverse environments of stomach and small intestine; most importantly, however, the introduced probiotic bacteria need to compete with the huge amounts of gut microbiota already established (Libertucci and Young, 2019; Zmora et al., 2018). Under physiological conditions, the colonisation resistance of established adult gut microbiota is an insurmountable obstacle and likely is a major mechanism limiting the "window of opportunity," during which manipulation of microbiota can enact long-term influence on host immunity and homeostasis in general (Gensollen et al., 2016; Torow and Hornef, 2017), to early postnatal period. Currently, alternative routes of probiotic administration for allergy prevention are being explored. Most importantly, various probiotics are considered for intranasal application (Mårtensson et al., 2016). Nasal mucosa is closely affiliated with respiratory immune tissue and represents a potent immune induction site for modulation of both respiratory and systemic immune responses (Pabst, 2015). Recently, it has been shown that intranasal application of EcO83 as well as *E. coli* Nissle 1917 can control and prevent allergic airway inflammation more effectively than oral supplementation (Sarate et al., 2019; Zwicker et al., 2018). An important caveat for potential use in human medicine, however, is that nasal mucosa is in close anatomical and physiological contact with the cranial cavity, and thus any application of live bacteria in this location may carry a risk of potentially severe infectious complications, particularly in subjects with compromised or underdeveloped immunity (e.g. newborns).

The data regarding probiotic use in the context of allergy remain inconclusive and further studies coupled with rigorously performed meta-analyses need to be carried out to determine strains conferring the most benefit. Although so far, most preparations use

single bacterial strains or a combination of several strains of related bacterial species, combination of different microbial entities with complementary metabolic and biological effects may be beneficial. Likewise, conditions such as timing, mode of application and dosage need to be optimised for maximal beneficial effect, with perinatal (combined prenatal and early postnatal) supplementation being proposed by current guidelines (Fiocchi et al., 2015). Therapeutic and adjuvant application of probiotics at a later age has likewise been proposed for allergy treatment, although perinatal modulation is much more likely to have lasting effects, as discussed above. Probiotic treatment of older infants, children and adults has been described in peanut allergen SIT (Loh and Tang, 2018; Tang et al., 2015), allergic rhinitis (Güvenç et al., 2016) and atopic dermatitis (Drago et al., 2012).

Finally, optimal choice of probiotic strains and particulars of supplementation will likely depend heavily on individual genetic, lifestyle and immunological factors, including the individual patient's pattern of allergic disorders. So far, the most persuasive arguments have been made for probiotic use in atopic dermatitis prevention (Fiocchi et al., 2015; Zuccotti et al., 2015), with the benefit of probiotics in other forms of allergy being hotly debated (Cuello-Garcia et al., 2015; Szajewska and Horvath, 2018).

In this thesis, we described a broadly preventive effect of EcO83 in allergy lasting up to the age of eight years (Hrdý et al., 2018). We propose that this effect is likely at least in part mediated via inducing maturation of DC, which leads to increased support of Treg and Th1 responses and a long-term increase in IL-10 and IFN-γ production in the treated neonates, helping the immune system to rebalance away from prenatal dominance of Th2 response. Supplementation with EcO83 thus seems like a useful approach for allergy prevention in the groups with higher risk of allergy.

5. Conclusions

5.1. We analysed population proportions and functionally relevant characteristics of Treg in cord blood of children of allergic mothers in an attempt to describe processes involved in early stages of allergy development and evaluate parameters that could be used for improving the limited options for early prediction of increased risk of allergy development.

a. Differences in the proportional composition of Treg pool have been observed between the high-risk and low-risk group, possibly signifying dysregulation in the high-risk children. Children of allergic mothers (with higher risk of allergy development) had larger proportion of total $CD25^{high}CD127^{low}$ $FoxP3^{+}$ Treg population among $CD4^{+}$ T cells. They also had larger $FoxP3^{+}Helios^{+}$ nTreg fraction, while the percentage of $FoxP3^{+}Helios^{-}$ iTreg among total Treg was lower than iTreg proportion among Treg of children of healthy mothers.

b. Analysis of cord blood parameters revealed signs of impaired regulatory capacity in the high-risk group at birth. Children of allergic mothers exhibit lower percentage of PD-1+ and IL-10+ cells among Treg, lower cord blood plasma levels of IL-10 and TGF-β, and impaired Treg suppressive function. Of possible relevance in this context is also our observation of higher expression of activation marker CD83 on moDC derived from mononuclear fraction of CBMC of children of allergic mothers, as well as the higher ability of these cells to induce T cell polarization to Th1, Th2 or Th17 branches upon coculture. This higher immune reactivity is in line with the previous observations by our group.

c. We re-evaluated cord blood Treg characteristics in high-risk and low-risk children with respect to presence or absence of allergic disease during childhood (6-10 years), observing differences in cell population proportions and regulatory cytokines between healthy and allergic children. Proportion of CD4+CD25+ T cells was higher in healthy children of healthy mothers compared with allergic children of healthy mothers, but no difference was observed between healthy and allergic children of allergic mothers, possibly hinting at different composition or role of this population between the high-risk and low-risk group. Furthermore, healthy children of healthy mothers showed increased IL-10 and TGF-β production and allergic children of allergic mothers had decreased TGF-β at birth.

Our data supports the hypothesis that children of allergic mothers (with higher risk of allergy) have delayed functional maturation of regulatory T cells in the perinatal period, reflected in decreased presence of markers associated with function, lower cord blood plasma levels of IL-10 and TGF-β and lower numbers of induced Treg. Such impaired regulatory capacity may be linked with the higher immune reactivity seen in moDC derived from CBMC of children of allergic mothers, as well as our previously published observations of higher immune reactivity of cord blood cells of children at high-risk of allergy development.

Furthermore, we conclude that analysis of function-associated markers of Treg and direct analyses of Treg function will be more useful for improving our understanding of early phases of allergy development than simple analysis of Treg proportion in circulatory $CD4^+$ T cells. Lastly, according to the results obtained upon re-analysis of data in the context of allergy development in the subjects,

combining evaluation of functional analyses of Treg with estimation of risk of allergy according to parental allergy status might be necessary for Treg to be useful for allergy prediction. The complexities of homeostasis and identification of the cells unfortunately make simple enumeration poorly informative. In addition to that, we have to account for substantial individual variability leading to difficulty to draw any simple unambiguous conclusion based on one study with limited number of participants. Further studies in this direction will be needed to resolve the issues with greater degree of certainty.

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

a. Our data revealed reduction in allergy incidence and differences in Treg characteristics and immune regulation in children of allergic mothers supplemented with EcO83 confirming previously published observations. Although no effect of colonisation on sensitization and specific IgE levels was seen, we observed decreased incidence of allergy. Furthermore, colonised children had substantially more $FoxP3^{+}Helios^{-}$ iTreg at the age of 8 years, particularly healthy colonised children. As iTreg generation occurs mostly in mucosal peripheral tissues, known to be extensively modulated by contact with microbiota, and iTreg include cells with TCR specific for environmental antigens including allergens, colonisation with EcO83 may favour more robust control of unwarranted reactivity against exogenous antigens, including Th2-mediated allergy.

b. Although colonisation with EcO83 did not influence IL-4 production, production of IL-10 and IFN-γ was substantially increased in

supplemented children of allergic mothers as opposed to non-supplemented children of allergic mothers at the age of 8 years. Indeed, concentration of IL-10 and IFN-γ reached levels found in the low-risk children, effectively normalising production of these cytokines. As IL-10 and IFN-γ are important cytokines involved in control of Th2 response, this increase in production is likely key to lower allergy incidence observed in the colonised children. Furthermore, we showed that IL-10 production was increased both in $CD25^{high}CD127^{low}$ Treg and non-Treg $CD4^+$ T cells (possibly including Tr1 cells) in the colonised group.

c. Stimulation of CBMC with EcO83 was able to strongly induce expression of IL-10 and IFN-γ in CBMC, detectable both on the level of mRNA and as protein in culture supernatants. EcO83 stimulation also led to upregulation of CD83 on moDC derived from CBMC and induced expression of regulatory effector molecules IDO and IL-10 in the moDC. Furthermore, moDC derived from CBMC of children of healthy mothers stimulated with EcO83 upregulated IL-10 expression much more strongly and were able to induce production of IL-10 in $CD4^+$ T cells.

Taken together, our data suggest that early postnatal EcO83 supplementation induces long-term regulatory effects in the high-risk children, leading to a decrease in allergy incidence detectable at the age of 8 years. Increase of iTreg population was seen, as well as normalisation of production of IL-10 and IFN-γ, likely major factors contributing to the clinical effect observed in this as well as previous studies. Increased production of IL-10 and IFN-γ by CBMC and increased maturation of moDC coupled with IDO and IL-10 upregulation were identified as possible early mechanisms which mediate these effects. Increased IL-10

and IFN-γ together with more appropriately polarizing antigen presentation by stimulated DC may help set up immunoregulatory conditions in the infant necessary for rebalancing immune polarization away from the prenatal Th2 bias. Nonetheless, more detailed studies need to be performed to conclusively determine the entire mechanistical basis of EcO83 function.

In conclusion, in the thesis we were able to address the questions posed in the aims we set for ourselves. Further studies will be needed to conclusively resolve the exact role of total Treg, iTreg and nTreg population dynamics in early regulation and allergy development in high-risk and low-risk children. Markers reflecting regulatory function of Treg show more promise than population proportions for use in allergy prediction, but analysis of the proposed Treg characteristics in the context of allergy development during childhood needs to be performed on a larger cohort of subjects to confirm this with higher certainty. Early postnatal supplementation with EcO83 can reduce incidence of allergy in children up to eight years old, with regulatory effect likely dependent on DC maturation and subsequent shift in regulation in favour of Treg and Th1 branches.

6. References

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Attachments

Attachment 1: Distinct characteristics of Tregs of newborns of healthy and allergic mothers.

Černý, V., Hrdý, J., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., and Prokešová, L.

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IF_{2018} = 2.776

Attachment 2: Value of cord blood Treg population properties and function-associated characteristics for predicting allergy development in childhood.

Černý, V., Petrásková, P., Novotná, O., Boráková, K., Prokešová, L., Kolářová, L., and Hrdý, J.

Cent. Eur. J. Immunol. (2019), accepted for publication.

IF_{2018} = 1.455

Attachment 3: Decreased allergy incidence in children supplemented with *E. coli* O83:K24:H31 and its possible modes of action.

Hrdý, J., Vlasáková, K., Černý, V., Súkeníková, L., Novotná, O., Petrásková, P., Boráková, K., Lodinová-Žádníková, R., Kolářová, L., and Prokešová, L.

Eur. J. Immunol. (2018) 48, 2015–2030. https://doi.org/10.1002/eji.201847636

IF_{2018} = 4.695

Attachment 4: Different capacity of *in vitro* generated myeloid dendritic cells of newborns of healthy and allergic mothers to respond to probiotic strain *E. coli* O83:K24:H31.

Súkeníková, L., Černý, V., Novotná, O., Petrásková, P., Boráková, K., Kolářová, L., Prokešová, L., and Hrdý, J.

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PLOS ONE

RESEARCH ARTICLE

Distinct characteristics of Tregs of newborns of healthy and allergic mothers

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Abstract

Allergic diseases represent a major issue in clinical and experimental immunology due to their high and increasing incidence worldwide. Allergy status of the mother remains the best predictor of an individual's increased risk of allergy development. Dysregulation of the balance between different branches of immune response, chiefly excessive polarization towards Th2, is the underlying cause of allergic diseases. Regulatory T cells (Tregs) play a pivotal role in the timely establishment of physiological immune polarization and are crucial for control of allergy. In our study we used flow cytometry to assess Tregs in cord blood of newborns of healthy (n = 121) and allergic (n = 108) mothers. We observed a higher percentage of Tregs ($CD4^+CD25^+CD127^{low}FoxP3^+$) in cord blood of children of allergic mothers. However, the percentage of cells expressing extracellular (PD-1, CTLA-4, GITR) and intracellular (IL-10, TGF-β) markers of function was lower (significantly for PD-1 and IL-10) within Tregs of these children. Furthermore, $Helios^-$ induced Tregs in the cord blood of children of allergic mothers were decreased. These results were supported by a decrease in plasma levels of IL-10 and TGF-β in cord blood of newborns of allergic mothers, implying lower tolerogenic capacity on the systemic level. Taken together, these findings reflect deficient function of Tregs in the group with higher risk of allergy development. This may be caused by a lower maturation status of the immune system, specifically Tregs, at birth. Such immaturity may represent an important mechanism involved in the increased risk of allergy in children of allergic mothers.

Introduction

Allergic diseases belong to the most common and important medical conditions. Despite intensive research, the early events leading to the development of allergy in predisposed infants remain to be conclusively elucidated. The hygiene hypothesis is a major theory, postulating that lower exposure to microbes typical for the more developed countries may delay the development of the immune system and alter the balance among immune response branches (e.g. Th1, Th2, Treg, Th17), facilitating allergy. Prenatally, T helper type 2 (Th2) response is

favoured to prevent undesirable reactivity towards maternal antigenic determinants foreign to the foetus [1]. Beginning after birth, a new physiological balance needs to be established upon contact with external environment, chiefly upon exposure to microbial stimuli. Persistence of the Th2 bias predisposes towards allergy development; Th1 and Th17 responses play important roles in anti-infectious immunity, but under certain conditions can lead to the development of autoimmune diseases. Regulatory T cells (Tregs) are the master T lymphocyte population overseeing this fine tuning and controlling potential development of pathological reactions including allergy-associated Th2 responses [2].

Human Tregs represent a population of $CD4^+$ T cells characterised by a typical set of cell surface markers ($CD4^+CD25^+CD127^{low}$) and intracellular markers ($FoxP3^+$, $Helios^+$). FoxP3 can be considered as a master transcription factor of Tregs responsible for their regulatory functions, and MFI of FoxP3 has been shown to correlate with Treg suppressive function [3]. Another hallmark of Tregs is a high dependence on IL-2. Tregs play an indispensable role in maintaining immunological reactions within physiological proportions, as evidenced by the severe autoimmune phenotype seen in FoxP3 deficient patients [4]. They are also essential for controlling allergy [2] and form the basis of allergen-specific immunotherapy [5]. Tregs exert their immunosuppressive function in a number of biologically significant ways, both through direct cell to cell contact (e.g. CTLA-4, LAG-3, PD-1, FasL) and remotely through the secretion of immunoregulatory cytokines (IL-10, TGF-β, IL-35) [6]. Measurement of these and other related markers is therefore routinely used to indirectly assess Treg´s suppressive capabilities and functional status.

Numerous Treg subpopulations differing in the details of their localization and function as well as expression of markers have been described, making proper identification strategies vital for data reproducibility [7]. Conventional $CD4^+CD25^+CD127^{low}FoxP3^+$ Tregs can be divided into two broad groups with distinct origin and function: natural Tregs (nTregs) arise in thymus, possess TCR specificity mainly towards autoantigens and have been described to express the Ikaros family transcription factor Helios [8–10] and occasionally also neuropilin-1 [11], while $Helios^-$ induced Tregs (iTregs) take on Treg phenotype upon interaction with environmental or self- antigens within the context of an immunosuppressive cytokine milieu (mainly TGF-β) on the periphery [12].

Many groups have so far tried to identify early prognostic markers which could be used to predict increased risk of allergy development, however with little conclusiveness. Maternal allergy remains the strongest and most reliable universally accepted established risk factor [13]. Among parameters studied in the cord blood were immunoglobulin (Ig)E levels [14,15], Th1 and Th2 cytokine proportions in plasma [16,17] as well as responsiveness of cord blood cells to various modes of stimulation [13,18,19]. Several groups including our own have reported correlation between proportional and functional characteristics of Tregs in cord blood and allergy status of the mother [7] or early allergy disorders in infants [20,21]. Our own observations revealed a lower presence of functional markers (IL-10, TGF-β, MFI of FoxP3) in Tregs from cord blood of children of allergic mothers as well as increased size of the population, possibly due to a compensatory upregulation caused by the dysfunctional nature of these cells [7]. We postulated that lower overall perinatal maturity of the immune system in children of allergic mothers may be the underlying cause of this. To further elucidate the relationships among functional phenotype of Tregs, their maturation status and risk of allergy development, we analysed Tregs and compared their populations as well as chosen surface (CTLA-4, PD-1, GITR) and intracellular (IL-10, TGF-β) markers of Tregs in cord blood of new-borns of allergic mothers (children with a relatively high risk of allergy development) and of healthy mothers (low-risk children). Importantly, we also compared the proportions of $Helios^+$ Tregs (putative nTregs) and $Helios^-$ Tregs (putative iTregs), as we postulated that since iTreg arise

mostly due to postnatal exposure to harmless exogenous antigens, their decreased number may reflect a lower overall maturation status of Tregs even on the level of cord blood.

Materials and methods

Subjects and sample collection

Healthy (n = 121) and allergic (n = 108) mothers with physiological pregnancies who delivered children vaginally at full term in the Institute for the Care of the Mother and Child in Prague, Czech Republic, were included for the study. There was no difference in pregnancy length between the two groups. Allergy status of the mother was determined based on clinical manifestation of allergy persisting for at least 24 months; allergy against respiratory and/or food allergens manifested by various individual combinations of symptoms (e.g. hay fever, conjunctivitis, eczema, bronchitis, asthma etc.), monitoring by an allergist, positive skin prick tests or positive specific IgE and anti-allergic treatment before pregnancy. The study was approved by the Ethical Committee of the Institute for the Care of Mother and Child (Prague, Czech Republic) and was carried out with a signed written informed consent of the mothers.

Cord blood (CB) samples (approx. 5 ml) were collected into sterile heparinized tubes immediately after birth via umbilical vein punction, as described previously [17]. CB plasma was obtained for cytokine and IgE detection. Mononuclear cell fraction was obtained from whole cord blood by density gradient centrifugation (Histopaque-1077; Sigma-Aldrich, St. Louis, MO, USA) for culture assays.

Flow cytometry

Whole blood samples were prepared and stained for flow cytometry as described in our previous studies [7]. Briefly, samples of whole blood were stained with the following antibodies against Treg surface markers: CD4 fluorescein isothiocyanate (FITC; clone RPA-T4; Becton Dickinson, Franklin Lakes, NJ, USA), CD25 peridinin chlorophyll-cyanin 5.5 (PerCP-Cy5.5; clone MEM-181; Exbio pls., Vestec, Czech Republic), and CD127 phycoerythrin-cyanin 7 (PE-Cy7; clone A019D5; BioLegend, San Diego, CA, USA). Staining and sample preparation were performed according to manufacturer's instructions using human regulatory T cell whole blood staining kit (eBioscience, San Diego, CA, USA). After fixation and permeabilization, the samples were stained with antibodies against Treg intracellular markers: FoxP3 phycoerythrin (PE; clone PCH101; Thermo Fisher Scientific, Waltham, MA, USA) and Helios allophycocyanin (APC; clone 22F6; BioLegend). In some experiments, non-stimulated whole blood samples were stained for the following surface markers associated with Treg function: CTLA-4 APC (clone L3D10), PD-1 allophycocyanin- cyanin 7 (APC-Cy7; clone EH12.2H7), GITR PE (clone 621), all from BioLegend. Non-stimulated whole blood samples treated with BD GolgiPlug (Becton Dickinson) for 6 hours were permeabilized after surface staining for Tregs and stained for intracellular expression of regulatory cytokines IL-10 PE (clone JES3-19F1) and TGF-β PerCP-Cy5.5 (clone BG/hLAP), both from BioLegend. Gating strategy used for estimation of Treg was described in greater detail previously [22]. Briefly, lymphocyte gate was set based on forward-scatter (FCS) and side-scatter (SSC) characteristics with doublets exclusion (FCS-A × FCS-H; S1A and S1B Fig). Tregs were gated from the lymphocyte gate as $CD4^{+}CD25^{+}CD127^{low}$ cells (S1C and S1D Fig) for analyses of Treg surface functional markers (FMO shown in S1E, S1G and S1I Fig, representative dot plots shown in F, H and J for GITR, PD-1 and CTLA-4, respectively), iTreg/nTreg ratios (unstained control shown in S1K Fig, representative dot plot of FoxP3 and Helios expression shown in L) and intracellular cytokines (FMO shown in S1M and S1O Fig, representative dot plots shown in N and P for IL-10 and TGF-β, respectively).

To confirm the validity of using Helios as a marker of nTregs by comparing its expression with another putative nTreg marker, neuropilin-1 (only several samples were stained), we stained cells with the following antibodies against surface markers: CD4 FITC (clone MEM-241, Exbio), CD25 PE (clone MEM-181, Exbio), neuropilin-1 APC/Fire750 (CD304; clone 12C2, BioLegend), followed by intracellular staining for FoxP3 APC (clone 3G3, Exbio) and Helios PE-Cy7 (clone 22F6, Exbio). TregFlowEx kit (Exbio) was used according to manufacturer's instructions for intracellular staining and preparation of these samples.

CFSE suppression of proliferation assay

A proliferation suppression assay was performed utilising coculture of magnetically isolated CFSE-stained target cells (non-Treg $CD4^+CD25^-CD127^+$ cells) with magnetically isolated Treg ($CD4^+CD25^+CD127^{low}$), as described previously [23,24].

EasySep™ Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (StemCell, Vancouver, BC, Canada) was used to magnetically isolate Tregs and target cells from cord blood mononuclear cells. The target cells were stained with 5 μM CFSE, plated into 24-well plates with or without Tregs and cultivated for 72 hours in RPMI medium (Sigma-Aldrich) supplemented with 10% FTS (Cambrex), gentamycin (Sigma-Aldrich, 40 mg/L) and L-glutamine (Sigma-Aldrich, 2mM). 20 ng of recombinant human IL-2 (PeproTech, Rocky Hill, NJ, USA), 1 μg of purified, functional grade human anti-CD3 (clone OKT3; ThermoFisherScientific) and 1 μg of purified, functional grade human anti-CD28 (clone CD28.2; ThermoFisherScientific) were added per 10^6 target cells to stimulate proliferation. 0.5×10^6 cells in total were seeded in each well. After 72h, cells were stained for CD4 (APC; clone MEM-241; Exbio) and analysed with flow cytometer.

ELISA

The plasma was stored at -20˚C. Levels of IL-10 and TGF-β were quantified by ELISA while specific IgE was measured immunoenzymatically by RISA (Ring-Immuno-Sorbent Assay), as described previously [25].

Data acquisition and statistics

Flow cytometry data were acquired on a BD FACSCanto flow cytometer using BD FACS Diva version 6.1.2 software (Becton Dickinson) and analysed using FlowJo 7·2.2. (TreeStar, Ashland, OR, USA). Results of ELISA and RISA assays were obtained using Genesis software. Statistical and graphical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA, USA). Differences between groups were compared using unpaired Student's t-test in case of data with normal distribution (total Treg, iTreg and nTreg ratios; surface markers of Treg function) and unparametric Mann-Whitney test for the rest of the data (proportions of IL-10+ Tregs and TGF-β+ Tregs, plasma levels of IL-10, TGF-β, sIgE). All results are presented as box plots with medians, 25th, and 75th percentiles as boxes and 10th and 90th percentiles as whiskers.

Results

The immunological characteristics of cord blood of children of allergic mothers (children with high risk of allergy development, n = 108) and healthy mothers (low-risk group, n = 121) were compared. Chiefly, nTreg and iTreg populations as well as total Treg proportion were assessed. Tregs were also tested for their surface functional markers (PD-1, CTLA-4, GITR) and intracellular cytokines (IL-10, TGF-β) to reveal possible differences in functional characteristics.

Such variations could contribute to the differential risk of allergy development in the two groups. Lastly, IL-10 and TGF-β levels in cord blood plasma of children as well as allergen-specific IgE levels in cord blood plasma of children and peripheral blood plasma of their mothers were tested.

IgE

As expected, peripheral blood of allergic mothers was found to entail increased titres of IgE specific for most common food (FX, p = 0.0450) and respiratory (DYNX, p = 0.0015) allergens (Fig 1A). Increased levels of respiratory (DYNX, p = 0.0026) but not food allergen specific IgE were also detected in cord blood of children of allergic mothers (Fig 1B). This could most likely be due to transplacental transport of maternal specific IgE into fetal circulation [26].

Treg population ratios

Using our gating strategy and a combination of surface and intracellular markers, we observed a significantly increased proportion of $CD25^{+}CD127^{low}FoxP3^{+}$ Tregs in $CD4^{+}$ T cells from the cord blood of children of allergic mothers (Fig 2A; p = 0.0361). After adding antibodies against Helios, a transcription factor characteristic for natural but not induced Tregs, we revealed a higher proportion of $FoxP3^{+}Helios^{+}$ nTregs (Fig 2B; p = 0.0149) and a lower proportion of $FoxP3^{+}Helios^{-}$ iTregs (Fig 2C; p = 0.0175) in $CD4^{+}CD25^{+}CD127^{low}$ Tregs of children of allergic mothers. We have tried to stain several samples for neuropilin-1, another putative marker of nTregs, in addition to Helios. Helios positive fraction of $CD25^{high}FoxP3^{+}$ Tregs expressed neuropilin at higher MFI compared with Helios negative cells (S2 Fig). Nevertheless, we also observed numerous $Helios^{+}neuropilin^{-}$ Treg, suggesting different dynamics and/or roles of these markers in the context of human regulatory T cells.

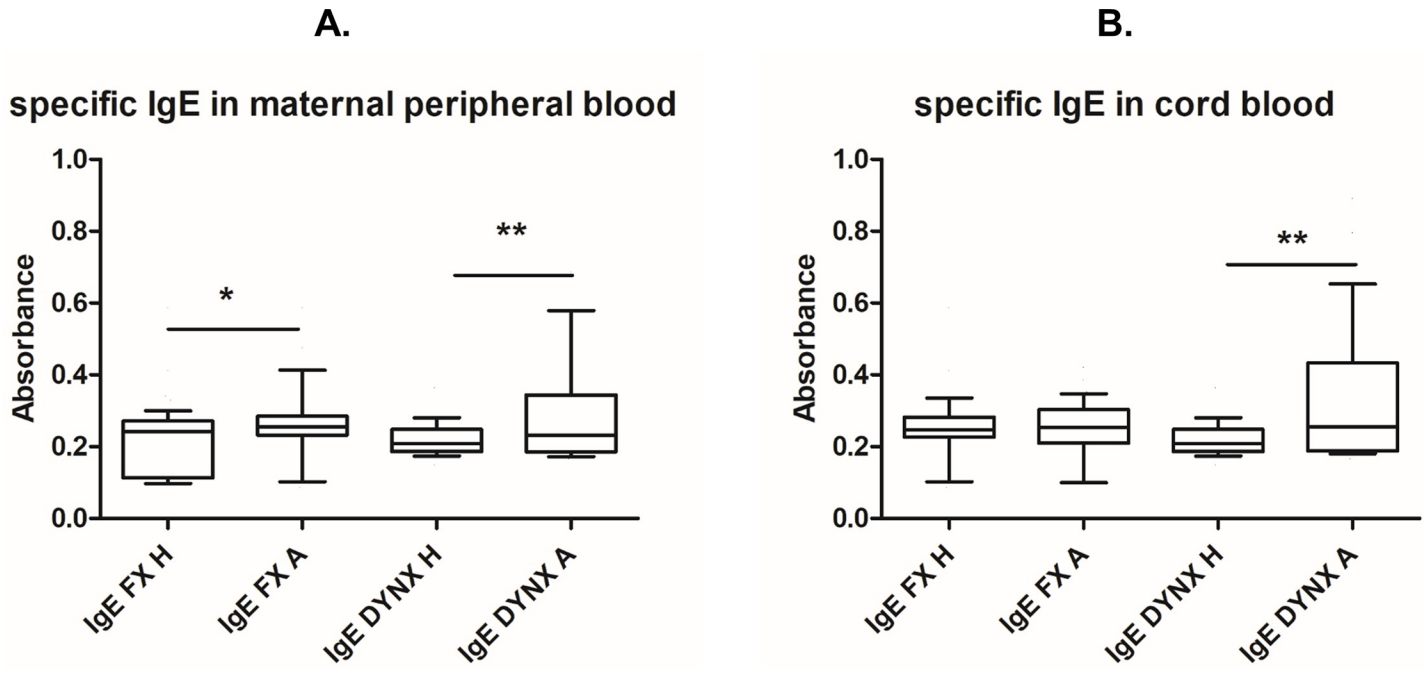

Fig 1. Specific IgE in maternal peripheral blood and cord blood. Samples of maternal peripheral blood and children's cord blood of healthy (H, n = 112) and allergic (A, n = 98) mothers were collected at the time of birth. Levels of IgE specific for common food (FX) and respiratory (DYNX) allergens were determined immunoenzymatically by RISA (Ring-Immuno-Sorbent Assay). (A) Specific IgE levels in maternal peripheral blood plasma. (B) Specific IgE levels in cord blood plasma. p values were calculated using the Mann-Whitney test.

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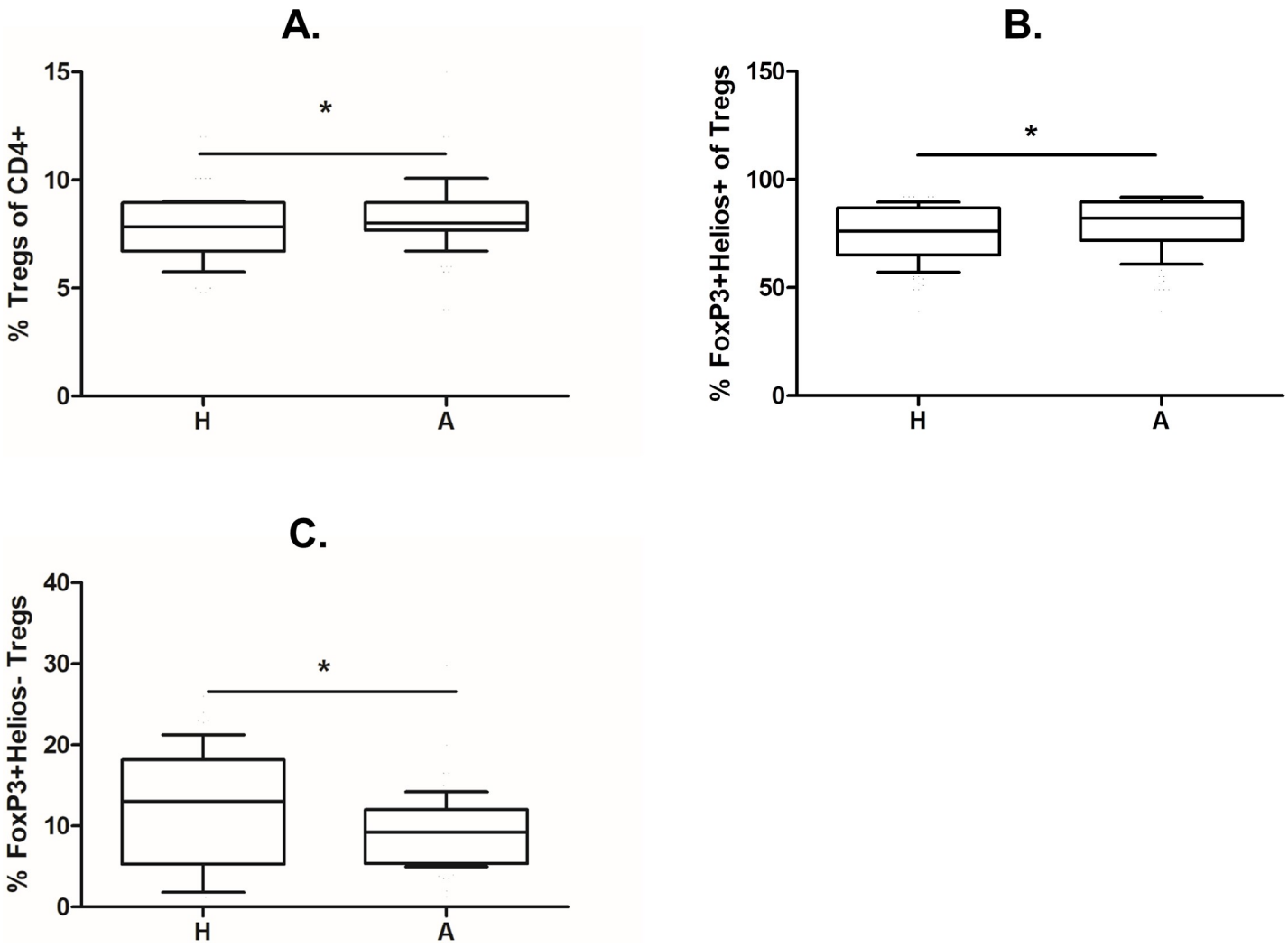

Fig 2. Proportions of total Treg population and nTreg and iTreg subpopulations in cord blood of children of allergic and healthy mothers. Samples of cord blood of children of healthy (H, n = 112) and allergic (A, n = 98) mothers were stained and analysed by flow cytometry. (A) Flow cytometry analysis showing the proportion of $CD25^+CD127^{low}FoxP3^+$ Tregs in the cord blood $CD4^+$ T cell population (p = 0.0361). (B) Flow cytometry analysis showing the proportion of $FoxP3^+Helios^+$ nTregs among $CD4^+CD25^+CD127^{low}$ Tregs in cord blood (p = 0.0149). (C) Flow cytometry analysis showing the proportion of $FoxP3^+Helios^-$ iTregs among $CD4^+CD25^+CD127^{low}$ Tregs in cord blood (p = 0.0175). p values were calculated using unpaired Student's t-test.

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Treg functional markers

To compare the expression of functional parameters, Treg surface markers of function CTLA-4, PD-1 and GITR were stained and evaluated as a percentage of $CD4^+CD25^+CD127^{low}$ Treg cells. A significantly higher proportion of PD-1^+ Tregs was found in cord blood of children of healthy mothers (Fig 3A; p = 0.0382). A similar trend, albeit not significant, can be seen for $CTLA^+$ Tregs (Fig 3B) and to a smaller extent also $GITR^+$ Tregs (Fig 3C).

Furthermore, intracellular presence of regulatory cytokines IL-10 and TGF-β was assessed using intracellular staining. The proportion of IL-10 and TGF-beta positive Tregs was analysed as described previously [22]. A significantly higher percentage of IL-10^+ Tregs was found in cord blood of children of healthy mothers compared with children of allergic mothers (Fig 4A; p = 0.0006). The same trend is discernible for TGF-β, although it is not significant (Fig 4B).

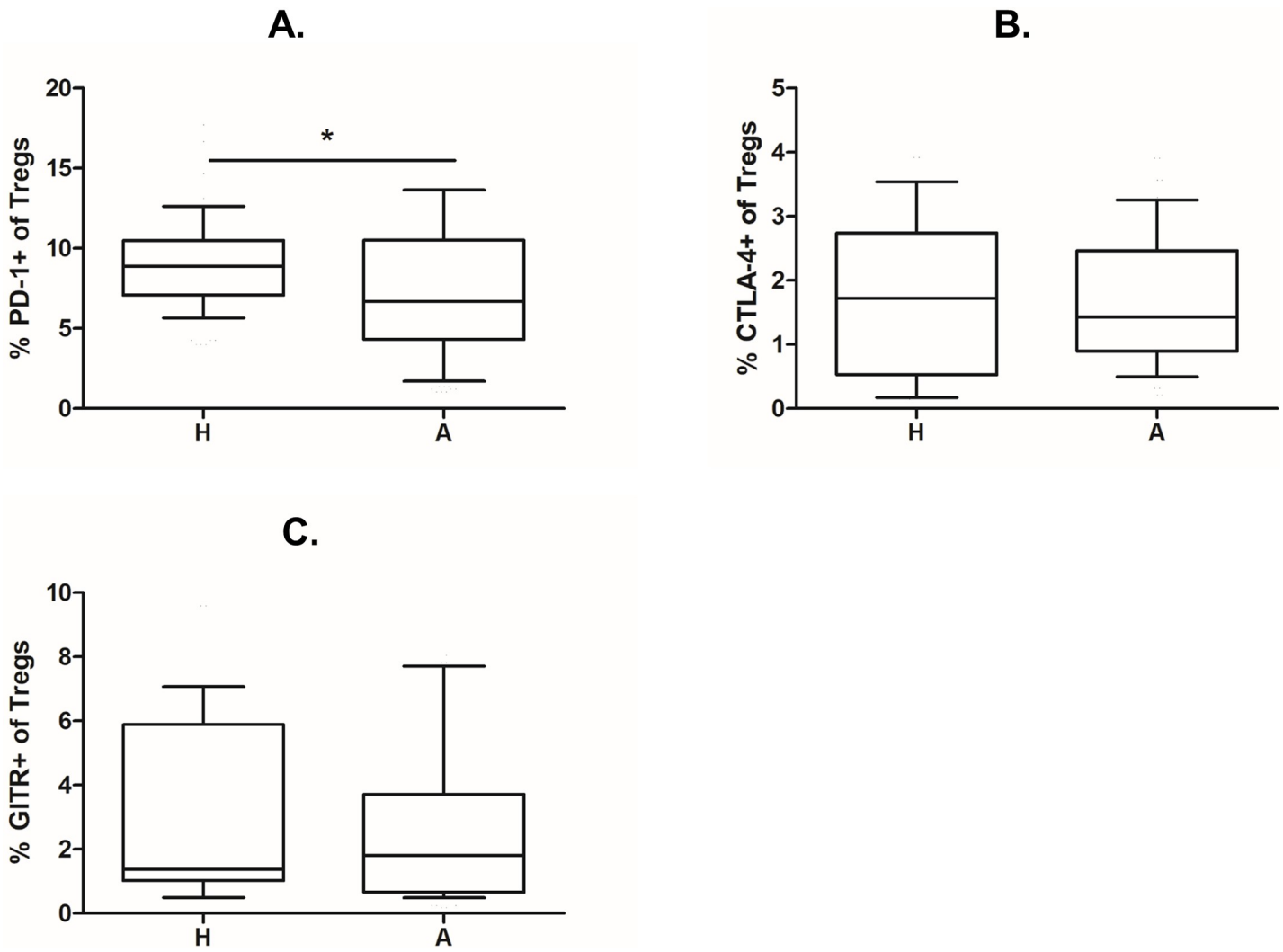

Fig 3. Expression of surface markers of Treg function in Tregs from cord blood of children of allergic and healthy mothers. Samples of cord blood of children of healthy (H, n = 112) and allergic (A, n = 98) mothers were stained and analysed by flow cytometry. (A) Flow cytometry analysis showing the proportion of PD-1^{+} cells among CD4^{+}CD25^{+}CD127^{low} Tregs in cord blood (p = 0.0382). (B) Flow cytometry analysis showing the proportion of CTLA-4^{+} cells among CD4^{+}CD25^{+}CD127^{low} Tregs in cord blood. (C) Flow cytometry analysis showing the proportion of GITR^{+} cells among CD4^{+}CD25^{+}CD127^{low} Tregs in cord blood. p values were calculated using unpaired Student's t-test.

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Treg functional assay

To further assess functional ability of Tregs from cord blood of newborns of allergic and healthy mothers, proliferation suppression assay based on coculture of CFSE-stained target cells (non-Treg CD4^{+}CD25^{-}CD127^{+} cells) with Tregs was performed on several samples (n = 19). Although the number of samples we were able to include was insufficient for proper statistical analysis, we observed a trend of impaired ability of Treg isolated from cord blood to suppress proliferation of target cells in comparison to Treg from peripheral blood of adults (S3C Fig). The suppressive function was notably less effective in Tregs isolated from cord blood of children of allergic mothers (S3B Fig) compared to newborns of healthy mothers (S3A Fig). A summary table with the percentage of proliferating cells and the number of cell divisions (S1 Table) shows extreme individual variability, which may be related to the inherent variability of cord blood in different clinical context of an individual pregnancy. Another

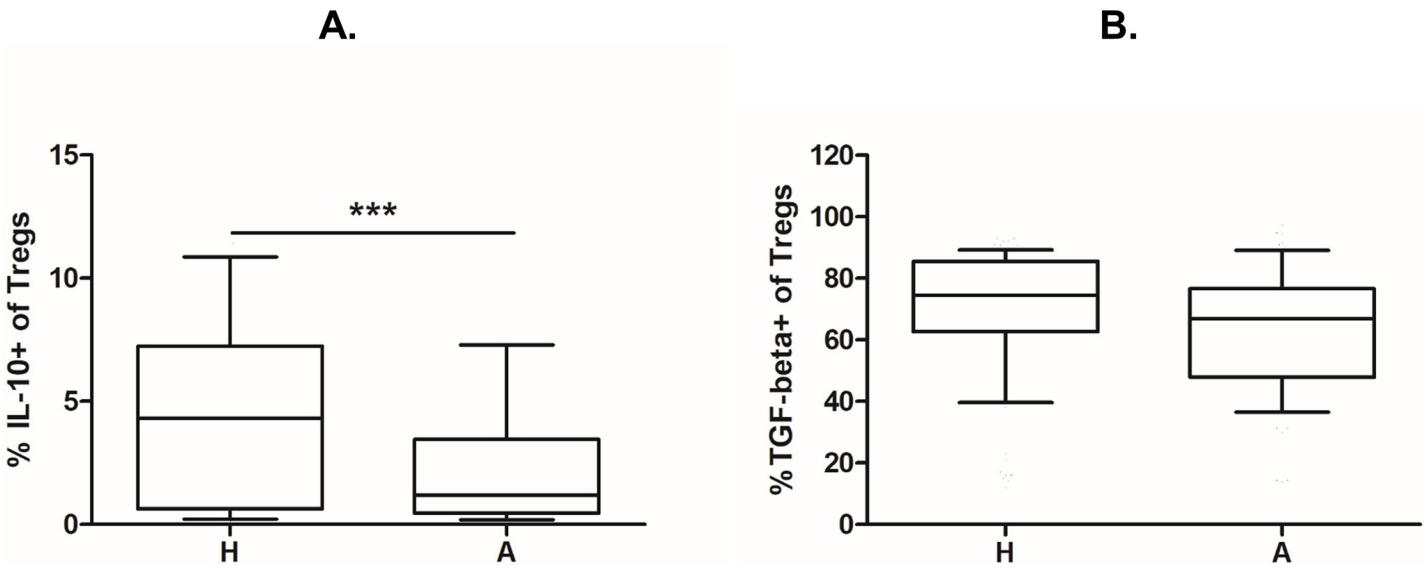

Fig 4. Intracellular expression of regulatory cytokines in Tregs from cord blood of children of allergic and healthy mothers. Samples of cord blood of children of healthy (H, n = 112) and allergic (A, n = 98) mothers were stained and analysed by flow cytometry for intracellular presence of cytokines IL-10 and TGF-β. (A) Flow cytometry analysis showing the proportion of IL-10^{+} cells among CD4^{+}CD25^{+}CD127^{low} Tregs in cord blood (p = 0. 0006). (B) Flow cytometry analysis showing the proportion of TGF-β^{+} cells among CD4^{+}CD25^{+}CD127^{low} Tregs in cord blood. p values were calculated using the Mann-Whitney test.

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factor may be the limited number of samples analysed. Additional studies would be warranted to confirm if distinct patterns of cord blood cells in the data could be traced by correlation with subsequent allergy development.

Regulatory cytokine levels in cord blood

In order to verify if the differences of immune regulation extend to the systemic level, IL-10 and TGF-β concentration in cord blood plasma was determined by ELISA. Significantly higher levels of both IL-10 and TGF-β were found in cord blood of children of healthy mothers than in the high-risk group of children of allergic mothers (Fig 5; p = 0.0009 and 0.0492, respectively).

Discussion

Regulatory T cells play a key role in establishment and maintenance of balance among different branches of the immune system both prenatally and postnatally [19,27]. Nevertheless, the exact mechanisms of Treg involvement in early stages of sensitization and allergy development have not been conclusively elucidated.

We found out differences between function-associated parameters and subpopulation characteristics of Tregs from cord blood of children of allergic mothers compared with children of healthy mothers. The decrease in some of the functional markers (PD-1, IL-10) points to a lower functional efficacy of Tregs from new-borns of allergic mothers [6]. This could be partially compensated by the increased number of Tregs in comparison with children of healthy mothers. Lower numbers of Helios-FoxP3+ Tregs, putative iTregs, in cord blood from children of allergic mothers may be another sign implying abnormal or delayed Treg development contributing to the increased risk of allergy development in these children. This seems to be in accordance with our current findings of decreased cord blood levels of regulatory cytokines IL-10 and TGF-β as well as higher cord blood levels of specific IgE in children of allergic mothers compared with children of healthy mothers.

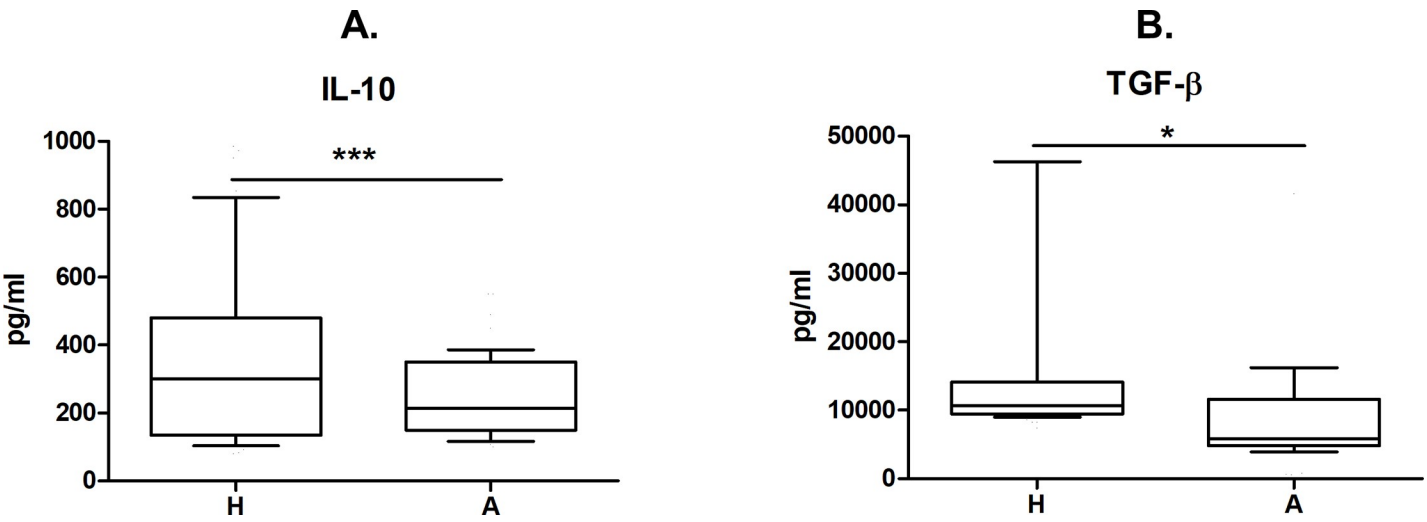

Fig 5. Regulatory cytokine levels in cord blood of children of allergic and healthy mothers. Samples of cord blood plasma of children of healthy (H, n = 112) and allergic (A, n = 98) mothers were collected at the time of birth. Levels of IL-10 (A, p = 0.0009) and TGF-β (B, p = 0. 0492) in plasma were analysed by ELISA. p values were calculated using the Mann-Whitney test.

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Children of allergic mothers exhibited an increased proportion of $CD4^+CD25^+CD127^{low}$-$FoxP3^+$ Tregs. This finding is consistent with our work published earlier [7] as well as with the data from some other groups [28,29], while some studies described no significant difference [30] or even observed an opposite trend [31]. Several factors may account for these discrepancies. Although identification of human Tregs as $CD25^+CD127^{low}FoxP3^+$ population within $CD4^+$ cells is now more or less commonly accepted, different gating strategies have previously been used for Treg identification, and slightly different population proportions were thus obtained based on gating hierarchy and markers used [7,28]. This can be further pronounced when different monoclonal antibody clones are used in flow cytometry [32]. Another important point is that beside bona fide Tregs ($CD4^+CD25^+CD127^{low}FoxP3^+$), activated effector T cells also upregulate CD25 expression [33]. $CD25^{high}$ population (approx. top 2% of expression) has been shown to correlate better with FoxP3 expression and regulatory phenotype [34], probably limiting the contamination of studied Treg sample with activated effector cells. FoxP3 transcription factor, previously thought exclusive for committed Tregs, has been described to be transiently upregulated upon activation of effector T cells [35]. Moreover, considering Tregs as only CD4+CD25+ [36] could lead to overestimation of Tregs especially in the group of newborns of allergic mothers. As we have shown, increased proliferation of cord blood cells [37] and CD25 is commonly accepted as a marker associated with activation. Epigenetic markers such as Treg-specific demethylated region (TSDR) are currently being proposed as better correlating with Treg commitment and functional activity [38].

Tregs are quite a diverse cell population with some degree of plasticity and numerous described subpopulations with varying functional roles in the context of different clinical settings and tissues [39,40]. The development of these subpopulations as well as regulatory capabilities is influenced by factors both intrinsic and external [19,20], including maternal health status, lifestyle and in utero exposure to microbial antigens, allergens or other environmental factors [19]. This could further compromise the comparability of data from different studies, specifically those carried out in rural [19,27,28] vs. urban environment [29].

Of possible importance in the context of allergy development is the proposed difference between thymus-derived natural Tregs (nTregs) and a peripheral population of induced Tregs

(iTregs) [8]. There is some controversy regarding the identification of these populations, and no reliable, indisputable distinguishing markers have been established so far. Helios, an Ikaros-family transcription factor, has been proposed as a marker specific for thymic-derived Tregs, i. e. nTregs, in humans [9] in conjunction with FoxP3 expression. We observed significantly higher proportion of FoxP3$^+$ Helios$^-$ iTregs in Tregs from cord blood of children of healthy mothers compared with the allergic group. Children of allergic mothers, in comparison, had higher proportion of FoxP3$^+$ Helios$^+$ nTregs in Treg population. Since iTregs are induced in periphery upon recognition of harmless environmental antigens under tolerogenic conditions, we assume they may play a vital role in downregulating inappropriate immune responses towards allergens. The lower percentage of iTregs among Tregs of children of allergic mothers may be a factor compromising early postnatal capacity of the immune system to establish tolerance to harmless environmental antigens. As these cells mainly arise upon exposure to environmental influences [12], i.e. later in the process of perinatal immune system maturation, we hypothesise that their relatively lower levels might imply a less mature immune system at birth in the group with higher relative risk of allergy development. To the best of our knowledge, these findings present the first published data regarding Helios expression in cord blood Tregs in the context of allergy.

The suitability of Helios as a marker specific for nTregs has been questioned by a number of studies performed in mice [41,42] as well as several studies involving human subjects [43,44]. Helios$^-$ cells have been found within the population of CD45RA+FoxP3+ Tregs, suggesting that antigen-naïve presumed nTregs may not universally exhibit the Foxp3+ Helios + expression pattern [44]. Likewise, Helios expression has been described in induced Tregs and even FoxP3- CD4+ and CD8+ cells [43]. Different explanations of the role of Helios have consequently been proposed.

It may be possible that Helios expression rises after exposure of the cell to cognate antigen and Helios may thus serve as an activation and proliferation marker [43], similar to FoxP3 itself [33]. The higher proportion of Helios+ FoxP3+ cells in unstimulated cord blood of the children of allergic mothers could then be viewed as a sign of generally higher reactivity of cells derived from cord blood of this group; these results are consistent with studies previously published by our group in this context [17,45–47]. This higher reactivity of cells in cord blood of the high-risk group may signify an immune milieu more prone to inflammation, including allergy.

Another hypothesis put forward is that Helios may play a role in Treg development and the establishment of a stable regulatory phenotype [48]. In such case, the higher expression of Helios we observed may in fact reflect the compensatory expansion of the Treg population brought about by their compromised function. Alternatively, we hypothesise that the lower maturity of the immune system of high-risk children at birth might conceivably mean that fewer stable, lineage-committed Tregs (with no further need of Helios expression) are present at birth. Again, this could also contribute to the higher risk of allergy development in these children.

To help contend with the challenge inherent in distinguishing iTregs from nTregs, we introduced staining for neuropilin-1, another proposed marker of nTregs [11]. Although the number of samples analysed was not sufficient for statistical analysis, we observed a slightly higher expression of neuropilin-1 expression on Helios+ Tregs, a trend consistent with both being considered putative nTreg markers [10]. As neuropilin-1 has been described to play a role in Treg stability maintenance [49], the mechanisms involved in its regulation may also be similar to those discussed earlier in the case of Helios. The full significance of neuropilin-1 expression on circulating human regulatory T cells is not yet clear, however, since most convincing studies were either performed in mice [11] or concerned with the context of lymph node resident Tregs [50].

In performing their regulatory functions, Tregs employ both secreted molecules (chiefly regulatory cytokines IL-10 and TGF-β) and surface proteins. In our study, we observed a significantly lower proportion of PD-1$^+$ Tregs in children of allergic mothers. The same trend is also hinted at for CTLA-4 and GITR, though the statistical power was insufficient for these smaller differences to be significant. PD-1 and CTLA-4 are two of the most ubiquitous Treg functional markers, serving as important coinhibitory molecules involved in cell to cell communication between Tregs and target cells [51], the so called immune checkpoints. They play a crucial role in regulation of autoimmune diseases [52,53] and allergy [54,55]. CTLA-4 involvement has been reported in food allergy [55] as well as the establishment of Th1 and Th2 balance [56], while PD-1 expression by Tregs was shown to play a major role in an animal model of antigen-induced asthma [57].

Targeting these molecules for inhibition also forms the basis of highly effective novel antitumor therapy–the "checkpoint blockade" [58]; the high incidence of immune-related adverse effects accompanying this treatment modality attests to their pronounced functional relevance [59]. GITR is likewise generally considered a marker of Treg function [60], albeit less well-defined functionally and likely serving a negative feedback role in the regulation of Treg homeostasis [61]. The lower expression of PD-1 and other surface markers in Tregs from cord blood of children of allergic mothers marks decreased functional ability of these cells, which in turn could contribute to increased risk of allergy development.

Children of allergic mothers also showed a significantly lower number of IL-10$^+$ Tregs, with the same trend visible for TGF-β. IL-10 and TGF-β are the two chief regulatory cytokines and have been proven to play crucial roles in controlling allergy and establishing tolerance to environmental antigens [62–64]. IL-10 production critically contributes to in vivo suppressive efficacy of Tregs [6], and IL-10 producing CD4 cells, termed Tr1 cells, are implicated in the success of allergen-specific immune therapy [5]. TGF-β is essential for establishing and maintaining tolerance to harmless environmental (e.g. microbial) antigens in the mucosal tissues [65], iTreg induction in the periphery [12,66] and the fine tuning of the mucosal immune system [67,68]. The decrease in intracellular IL-10 as well as lower plasma levels observed in allergic children are in keeping with our previously published observations–higher proliferation activity of CD4+ T cells [69] and increased activation of dendritic cells [47] isolated from cord blood of children of allergic mothers.

To assess the suppressive capacity of cord blood Tregs directly, we also performed a proliferation inhibition assay. Although the data is only preliminary so far, we could demonstrate a notable deficiency of suppressive function of Tregs isolated from cord blood compared with Tregs isolated from adult peripheral blood. This deficiency was more pronounced in Tregs from cord blood of a newborn of allergic mother compared with Tregs from cord blood of a child of a healthy mother. Although these findings need confirmation with further experiments, they could support our proposed hypothesis regarding functional maturity of Tregs of children of allergy mothers.

Impaired Treg function, evidenced by lower functional marker expression, as well as smaller number of induced Tregs in these children could compromise the capacity of their immune systems to correctly set up the immune system balance in early postnatal period. Perinatal period is critical for this fine tuning as the organism first encounters numerous environmental factors, including microbial antigens, as well as potential food and respiratory antigens. Taken together, our results imply that the immune system of children of allergic mothers tends to be more immature at the time of birth, and therefore more prone to developing dysbalance such as allergy. The increased proportion of total Tregs seen in these children is then likely an attempt of the system to compensate for the lower functional capabilities by upregulating the population size. Nevertheless, several points still need to be addressed. Since the flow

cytometric analysis of intracellular cytokines and surface markers presents an indirect view of Treg suppressive efficiency, these observations need to be confirmed by functional assays. Preliminary data shown in the article support positive correlation between impaired production of immunoregulatory cytokines and lower capacity to limit proliferation of effector CD4+ T cells. Nevertheless, this needs to be confirmed on a larger scale to bring definitive insight into the suggested difference of Treg functional capacity between high-risk and low-risk groups. In addition to that, our data suggest that neonatal Tregs have a lower immunosuppressive capacity in comparison to adult ones. There is also a pressing need of further research into markers characteristic for natural and induced Tregs. Presently, there are no universally accepted markers capable of reliably distinguishing the two populations, greatly complicating any analysis of their respective importance in different contexts. The conundrum regarding the role and function of Helios within the context of nTreg and iTreg subpopulations needs to be resolved before a conclusive picture of its possible significance for allergy prediction can be drawn. Lastly, long-term prospective monitoring of children will be necessary for assessment of the predictive value of individual findings and perinatal Treg insufficiency as a whole for actual future development of allergy.

Supporting information

S1 Fig. Gating strategy, FMO controls and representative dot plots of surface and intracellular markers. Cord blood samples were stained and analysed by flow cytometry. A-D: Gating strategy of $CD4^+CD25^+CD127^{low}$ cells.E-P: FMO controls and representative dot plots of $CD4^+CD25^+CD127^{low}$ cells stained for surface (E-J) and intracellular (K-P) markers. E, G, I: FMO controls for GITR, PD-1 and CTLA-4 staining. F, H, J: Representative dot plots of GITR, PD-1, CTLA-4. K: Control sample unstained for FoxP3 and Helios. L: Representative dot plot of intracellular staining for FoxP3 and Helios. M, O: FMO control for intracellular staining of IL-10 and TGF-β. N, P: Representative dot plot of intracellular staining for IL-10 and TGF-β.
(TIF)

S2 Fig. Representative histograms of staining for neuropilin-1. Several whole blood samples were stained for CD4, CD25, neuropilin-1, FoxP3 and Helios and analysed by flow cytometry. A: $CD25^{high}FoxP3^+$ cells were gated into $Helios^+$ and $Helios^-$ populations. B: Expression of neuropilin-1 on $Helios^+$ (blue) and $Helios^-$ (red) cells. FMO control for neuropilin-1 shown in black.
(TIF)

S3 Fig. Suppression of proliferation of CFSE-stained non-Treg cells. $CD4^+CD25^-CD127^+$ target cells were magnetically isolated from cord blood mononuclear cells (n = 19), stained with 5 μM CFSE and cocultured with $CD4^+CD25^+CD127^{low}$ Treg cells at 1:5 Treg:target cell ratio. After 72 hours, cells were harvested, stained for CD4 and analysed by flow cytometry. Representative histograms show unstimulated control cells (blue), anti-CD3/CD28 stimulated control cells (red) and stimulated cells cocultured with Tregs at 1:5 Treg:target ratio (orange). A: Cells isolated from cord blood of a newborn of a healthy mother. B: Cells isolated from cord blood of a newborn of an allergic mother. C: Cells isolated from adult peripheral blood.
(TIF)

S1 Table. Summary table of data from CFSE-based suppression assays. $CD4^+CD25^-CD127^+$ target cells were magnetically isolated from cord blood mononuclear cells (n = 19), stained with 5 μM CFSE and cocultured with $CD4^+CD25^+CD127^{low}$ Treg cells at 1:5 Treg:target cell ratio. After 72 hours, cells were harvested, stained for CD4 and analysed by flow cytometry. Table shows percentage of cells which went through at least one round of cell division (Divided

cells), percentage of cells which did not proliferate (Undivided cells) and the number of peaks representing cell divisions in each sample (Number of generations). For each sample, allergy status is shown (A–children of allergic mothers, H–children of healthy mothers) and three conditions are included: Tregs cocultured with target cells at 1:5 Treg:target ratio; target cells stimulated with CD3 and CD28 monoclonal antibodies and IL-2; and unstimulated target cells, with only IL-2 added.
(PDF)

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Title page

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

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1. Introduction

Allergy remains one of the most common medical conditions, with significant clinical and economical relevance. Allergic diseases represent a highly variable, multifactorial group of disorders characterized by immune dysregulation and failure to develop tolerance towards innocuous environmental antigens. This underlying dysbalance has its roots as early as in the perinatal period. Prenatally, the foetus is maintained under Th2 bias to prevent unwanted reactivity towards antigenically foreign maternal determinants[1]. During the course of pregnancy, immunosuppressive environment supporting regulatory subpopulations is present in uterus under hormonal control[2]**.** Postnatally, new balance among the branches of immune system needs to be established – persisting Th2 predominance promotes sensitization and allergy development, while Th1 and Th17 branches play crucial roles in anti-infectious immunity but unchecked can cause autoimmune or inflammatory diseases[3].

Generally, neonatal immune system is immature. Proper development of immune responses together with setting tolerance to food antigens and compounds of microbiota is therefore necessary. This fine tuning of immunity in the early postnatal period is highly regulated and involves both intrinsic and extrinsic factors, chiefly exposure to microbial stimuli[4], consistent with the generally accepted hygiene hypothesis[5]. Intrinsically, regulatory T cells (Tregs) play a key role. This crucial immunomodulatory population of Th lymphocytes serves as master regulators capable of finely tuning balance among the other branches of immune response, maintaining peripheral tolerance and suppressing pathological immune reactions, including Th2-based allergy[3].

Human Tregs are commonly described as $CD4^{+}CD25^{high}CD127^{low}$ population, further characterized by high dependence on IL-2, expression of lineage-specific transcription factor FoxP3 and demethylation of specific sequences within *foxp3* promoter region (Treg-specific demethylated region – TSDR)[6,7]. The functional relevance of these cells is evidenced by severe autoimmunity as well as allergy accompanying deficiency or impaired function of these cells[8,9]. Tregs and their various subpopulations are thus indispensable for controlling allergic diseases both physiologically[3,10] and in the context of specific allergen immunotherapy[11].

Regulatory T cells utilize numerous mechanisms for their suppressive and immunomodulatory functions, both contact-dependent (presence of cell surface molecules like e.g. CTLA-4, PD-1, LAG3, FasL) and remote (secretion of regulatory

cytokines IL-10, TGF-β and IL-35)[12,13]. Assessment of such Treg-associated markers is commonly used to indirectly assess functional capacity of Tregs, both on the level of individual cells (measured via flow cytometry, e.g. presence of surface or intracellular markers, median of fluorescence intensity [MFI] of FoxP3[14]) and systemically (e.g. levels of regulatory cytokines in serum measured by ELISA[15,16]).

Numerous subpopulations of Tregs were identified by flow cytometry, based upon differential expression of various surface and/or intracellular markers, as well as different biological functions. Gating strategies, sample preparation (particularly antibody clone selection), genetic background, clinical and environmental context and other factors can significantly influence Tregs, introducing discrepancies into published results. Therefore, it is vital to strive for consistency and to carefully consider the epidemiological and methodical aspects when interpreting studies published by different groups[17].

Despite marked progress in understanding the early processes involved in sensitization and allergy development, there is still lack of definite consensus regarding reliable early predictors of an individual's increased risk of allergy. Numerous markers have been analysed in cord blood, including cord blood IgE levels[18], levels of cytokines including IL-10 and TGF-β in cord blood[15] and neonatal peripheral blood[19] plasma, as well as reactivity of cord blood cells to various stimuli, with limited conclusiveness when taken together[16,20–23]. So far, the allergy status of the mother remains the strongest unambiguously accepted predictive factor[23].

Several studies have correlated population characteristics and functional properties of Tregs with atopic diseases in small infants[16,24–27]. In our own work published by Hrdý in 2012[17], we reported lower presence of intracellular markers associated with Treg function (IL-10, TGF-β, MFI of FoxP3) in Tregs from cord blood of children at higher risk of allergy development (based on maternal allergy status). Furthermore, we observed a slightly larger population of Tregs in cord blood of children of allergic mothers, possibly due to compensatory expansion of the population. We concluded by stating the importance of utilizing functional studies and above all the correlation of findings obtained from cord blood samples with the actual development of allergy in later life. In the current study, we perform retrospective analysis of a subset of data from the 2012 report, stratifying the previous findings according to the clinical allergy status of the children at the age of 6 to 10 years.

2. Materials and Methods

2.1 Subjects

The original study included a total of 153 healthy and allergic mothers with physiological pregnancies and children delivered vaginally at full term in the Institute for the Care of Mother and Child in Prague, Czech Republic[17]. The subjects were divided into two groups according to maternal allergic status, based on clinical manifestation of allergy persisting for over two years; monitoring by an allergist; positive skin prick tests and/or specific IgE antibodies; and anti-allergic treatment administered before pregnancy. The study including the current follow-up was approved by the Ethical Committee of the Institute for the Care of Mother and Child (Prague, Czech Republic) and carried out with the signed written informed consent of the mothers.

For the current follow-up study, the families originally included were contacted with a questionnaire in order to collect clinical data regarding manifestation of allergy in the children at the age of 6-10 years, confirmed by clinical examination by an allergist. A total of 39 responding subjects were included in the analysis, with the subjects subdivided into four groups based on combination of the allergy status of the mothers and the children themselves: allergic children of allergic mothers (A/A; n=8), healthy children of allergic mothers (H/A; n=12), allergic children of healthy mothers (A/H; n=7) and healthy children of healthy mothers (H/H; n=12).

2.2 Cord blood samples

Between 10 and 20 ml of umbilical cord blood were collected into sterile heparinised tubes for analysis of Treg population characteristics as well as intracellular presence of transcription factor FoxP3 and regulatory cytokines IL-10 and TGF-β by flow cytometry.

2.3 Flow cytometry

Whole blood samples were prepared and stained for flow cytometry analyses as described previously[17]. Briefly, samples of whole blood were stained for cell surface markers using the following antibodies: CD4-FITC, clone RPA-T4, cat. no. 555346; CD25- PE-Cy7, clone M-A251, cat. no. 557741; and CD127-Alexa 647, clone HIL-7R-M21, cat. no. 558598, all from Becton Dickinson (Franklin Lakes, NJ, USA). For intracellular staining, following antibodies were used: FoxP3-PE (clone PCH101, cat. no. 12-4776-41A; eBioscience, San Diego, CA, USA); IL-10-PE (clone JES3-19F1, cat. no. 506804) and TGF-β-PerCP (clone BG/hLAP, cat. no. 341803; both from BioLegend).

Proportion of Tregs and percentage IL-10 and TGF-β positive Tregs were determined as described previously[17]. In the current study, data were re-analysed to obtain proportion of Tregs characterised as $CD4^{+}FoxP3^{+}$ using gating strategy shown in the Supplements (S Fig. 1). Briefly, lymphocytes were gated based on forward-scatter (FSC) and side-scatter (SSC) characteristics, followed by doublets exclusion based on FSC-A × FSC-H. $CD4^{+}$ T cells were identified and Tregs then gated from this population as T cell subset positive for FoxP3.

2.4 Data acquisition and statistics

Flow cytometry data were acquired using BD FACSCanto II flow cytometer using BD FACS Diva version 6.1.2 software (Becton Dickinson) and analysed using FlowJo 7.2.2 (TreeStar. Ashland, OR, USA). Statistical and graphical analyses were performed using GraphPad Prism 6.0 (Graph Pad Software, La Jolla, CA, USA). Differences between the groups were compared using unpaired Student's *t*-test in the case of normally distributed data (Treg ratios, MFI of FoxP3) and Mann-Whitney non-parametric test for the proportions of $IL\text{-}10^{+}$ Tregs and $TGF\text{-}\beta^{+}$ Tregs.

2.5 ELISA

Concentrations of regulatory cytokines IL-10 and TGF-β in the sera of cord blood were quantified by an enzyme-linked immunosorbent assay (ELISA) as described previously[15]. Briefly, primary (MAB 240) and secondary biotinylated (BAF 240) antibodies and recombinant protein (240-IL) for TGF-β and Detection kit for IL-10 (DY217-B) were purchased from R&D Systems. Concentration of cytokines were calculated from calibration curves and expressed in pg/ml using software KIM (Schoeller Instruments).

3. Results

The original study analysed immunological characteristics of Tregs from cord blood in the context of expected risk of allergy development, based on maternal allergy status[17]. Current follow-up allowed us to correlate the data with actual allergy status of the children, an aspect critically important for considering proportion and functional characteristics of Treg as possible predictive markers.

The proportion of Tregs, MFI of FoxP3 and intracellular presence of regulatory cytokines (IL-10 and TGF-β) were compared among the healthy and allergic children of healthy mothers and allergic mothers, respectively.

3.1 Treg ratio

When we used the gating strategy described earlier[17] and Tregs were considered as $CD4^+CD25^{high}CD127^{low}FoxP3^+$, no significant difference was found between the healthy and allergic children regardless of maternal allergy status (Fig. 1A). However, when only two-colour flow cytometry analysis ($CD4^+CD25^+$) was performed, healthy children had significantly increased proportion of these cells (p= 0.0495) (Fig. 1B). Proportion of Tregs considered as $CD4^+FoxP3^+$ was not different in cord blood of children of healthy and allergic mothers, Fig. 1C).

After further dividing the two groups of children according to maternal allergy status, there was no difference in $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs (Fig. 2A). The increase of $CD4^+CD25^+$ cells in healthy children has been driven mainly by the group of healthy children of healthy mothers (i.e., from the low-risk group; H/H), who had increased proportion of these cells compared both with allergic children of healthy mothers (A/H; p=0.0008) and with healthy children of allergic mothers (H/A; p=0.0418) (Fig. 2B). Notably, in the group of children of allergic mothers (i.e., the high-risk group) no trend towards lower population of $CD4^+CD25^+$ cells in allergic children is discernible, possibly hinting at a different composition or role of this population between the low-risk and high-risk groups. In addition to that, comparison of Tregs based on positivity for CD4 and FoxP3 revealed significantly increased proportion of $CD4^+FoxP3^+$ Tregs in the group of healthy children of healthy mothers in comparison to healthy children of healthy mothers (p = 0.0392) (Fig. 2C).

As the observed trends in Treg proportions were somewhat inconsistent with the original findings and other studies published previously[17,25] and our current cohort represented a smaller subset of the subjects included in the original study, we decided to confirm whether the originally described differences between children of allergic and healthy mothers are detectable in our current data. First, we compared Treg proportions between the two groups. While difference in the proportion of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ does not reach statistical significance, there is a noticeable trend towards higher numbers of these cells in children of healthy mothers (p=0.0559) (supplementary Fig. S2A). The proportion of $CD4^+CD25^+$ subpopulation is comparable in both groups (supplementary Fig. S2B). Similarly, no significant difference

was observed between groups of children of healthy and allergic mothers in Tregs characterised as $CD4^+FoxP3^+$ cells (supplementary Fig. S2C).

3.2 MFI of FoxP3

The original study included the MFI of FoxP3 as one of markers characterising Treg function[14,17]. After analysing the data divided according to the child's allergy status, we found no difference in MFI of FoxP3 between the two basic groups (healthy children versus allergic children) (Fig. 3A). Further subdivision according to the allergy status of the mother likewise revealed no difference among the groups (Fig. 3B), implying that MFI of FoxP3 may not associate with the risk of allergy development strongly enough to serve as a useful predictive factor.

3.3 Intracellular regulatory cytokines IL-10 and TGF-β

Further, we investigated whether there was lower intracellular presence of the major regulatory cytokines IL-10 and TGF-β in Treg of cord blood of children who developed allergy. No difference was found between the two basic groups for either IL-10 (Fig. 4A) or TGF-β (Fig. 4B), although a tendency toward higher values can be discerned in the healthy children. Upon further division of the samples based on the allergy status of the mothers, we uncovered significantly higher proportion of $IL\text{-}10^+$ Tregs in cord blood of healthy children of healthy mothers (H/H) compared with both groups of high-risk children: healthy children of allergic mothers (H/A; p=0.0146) and allergic children of allergic mothers (A/A; p=0.0306) (Fig. 5A). Furthermore, lower proportion of $TGF\text{-}\beta^+$ Tregs was observed in allergic children of allergic mothers (A/A) compared with all the other groups, i.e. allergic children of healthy mothers (A/H; p=0.0205), healthy children of allergic mothers (H/A; p=0.0311) and healthy children of healthy mothers (H/H; p=0.02) (Fig. 5B). These findings are consistent with the functional importance of these cytokines in allergy and support the notion that their decreased intracellular presence is an important factor for the actual development of allergy, especially within the high-risk group of children of allergic mothers.

To see if the trends of the markers of Treg function described in the original study can be confirmed in the current smaller subgroup of subjects, we compared MFI of FoxP3 and intracellular presence of IL-10 and TGF-β in Tregs from cord blood of children of allergic mothers and children of healthy mothers, regardless of the allergy status of the

children themselves. While MFI of FoxP3 did not differ between the groups (supplementary Fig. S3A), the proportions of both IL-10^+ and TGF-β^+ Tregs were significantly lower in the group of children of allergic mothers, regardless of their own allergy status (p =0.0173 and 0.0444, respectively) (supplementary Fig. S3B and supplementary Fig. S3C, respectively), consistent with the results seen in the larger cohort[17].

Lastly, concentration of cytokines with regulatory functions (IL-10, TGF-β) was determined in cord blood sera of newborns of healthy and allergic mothers. Levels of IL-10 in cord blood sera of healthy children was not significantly increased in comparison to concentration of IL-10 in cord blood sera of children suffering from allergic diseases. There is only non-significant trend to increased values (p = 0.0681) (Fig. 6A). After division of children according to their allergy status and allergic status of their mothers, only significantly higher levels of IL-10 were observed in cord blood sera of healthy children of healthy mothers in comparison to allergic children of allergic mothers (p = 0.0422) (Fig. 6B). No difference was found in concentration of TGF-β in cord blood sera of healthy and allergic children (Fig. 6C). After subdivision of children according to both their and maternal allergy status, elevated concentration of TGF-β was found in cord blood sera of healthy children of healthy mothers in comparison to allergic children of allergy mothers (p = 0.015) and healthy children of allergic mothers (p = 0.0402) (Fig. 6D).

4. Discussion

It is well documented that Tregs are critically responsible for immune system regulation, evidenced by the severe autoimmune, inflammatory and allergic disorders accompanying their deficiency or dysfunction[8,9,28]. They play a key role in induction and maintenance of immune tolerance, from prenatal period through the infancy into adulthood[3,29]. Numerous studies aimed to uncover whether analysis of these cells in cord blood, i.e. at the moment of birth, could be used to predict increased risk of allergy, a notion that can only be confirmed upon correlating these parameters with actual allergy development later in life[17,21,22,24,25].

In the original study, we measured proportional characteristics and parameters associated with Treg function in samples of cord blood using flow cytometry and analysed the data in the context of allergy development risk according to the allergy status of the mother[17], a commonly accepted predictive marker of increased risk of allergy

development[23]. In the current follow-up, we correlated the data with clinical manifestation of allergy in childhood in a subset of the original subjects. One limitation we encountered was the low number of subjects we were able to include for the retrospective analysis – only 39 out of the 153 subjects involved in the previous study. Several factors contributed to the high drop-out rate between the original study and current follow-up. Firstly, a number of questionnaires failed to reach the subjects due to changes in the mailing address. Furthermore, a portion of recipients declined participation in the follow-up study. Finally, several respondents returned incompletely filled questionnaires which failed to provide information necessary for inclusion in analysis. This unfortunately limited the statistical power of our analyses and might account for some of the inconsistencies we observed.

While we did not see any difference in Treg proportions between healthy and allergic children when Tregs were identified as $CD4^+CD25^{high}CD127^{low}FoxP3^+$ or only $CD4^+FoxP3^+$ cells, we found out that the population of $CD4^+CD25^+$ cells was increased in the healthy group. This effect was mainly due to healthy children from the low-risk group, which had higher proportion of these cells than allergic children from the low-risk group as well as healthy children from the high-risk group, with the allergic children from the high-risk group showing notably higher variance.

Although these results are supported by some published data[24,26], they are somewhat surprising in the context of the original study, where we found that $CD4^+CD25^+$ cells were higher in the high-risk group and hypothesised that these cells might in fact be activated CD4 T cells rather than *bona fide* Tregs[17]. The discrepancy might be attributable to the limited number of subjects available for the retrospective study. Indeed, when the current subjects were stratified according to maternal allergy status without regard to allergy of the children, the original findings could not be confirmed, indicating that results strongly depend on selection of samples within the cohort, as well as the number of subjects analysed.

Further inconsistencies may also be due to inherent properties of Tregs and imperfect identification or understanding of the cells. Regulatory T cells represent a highly heterogeneous population, which has led to considerably diverse and sometimes contradictory reports. While some groups including ours have previously observed an increase in Tregs in cord blood of children of allergic mothers[17,25,30], in other studies, no difference was found[31] or even the opposite trend was reported[24,26]. Exacerbating this are confounding factors such as differences in gating strategy and identification of

Tregs[17], antibody clones used, especially in the case of FoxP3[32]; diversity of studied cohorts, particularly considering different genetic background and environment[21,22]; and methodical inconsistencies, including sample preparation and cohort sizes[17,33].

Moreover, while conventional Tregs are now generally accepted to be $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ cells in humans, numerous other subpopulations have been described in the literature. Expression of transcription factor FoxP3 is considered lineage specific for Tregs and FoxP3 MFI has been linked to suppressive function[14]. However, it has been shown that FoxP3 can be transiently induced by cell activation in non-Treg cells[34] and also that $FoxP3^{-}$ Treg cell populations with suppressive function both exist[35] and play a crucial role in the success of specific allergen immunotherapy[11,35]. Currently, epigenetic analysis of demethylation of TSDR in the promotor area of *foxp3* gene is proposed to better correspond to Treg phenotype stability and lineage commitment[6,7]. Furthermore, while CD25 is the original marker first used to identify Tregs[36], it is also upregulated on activated non-Treg T cells[34], and it has been shown that $CD25^{high}$ (approximately the top 2% of CD25 positivity) better corresponds with *bona fide* Treg phenotype[37], whereas defining Tregs solely as $CD4^{+}CD25^{+}$ might include activated T cells.

Considering the diversity of Treg subpopulations, possible contamination by activated T cells and also marked plasticity of regulatory T cells[38,39], it is interesting to note that contrary to the situation observed in the groups of low-risk children, the group of children of allergic mothers, shows no tendency towards higher proportion of $CD4^{+}CD25^{+}$ cells in the healthy children. This can possibly be due to the higher variance seen among the allergic subjects from this group. It is perhaps conceivable that the ratios of particular Treg subpopulations within and/or functional significance of the observed $CD4^{+}CD25^{+}$ population might differ between the low-risk and the high-risk groups as defined by maternal allergy status, similar to how some studies report different results based on the inherent risk due to environmental factors (e.g. urban vs. farming environment[21,22]). Utilising alternative methods of Treg characterisation (such as epigenetic analysis of TSDR) or adding more cell markers associated with Tregs into the panel (e.g. CTLA-4, PD-1, LAG3, CD39 and others[12]) in future studies to better characterise the observed populations would be necessary to conclusively confirm or deny this possibility.

In light of the abovementioned limitations and confounding factors, our findings suggest that simple analysis of proportion of Tregs might not be reliable enough to give

a representative picture of perinatal immune status. Functional studies and markers associated with Treg function seem to provide better insight. While in our study there was no significant difference in MFI of FoxP3 between the healthy and allergic children, both IL-10^+ and TGF-β^+ Tregs have been higher in healthy children. Specifically, intracellular presence of IL-10 in Tregs was significantly higher in healthy children of healthy mothers compared with both groups of children of allergic mothers and intracellular presence of TGF-β was significantly lower in allergic children of allergic mothers than in all other groups. Taken together with the originally described increased intracellular presence of these cytokines in Tregs in cord blood of children of healthy mothers, the current results indicate that lower IL-10 and TGF-β intracellular presence in Tregs correlates with future allergy development, particularly in the high-risk group of children of allergic mothers. This observation suggests that lower levels of regulatory cytokines could be one of the promising predictive markers indicating increased risk of allergy development, is in accordance with previously published data[19,20].

Our findings are also consistent with the well-established role of these cytokines in immune regulation, including inducing and maintaining tolerance to environmental antigens and control of allergy[10]. IL-10 is the primary effector molecule involved in contact-independent suppression by Tregs[12] and plays an indispensable role in the success of allergen-specific immunotherapy[11,40]. TGF-β plays a vital role in the context of mucosal immunity, including establishment and maintenance of peripheral tolerance towards microbial antigens as well as allergens[41,42], inducing Tregs in the periphery[42] and generally tuning the mucosal immune system toward tolerogenic conditions[43,44].

Collectively, our data support the hypothesis that evaluation of early postnatal Treg function, such as intracellular presence of regulatory cytokines, might be more useful in predicting allergy development than simple analyses of Treg population proportions, warranting further research using direct functional assays, similar to studies already published[24,30]. It might also benefit future studies to include other markers associated with Treg function, such as surface molecules CTLA-4 or PD-1[45–48], as some studies have already done[30]. Size of the studied cohorts is also a common limiting factor which together with high heterogeneity of allergic diseases may contribute to the inconsistencies reported in literature[17,33]. Furthermore, reliable identification of stable Treg phenotype using novel techniques, chiefly epigenetic analysis of TSDR demethylation status, might shed some light into the conflicting reports regarding population proportions of Tregs and

their relevance between different subject groups at higher/lower risk of allergy development. Of course, the final verdict regarding the real benefit of the proposed approaches for early prediction of allergy will only be possible upon correlating the data measured at the time of birth with the actual development of allergy later in life. So far, while there is a multitude of studies which characterise Treg populations and functional parameters in cord blood, relatively few studies correlate their findings with clinical data obtained later during life[24].

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Conflicts of Interest

The authors declare no commercial or financial conflict of interest.

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Figure 1.

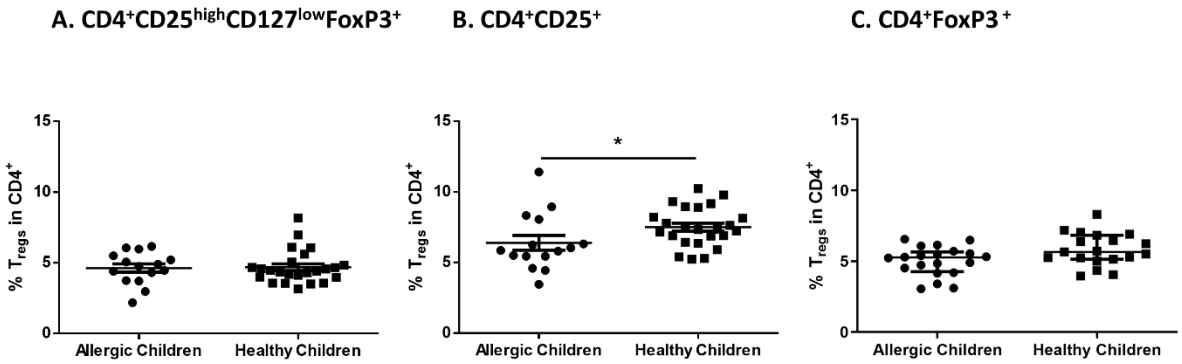


Figure 1. Proportions of regulatory T cells (Tregs) in cord blood evaluated according to the children's allergy status.

Samples of cord blood of allergic children (n=15) and healthy children (n=24) were stained and analysed by flow cytometry. (A) Four-color cytometry analysis of $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ Tregs; (B) Two-color cytometry analysis of $CD4^{+}CD25^{+}$ cells; (C) Two-color cytometry analysis of $CD4^{+}FoxP3^{+}$ Tregs; *$p \leq 0.05$

Figure 2.

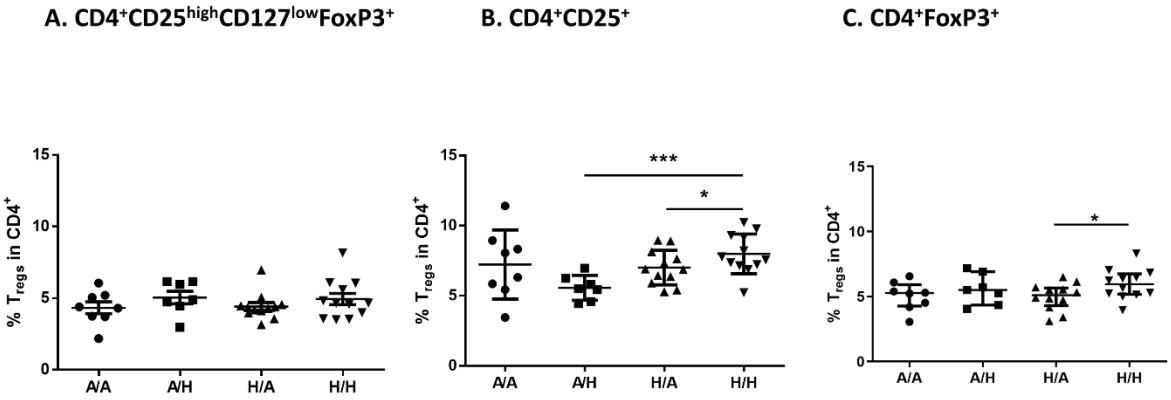


Figure 2. Proportions of regulatory T cells (Tregs) in cord blood evaluated according to the children's allergy status and maternal allergy status.

Samples of cord blood of allergic children of allergic mothers (A/A, n=8), allergic children of healthy mothers (A/H, n=7), healthy children of allergic mothers (H/A, n=12) and healthy children of healthy mothers (H/H, n=12) were stained and analysed by flow cytometry. (A) Four-color cytometry analysis of $CD4^{+}CD25^{high}CD127^{low}FoxP3^{+}$ Tregs; (B) Two-color cytometry analysis of $CD4^{+}CD25^{+}$ cells; (C) Two-color cytometry analysis of $CD4^{+}FoxP3^{+}$ Tregs; *$p \leq 0.05$; ***$p \leq 0.001$

Figure 3.

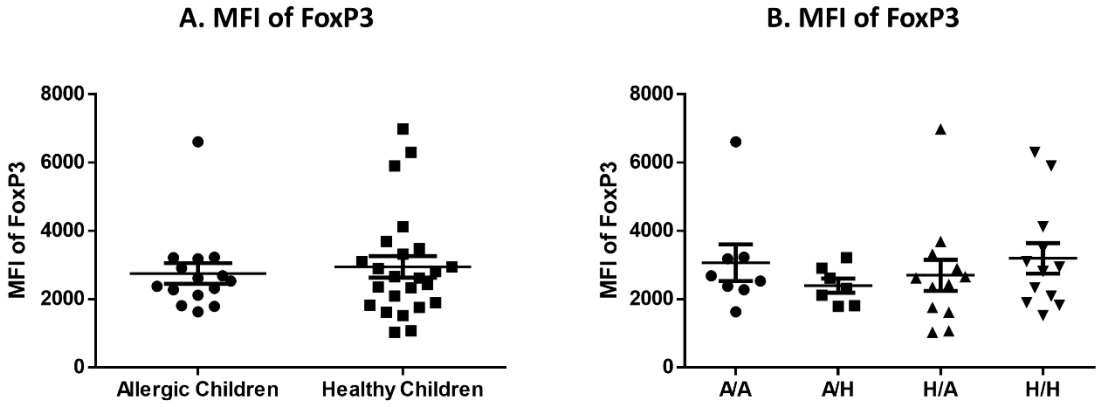


Figure 3. Median of fluorescence intensity in regulatory T cells (Tregs) in cord blood. Cord blood samples were stained and analysed by flow cytometry.

(A) Median of fluorescence intensity (MFI) of FoxP3 comparison in cord blood of allergic children (n=15) and healthy children (n=24); (B) MFI of FoxP3 comparison in cord blood of allergic children of allergic mothers (A/A, n=8), allergic children of healthy mothers (A/H, n=7), healthy children of allergic mothers (H/A, n=12) and healthy children of healthy mothers (H/H, n=12).

Figure 4.

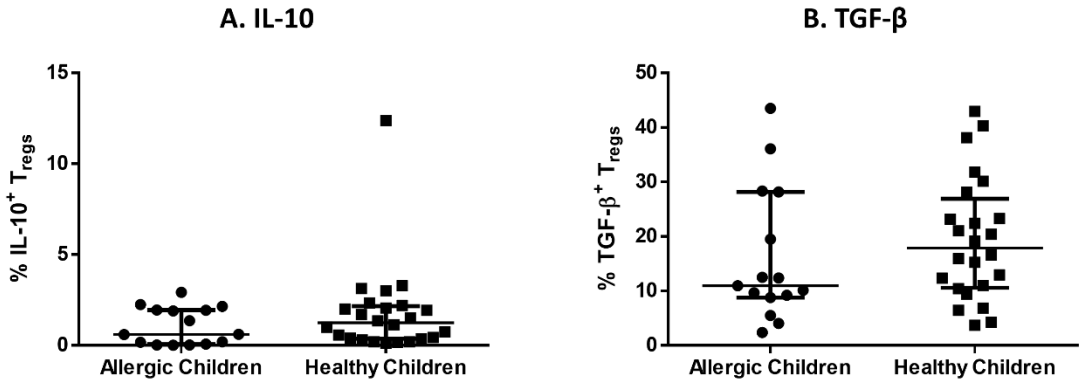


Figure 4. Intracellular presence of regulatory cytokines IL-10 and TGF-β in regulatory T cells (Tregs) in cord blood evaluated according to the children's allergy status.

Samples of cord blood of allergic children (n=15) and healthy children (n=24) were stained and

analysed by flow cytometry. (A) Percentage of IL-10^{+} Tregs ($CD4^{+}CD25^{high}CD127^{low}$); (B) Percentage of TGF-β^{+} Tregs ($CD4^{+}CD25^{high}CD127^{low}$).

Figure 5.

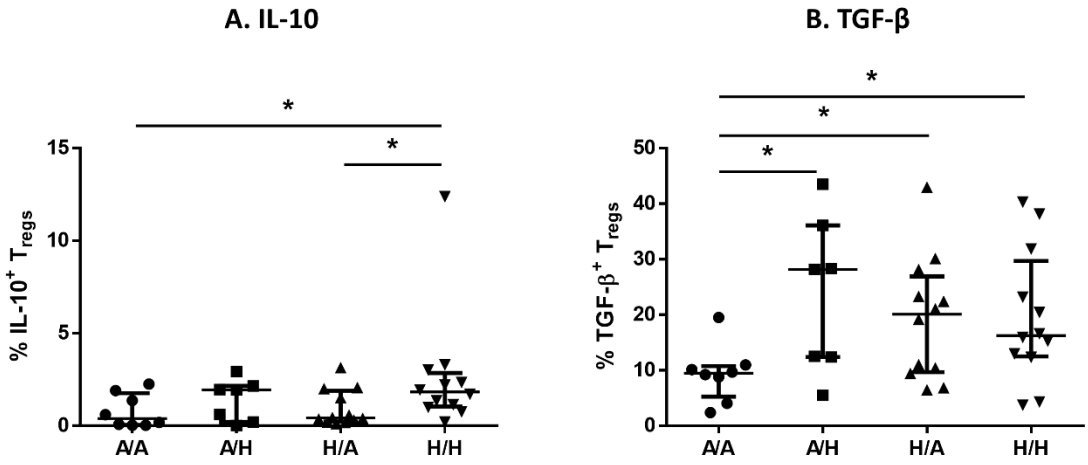


Figure 5. Intracellular presence of regulatory cytokines IL-10 and TGF-β in regulatory T cells (Tregs) in cord blood evaluated according to the children's allergy status and maternal allergy status.

Samples of cord blood of allergic children of allergic mothers (A/A, n=8), allergic children of healthy mothers (A/H, n=7), healthy children of allergic mothers (H/A, n=12) and healthy children of healthy mothers (H/H, n=12) were stained and analysed by flow cytometry. (A) Percentage of IL-10^{+} Tregs ($CD4^{+}CD25^{high}CD127^{low}$); (B) Percentage of TGF-β^{+} Tregs
($CD4^{+}CD25^{high}CD127^{low}$); *$p \leq 0.05$

Figure 6.

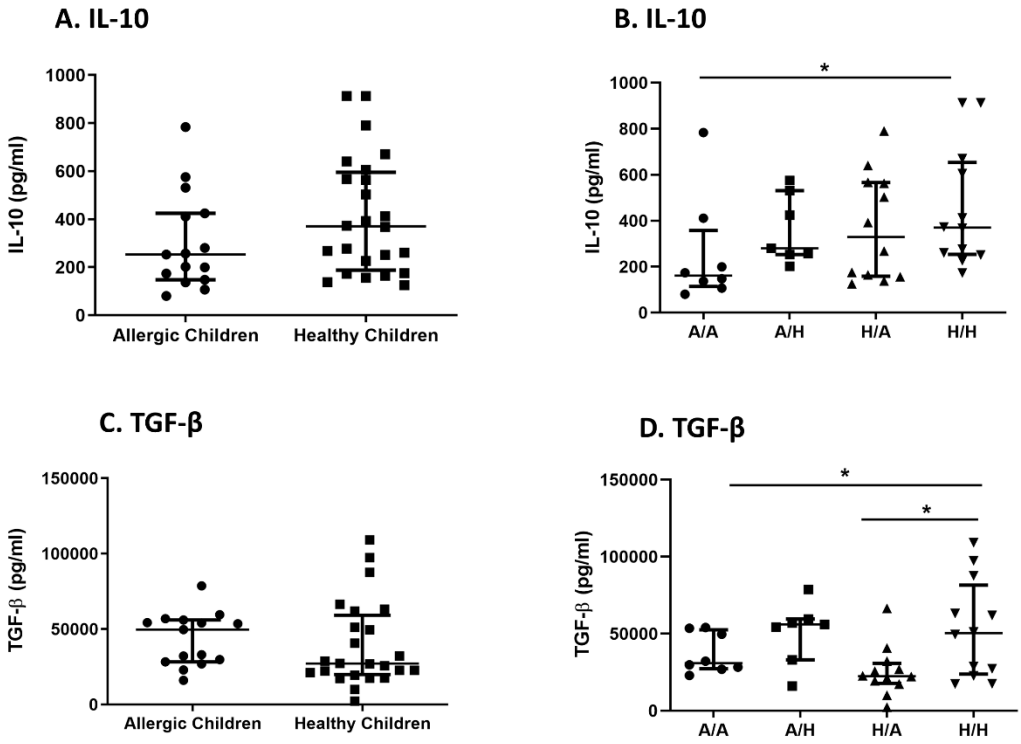
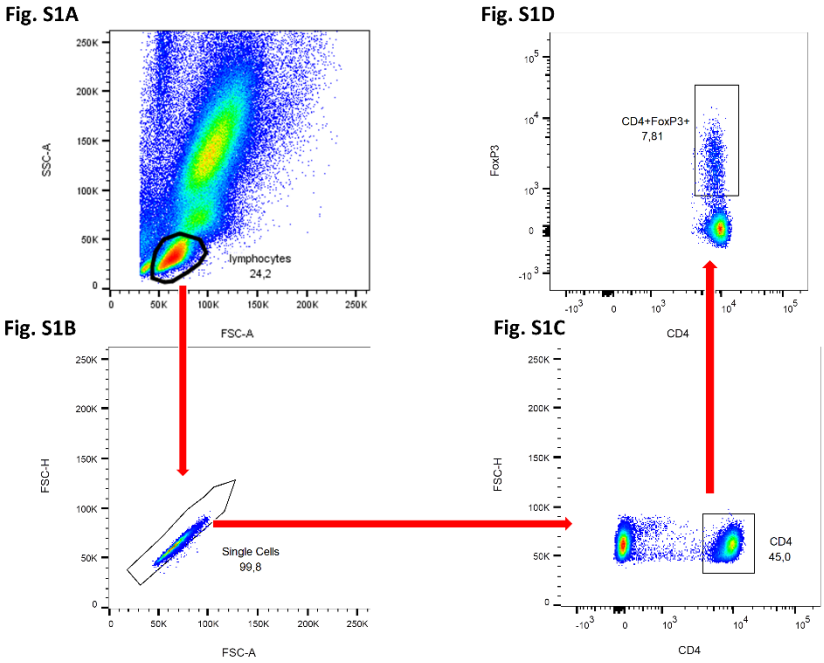


Figure 6. Cord blood serum levels of regulatory cytokines IL-10 and TGF-β evaluated

according to the children's allergy status and maternal allergy status.

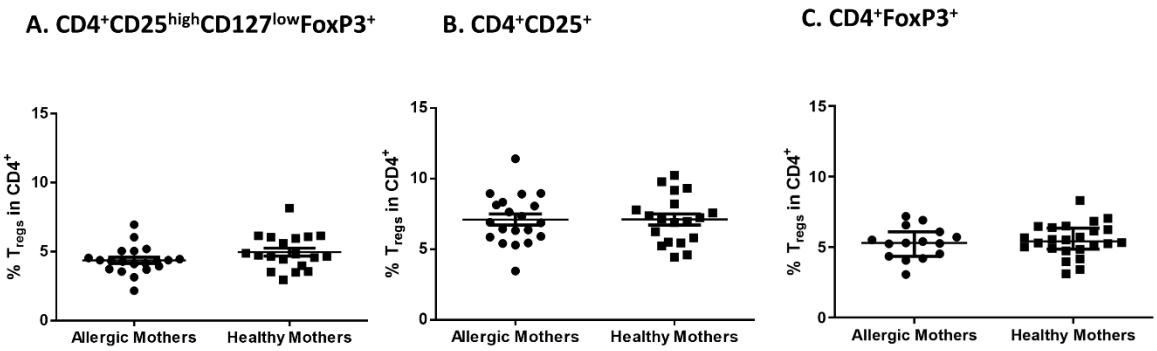
Levels of regulatory cytokines IL-10 and TGF-β in cord blood sera were determined using ELISA. (A) Concentration of IL-10 in cord blood sera of allergic children (n=15) and healthy children (n=24); (B) Concentration of IL-10 in cord blood sera of allergic children of allergic mothers (A/A, n=8), allergic children of healthy mothers (A/H, n=7), healthy children of allergic mothers (H/A, n=12) and healthy children of healthy mothers (H/H, n=12); (C) Concentration of TGF-β in cord blood sera of allergic children (n=15) and healthy children (n=24); (D) Concentration of TGF-β in cord blood sera of allergic children of allergic mothers (A/A, n=8), allergic children of healthy mothers (A/H, n=7), healthy children of allergic mothers (H/A, n=12) and healthy children of healthy mothers (H/H, n=12); *$p \leq 0.05$

Figure S1.



Supplementary figure 1. Gating strategy used for $CD4^+FoxP3^+$ Treg proportion determination. Gating strategy for estimation of $CD4^+FoxP3^+$ Treg in cord blood. According to the size (FSC-A) and complexity (SSC-A), lymphocyte gate was set (Fig. S1A) followed by doublet discrimination (Fig. S1B). $CD4^+$ T cells were selected (Fig. S1C) and population of $CD4^+FoxP3^+$ Treg was gated as shown in Figure S1D.

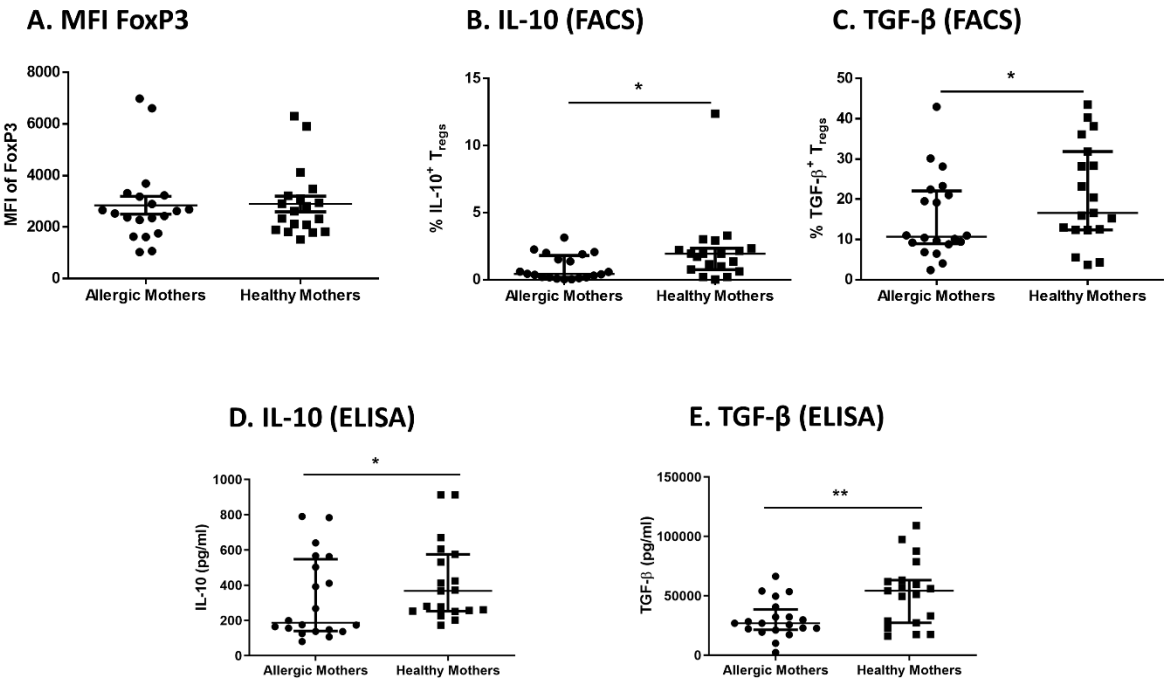
Figure S2.



Supplementary figure 2. Proportions of regulatory T cells (Tregs) in cord blood evaluated according to the maternal allergy status.

Samples of cord blood of children of allergic mothers (n=20) and children of healthy mothers (n=19) were stained and analysed by flow cytometry. (A) Four-color cytometry analysis of $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Tregs; (B) Two-color cytometry analysis of $CD4^+CD25^+$ cells; (C) Two-color cytometry analysis of $CD4^+FoxP3^+$ Tregs.

Figure S3.



Supplementary figure 3. Analysis of markers associated with Treg function in cord blood evaluated according to the maternal allergy status.

(A) – (C) Samples of cord blood of children of allergic mothers (n=20) and children of healthy mothers (n=19) were stained and analysed by flow cytometry. (A) MFI of FoxP3; (B) Percentage of IL-10^{+} Tregs (CD4^{+}CD25^{high}CD127^{low}); (C) Percentage of TGF-β^{+} Tregs (CD4^{+}CD25^{high}CD127^{low}); (D) – (E) Levels of regulatory cytokines IL-10 and TGF-β in cord blood sera of children of allergic mothers (n=20) and children of healthy mothers (n=19) were determined using ELISA. (D) Cord blood serum concentration of IL-10; (E) Cord blood serum concentration of TGF-β; *$p \leq 0.05$; **$p \leq 0.01$

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Research Article

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

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The growing knowledge of the key role of microbiota in the maturation of neonatal immune system suggests that manipulation of microbiota could be exploited in hampering allergy development. In this study, *Escherichia coli* O83:K24:H31 (EcO83) was administered to newborns that were followed prospectively. Several immunological characteristics (cytokines, specific IgE, total T regulatory cells (Treg) and subpopulation of natural Treg (nTreg) and induced Treg (iTreg)) were tested in peripheral blood of 8-year-old children. Incidence of allergic disease was decreased in EcO83 supplemented children and significantly elevated levels of IL-10 and IFN-ɣ were detected in serum of EcO83 supplemented children. Probiotic supplementation did not influence the numbers of the total Treg population but their functional capacity (intracellular expression of IL-10) was significantly increased in children supplemented with EcO83 in comparison to non-supplemented children. Morover, decreased proportion of iTreg was present in peripheral blood of non-supplemented in comparison to EcO83 supplemented children. Finally, stimulation of cord blood cells with EcO83 promoted both gene expression and secretion of IL-10 and IFN-ɣ suggesting that beneficial effect of EcO83 in prevention of allergy development could be mediated by promotion of regulatory responses (by IL-10) and Th1 immune response (by IFN-ɣ).

Keywords: allergy · cord blood · *E. coli* · probiotics · Treg

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

There is growing knowledge of the importance of mutual interaction between microbiota and host immune system. Microbiota plays a key role in the development of newborn immune system when relatively sterile newborn is becoming gradually colonized by a broad spectra of microorganisms [1, 2]. Microbiota becomes stable at the age of 2–3 years [3], therefore early intervention targeting modification of microbiota composition will be more likely successful and easier than correcting the dysbiosis in adulthood. Especially pre-term born children or children delivered by caesarean section have altered microbiota composition [4] and they will be potentially ideal target groups for microbiota correction

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(e.g. by probiotic supplementation). It is important to emphasize that distinct bacterial species are predominant at different time points of newborn´s ontogeny depending on whether child is breastfed or formula fed [4, 5]. The first colonizers come from the birth canal helping neonate to metabolize oligosaccharides present in maternal milk. Substantial changes occur when solid food is introduced. Generally, delayed maturation of neonatal immune system together with prolonged Th2 predominance contribute to the increased risk of allergy development [6]. Despite an intensive search of prognostic markers for allergy in cord blood (e.g. specific IgE [7], cytokines [8–10], cord blood cell proliferation [11], proportion and functional capacity of T regulatory cells [12, 13]), maternal allergy status seems to be the most reliable one [14].

Distinct lung microbiota composition has been described in patients suffering from asthma [15] but contradictory results were published regarding the comparison of gut microbiota between healthy and allergic patients [16]. Although it seems that gut microbiota modification will be an easy task, it is not clear what kind of probiotic bacteria should be used and what will be the best timing of probiotic supplementation. Different researcher groups tried to lower allergy incidence in children by administration of various probiotic strains or mixtures either prenatally to pregnant women [17, 18] or postnatally to neonates [19, 20]. Timing, dose of probiotics and selection of suitable strain are the most important things to be considered. In addition to that, several studies tried to apply probiotics to nasal cavity in mice [21] and human [22] indicating that the site of application will be important as well.

In our study, newborns of allergic mothers were considered to be at a higher risk for allergy development. Probiotic supplementation was used as a preventive measure to lower/prevent allergy development later in children life. The probiotic strain *Escherichia coli* O83:K24:H31 (EcO83) was selected based on previous positive reports [20, 23, 24] and similarly to prior results the early administration of EcO83 significantly lowered allergy development in the current report. Several selected immune characteristics were influenced by early postnatal EcO83 supplementation. Notably, EcO83 supplemented children have increased proportion of iTregs and elevated concentration of IL-10 and IFN-ɣ in peripheral blood.

To describe the possible mechanism how EcO83 could influence immature immune system, newborn cord blood mononuclear cells (CBMC) were stimulated in vitro. CBMC stimulation by EcO83 promoted gene expression and secretion of IL-10 and IFN-ɣ. It seems that the beneficial effect of EcO83 could be mediated via IL-10 supporting suppressive immunoregulatory responses and by IFN-ɣ promotion leading to enhancement of Th1 function.

Results

Decreased allergy incidence in probiotic supplemented children

EcO83 supplementation decreased allergy incidence in children of allergic mothers. During regular visit, allergy status of 8-year-old children reported by parents was confirmed by allergist and/or positive skin prick test and/or detection of specific IgE antibodies. Children suffered from different allergic diseases including rhinoconjunctivitis, asthma, atopic eczema or combination of more allergic diseases. Detailed information regarding allergy status of every allergic child participating in this study is provided in Supporting Information Table S1. In the group of non-colonized children of healthy mothers (NC H), 4 children out of 25 children (16%) who visited regular check at 8 years were allergic. In the group of non-colonized children of allergic mothers (NC A), 11 children out of 36 children (30.6%) suffered from allergy. In the group of probiotic supplemented children of allergic mothers (C A), 8 out of 46 children (17.4%) were allergic. Proportion of allergic children in the EcO83 supplemented group was similar with the group of children with low risk for allergy development (children of healthy mothers) and significantly lower in comparison with non-supplemented children of allergic mothers.

Detection of specific IgE

Probiotic supplementation has not downregulated specific IgE production in the group of children at high risk for allergy development in comparison to non-supplemented ones. All children were screened for specific IgE antibodies against the mixture of most common food allergens (FX 4) and most common respiratory allergens (DYNX 1) [25]. Three basic groups of eight year old children (non-colonized children of healthy mothers - NC H, non-colonized children of allergic mothers - NC A, colonized children of allergic mothers - C A) were divided according to the allergy status into 6 subgroups (see Supporting Information Table S2). No difference was observed when comparing specific IgE antibodies against food and respiratory allergens among basic three groups (Fig. 1A and B). No significant difference among groups was detected for food allergen specific IgE when comparing children according to their allergy status (Fig. 1C) but increased values of specific IgE against respiratory allergens were observed in plasma of allergic non-colonized children of healthy mothers (A NC H) in comparison to non-allergic (healthy) non-colonized children of healthy mothers (H NC H) (5.83 ± 5.6; 0.33 ± 0.07, respectively, $p = 0.0372$). Similarly, higher values of specific IgE against respiratory allergens were measured in allergic non-colonized children of allergic mothers (A NC A) in comparison to healthy non-colonized children of allergic mothers (H NC A) (2.17 ± 0.64; 0.56 ± 0.20, respectively, $p = 0.044$). Elevated values of specific IgE were detected in allergic colonized children of allergic mothers (A C A) in comparison to healthy colonized children of allergic mothers (H C A) (7.14 ± 2.33; 0.41 ± 0.09, respectively, $p = 0.00001$). Surprisingly, significantly increased specific IgE against respiratory allergens was determined in plasma of A C A in comparison to A NC A (7.14 ± 2.33; 2.17 ± 0.64, respectively, $p = 0.0304$), Fig. 1D. When comparing IgE against food allergens, no difference was detected between healthy (H) and allergic (A) children regardless of their mothers' allergy status or probiotic supplementation, Fig. 1E. Specific IgE

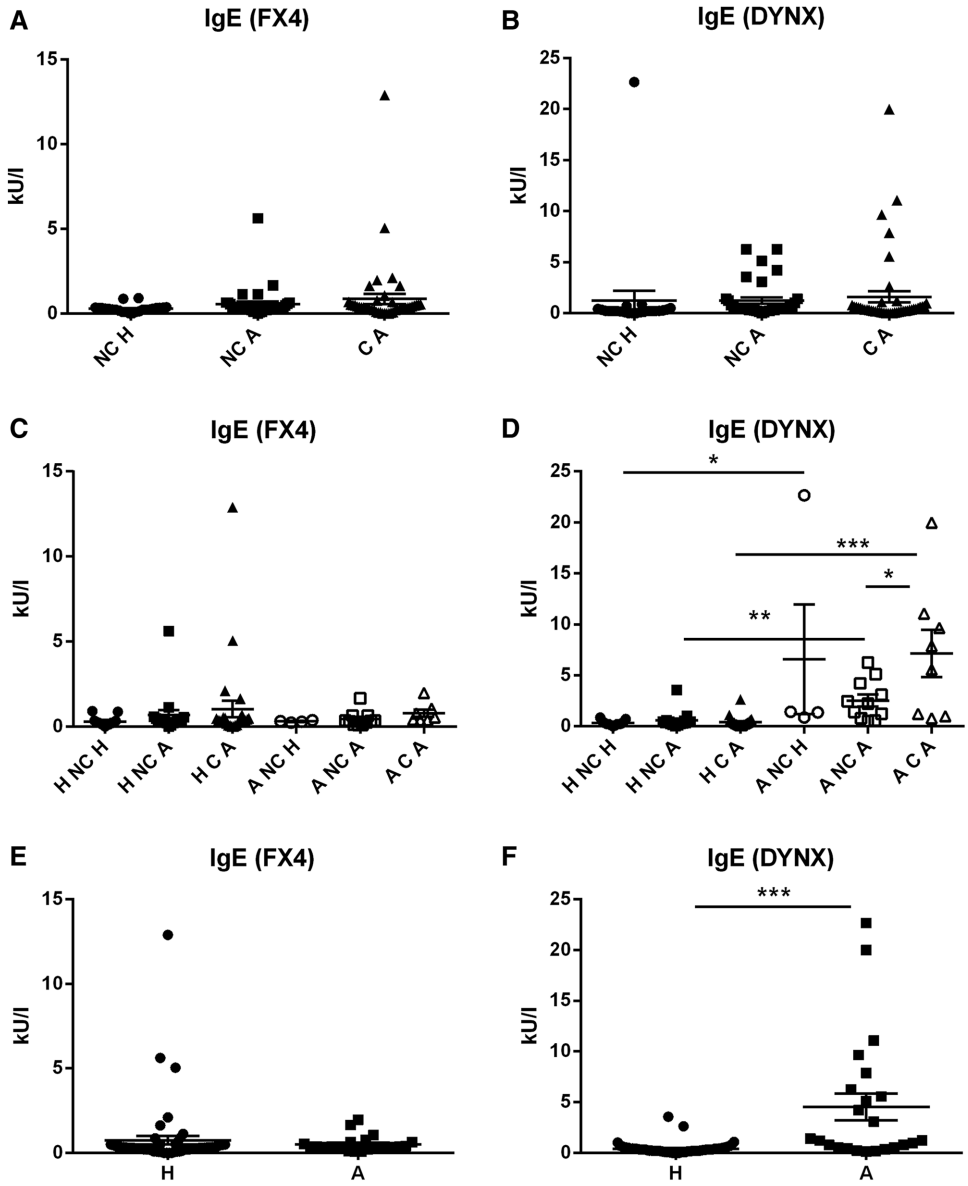

Figure 1. Detection of specific IgE antibodies. 107 eight year old children were individually screened for specific IgE antibodies in sera by ELISA in duplicates against a mixture of food allergens (FX4) and air allergens (DYNX). NC H – non-colonized children of healthy mothers, NC A – non-colonized children of allergic mothers, C A – *E. coli* O83:K24:H31 colonized children of allergic mothers, H NC H – healthy non-colonized children of healthy mothers, H NC A – healthy non-colonized children of allergic mothers, H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers, A NC H – allergic non-colonized children of healthy mothers, A NC A – allergic non-colonized children of allergic mothers, A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers, H – healthy children, A – allergic children. Data were analyzed using unpaired t-test and results are presented as a mean with error bars representing + SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

against respiratory allergens was significantly increased in A group in comparison to H group (4.54 ± 1.30; 0.44 ± 0.07, respectively, $p = 0.0001$), Fig. 1F regardless of the mother´s allergy.

Detection of specific IgG4

No significant difference in specific IgG4 against common respiratory and food allergens was observed among the basic three groups (Supporting Information Fig. 3A, 3B). It is important to mention that concentration of specific IgG4 against respiratory allergens was generally lower and under detection limit in several samples. After separation of children according to their allergy status, significantly lower levels of specific IgG4 against food allergens ($p = 0.0432$) was detected in allergic non-supplemented children of allergic mothers in comparison with non-allergic non-supplemented children of allergic mothers, Supporting Information Fig. 3C. Allergic non-supplemented children of healthy mothers have lower specific IgG4 against food allergens compared with non-allergic non-supplemented children of healthy mothers,

$p = 0.0336$, Supporting Information Fig. 3C. No difference in IgG4 against food allergens was observed among the groups of children based on probiotic supplementation and children allergy status, Supporting Information Fig. 3D. Specific IgG4 against food allergens was significantly lower in children suffering from allergy in comparison with non-allergic children ($p = 0.0427$), Supporting Information Fig. 3E. No difference between healthy and allergic children was observed in specific IgG4 against respiratory allergens, Supporting Information Fig. 3F.

Proportion of natural and induced Treg

Increased proportion of induced Tregs was detected in peripheral blood of probiotic supplemented children. Interestingly, decreased numbers of iTreg were detected in children suffering from allergy. We were interested whether early probiotic administration can influence induction of Treg and whether it will be reflected by an increased proportion of induced Treg (iTreg) versus natural Treg (nTreg) in 8-year-old children. As mentioned above, we were not able to detect significantly different percentages of total population of Treg ($CD4^+CD25^{high}FoxP3^+$) in peripheral blood among different groups based on maternal and/or children allergy status or probiotic supplementation (Fig. 2A–C).

No difference in proportion of nTreg ($CD4^+CD25^{high}FoxP3^+Helios^+$) among basic groups of non-colonized children of healthy mothers (NC H), non-colonized (NC A) and colonized (C A) children of allergic mothers was detected (Fig. 2D) but the abundance of nTreg was higher in allergic EcO83 supplemented children of allergic mothers (A C A) in comparison with healthy EcO83 supplemented children of allergic mothers (H C A) ($73.22 \pm 4.81\%$; $49.16 \pm 3.21\%$, respectively, $p = 0.0032$), Fig. 2E. Allergic children (A) have higher proportion of nTreg in comparison with healthy ones (H) ($66.86 \pm 3.33\%$; $53.01 \pm 2.34\%$, respectively, $p = 0.0034$), Fig. 2F. regardless of maternal allergy status and probiotic supplementation.

Proportion of iTreg was higher in a group of probiotic colonized children (C A) in comparison with non-colonized children of healthy mothers (NC H) ($35.01 \pm 2.71\%$; $24.86 \pm 2.71\%$, respectively, $p = 0.0198$) and non-colonized children of allergic mothers (NC A) ($35.01 \pm 2.71\%$; $25.27 \pm 2.29\%$, respectively, $p = 0.0085$), Fig. 2G. Healthy probiotic supplemented children of allergic mothers (H C A) have significantly elevated proportion of iTreg in comparison with healthy non-colonized children of healthy mothers ($38.24 \pm 2.96\%$; $25.51 \pm 3.23\%$, respectively, $p = 0.0095$) and healthy non-colonized children of allergic mothers ($38.24 \pm 2.96\%$; $25.73 \pm 2.80\%$, respectively, $p = 0.0041$). Allergic EcO83 supplemented children of allergic mothers (A C A) have lower proportion of iTreg in comparison to healthy ones (H C A) ($19.26 \pm 2.67\%$; $38.24 \pm 2.96\%$, respectively, $p = 0.0069$), Fig. 2H. Children suffering from allergy without respect to maternal allergy status and probiotic supplementation have significantly decreased proportion of iTreg in comparison to healthy children ($21.97 \pm 2.14\%$; $31.22 \pm 1.87\%$, respectively, $p = 0.0087$), Fig. 2I.

Percentage of IL-10 producing $CD4^+$ T cells and Treg in peripheral blood of 8-year-old children

EcO83 supplementation promoted secretion of immunoregulatory cytokine IL-10 in both Treg and total $CD4^+$ T lymphocytes. The capacity of Treg and $CD4^+$ T cells to produce IL-10 was characterized using flow cytometry. Intracellular presence of IL-10 in Tregs was lower in non-supplemented children of allergic mothers (NC A) in comparison with non-supplemented children of healthy mothers (NC H) (0.1; IQR 0.06-1.06% versus 1.77; IQR 0.95–5.34%, $p = 0.0003$) and supplemented children of allergic mothers (C A) (0.1; IQR 0.06–1.06% versus 1.77; IQR 1.04–6.06%, $p = 0.0002$), Fig. 3A. When intracellular presence of IL-10 was analyzed according to allergy status of children, still significantly lower presence of IL-10 in Treg was detected in healthy non-supplemented children of allergic mothers (H NC A) in comparison with healthy non-supplemented children of healthy mothers (H NC H) (0.06; IQR 0.04–1.77% versus 3.65; IQR 1.05–6.28%, $p = 0.0039$) and healthy EcO83 supplemented children of allergic mothers (H C A) (0.06; IQR 0.04–1.77% versus 2.04; IQR 1.06–6.96%, $p = 0.0032$). Significantly decreased intracellular presence of IL-10 was observed in Treg of allergic non-supplemented children (A NC H) in comparison with healthy non-supplemented children of healthy mothers (H NC H) (0.65; IQR 0.36–1.93% versus 3.65; IQR 1.05–6.28%, $p = 0.0402$). Similarly, allergic EcO83 supplemented children of allergic mothers (A C A) exerted lower levels of IL-10 in Treg in comparison with healthy EcO83 supplemented children of allergic mothers (H C A) (1.05; IQR 1.01-1.07% versus 2.04; IQR 1.06–6.96%, $p = 0.0052$), Fig. 3B. Significantly lower intracellular presence of IL-10 was measured in children suffering from allergy (A) in comparison with healthy ones (H) (1.03; IQR 0.15–1.05% versus 1.77; IQR 0.4–6.06%, $p = 0.0011$), Fig. 3C. regardless of the mother's allergy.

To characterize the capacity of other CD4 subpopulations to produce IL-10 (particularly supposed Tr1 subpopulation), the proportion of $IL\text{-}10^+$ $CD4^+$ cells was analyzed in total $CD4^+$ population excluding Treg $CD4^+CD25^{high}CD127^{low}$. Impaired production of IL-10 was detected in $CD4^+$ T cells of non-supplemented children of allergic mothers (NC A) in comparison with non-supplemented children of healthy mothers (NC H) (0.5; IQR 0.04–2.00% versus 2.01, IQR 0.37–4.49%, $p = 0.0102$) and EcO83 supplemented children of allergic mothers (C A) (0.5; IQR 0.04–2.00% versus 3.00, IQR 1.23–6.39%, $p = 0.0001$), Fig. 3D. After sorting children according to their allergic status, still more reduced presence of IL-10 is obvious in healthy non-colonized children of allergic mothers (H NC A) in comparison with healthy non-colonized children of healthy mothers (H NC H) (0.07; IQR 0.01–3.07% versus 3.02, IQR 0.57–5.89%, $p = 0.0073$) and healthy EcO83 colonized children of allergic mothers (H C A) (0.07; IQR 0.01–3.07% versus 4.05, IQR 1.61–8.42%, $p = 0.0002$). Allergic EcO83 colonized children of allergic mothers (A C A) have lower numbers of $IL\text{-}10^+CD4^+$ T cells in comparison with H C A (1.5; IQR 0.42-2.11% versus 4.05, IQR 1.61–8.42%, $p = 0.0048$), Fig. 3E. $CD4^+$ T cells of children suffering from allergy produce less IL-10

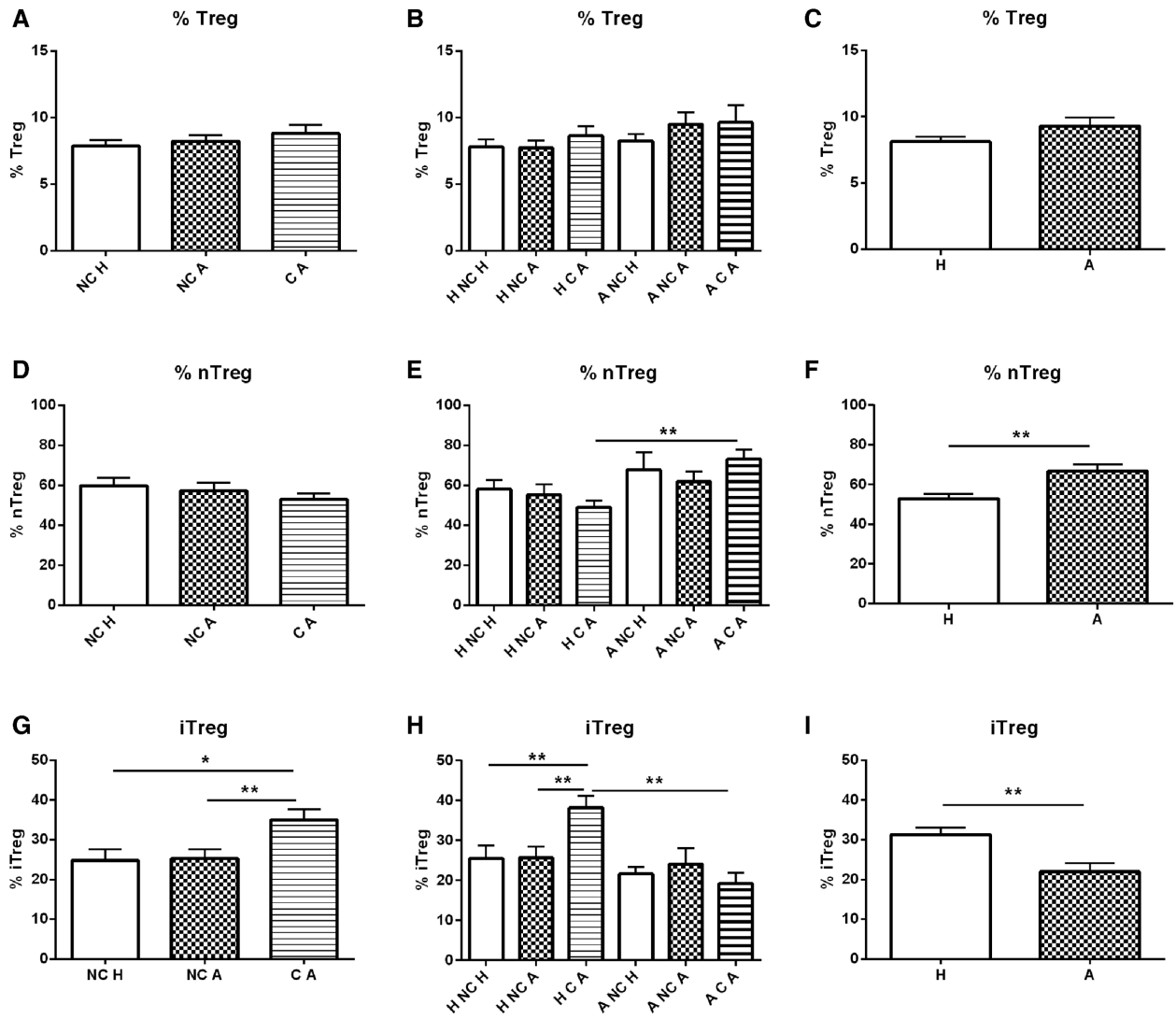

Figure 2. Characterization of Tregs in peripheral blood of 8-year-old children. Proportion of Tregs in blood was detected using flow cytometry at 107 children during regular check at eight years of age. After staining of cell surface markers CD4 and CD25 and intranuclear transcription markers FoxP3 and Helios, subpopulations of induced (iTreg) and natural (nTreg) T regulatory cells were analyzed by flow cytometry using gating strategy indicated in Supporting Information Fig. 1. NC H – non-colonized children of healthy mothers, NC A – non-colonized children of allergic mothers, C A – *E. coli* O83:K24:H31 colonized children of allergic mothers, H NC H – healthy non-colonized children of healthy mothers, H NC A – healthy non-colonized children of allergic mothers, H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers, A NC H – allergic non-colonized children of heathy mothers, A NC A – allergic non-colonized children of allergic mothers, A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers, H – healthy children, A – allergic children. Results were statistically analyzed using unpaired t-test and data are displayed as a mean +SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

than healthy children (1; IQR 0.34–2% versus 3; IQR 0.13–6.27%, $p = 0.0452$).

We were not able to detect significant differences in the presence of TGF-beta among the groups followed (data not shown).

Cytokines in plasma of peripheral blood of 8-year-old children

Probiotic supplementation elevated immunoregulatory cytokine IL-10 and Th1 cytokine IFN-γ in peripheral blood of eight year old children. In line with the literature, decreased concentrations of IFN-γ and IL-10 and elevated levels of IL-4 were present in sera of allergic children in comparison with healthy children. Cytokines typical for Th1 (IFN-γ), Th2 (IL-4) and Treg (IL-10) were followed by ELISA. Concentration of IFN-γ was significantly decreased in non-supplemented children of allergic mothers (NC A) in comparison with non-supplemented children of healthy mothers (NC H) (235.6±61.45 pg/mL; 773.8±274.2 pg/mL, respectively, $p = 0.02$). Non-supplemented children of allergic mothers (NC A) have decreased values of IFN-γ in comparison with EcO83 supplemented children of allergic mothers (C A) (235.6 ± 61.45 pg/mL; 841.9 ± 204.4 pg/mL, respectively, $p = 0.008$) documenting the capacity of EcO83 to promote IFN-γ production

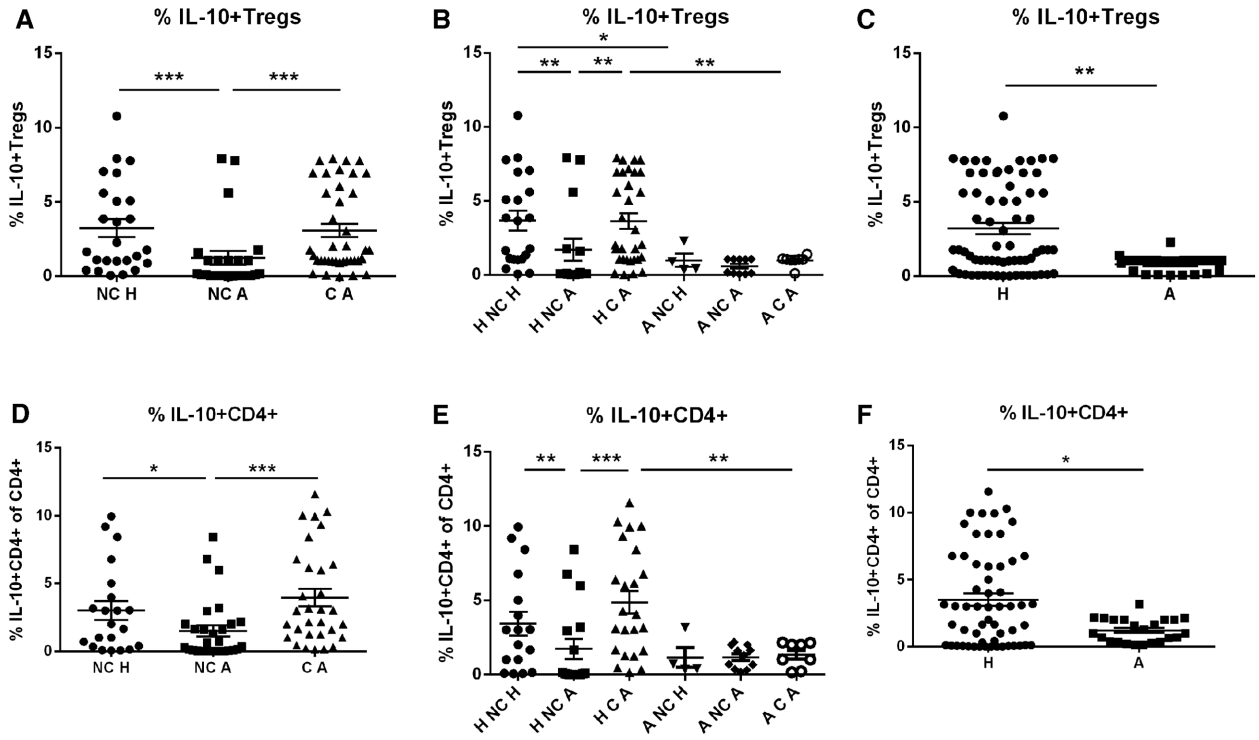

Figure 3. Detection of intracellular IL-10 in Tregs and CD4$^+$ T cells. The effect of *E. coli* O83:K24:H31 on IL-10 production was analyzed using flow cytometry in both Tregs and CD4$^+$ T cells in relation to allergy status of 107 8-year-old children. Samples of peripheral blood of children were incubated with BD GolgiPlug for preventing extracellular cytokine secretion for 5 h (107 individual samples). Then cell surface markers CD4, CD25, CD127 were stained by fluorochrome conjugated antibodies followed by red blood cell lysis. Presence of intracellular IL-10 was detected using Transcription factor buffer set. Gating strategy is showed at Supporting Information Fig. 2. NC H – non-colonized children of healthy mothers, NC A – non-colonized children of allergic mothers, C A – *E. coli* O83:K24:H31 colonized children of allergic mothers, H NC H – healthy non-colonized children of healthy mothers, H NC A – healthy non-colonized children of allergic mothers, H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers, A NC H – allergic non-colonized children of heathy mothers, A NC A – allergic non-colonized children of allergic mothers, A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers, H – healthy children, A – allergic children. Data were evaluated in FlowJo and statistical significance was tested using nonparametric Mann–Whitney test. Results are presented as a median with interquartile range. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

which is comparable with children with the low risk for allergy development, Fig. 4A. After separation of children according to their allergy status, significantly lower concentration of IFN-γ was detected in allergic non-colonized children of healthy mothers (A NC H) in comparison with healthy non-colonized children of healthy mothers (H NC H) (81.86 ± 22.63 pg/mL; 899.5 ± 317.6 pg/mL, respectively, $p = 0.0161$). Allergic non-colonized children of allergic mothers (A NC A) have lower levels of IFN-γ in comparison with healthy non-colonized children of allergic mothers (H NC A) (81.91 ± 17.92 pg/mL; 316.2 ± 88.83 pg/mL, respectively, $p = 0.0347$) and similarly, allergic EcO83 colonized children of allergic mothers (A C A) have decreased levels of IFN-γ in comparison with healthy EcO83 colonized children of allergic mothers (H C A) (127.4 ± 41.85 pg/mL; 1001 ± 242.3 pg/mL, respectively, $p = 0.05$), Fig. 4B. In addition to that, H NC A have still significantly lower IFN-γ in comparison with H NC H (316.2 ± 88.83 pg/mL; 899.5 ± 317.6 pg/mL, respectively, $p = 0.0453$) and more interestingly in comparison with H C A (316.2 ± 88.83 pg/mL; 1001 ± 242.3 pg/mL, respectively, $p = 0.02$), Fig. 4B. Decreased levels of IFN-γ were observed in children suffering from allergy in comparison with healthy ones (97.76±17.24 pg/mL; 870.4±164.3 pg/mL, respectively, $p = 0.0041$) as expected, Fig. 4C. regardless of the mother´s allergy.

Concentration of IL-4 was increased in non-supplemented children of allergic mothers (NC A) in comparison with non-supplemented children of healthy ones (NC H) (1377±283 pg/mL; 530.8±120.8 pg/mL, respectively, $p = 0.011$). Increased concentration of IL-4 was detected in plasma of EcO83 supplemented children of allergic mothers (C A) in comparison with non-supplemented children of healthy mothers (NC H) (1102 ± 174.8 pg/mL; 530.8 ± 120.8 pg/mL, respectively, $p = 0.0138$), Fig. 4D, documenting that EcO83 supplementation has not influenced levels of Th2 cytokine IL-4. After division of children according to the allergy status, significantly increased concentration of IL-4 was measured in plasma of allergic non-colonized children of healthy mothers (A NC H) in comparison with healthy non-colonized children of healthy mothers (H NC H) (1103 ± 371 pg/mL; 410.2 ± 110 pg/mL, respectively, $p = 0.0128$), allergic non-colonized children of allergic mothers (A NC A) in comparison with healthy non-colonized children of allergic mothers (H NC A) (2085 ± 644.3 pg/mL; 1038 ± 267 pg/mL;

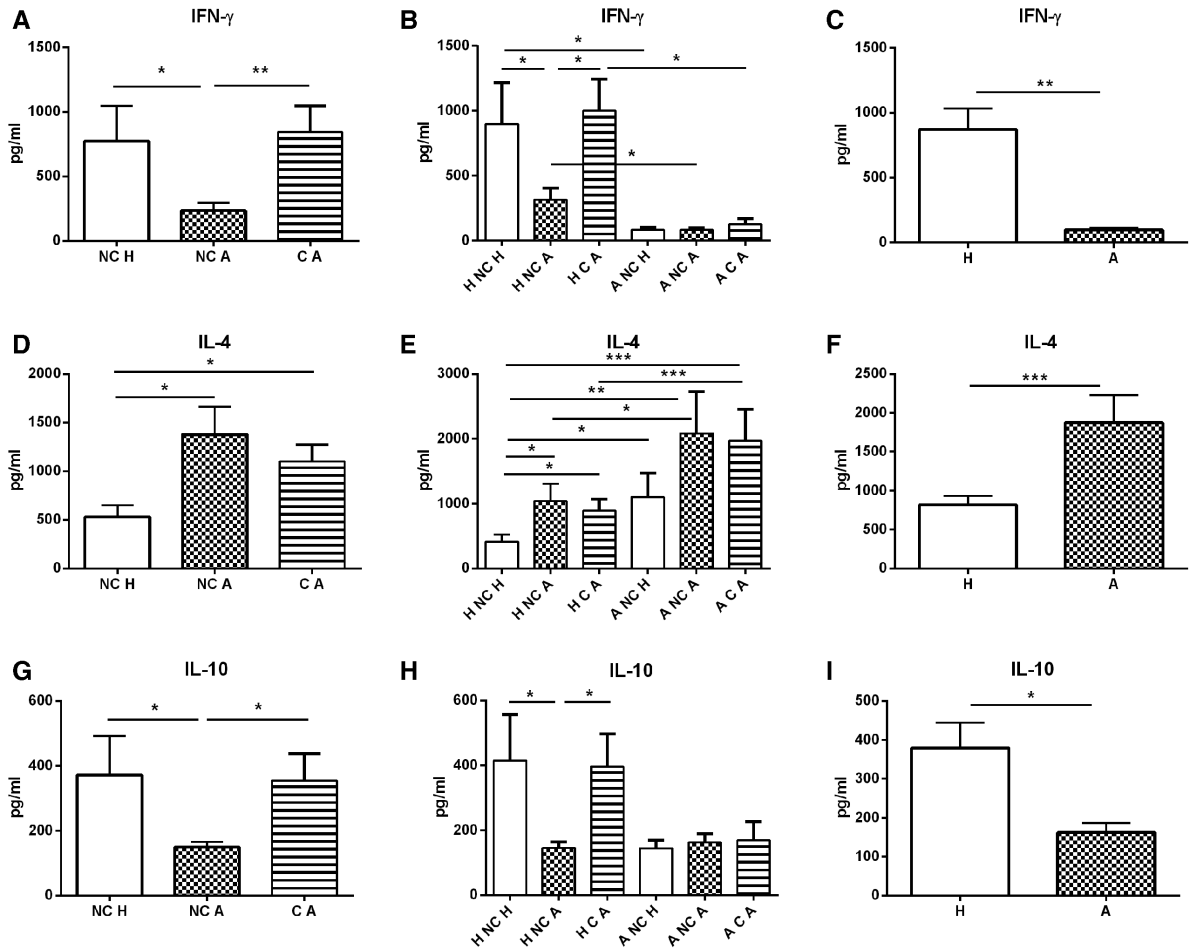

Figure 4. Detection of cytokines in plasma of 8-year-old children. Cytokines characteristic for Th1 (IFN-γ), Th2 (IL-4) and Tregs (IL-10) were measured in plasma of probiotic supplemented and non-supplemented 107 8-year-old children collected during regular checks by ELISA using primary and biotinylated antibodies. Assays were ran in duplicates. NC H – non-colonized children of healthy mothers, NC A – non-colonized children of allergic mothers, C A – *E. coli* O83:K24:H31 colonized children of allergic mothers, H NC H – healthy non-colonized children of healthy mothers, H NC A – healthy non-colonized children of allergic mothers, H C A – healthy *E. coli* O83:K24:H31 colonized children of allergic mothers, A NC H – allergic non-colonized children of healthy mothers, A NC A – allergic non-colonized children of allergic mothers, A C A – allergic *E. coli* O83:K24:H31 colonized children of allergic mothers, H – healthy children, A – allergic children. Data were statistically evaluated using unpaired *t*-test and presented as mean +SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

respectively, $p = 0.0417$) and allergic EcO83 supplemented children of allergic mothers (A C A) in comparison with healthy EcO83 supplemented children of allergic mothers (H C A) (1970 ± 480.1 pg/mL; 897.3 ± 169.5 pg/mL, respectively, $p = 0.007$), Fig. 4E. Non-colonized healthy children of allergic mothers (both EcO83 supplemented and non-supplemented) have increased values of IL-4 in comparison to non-colonized healthy children of healthy mothers (H NC H) (1038 ± 267 pg/mL; 410.2 ± 110 pg/mL, respectively, $p = 0.025$). Healthy EcO83 supplemented children of allergic mothers have higher concentration of IL-4 in plasma in comparison with non-colonized healthy children of healthy mothers (H NC H) (897.3 ± 169.5 pg/mL; 410.2 ± 110 pg/mL, respectively, $p = 0.0247$), Fig. 4E. Increased levels of IL-4 in plasma of children suffering from allergy in comparison to healthy ones were detected (1875 ± 353.3 pg/mL; 818.2 ± 116.2 pg/mL, respectively, $p = 0.0002$), Fig. 4F regardless of the mother's allergy.

Significantly lower concentration of IL-10 was observed in non-colonized children of allergic mothers (NC A) in comparison with non-colonized children of healthy mothers (NC H) (149.9 ± 15.53 pg/mL; 371.9 ± 120.7 pg/mL, respectively, $p = 0.0179$). Importantly, probiotic supplemented children (C A) have substantially increased levels of IL-10 in comparison with NC A (354.7 ± 83.58 pg/mL; 149.9 ± 15.53 pg/mL, respectively, $p = 0.016$), Fig. 4G. After division of children according to their allergy status, significantly lower IL-10 was observed in plasma of healthy non-colonized children of allergic mothers (H NC A) in comparison to healthy non-colonized children of healthy mothers (H NC H) (145.9 ± 18.66 pg/mL; 415.1 ± 142.2 pg/mL, respectively, $p = 0.0196$) and healthy EcO83 colonized children of allergic mothers (H C A) (145.9 ± 18.66 pg/mL; 397.1 ± 100.8 pg/mL, respectively, $p = 0.0173$). Allergic non-colonized children of healthy mothers (A NC H) and allergic EcO83 colonized children of allergic mothers (A C A) have lower levels of

IL-10 in comparison to healthy non-colonized children of healthy mothers (H NC H) and healthy EcO83 colonized children (H C A), respectively, but it does not reached statistical significance probably due to the low number of allergic children and high individual variability in the groups of H NC H and H C A, Fig. 4H. Concentration of IL-10 was significantly decreased in plasma of allergic children (A) in comparison with healthy ones (H) (162.1 ± 24.88 pg/mL; 379.9 ± 64.15 pg/mL, respectively, $p = 0.0492$), Fig. 4I. regardless of the mother´s allergy.

Changes in gene expression of cytokines in CBMC after EcO83 stimulation

EcO83 promotes IL-10 and IFN-ɣ expression in CBMC in vitro. To evaluate the effect of EcO83 on CBMC in vitro, cytokines characteristic for Th1 (IFN-ɣ), Th2 (IL-4) and Treg (IL-10) were estimated by qPCR. IFN-ɣ was increased in LPS, EcO83 and EcN stimulated CBMC in comparison to nonstimulated CBMC ((32.92; IQR 7.69-176.5 versus 0.88; IQR 0.63-1.77, respectively, $p = 0.00001$); ((33.72; IQR 11.4-103.5 versus 0.88; IQR 0.63-1.77, respectively, $p = 0.00001$)) and ((4.94; IQR 3–16.7 versus 0.88; IQR 0.63-1.77, respectively, $p = 0.0002$)) Fig. 5A. To compare possible different reactivity of CBMC of neonates of healthy mothers and CBMC of newborns of allergic mothers, children were divided into two subgroups based on maternal allergy status. Significantly increased IFN-ɣ expression was observed after LPS (healthy group: 33.73; IQR 12.4-218.4 versus 0.95, IQR 0.59-1.83, $p = 0.00001$, allergic group: 24.77, IQR 7.69-196.7 versus 0.88, IQR 0.73-1.67, $p = 0.00001$) and EcO83 stimulation (healthy group: 27.44, IQR 11.69-108 versus 0.95, IQR 0.59-1.83, $p = 0.00001$, allergic group: 45.23, IQR 10.89-103.4 versus 0.88, IQR 0.73-1.67, $p = 0.00001$) without any significant difference between children of healthy and allergic mothers. After EcN stimulation, significantly increased gene expression of IFN-γ was detected only in CBMC of newborns of healthy mothers (14.92; IQR 11.7-27.4 versus 0.95, IQR 0.59-1.83, $p = 0.00001$), Fig. 5B.

No significant effect of EcO83 and EcN on IL-4 expression in CBMC was detected, Fig. 5C. Moreover, CBMC of children of healthy and allergic mothers have similar IL-4 expression, Fig. 5D.

Gene expression of IL-10 was elevated after LPS (6.38, IQR 3.02-7.19 versus 0.88, IQR 0.65-1.35, $p = 0.00051$), EcO83 (8.12, IQR 5.025-13.96 versus 0.88, IQR 0.65-1.35, $p = 0.00001$) and EcN (3, IQR 1.2-7.9 versus 0.88, IQR 0.65-1.35, $p = 0.0082$) stimulation in comparison to non-stimulated CBMC. Gene expression of IL-10 in CBMC was significantly higher after EcO83 stimulation in comparison to the effect of LPS (8.12, IQR 5.025-13.96 versus 6.38, IQR 3.02-7.19, $p = 0.0374$), Fig. 5E. After division of children according to the maternal allergy status, still increased values of IL-10 expression was observed after LPS (healthy group 5.56, IQR 1.83-8.45 versus 1, IQR 0.75-1.24, $p = 0.003$, allergy group: 7.28, IQR 4.97-12.13 versus 1.07, IQR 0.67-1.81, $p = 0.0015$) and EcO83 (healthy group: 10.26, IQR 4.81-14.33 versus 1, IQR 0.75-1.24, $p = 0.0001$, allergy group: 8.74, IQR 6.73-18.66 versus 1.07, IQR 0.67-1.81, $p = 0.0001$) stimulation but significantly increased values of IL-10 after EcO83 stimulation in comparison with LPS were observed only in CBMC of children of healthy mothers (10.26, IQR 4.81-14.33 versus 5.56, IQR 1.83-8.45, $p = 0.0497$). After EcN stimulation, significantly increased gene expression of IL-10 was observed only in CBMC of neonates of healthy mothers (6.2, IQR 3.5-8.2 versus 1, IQR 0.75-1.24, $p = 0.0026$), Fig. 5F.

Secretion of cytokines by EcO83 stimulated CBMC

Significantly elevated concentration of IFN-ɣ and IL-10 was observed in cell culture supernatants of CBMC stimulated with EcO83. The capacity of EcO83 stimulation to promote cytokine production was tested in cell culture supernatants by ELISA. IFN-ɣ was increased after LPS (54.62, IQR 3.9-92.18 pg/mL; 33.62, IQR 3.9-92.18 pg/mL, respectively, $p = 0.0425$) and EcO83 stimulation in comparison to non-stimulated CBMC (50.96, IQR 14.19-94.94 pg/mL; 33.62, IQR 3.9-92.18 pg/mL, respectively, $p = 0.0358$) stimulation in comparison with non-stimulated CBMC. Interestingly, secretion of IFN-γ by CBMC was increased after EcO83 stimulation in comparison with EcN stimulation (50.96, IQR 14.19-94.94 pg/mL; 15.5, IQR 3.9-37.5 pg/mL, respectively, $p = 0.0057$). Production of IFN-γ after EcN stimulation was lower in comparison to LPS as well (15.5, IQR 3.9–37.5 pg/mL; 54.62, IQR 3.9–92.18 pg/mL, respectively, $p = 0.0026$), Fig. 6A. After division of children according to the maternal allergy status, only significantly increased IFN-γ production was observed after EcO83 stimulation of CBMC of newborns of allergic mothers in comparison to control CBMC (64.99, IQR 10.6-100.6 pg/mL; 12.2, IQR 3.9-52.47 pg/mL; $p = 0.035$). Promotion of IFN-γ secretion by CBMC of neonates of healthy mothers after LPS and EcO83 stimulation was on the border of significance, Fig. 6B.

No difference in IL-4 secretion by CBMC stimulated with either LPS or EcO83 and EcN was observed, Fig. 6C, but after sorting children according to the maternal allergy status, significantly increased concentration of IL-4 was detected in cell culture supernatants of non-stimulated (166.2, IQR 3.9-384.8; 3.9 pg/mL, IQR 3.9-62.28 pg/mL, $p = 0.0239$) and LPS (238.4, IQR 3.9-493.6 pg/mL, 3.9, IQR 3.9-134 pg/mL, $p = 0.0175$), EcO83 (240.4, IQR 39.86-398.4 pg/mL, 3.9, IQR 3.9-132 pg/mL, $p = 0.026$) and EcN (238.8, IQR 3.9-442.6 pg/mL, 3.9, IQR 3.9-63 pg/mL, $p = 0.0038$) stimulated CBMC of children of allergic mothers in comparison with healthy ones, Fig. 6D.

Stimulation of CBMC by LPS (1020, IQR 584.3-1465 pg/mL; 76.42, IQR 52.26-110.7 pg/mL, $p = 0.0001$), EcO83 (1293, IQR 934.2-2528 pg/mL; 76.42, IQR 52.26-110.7 pg/mL, $p = 0.0001$) and EcN (642.4, IQR 232.7-1322 pg/mL; 76.42, IQR 52.26-110.7 pg/mL, $p = 0.0001$) increased IL-10 secretion with EcO83 being more efficient in comparison to LPS (1293, IQR 934.2-2528 pg/mL; 1020, IQR 584.3-1465 pg/mL, $p = 0.0161$) and EcN (1293, IQR 934.2-2528 pg/mL;642.4, IQR 232.7-1322 pg/mL, $p = 0.002$), Fig. 6E. After separation of children according to maternal allergy status, significantly increased levels of IL-10 were observed in LPS (733.6, IQR

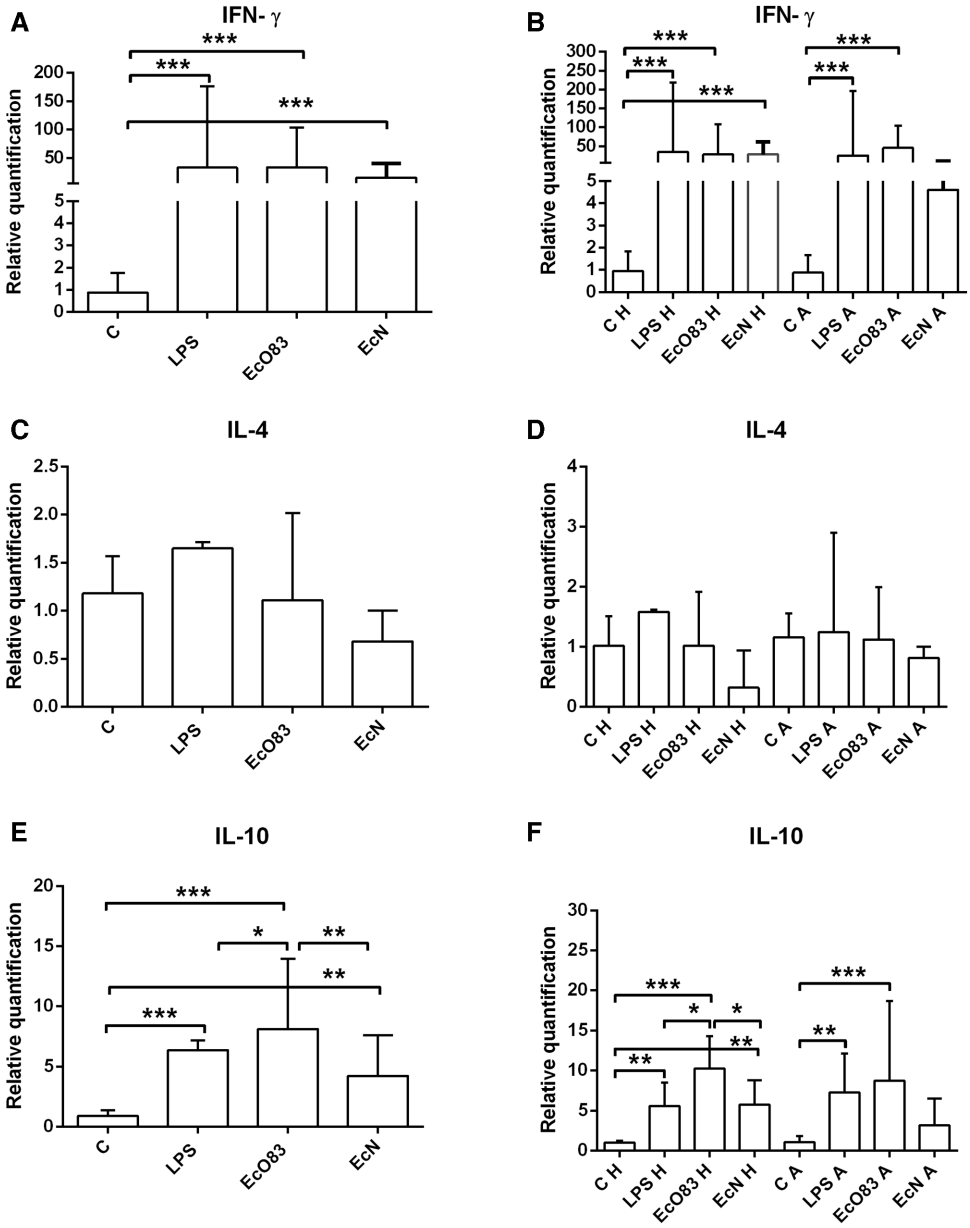

Figure 5. The effect of probiotic strain *E. coli* O83:K24:H31 on gene expression of cytokines. The capacity of *E. coli* O83:K24:H31 to change gene expression in CBMC was evaluated using quantitative real-time PCR. Results represent 59 individual experiments (32 cord blood samples of newborns of healthy mothers and 27 cord blood samples of newborns of allergic mothers). After stimulation of CBMC with bacterial strains or LPS, RNA was extracted followed by reverse transcription and quantitative real-time PCR using TaqMan reagents. PCR reaction were ran in duplicates. Actin Beta was used as endogenous control for normalization ($2^{-\Delta\Delta ct}$) of gene expression of target genes.C – control (non-stimulated) CBMC, LPS – lipopolysaccharide stimulated CBMC, EcO83 - *E. coli* O83:K24:H31 stimulated CBMC, C H control CBMC of newborns of healthy mothers, LPS H - lipopolysaccharide stimulated CBMC of newborns of healthy mothers, EcO83 H - *E. coli* O83:K24:H31 stimulated CBMC of newborns of healthy mothers, C A - control CBMC of newborns of allergic mothers, LPS A - LPS H - lipopolysaccharide stimulated CBMC of newborns of allergic mothers, EcO83 A - *E. coli* O83:K24:H31 stimulated CBMC of newborns of allergic mothers. Non-parametric Mann-Whitney test was employed for statistical evaluation of gene expression. Results are presented as median with interquartile range. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

422.3-1407 pg/mL; 62.2, IQR 42.5-112.6 pg/mL, $p = 0.0001$), EcO83 (1159, IQR 757.4-1304 pg/mL; 62.2, IQR 42.5-112.6 pg/mL, $p = 0.00001$) and EcN (584.3, IQR 219.8-1176 pg/mL; 62.2, IQR 42.5-112.6 pg/mL, $p = 0.0079$) stimulated CBMC of children of healthy mothers and LPS (1271, IQR 773.5-1565 pg/mL; 88.06, IQR 58.92-152.4 pg/mL, $p = 0.00001$), EcO83 (2370, IQR 1300–2660 pg/mL; 88.06, IQR 58.92-152.4 pg/mL, $p = 0.00001$) and EcN (781, IQR 237–1545 pg/mL; 88.06, IQR 58.92-152.4 pg/mL, $p = 0.00019$) stimulated CBMC of children of allergic mothers. Significantly increased production of IL-10 by EcO83 stimulated CBMC in comparison to LPS stimulated CBMC was observed only in children of allergic mothers

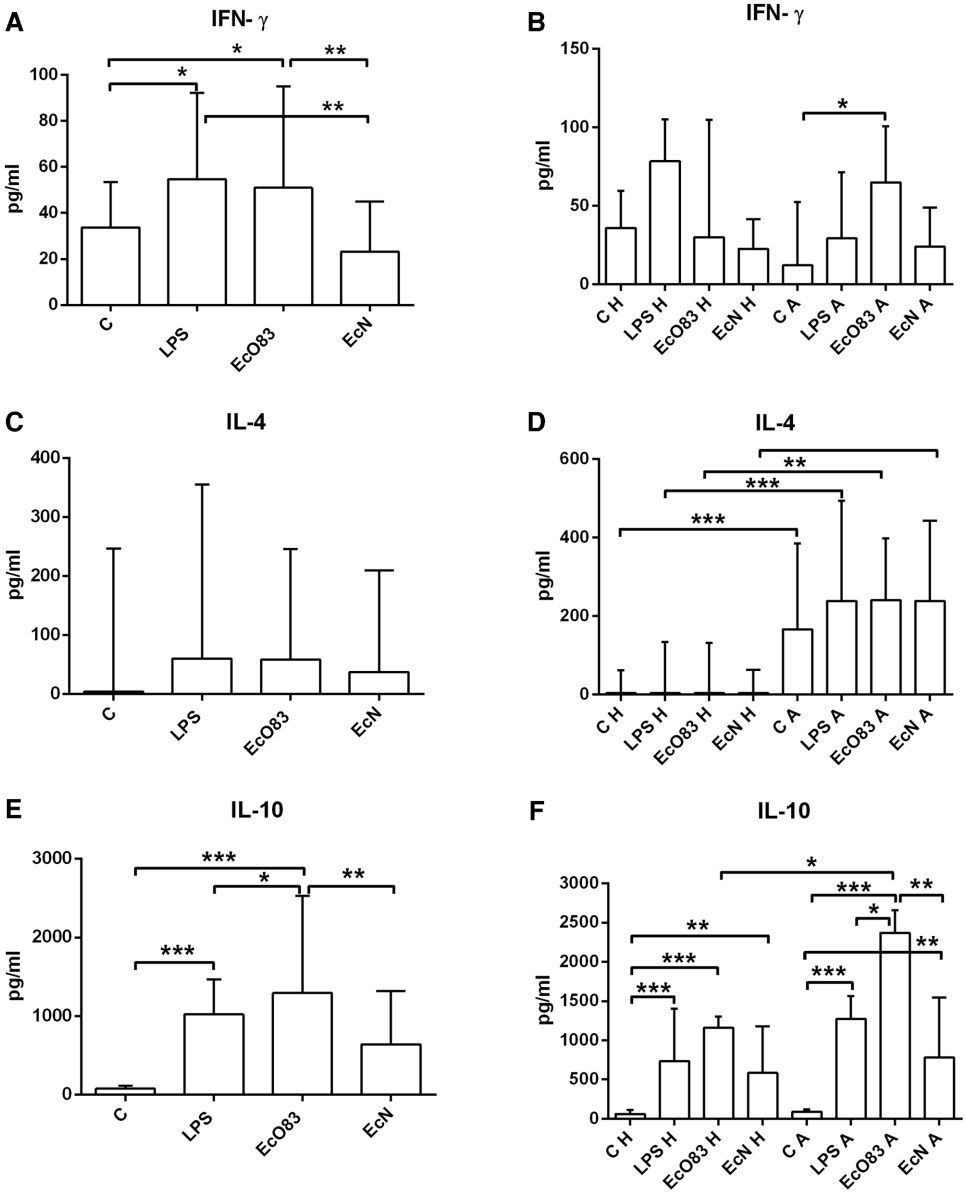

Figure 6. The effect of *E. coli* O83:K24:H31 on secretion of cytokines. Cytokines in cell culture supernatants of *E. coli* O83:K24:H31 and LPS stimulated CBMC were inspected by ELISA. Results represent 59 individual experiments (32 cord blood samples of neonates of healthy mothers and 27 cord blood samples of neonates of allergic mothers). After CBMC stimulation, cell culture supernatants were harvested and frozen for detection of cytokines by ELISA. Primary and biotinylated secondary antibodies for detection of IL-4, IL-10 and IFN-γ were used. Experiments were assayed in duplicates. C – control (non-stimulated) CBMC, LPS – lipopolysaccharide stimulated CBMC, EcO83 - *E. coli* O83:K24:H31 stimulated CBMC, C H control CBMC of newborns of healthy mothers, LPS H - lipopolysaccharide stimulated CBMC of newborns of healthy mothers, EcO83 H - *E. coli* O83:K24:H31 stimulated CBMC of newborns of healthy mothers, C A - control CBMC of newborns of allergic mothers, LPS A - LPS H - lipopolysaccharide stimulated CBMC of newborns of allergic mothers, EcO83 A - *E. coli* O83:K24:H31 stimulated CBMC of newborns of allergic mothers, Results were statistically evaluated using non-parametric Mann-Whitney test and are displayed as median with interquartile range. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

(2370, IQR 1300–2660 pg/mL; 1271, IQR 773.5-1565 pg/mL, $p = 0.0203$). Moreover, EcO83 stimulated CBMC of children of allergic mothers produced more IL-10 in comparison with children of healthy mothers (2370, IQR 1300–2660 pg/mL; 1159, IQR 757.4-1304 pg/mL, $p = 0.0152$). In addition to that, IL-10 secretion by EcO83 stimulated CBMC of neonates of allergic mothers was higher in comparison to EcN stimulation (2370, IQR 1300–2660 pg/mL; 781, IQR 237–1545 pg/mL, $p = 0.0046$), Fig. 6F.

Discussion

Early supplementation of newborns at high risk of allergy (children of allergic mothers) with probiotic EcO83 reduced development of allergic diseases in comparison to non-supplemented children. Eight year old probiotic supplemented children have, in comparison of non-supplemented children, increased proportion of IL-10^{+}Tregs and CD4^{+} T cells (excluding FoxP3^{+}Tregs),

elevated values of IFN-γ and IL-10 in plasma of peripheral blood suggesting that EcO83 supplementation promoted the function of both Treg and Th1 cells. The capacity of EcO83 to support IFN-γ and IL-10 expression and secretion was confirmed in vitro when stimulating CBMC. We were not able to detect the effect of EcO83 on the concentration of IL-4 in plasma of probiotic supplemented children. Similarly, EcO83 did not influence gene expression of IL-4 in CBMC but the production of IL-4 was increased in cell culture supernatants of CBMC of newborns of allergic mothers indicating increased Th2 immune response in predisposed children already at the time of birth.

Numerous studies using various probiotic strains described contradictory results regarding the protective effect of probiotic supplementation in allergy prevention in children [26]. Generally, early colonizing bacteria are expected to promote neonatal immune system maturation [27] and set a balance among particular immune responses [28]. It is important to emphasize that the key factors contributing to the successful preventive measure are careful probiotic strain selection, suitable dose of bacteria administered and especially timing of probiotic administration. Interestingly, usage of one probiotic strain could lead to contradictory results when used in different countries in slightly different settings [29–31]. According to the literature, it seems that either prenatal supplementation of pregnant women [17, 18] or early postnatal [19, 20, 23] supplementation of newborns by probiotics are the most successful strategies preventing allergy development. In our study, the first dose of EcO83 was administered within 48 hrs after delivery and children were followed prospectively. We suppose that immunomodulatory effect of EcO83 in the critical perinatal period could have long lasting effect even after eventual disappearance EcO83 from the intestinal microbiota of supplemented children later in life. Appropriate probiotics can probably exert their anti-allergy effect only when applied before the individual microbiota is definitely settled.

Newborns have not sufficiently developed Th1 immune responses and this situation can promote the allergy development and therefore it is critical for allergy prevention to support Th1 immune response during maturation of neonatal immune system together with setting tolerance to a broad spectra of antigens including food antigens and microbial compounds of residing microbiota. We assumed that one of possible mechanisms behind the effect of probiotic supplementation in allergy prevention could be the potential of probiotic bacteria to increase proportion and functional capacity of Tregs. The potential of bacteria to induce Treg has been demonstrated both in vitro [32] and in vivo [33]. The key role of Tregs in setting and maintaining the tolerance to allergens has been shown in mouse model [34] and in human studies [35]. We were not able to detect increased numbers of Tregs ($CD4^+CD25^+CD127^{low}FoxP3^+$) in peripheral blood of eight year old children supplemented with EcO83 in contrary to the study using *Bifidobacterium infantis* [33] but rather proportion of $IL\text{-}10^+$ Tregs and $IL\text{-}10^+CD4^+$ T cells were heightened . IL-10 plays a key role in successful allergen immunotherapy [36] or limitation of the severity of allergic airway inflammation documented in an experimental model where IL-10 was deleted in Tregs [36]. Therefore, the capacity of probiotic strain to support IL-10 production is highly desirable and can be considered as one of the mechanisms contributing to the prevention of allergy origination.

Contradictory information regarding the proportion of Tregs in patients suffering from allergy were published [37–39]. Previously, we have shown increased values of Tregs in peripheral blood of six year old children suffering from allergy [24]. In the current report, only non-significantly increased proportion of Tregs was found in peripheral blood of allergic children. This discrepancy could be caused by sampling at different part of allergy season [40], different number of children inspected in the current study and the usage of other kit for Treg detection together with altered panel of antibodies because more parameters were tested in the present work. Especially utilization of distinct clones of antibody against FoxP3 could lead to the controversy on Treg proportion as every clone binds to various epitopes on FoxP3 which can be influenced by activation status of Tregs and this could further contribute to the inconsistency [12, 41–43].

No induction of Tregs ($CD4^+FoxP3^+$) was observed after stimulation of CBMC with EcO83 in vitro (data not shown). On the other hand, EcO83 promoted gene expression and secretion of IL-10 in CBMC. We were able to prove in our previous work that probiotic bacteria primed dendritic cells (DC) did not promote induction of Tregs but rather supported immunoregulatory cytokine IL-10 secretion from both DC and $CD4^+$ T cells cocultured with EcO83 primed DC in vitro [44]. The capacity of EcO83 to promote IL-10 is vital for the development of tolerance to food antigens and microbiota compounds by neonatal immune system. It is important to emphasize that we considered Tregs as $CD4^+FoxP3^+$ T cells (in in vitro experiments) but other $CD4^+$ regulatory subpopulations without FoxP3 expression were described. Notably, Tr1 represents a subpopulation of $CD4^+$ T cells not expressing FoxP3 but producing a large amount of IL-10 [45]. The crucial role of Tr1 ($CD4^+IL\text{-}10^+$) was observed in management of allergic diseases especially during the successful specific allergen immunotherapy [46, 47]. In agreement with previous observation [24], we have detected increased proportion of IL-10 producing Tregs ($CD4^+CD25^{high}CD127^{low}FoxP3^+$) in peripheral blood of probiotic supplemented children of allergic mothers in comparison to non-supplemented children of allergic mothers. It was difficult to properly characterize and distinguish Tr1 from other Treg subpopulations or IL-10 producing CD4 T-lymphocytes due to lack of Tr1 specific markers. Nowadays, usage of additional markers (CD49b, lymphocyte-activation gene 3 - LAG3) enable identification of Tr1 more precisely [48]. Based on our observation of increased proportion of $IL\text{-}10^+FoxP3\text{-}CD4^+$ T cells, we are suggesting that EcO83 could promote Tr1 origination but implementation of recently described markers CD49b and LAG3 would be beneficial for clear conclusion.

Induced Treg (iTreg) are believed to be more important in setting tolerance to allergens than nTregs which are rather responsible for maintaining tolerance to autoantigens [49]. As expected, allergic children have lower proportion of iTreg ($CD4^+CD25^{high}CD127^{low}FoxP3^+Helios^-$) suggesting impaired generation of iTreg together with lower IL-10 production but

there was no significant difference in total Treg population ($CD4^+CD25^{high}CD127^{low}FoxP3^+$). Increased proportion of nTreg in allergic children compared to non-allergic ones could be simply explained as a consequence of decreased percentage of iTreg in allergic children because frequencies of both iTreg and nTreg are reported as a proportion of total population of Treg. As mentioned above, various subpopulations of iTreg were described including Tr1 ($CD4^+IL\text{-}10^+FoxP3-$). We have shown that both subpopulations of iTreg ($CD4^+CD25^{high}CD127^{low}FoxP3^+Helios^-$ and Tr1) were downregulated in children suffering from allergy and probiotic supplementation promoted induction of iTreg. The potential of probiotics to support iTreg origination was reported previously [50].

There is no doubt about the critical role of immunoregulation in allergy prevention. EcO83 induced IL-10 could preclude development of clinical signs of allergic diseases. This is supported by the fact that probiotic supplemented children at high risk for allergy development have still elevated levels of specific IgE, especially against food allergens, without any clinical signs of allergy. Similarly, no change in venom specific IgE during successful venom immunotherapy was observed but increase in IgG4 was described [51]. Nouri-Aria et al. reported that increased IL-10 production during grass pollen immunotherapy promoted IgG4 secretion [52]. Increased levels of IL-10 and IFN-γ secretion after EcO83 stimulation is in agreement with previous work published by Cosmi et al. on PBMC of patients after sublingual immunotherapy [53]. These observations highlighted the importance of balanced immunoregulation and particularly IL-10 production induced by EcO83 in prevention of allergy development in probiotic supplemented children.

The capacity of EcO83 to enhance Th1 immune response being limited at the time of delivery could be other mechanism involved in prevention of allergy development in predisposed children supplemented with EcO83. The importance of early induction of Th1 is highlighted by Prescott et al. stating that delayed maturation of neonatal immune system together with prolonged increased Th2 immune response facilitate allergy origination [6]. Both cord blood sera of probiotic supplemented children and cell cultures stimulated with EcO83 have increased levels of IFN-γ. In agreement with our observation, the capacity of various probiotic bacteria to induce Th1 immune response was reported. The capacity of EcO83 to induce immune responses in vitro was compared with other probiotic strain EcN commonly used for treatment of gastrointestinal disorders. In our experimental settings, EcO83 has a superior capacity to promote IL-10 and IFN-γ secretion by CBMC. We acknowledge that the time interval for determination of gene expression of cytokines could be shorter but we have decided to analyse gene expression after 20hrs of stimulation because at this time gene expression of IL-10 after EcO83 stimulation was the most prominent and expression of IFN-γ was still on the high level in our pilot experiments. It is possible different time intervals are optimal for EcN stimulation. In addition to that, analysis of more cytokines representing other immune responses (e.g. Th9, Th17, Th22) would be helpful to better understand the effect of EcO83 on immature neonatal immune system.

Neonatal immune system can be characterized as immature with Th2 bias. Therefore, equilibration of Th1 and Th2 together with tuning of immunoregulatory responses is highly desirable during the first months of life. We hypothesized that EcO83 supplementation promotes neonatal immune system maturation via promotion of Th1 and Treg while suppressing Th2. Contrary to our hypothesis, there was no effect of EcO83 on the decrease of IL-4 both in vitro and probiotic supplemented children. In our study, elevated concentration of IL-4 in peripheral blood was detected. Similar concentration of IL-4 was reported by Hohnoki et al. [54] although majority of reports indicate levels of IL-4 within the range 1–10pg/mL. Some other probiotic strains (e.g. *Lactobacillus acidophilus* CCFM137 [55, 56], *Lactobacillus plantarum* [57], *Lactobacillus paracasei* L9 [58]) pose the capacity to downregulate Th2 immune response indicating that immunomodulatory properties are strongly strain dependent. Increased levels of IL-4 were detected in cell culture supernatants of CBMC of newborns of allergic mothers in comparison to infants of healthy mothers. This observation is in line with the work reported previously [59] confirming that the immune system of newborns of allergic mothers is altered and thus predisposing children to later allergy development.

Until now, the exact mechanisms of mutual interaction between microbiota and host immune system are not fully understood despite growing body of evidence that microbiota residing in neonatal intestinal tract early after the birth plays a key role in setting proper immune effector responses as well as tolerance to a broad spectra of antigens from environment and compounds of microbiome. There is a need for further studies on a larger cohort to confirm our observation of the capacity of EcO83 to promote immunoregulation together with elevation of Th1 immune responses. Probably, it would be interesting to use a mixture of strains instead of one single strain. This mixture should include probiotic bacteria with the capacity to downregulate Th2 representing more effective and universal probiotic supplement to be used as a preventive measure limiting allergy development and promoting proper immune system maturation.

Conclusion

We have shown the preventive effect of early supplementation of newborns with EcO83 on allergy development. It is suggested that beneficial effect of EcO83 could be mediated by promoting maturation of neonatal immune system including setting appropriate immunoregulatory responses and induction of Th1 immune response. Children supplemented with EcO83 have increased numbers of Treg and $CD4^+$ T lymphocytes secreting IL-10. To contribute to the elucidation of mechanisms of the beneficial effect of EcO83 on immature neonatal immune system, the stimulation of CBMC with EcO83 was followed and its stimulatory effect on expression and secretion of IL-10 and IFN-γ was demonstrated. Further studies are needed to confirm our observation and clarify the effect of EcO83 on immature neonatal immune system.

Materials and methods

Subjects

Healthy and allergic mothers without any substantial complication during the course of pregnancy and children delivered vaginally at full term in the Institute for the Care of Mother and Child in Prague, Czech Republic were included into the study after the signed written informed consent. As allergic mother was considered pregnant woman with clinical manifestation of allergy persisting for period longer than 24 months before pregnancy. Allergic diseases of mothers were heterogeneous - various kinds of food and pollen allergy, allergy to insect (including bee and wasp venom), eczema, asthma or combination of more allergic symptoms (including contact dermatitis). For the statistical evaluation, the differences among allergic symptoms were not considered. After division into particular subgroups according to the type of allergic disease, the low number of individuals in every group prevented valid statistical analyses. Mothers suffering from diabetes, autoimmune disease, all forms of coeliac disease, cancer or pregnant women with recorded transfusions, repeated abortions, conceived after in vitro fertilization or with multiparous pregnancy were excluded from the study. The study was approved by the Ethical Committee of the Institute for the Care of Mother and Child (Prague, Czech Republic).

Long-term effect of early postnatal supplementation with EcO83 on allergy prevention

Children of allergic mothers (considered to be at higher risk for allergy development) were supplemented with Colinfant Newborn (kindly provided by Dyntec, plc.) containing probiotic strain *Escherichia coli* O83:K24:H31 (EcO83). The first probiotic dose was given within 48 h after the delivery, in total, 12 doses were administered every third day; one dose consisting of approximately 1.2×10^8 colony forming units (CFU) of EcO83 ($0.8 \times 10^8 - 1.6 \times 10^8$ CFU)). Non-supplemented children of healthy and allergic mothers were involved as well. Children were born during 2001–2006 and were followed prospectively. Their peripheral blood was collected during regular check every year. Here, we are presenting data of 8-year-old children. There were three basic groups of children as follow: non-colonized children of healthy mothers (NC H); non-colonized children of allergic mothers (NC A) and EcO83 colonized children of allergic mothers (C A). In the group of non-supplemented children of healthy mothers, we were able to recruit 25 children for regular check from original 45 children involved in the study, in the group of non-supplemented children of allergic mothers 36 individuals (from original 57 children) came to regular check and 46 probiotic supplemented children of allergic mothers (from original 56 children) visited check at the age of 8 years. Based on children allergy status at the age of 8 years, three basic groups (NC H; NC A; C A) were divided into six subgroups characterized in Supporting Information Table 2. Allergy status of 8-year-old children was followed by allergist (parents reports of allergy manifestation was confirmed by allergist either by positive skin prick tests and/or positive specific IgE antibodies). Allergy status of children is presented in Supporting Information Table 1. Immunological characteristics of peripheral blood of children were evaluated also only according to the children allergy status regardless of maternal allergy status or probiotic supplementation (healthy children – H; allergic children – A).

Characterization of the effect of EcO83 on cord blood cells in vitro

To better understand the mechanism of the beneficial effect of EcO83, the immunomodulatory capacity of EcO83 was characterized in vitro. Cord blood of newborns of healthy and allergic mothers delivered vaginally or by cesarean section at full term was collected during September 2015 and June 2017.

Newborns were divided into two groups based on the maternal allergy status: Group 1 consists of 32 infants of healthy mothers (non-allergic, children with relatively low risk for allergy development) and Group 2 consists of 27 children of allergic mothers (children at higher risk for allergy development). No significant difference between two groups was observed in birth weight and length, sex, mode of delivery (vaginally or caesarean section) and Apgar score. Mononuclear fraction of cord blood cells (CBMC) was isolated by gradient centrifugation. To characterize the effect of EcO83 on neonatal immune system, CBMC were stimulated by EcO83 in vitro and cytokines typical for Th1 (IFN-γ), Th2 (IL-4) and Treg (IL-10) were detected by qPCR and ELISA. The capacity of EcO83 to promote Treg induction was tested by flow cytometry. The capacity of EcO83 to induce immune response was compared with other probiotic strain *E. coli* Nissle 1917 (EcN) (kindly provided by Ulrich Sonnenborn, ArdeyPharm).

Detection of specific IgE

Specific IgE and IgG4 against a mixture of common respiratory allergens (DYNX 1 recognizing antigens of timothy grass (*Phleum pratense*), rye (*Secale cereale*), birch (*Betula* spec.), mugwort (*Artemisia vulgaris*), mite (*Dermatophagoides pteronyssinus*), cat hair+epithelium (*Felis domestica*), dog hair+epithelium (*Canis familiaris*), mold (*Cladosporidium herbarum*)) and food allergens (FX 04 recognizing antigens of hen´s egg white, cow´s milk, wheat flour, peanuts, soyabean) was detected in sera of peripheral blood of all eight year old children (both healthy and allergic) by ELISA as described by Prokesova et al. [25]. Reagents for detection of specific IgE and IgG4 were produced by Dr. Fooke Laboratorien and distributed by Dynex Laboratories.

Characterization of T regulatory cells (Treg) in peripheral blood

Peripheral blood of 8-year-old children was collected in heparinized tubes and proportion of Treg was determined using

TregFlowEx® Kit (cat. no. 7417, Exbio, plc.) optimized for detection of Treg in heparinized whole blood. Kit contains all necessary solutions for cell permeabilization and fixation, antibodies against cell surface markers (CD4 FITC ED7417-4, clone MEM-241, CD25 PE ED7417-4, MEM-181) and FoxP3 (APC ED7417-5, clone 3G3). Tregs were stained according to the manufacturer's recommendation with the addition of PE-Cy7 labeled antibody against Helios (clone 22F6, cat. no. T7-771-T100, Exbio, plc.). To estimate whole population of Treg, cells were characterized as $CD4^+CD25^{high}$FoxP3. To distinguish between natural (n) Treg and induced (i) Treg, presence of transcription factor Helios considered to be a marker on nTreg was followed. Proportion of nTregs and iTregs was analyses according to the gating strategy indicated at Supporting Information Fig. 1. Peripheral blood samples treated with BD GolgiPlug (cat. no. 555029) preventing extracellular secretion of cytokines for 5 h were stained against CD4 (FITC, clone RPA-T4, cat. no. 555346), CD25 (PE-Cy7, clone M-A251, cat. no. 557741), CD127 (Alexa 647, clone HIL-7R-M21, cat. no. 558 598) followed by permeabilization and fixation using Transcription Factor Buffer Set (cat. no. 562 574), all BD Biosciences, and then stained intracellularly for cytokines IL-10 (PE, clone JES3-19F1, cat. no. 506 804) and TGF-β (PerCP-Cy5.5, clone BG/hLAP, cat. no. 341803), both purchased from BioLegend. Samples were acquired using BD FACS CantoII (BD Biosciences) and proportion of $IL\text{-}10^+$Tregs was analyzed according to the gating strategy documented at Supporting Information Fig. 2. Generally, at least 1 million of events were recorded for future data analyses using FlowJo software.

Detection of cytokines in peripheral blood of 8-year-old children and cell culture supernatants

Cytokines in plasma of peripheral blood of 8-year-old children and cytokines released by non-stimulated and stimulated CBMC after 3-day incubation with EcO83 or LPS (1 μg/mL, cat. no. L2654-1MG; Sigma-Aldrich) were detected by ELISA. Reagents for IL-4, IL-10, IFN-γ measurement were purchased from R&D System (IL-4: primary antibody MAB 604, biotinylated secondary antibody BAF 204, recombinant standard protein 204-IL; IFN-γ: MAB 2852, BAF 285, 285-IF; IL-10: DUO SET DY217B). The results were read from calibration curve in picograms per milliliter. If the concentration of concrete cytokine was below the detection limit the value corresponding to the half of detection limit was applied.

Cord blood processing

Cord blood was collected and processed as described before [9, 60]. Briefly, cord blood was collected in sterile heparinized tubes and CBMC were isolated by gradient centrifugation using Histopaque. CBMC were seeded at concentration 10^6 cells/mL and stimulated with LPS (1 μg/mL) or EcO83 in ratio 10 bacterial cells: 1 CBMC for 20 h (followed by RNA extraction and gene expression analyses or flow cytometry measurement of Treg induction or intracellular presence of IL-10 as described above for whole blood samples) or 3 days (estimation of cytokine concentration in cell culture supernatants) in the incubator with regulated CO_2 atmosphere (5.5% CO_2) at 37°C. We adhered with the flow cytometry analyses to the guidelines proposed by Cossarizza et al. [61].

Gene expression of cytokines

Gene expression of cytokines in CBMC was performed as described previously [9]. Briefly, total RNA was isolated from CBMC stimulated with EcO83 or LPS after 20 h using RNeasy Mini Kit (Qiagen) followed by reverse transcription. Gene expression was tested using TaqMan probes (IL-4 Hs 00174122_m1, IL-10 Hs 00174086_m1, IFN-γ Hs 00174143_m1, actin beta Hs 99999903_m1 was used as an endogenous control; all Applied Biosystems). PCR reactions were run and data analyzed as described by Hrdý et al. [9, 60]).

Statistics

The impact of EcO83 supplementation on allergy incidence between groups was evaluated using Wilcoxon sign rank test. Differences between groups were evaluated using the unpaired t-test for normally distributed data (concentration of specific IgE, proportion of Tregs and its subpopulations, concentration of cytokines in plasma of eight year old children); the non-parametric Mann–Whitney test was utilized for statistical evaluation of proportion of $IL\text{-}10^+$Tregs, $IL\text{-}10^+CD4$ T cells, gene expression in CBMC and cytokine concentrations in cell culture supernatants. Statistical significance was set at $p \leq 0.05$. Results are expressed as mean ± standard error of the mean (SEM) for data with normal distribution (concentration of specific IgE, proportion of Tregs and their subpopulations, concentration of cytokines in plasma of eight year old children). Data without normal distribution are presented as median with interquartile range (IQR) (intracellular presence of IL-10 in Tregs and $CD4^+$ T cells, gene expression in CBMC and cytokine concentrations in cell culture supernatants).

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Abbreviations: **CBMC**: cord blood mononuclear cells · **CFU**: colony forming units · **EcO83**: *Escherichia coli* O83:K24:H31 · **IQR**: interquartile range · **nTreg**: natural Treg · **Treg**: T regulatory cells · **iTreg**: induced Treg

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

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Cytokine

ABSTRACT

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

1. Introduction

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

Different researcher groups tried to propose some immunological characteristics of cord blood as prognostic markers pointing to increased risk of allergy development (e.g. IgE levels [2], cytokines present in cord blood sera [3–5], the capacity of cord blood mononuclear cells (CBMC) to release cytokines [5], number and activity of T regulatory cells (Treg) [6–8]). Nevertheless, contradictory conclusions exist in the literature and it is therefore difficult to find a consensus on one or a group of reliable prognostic markers in cord blood pointing to increased allergy development. It seems that the most reliable marker indicating increased risk of future allergy development is the allergic status of the mother [9].

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

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

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

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

2. Material and methods

2.1. Subjects

Healthy and allergic mothers without any substantial complication during the course of pregnancy and children delivered by caesarean section at full term in the Institute for the Care of Mother and Child in Prague, Czech Republic from August 2015 to December 2016 were included into the study after signed written informed consent. Mothers involved in the current study were from the capital and suburban areas. The age was not significantly different between non-allergic mothers (NA) (32.8 ± 4.8 years) and allergic mothers (A) (32.7 ± 3.7 years). The length and birth weight of newborns were not different either (on average 49.4 ± 2.3 cm A, 49.9 ± 2.4 cm NA; 3.488 ± 508.5 kg A, 3.227 ± 395.1 kg NA). The length of the pregnancy was similar as well (39 w 3/7 ± 0.5 days A and 39 w 1/7 ± 8.6 days NA). Other factors possibly influencing cord blood mononuclear cell reactivity, like smoking, diet (vegetarian, raw, etc.) did not differ between healthy and allergic groups. The diagnosis of allergy in mothers was based on the clinical manifestation of allergy persisting for longer than 24 months (allergy against respiratory and food allergens manifested by various individual combinations of hay fever, conjunctivitis, bronchitis, asthma, eczema or possible other allergic manifestation), monitored by an allergist, positive skin prick tests or positive specific IgE antibodies and anti-allergic treatment before pregnancy. Detailed list of type or combination of allergies of allergic mothers involved in the study is provided in Table 1. Only two women used anti-allergic drugs during the whole course of pregnancy (from Table 1 patient 2 used Claritine and patient 16 used Zyrtec (cetirizine)). The mothers with previous miscarriages, diabetes, transplantation, transfusion, serious surgery and any health problem during the course of pregnancy were excluded from the study. The study was approved by the Ethical Committee of the Institute for the Care of Mother and Child (Prague, Czech Republic). A total of 65 maternal–child pairs were included in the current study. Based on maternal allergy status, the children were divided into two groups: 43 children NA (22 females, 21 males) and 22A (11 females, 11 males).

Table 1
List of mothers included in allergy group and kind of confirmed allergy.

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

2.2. Cord blood sampling

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

2.3. Cord blood cell isolation and dendritic cell generation

Cord blood mononuclear cells (CBMC) were isolated by gradient centrifugation using Histopaque (Sigma-Aldrich). Dendritic cells (monocyte derived dendritic cells; moDC) were derived from adherent fraction of CBMC as described previously [25]. Briefly, after 1 h cultivation of CBMC in cell culture flasks in the incubator with regulated CO_2 atmosphere, nonadherent CBMC were washed out and adherent CBMC were cultured for 6 days with rhIL-4 and rhGM-CSF.

2.4. Induction and characterisation of maturation of moDC

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

2.4.1. Flow cytometry analyses of maturational status of moDC

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

2.4.2. Detection of cytokines produced by moDC

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

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

2.4.3. Gene expression of cytokines

Total RNA was isolated with the RNeasy Minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of RNA was assessed by the ratio of absorbance at 260 nm and 280 nm, and was in the range of 1.9–2.2. The total RNA concentration was estimated by spectrophotometric measurement at 260 nm, assuming that 40 μg of RNA per millilitre equals one absorbance unit. RNA was stored in aliquots at −20 °C until used for reverse transcription. Isolated mRNA was converted to cDNA using reverse transcription reagents (Applied Biosystems/Life Technologies, Prague, Czech Republic), according to the manufacturer's instructions. A reaction mix for quantitative real-time polymerase chain reaction (qPCR) was made with a TaqMan Universal PCR master mix, RNase-free water, and Assays on Demand gene expression products for IL-6 (Hs00174131_m1), IL-10 (Hs00174086_m1), TNF-alpha (Hs00174128_m1), indolamine 2, 3–dioxygenase (IDO) Hs00984148_m1; all Applied Biosystems. Cyclophilin A (peptidylprolyl isomerase A, Hs99999904_m1) was used as an endogenous control (Applied Biosystems). PCR reactions were run and data analysed as described by Hrdý et al. [25].

2.5. Characterisation of immune responses

Bacteria primed moDC were cocultured with naïve CD4+ T lymphocytes for 7 days. CD4+ T cells were isolated from non-adherent fraction of CBMC by CD4+ T Cell Isolation Kit (130-096-533) from Miltenyi Biotec according to the manufactureŕs recommendation. moDC: CD4 ratio was 1: 10. On day 4, anti CD3 (16-0037-85) and anti CD28 (16-0289-85), both eBioscience, were added at the final concentration 1 μg/ml. Additional stimulation of cells and prevention of extracellular transportation was performed by Leukocyte Activation Cocktail (LAC; 550583 BD Biosciences) which was added on day 7. After 5 h, the cells were harvested for further FACS analysis.

2.5.1. Flow cytometry analyses of induced immune responses

Intracellular staining of transcription factors and cytokines typical for Th1 (T-bet, 561266; IFN-gamma, 560704, both BD Biosciences), Th2 (GATA3, 653804, IL-4, 500824, IL-13, 501907, all BioLegend), Th17 (RORgt, 563621, IL-17A, 560799, both BD Biosciences, IL-22, 366704, BioLegend) and Tregs (FoxP3, 1A-601-C100, Exbio, Plc., IL-10, 501420, BioLegend) was performed after cell surface staining by antibody against CD4 (T4-359-T100, Exbio, Plc.) for 20 min, then washed twice with PBS containing 1% BSA, followed by fixation and permeabilization (BD Pharmingen Transcription Factors Buffer Set, 562574) according to the manufactureŕs recommendation. Cells were immediately acquired using BD FACS Canto II. (Becton Dickinson, Franklin Lakes, NJ).

2.6. Statistics

Differences between groups were evaluated using the unpaired *t*-test for normally distributed data (age of the mothers, birth weight and length of newborns, length of pregnancy, activation markers and gene expression in moDC); the non-parametric Mann-Whitney test was utilized for comparing cytokine concentrations in cell culture supernatants and Wilcoxon signed rank test was employed for statistical analyses of intracellular presence of cytokines and transcription factors detected in CD4 T cells. Statistical significance was set at $P \leq 0.05$. For statistical evaluation of ELISA results, the values under the detection level were given as half of the detection limit. Results of data with normal distribution are expressed as mean + standard deviation (SD) and data with non-normal distribution are displayed by box plots showing median with minimum and maximum values of 95% confidence interval (CI) (2.5–97.5 percentile).

3. Results

3.1. moDC

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

3.1.1. Detection of activation markers

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

3.1.2. Gene expression in moDC

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

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

3.1.3. Secretion of cytokines by moDC

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

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

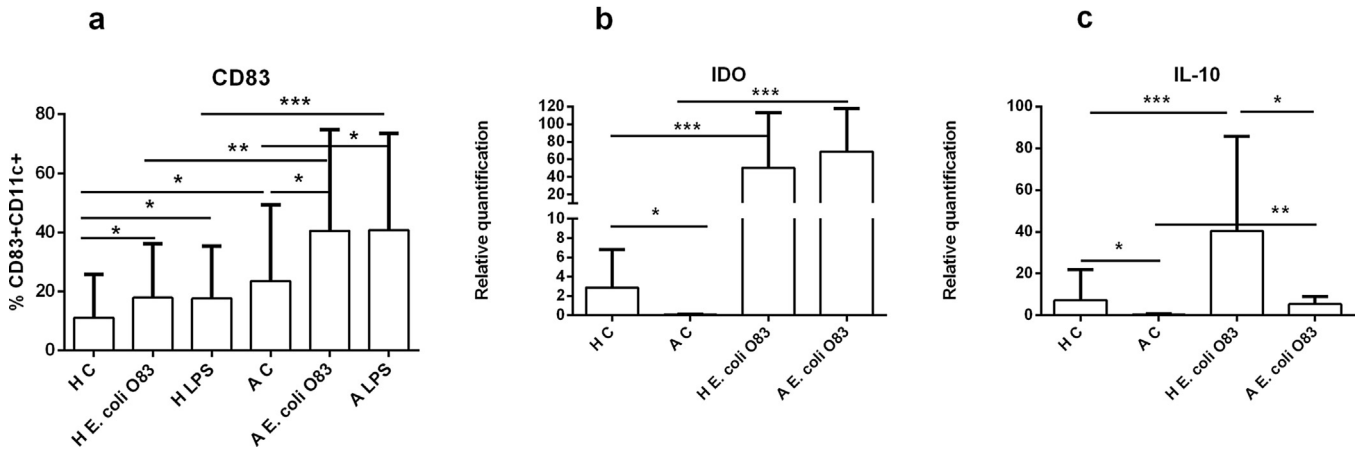

Fig. 1. The capacity of probiotic strain to induce maturation and gene expression in moDC. Activation markers indicating maturation status of moDC were followed by flow cytometry after 24 h stimulation. Gene expression of indolamine 2, 3- dioxygenase (IDO) and IL-10 in moDC was measured after 5 h stimulation by quantitative real-time PCR (qPCR).
a) cell surface presence of CD83 on moDC of newborns sorted according to the maternal allergy status
b) Gene expression of IDO in moDC of newborns separated according to allergy status of their mothers
c) Gene expression of IL-10 in moDC of newborns separated according to allergy status of their mothers
HC – nonstimulated moDC of newborns of nonallergic mothers − DC (NA)
H *E. coli* O83 – DC (NA) stimulated with *E. coli* O83
H LPS – DC (NA) stimulated with LPS
A C – nonstimulated moDC of newborns of allergic mothers − DC (A)
A *E. coli* O83–DC (A) stimulated with *E. coli* O83
A LPS – DC (A) stimulated with LPS
*p ≤ 0.05
** p ≤ 0.01
*** p ≤ 0.001

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

The capacity of DC to secrete IL-6 in response *E. coli* O83 or LPS was not different between healthy and allergic group. Concentration of IL-6 was increased in supernatants of *E. coli* O83 (842 (696.1–1754) pg/ml vs. 110.7 (81.4–170.6), respectively, p = 0.0001) and LPS (1107 (768.8–1587) pg/ml vs. 110.7 (81.4–170.6), respectively, p = 0.0001) stimulated DC (NA) and *E. coli* O83 (856 (693.5–1827) pg/ml vs. 118.1 (36.9–160.5) pg/ml, respectively, p = 0.0005) and LPS (1107 (769.8–1587) pg/ml vs. 118.1 (36.9–160.5) pg/ml, respectively, p = 0.0001) stimulated DC (A), Fig. 2c.

3.2. Induction of immune responses by probiotic primed moDC

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

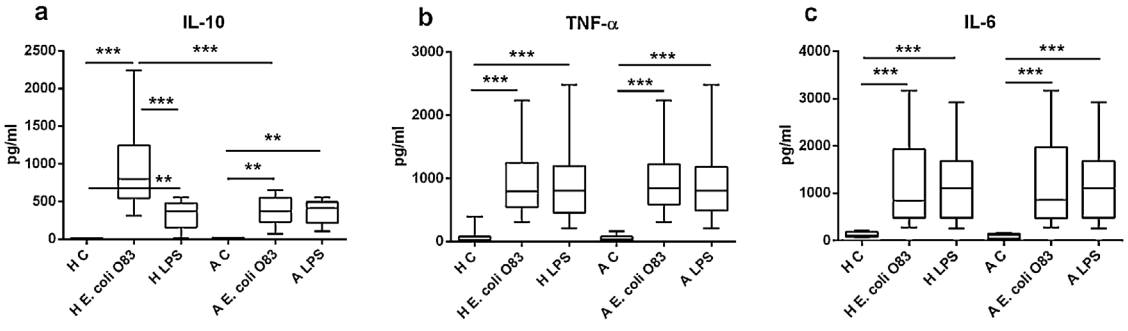

Fig. 2. Detection of cytokines in cell culture supernatants of moDC. To evaluate the capacity of *E. coli* O83 to promote secretion of cytokines, moDC were stimulated 24 h and cytokines were detected by ELISA.
a) IL-10 concentration in moDC supernatants after division of moDC according to maternal allergy status of newborns (MASN).
b) TNF- alpha concentration in moDC supernatants after division of moDC according to MASN.
c) IL-6 concentration in moDC supernatants after division of moDC according to MASN.
HC – nonstimulated moDC of newborns of nonallergic mothers − DC (NA)
H *E. coli* O83 – DC (NA) stimulated with *E. coli* O83
H LPS – DC (NA) stimulated with LPS
A C – nonstimulated moDC of newborns of allergic mothers – DC (A)
A *E. coli* O83–DC (A) stimulated with *E. coli* O83
A LPS – DC (A) stimulated with LPS
** p ≤ 0.01
*** p ≤ 0.001

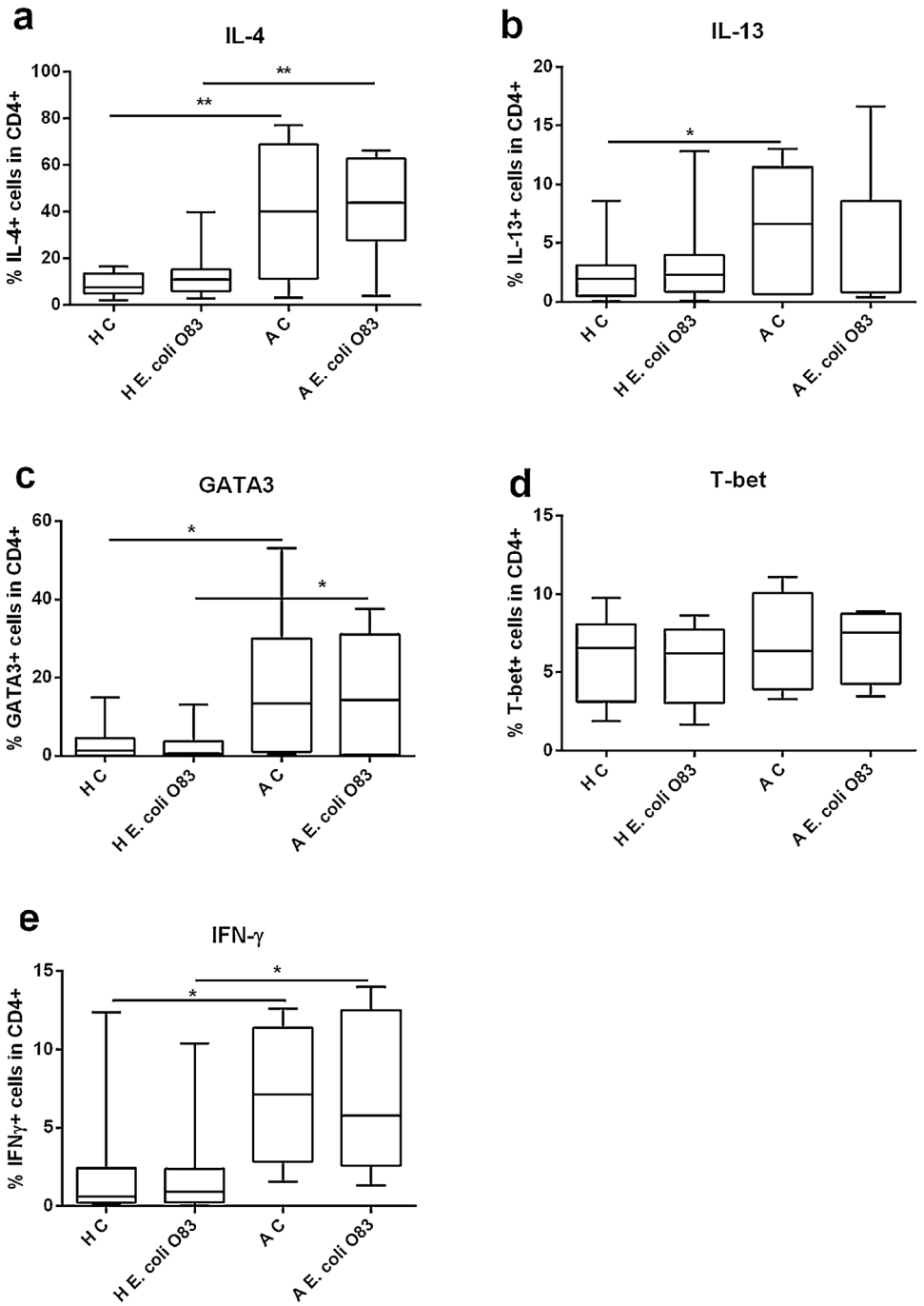

Fig. 3. Evaluation of the capacity of *E. coli* O83 stimulated moDC to induce Th1 or Th2 immune responses. Control or stimulated moDC were cocultured with naïve CD4 T cells for 7 days. On day 7, Leukocyte Activation Cocktail was added and the presence of intracellular cytokines and transcription factors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

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

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

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

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

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

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

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

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

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

*$p \leq 0.05$

** $p \leq 0.01$

induce Th1 (T-bet, IFN-gamma) and Th2 (GATA3, IL-4, IL-13) responses (data not shown). When the data were compared according to the maternal allergy status, increased values of Th2 (IL-4, 40.1 (19–61.2) vs. 7.7 (5.8–11.6) % IL–4 + CD4 T cells, respectively, p = 0.0076; IL-13, 7.5 (3.5–9.9) vs. 2 (1–3.9) % IL–13 + CD4 + T cells, respectively, p = 0.0478; GATA3, 13.4 (6.1–30.9) vs. 1.3 (0.6–4.8) % GATA3+ CD4 + T cells, respectively, p = 0.0423, Fig. 3a–c) immune response were detected in CD4+ T cells co-cultured with control DC (A) in

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

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

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

4. Discussion

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

In addition to that, lower expression and secretion of IL-10 in *E. coli* O83 stimulated moDC of newborns of allergic mothers indicate insufficient regulatory function which could support the onset of inappropriate immune responses. The capacity of probiotic bacteria to induce regulatory cytokine IL-10 was described previously by Niers et al. [33]. Reduced secretion of IL-10 from Treg of the offspring of allergic mothers was reported by other authors [8,34] including our group [6]. In addition to that, we have detected increased expression of indol-amine 2, 3–dioxygenase (IDO) in moDC stimulated with *E. coli* O83 but nonstimulated moDC of newborns of allergic mothers have lower expression of IDO suggesting inferior regulatory potential of these cells. The importance of IDO in controlling allergic inflammatory responses was proposed [35–37]. It is possible to speculate that DC of newborns of allergic mothers are more prone to initiate inadequate immune responses to relatively innocuous environmental antigens due to the decreased regulatory responses.

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

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

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

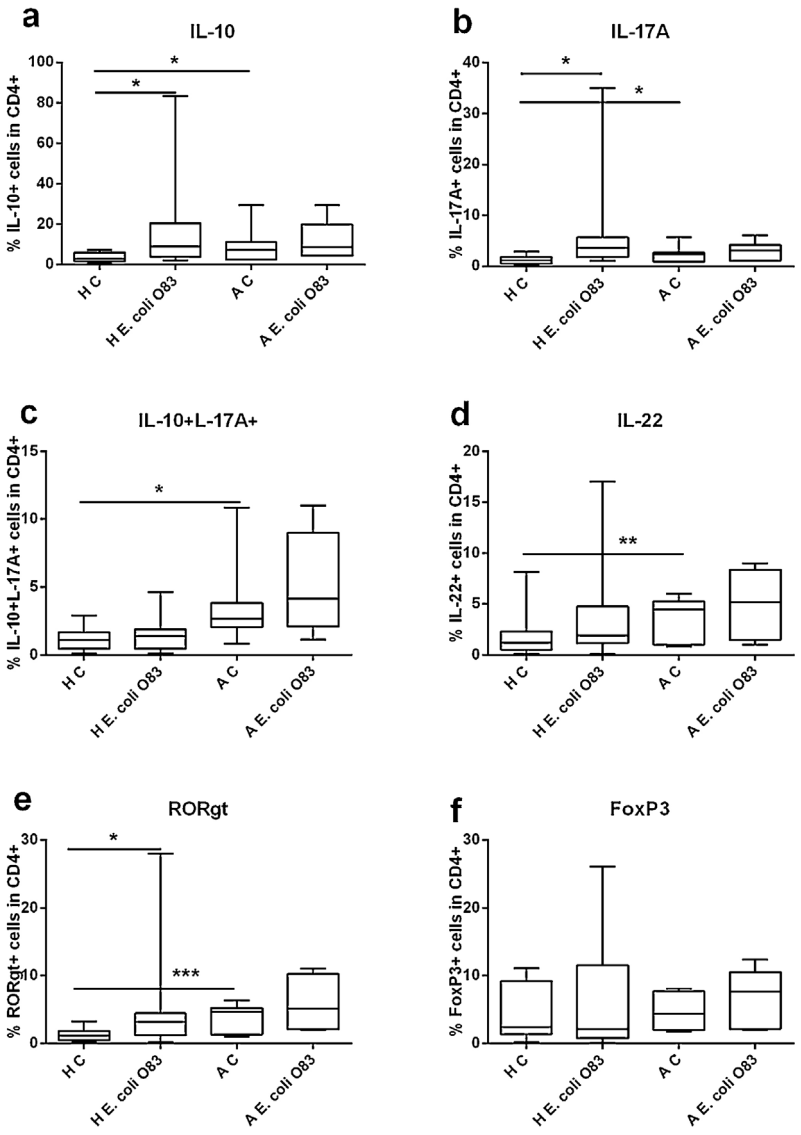

Fig. 4. Evaluation of the capacity of *E. coli* O83 stimulated moDC to induce Th17 or Treg immune responses. Control or stimulated moDC were cocultured with naïve CD4 T cells for 7 days. On day 7, Leukocyte Activation Cocktail was added and presence of intracellular cytokines and transcription factors were detected by flow cytometry.
a) Percentage of IL-10 positive CD4 T cells according to maternal allergy status (MAS)
b) Percentage of IL-17A positive CD4 T cells according to MASN
c) Percentage of double positive IL–10 + IL-17A+ CD4 T cells according to MASN
d) Percentage of IL-22 positive CD4 T cells according to MASN
e) Percentage of RORgt positive CD4 T cells according to MASN
f) Percentage of FoxP3 positive CD4 T cells according to MASN
H C – nonstimulated moDC of nonallergic mothers (DC (NA)) cocultivated with naïve CD4 T cells of newborns of healthy mothers (CD4 (NA))
H *E. coli* O83–*E. coli* O83 stimulated DC (NA) cocultivated with naïve CD4 (NA)
A C – nonstimulated moDC of allergic mothers (DC (NA)) cocultivated with naïve CD4 T cells of newborns of allergic mothers (CD4 (A))
A *E. coli* O83–*E. coli* O83 stimulated DC (NA) cocultivated with naïve CD4 (A)
*$p \leq 0.05$
** $p \leq 0.01$
*** $p \leq 0.001$

general conclusion about how the probiotic bacteria work.

5. Conclusions

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

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