CZECH TECHNICAL UNIVERSITY IN PRAGUE

Faculty of biomedical engineering
Department of Specializations in Health Service

The Immunomodulatory Capacity of HLY$^+$ and HLY$^-$ Mutant of *Escherichia coli* O83:K24:H31

Imunomodulační vlastnosti HLY$^+$ a HLY$^-$ mutanty *Escherichia coli* O83:K24:H31

Bachelor's thesis

Study programme: Specialization in Health Service
Study branch: Medical Laboratory Technician

Supervisor: RNDr. Jiří Hrdý, Ph.D.

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Bachelor thesis assignment

Student:	**Nella Bukáčková**
Study branch:	Medical Laboratory Technician
Title:	**The Immunomodulatory Capacity of HLY+ and HLY- mutant of Escherichia coli O83:K24:H31**
Title in Czech:	Imunomodulační vlastnosti HLY+ a HLY- mutanty Escherichia coli O83:K24:H31

Instructions for processing:

The subject of bachelor thesis (BT) is comparing the immunomodulatory capacity of Escherichia coli O83:K24:H31 (Ec083) with hemolysin (HLY+) included in vaccine Colifant Newborn and mutant without hemolysin (HLY-) on dendritic cells (DC) of newborns.
The theoretical part of BT focuses on characterization of DC, subtypes of DC, describes DC activation, maturation, antigen recognition and processing. Then BT describes allergy, characterization of probiotics and their possible impact on prevention of allergic diseases with a particular focus on Ec083.
BT will focus on toxin hemolysin and its impact on human body and possible benefit of mutant Ec083 without hemolysin.
In the practical part of BT, immunomodulatory activities of Ec083 HLY+ and HLY- on DC generated from cord blood precursors will be compared by flow cytometry. Results obtained could indicate if Ec083 HLY- can have same convenient results to an immune system of a newborn as a currently used type.

List of Literature:

[1] LODINOVÁ-ŽÁDNÍKOVÁ, R., PROKEŠOVÁ L., KOCOURKOVÁ I., HRDÝ J. a ŽIŽKA J., Prevention of Allergy in Infants of Allergic Mothers by Probiotic Escherichia coli, International Archive of Allergy and Immunology., číslo 153(2), 2010, 201-6 s., ISSN 1423-0097
[2] SÚKENÍKOVÁ L., ČERNÝ V., NOVOTNÁ O., PETRÁSKOVÁ P., BORÁKOVÁ K., KOLÁŘOVÁ L., PROKEŠOVÁ L., HRDÝ J. , 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. Immunology Lett, 2017 May 26. , pii: S0165-2478(17)30110-4. doi:10.1016/j.imlet.2017.05.013. PubMed PMID: 28554713.

Validity of assignment until date:	13.09.2019
Supervisor of bachelor thesis:	RNDr. Jiří Hrdý, Ph.D.

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In Kladno, 25.10.2017

České vysoké učení technické v Praze, Fakulta biomedicínského inženýrství

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Zadání bakalářské práce

Student:	**Nella Bukáčková**
Obor:	Zdravotní laborant
Téma:	**Imunomodulační vlastnosti HLY+ a HLY- mutanty Escherichia coli O83:K24:H31**
Téma anglicky:	The Imunomodulatory Capacity of HLY+ and HLY- Mutant of Escherichia Coli O83:K24:H31

Zásady pro vypracování:

Předmětem bakalářské práce bude porovnání imunomodulačních vlastností Escherichia coli O83:K24:H31 (EcO83) s hemolyzinem (HLY+) obsažené v probiotické vakcíně Colinfant Newborn a mutanty bez hemolyzinu (HLY-) na dendritické buňky (DC) novorozenců.
Teoretická část bakalářské práce bude věnována charakteristice DC a jejich subpopulací, způsobu aktivace, maturace, rozpoznání a zpracování antigenu. Dále bude pojednávat o alergii, charakteristice probiotik a jejich možnému účinku při prevenci vzniku alergických onemocnění se speciálním zaměřením na EcO83. Bakalářská práce se bude věnovat toxinu hemolysinu a jeho vlivu na lidský organismus a v čem by mohl spočívat přínos mutanty EcO83 bez hemolyzinu.
V praktické části bakalářské práce bude porovnána imunomodulační aktivita EcO83 HLY+ a HLY- na DC získané z prekurzorů pupečníkové krve. Schopnost EcO83 HLY+ a HLY- podporovat maturaci DC bude testována pomocí průtokové cytometrie. Získané výsledky by mohly naznačit, zda by EcO83 HLY- mohla mít stejně prospěšné účinky na nezralý novorozenecký imunitní systém jako současně užívaný kmen.

Seznam odborné literatury:

[1] LODINOVÁ-ŽADNÍKOVÁ, R., PROKEŠOVÁ L., KOCOURKOVÁ I., HRDÝ J. a ŽIŽKA J., Prevention of Allergy in Infants of Allergic Mothers by Probiotic Escherichia coli, International Archive of Allergy and Immunology., číslo 153(2), 2010, 201-6 s., ISSN 1423-0097
[2] GOLDSBY, Richard A., Thomas J. KINDT a Barbara Anne OSBORNE, Kuby immunology, ed. 4th ed., New York: W. H. Freeman and Company, c2000, ISBN 0-7167-3331-5
[3] SÚKENÍKOVÁ L., ČERNÝ V., NOVOTNÁ O., PETRÁSKOVÁ P., BORÁKOVÁ K., KOLÁŘOVÁ L., PROKEŠOVÁ L., HRDÝ J. , 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. Immunology Lett, 2017 May 26. , pii: S0165-2478(17)30110-4. doi:10.1016/j.imlet.2017.05.013. PubMed PMID: 28554713.

Zadání platné do: 13.09.2019

Vedoucí: RNDr. Jiří Hrdý, Ph.D.

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děkan

V Kladně dne 25.10.2017

Declaration

I hereby declare that I have completed this bachelor's thesis with topic the immunomodulatory capacity of HLY^+ and HLY^- mutant of *Escherichia coli* O83:K24:H31 independently and I have included a full list of used references.

I do not have a compelling reason against the use of the thesis within the meaning of Section 60 of the Act No 121/2000 Coll., on copyright, rights related to copyright and amending some laws (Copyright Act).

In Kladno, May 18, 2018

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Nella Bukáčková

Acknowledgments

With this text, I would like to thank my supervisor RNDr. Jiří Hrdý, Ph.D. for enabling generous working conditions and providing the necessary material for our work, for professional guidance and factual comments on my work. I also have a great thanks to the members of the Institute of Immunology and Microbiology in Prague, especially to Mgr. Lenka Súkeníková for patience and munificent help in processing the practical part of my bachelor's thesis.

Abstract

Considering the immature immune system of the newborn, microbial stimulation is a significant benefit in providing the physiological functions of the immune system. The influential action of the intestinal microorganisms is described in many medical disciplines. Reduced natural bacterial stimuli could support the distribution of homeostasis leading to the stimulation of Th2 immunological response and the development of allergic diseases whose incidence is increasing.

Previous studies have described the beneficial properties of *Escherichia coli* O83:K24:H31 (*E. coli* O83) used as probiotic administered to the newborns to prevent allergy rise. *E. coli* O83 is Gram negative bacteria containing hemolysin as a virulence factor. Toxin hemolysin is one of the cytolysins that can cause cell cytolysis. Because of the character of the sensitive recipients group of probiotics it would be desirable to administer these bacteria without hemolysin (HLY^-). To confirm similar quality of *E. coli* O83 HLY^-, the effect of both variants of *E. coli* O83 on induction of maturation of dendritic cells derived from monocyte precursors from cord blood of the newborns was compared *in vitro*.

The ability of *E. coli* O83 without hemolysin to stimulate *in vitro* cultured dendritic cells from umbilical cord blood precursors was assessed by the flow cytometry. The results demonstrated the analogous stimulatory capacity of *E. coli* HLY^- as stimulatory properties of *E. coli* HLY^+ on moDCs. CD83 was used as a marker indicating dendritic cell maturation.

Key words

allergy; dendritic cell; *Escherichia coli* O83:K24:H31; hemolysin; probiotic

Abstrakt

Vzhledem k nezralosti imunitního systému novorozence je mikrobiální stimulace značným přínosem při zajišťování fyziologických funkcí imunity. Vlivné působení střevních mikroorganismů je popsáno v mnoha medicínských odvětvích. Snížením přirozených bakteriálních stimulů může docházet k narušení rovnováhy vedoucí ke stimulaci Th2 imunologické odpovědi a rozvoji alergických onemocnění, jejichž incidence se stále zvyšuje.

Předchozí studie popsali výhodné vlastnosti bakterie *Escherichia coli* O83:K24:H31 (*E. coli* O83), užité jako probiotikum, při podání novorozencům, jako prevence vzniku alergií. *E. coli* O83 je gram negativní bakterie, obsahující virulentní faktor hemolyzin. Toxin hemolyzin patří mezi cytolysiny, které mohou vyvolat cytolýzu buněk. Vzhledem k charakteru citlivé skupiny příjemců těchto probiotik, by mohlo být žádoucí podávání této bakterie bez přítomnosti toxinu hemolysinu (HLY^-). Pro potvrzení obdobné kvality *E. coli* O83 HLY^-, byl porovnán vliv těchto dvou variant *E. coli* O83 na indukci maturace dendritických buněk získaných z monocytů (moDCs) z pupečníkové krve novorozenců *in vitro*.

Způsobilost *E. coli* O83 bez hemolyzinu stimulovat *in vitro* kultivované dendritické buňky z prekurzorů v pupečníkové krvi byla posouzena pomocí průtokové cytometrie. Výsledky prokázaly analogické imunostimulační vlastnosti *E. coli* O83 HLY^-, jako jsou stimulační vlastnosti *E. coli* O83 HLY^+ na moDCs. Jako znak poukazující na maturaci dendritických buněk byl použit CD83.

Klíčová slova

alergie; dendritické buňky; *Escherichia coli* O83:K24:H31; hemolysin; probiotika

Table of contents

1 INTRODUCTION

Allergy is a disease affecting a large part of the population from developed countries. Allergy often occurs in the early age of children and the number of affected individuals has risen every year. At this time, there is no known drug that directly treats allergy. One of the commonly used treatment are only alleviate clinical signs. Given this, prevention and treatment of allergy are the most important topics of research and medicine.

Allergy is probably caused by combination of genetic predispositions with the influence of the external environment. One of the external effects which influences the immune system is commensal microbiome. Microbial composition directly influences the immune system, including maturation of immune cells of newborn or development of hypersensitivity. Due to the trend of today - the decrease/the lack of bacterial and viral infections, is Th1 response is reduced and the Th2 response supporting hypersensitivities (allergy) is promoted. That is the reason of exploring probiotics with properties such as increasing the maturation of the newborn immune system, including physiological Th1 response, and reducing the likelihood of an allergic disease outbreak. The probiotic strain of *Escherichia coli* O83:K24:H31 was already tested and the reduction of incidence of allergy in children supplemented with this probiotic strain was confirmed.

Unlike the most explored probiotic strain *Escherichia coli* Nissle 1917, the *Escherichia coli* O83:K24:H31 contains a virulent factor α-hemolysin. Hemolysin is cytolysin which binds membrane, disturb the stability of membrane and cause lysis of the cell. Hemolysin might cause the damage to cells of the intestinal tract of the newborns.

The expected benefit of this work is the creation of *in vitro* model demonstrating the properties of *Escherichia coli* O83:K24:H31 without hemolysin stimulating monocyte-derived dendritic cells isolated from umbilical cord blood.

2 CURRENT STATE

2.1 Allergy

Allergic diseases belong to one of the most common diseases. Allergy manifests in predisposed individuals when their immune system develops set effector immune responses to relatively innocuous environmental antigens called allergens (e.g., pollen, dust, mites) [1]. Majority of the immune-mediated disorders is caused by the failure of the immune system to set and maintain tolerance to relatively innocuous exoantigens (pollen, food) or compounds of microbiota. Impaired tolerance could lead to disorders characterized by a long-term inflammatory reaction transforming into chronic forms (known as 'a chronic inflammation') [2].

The allergic response (hypersensitivity type I) is based on a production of antibody (Ab) also known as an immunoglobulin (Ig), specifically immunoglobulin E (IgE) which bind to the high-affinity receptors (FcϵRI) presents on mast cells or basophils. Even an insignificant concentration of IgE can inflict the FcϵRI and linger, before later reuniting with an allergen. Eosinophils (also lymphocytes and monocytes) express low-affinity receptors CD 23 (FcϵRII) binding IgE only at higher concentrations and regulating IgE production by B cells. The origin and physiological function of inflammation are to protect organism against infection and help the body to heal by locating the source of infection and removing the agent or destroyed the tissue elements. Pathological role of inflammation principally leads to the dysfunction or disorder of an organ or a tissue [1, 2, 3].

The process of allergic reaction starts with sensitization being similar to the normal immune response development. Allergen is recognized and presented by antigen-presenting cells (APC). Regularly, this happens in a tissue where allergen penetrates the mucosa and gets in contact with APC. The antigen is processed into peptide fragments and presented on the surface of the cells by class II major histocompatibility complex (MHC II) proteins. MHC is in human cells also called human leukocyte antigen (HLA), is divided into subgroups. MHC II, mentioned earlier, is characterized by presentation of exoantigen by APC, on the contrary, class

I major histocompatibility complex (MHC I) is presented on all body origin cells (and virus or malignant proteins) [1, 3, 4].

For the following development of a reaction, the presentation of the allergen is fundamental. It may induce proliferation of Th1 and form a specific T lymphocyte or, due to the form of presentation; activate proliferation of Th2 and the production of IgE. The first encounter of allergen with the body is without clinical signs. IgE binds to its receptors on basophils or mast cells. After encounter with appropriate allergen, the allergen interacts directly with IgE presented on mast cells and basophils. Following, the interaction of the allergen with IgE, FcϵRII are cross-linked leading to the activation of mast cells and release of mediators with pro-inflammatory substances promoting acute phase of allergic inflammation. Pro-inflammatory and also acute phase reactants are significant for acute inflammation. Major protein released after initiation of inflammatory responses is C-reactive protein (CRP) which increases its serum level in 5-10 hours, 10-1000 times. Cytokines such as interleukin 1 beta (IL-1β) or tumor necrosis factor α (TNF-α) are relevant for production of acute phase response proteins. Inflammation is also accompanied by a fever which is caused by the influence of prostaglandin E2 in a thermoregulatory center of the hypothalamus. The production of prostaglandin E2 is induced by the pyrogens. The pyrogens are e.g., lipopolysaccharides from bacteria (from external environment) and TNF-α, IL-1, and IL-6 (internal environment). Other cytokines involved in acute inflammation are IL-6, IL-8, and molecules of complement C3, C4, etc. The late phase mediators are interferon γ (IFN-γ), IL-2, IL-4 or IL-5 which activate inflammatory cells and induce production of IL-1β, IL-12, and TNF-α [1, 3].

The next phase of allergic reaction is an acute phase of allergic reaction followed by late phase. The acute (or early) response begins immediately, often mere minutes after allergen encounter. Antigen interacts with IgE already bound to FcϵRI leading to secretion of substances (such as histamines), then heparin or proteases from granule of mast cells [1, 3]. The released mediators influence surrounding tissue and other immune cells causing inflammatory symptoms. Release of histamine, leukotrienes, and prostaglandins leads to smooth muscle contraction, vasodilation; increased vascular permeability and stimulation of nerve endings, etc.

The vasodilation together with elevated vascular permeability allows immune cells to permeate the vessel wall and migrate to instead of inflammation [2]. Production of these primary mediators is present in food allergies, bee venom, anaphylactic shock or hay fever. The late response (hours to days) begins by the production of secondary mediators, being represented mainly by a metabolites of an arachidonic acid such a prostanoids, thromboxanes by mast cells, leukotrienes, platelet-activating factor (PAF) by neutrophils, IFN- γ, IL-1, IL-2, IL-4, IL-5, IL-12 and TNF- α [1; 2; 3]. The function of these substances is pro-inflammatory and has a chemotactic effect on cells like eosinophils, thrombocytes, neutrophils, and lymphocytes. As a late response is typical eczema. [1, 3].

The allergy appears as a combination of genetic predisposition for developing allergy and environmental factors. It can be located in a specific organ like the skin (eczema), the nasal mucosa (hay fever) or it can be systemic (systemic anaphylaxis) [1, 3]. Localized hypersensitivity reactions (hay fever) mostly settle the epithelial surface, as it is the first point of contact for an allergen. A systemic hypersensitivity reaction (food allergy, drug allergy) usually penetrates directly into the blood system. The most severe form: anaphylactic shock, is accompanied by a high concentration of histamine, heparin, leukotrienes, platelet activating factor (PAF), TNF-α, IL-4, IL-5, IL-6, IL-13, etc. If anaphylactic shock occurs without immediate medical aid, the death of a patient may occur 2-4 minutes after the encounter of antigen/allergen. The most common allergens causing anaphylaxis are drugs (e.g., penicillin, insulin) or food (e.g., nuts, seafood). Epinephrine is the most common immediate remedy to soothe the symptoms of anaphylaxis [2, 5].

Genetic predisposition to allergy is prevalent in half of the patients. The heritability of genetic disease differs: for asthma is genetic predisposition in the range of 35% and 95%, for allergic rhinitis 33% and 91%, for total serum of IgE levels 35% and 84%, for atopic dermatitis 71% and 84% and for bronchial hyperresponsiveness 30% and 66% [6]. According to the epidemiological data, the risk of allergy in infants without genetic predisposition is 20%, in a family with one affected parent 30 – 40% and with both parents suffering from allergy reach 75 – 80%. The risk of allergic disease development is about four times higher if it is affected by the mother. Allergy has polygenic inheritance, so individual does not

inherit allergy directly, the only predisposition for allergy manifestation [7, 8]. Essentially, genes code proteins, which are significant for the initiation of an allergy and located on various chromosomes. This genetic information can be divisible into three groups. The first group influences the recognition and processing antigen, such as coding MHC II molecules and T cells receptors (TCR). The second supports the production of IgE by cytokines like IL-4, IL-5, IL-13, TNF, etc. The last one consists of genes which influence the determination of organ which will be affected [1] For example, filaggrin (FLG) is a crucial protein for the skin protection. The epidermis is a component of the innate immune protection, as it keeps water within the body and averts a pathogens effort to penetrate inside the body [9]. One of the possibilities of labeling and searching for the heritability of allergy marker is the use of single nucleotide polymorphism (SNP). The disparity of the population is assured by a single base-pair variation in DNA sequence of individuals. With the use of SNPs is possible to locate genes which may be the basis for differences in individual responses to disease. For example, the risk alleles, guanine, of IL-4Rα rs1805010 and rs1801275 SNPs are more likely associated with asthma than alleles adenine. The major locations of other genes initiating inflammation are rs1800925 and rs20541 SNPs of IL-13, rs1800629 and rs361525 of TNF-α. Conversely, IL-10 which has an anti-inflammatory effect and therefore preventing allergy development has SNP rs1800872 [10, 11, 12]. Until now, no gene which causes allergy without stimuli, has been discovered. Genetic predisposition together with the combination of the effect of environmental factors play a critical role in allergy development [1].

The frequency of diagnosed allergy in past 22 years, in the Czech Republic, is indicated in the Figure 1.In the year 1996, it was detected 17% of children suffered from allergic diseases and asthma was diagnosed in 4% of children. In the year 2001 allergy incidence was increased to 25%, representing a rise of 5%. In 2006 there was a further increment of incidence of allergic diseases, up to 32% for allergic disease in general, with an 8% rise in allergic asthma in children. The presence of allergies had still a growing tendency. The generally accepted hygiene hypothesis, proposed by Strachan in 1989, is trying to explain the increase of asthma, multiple sclerosis, obesity, etc. by lower exposition to parasites and limited microbial burden. Based on the fact, that in the last one hundred years as a consequence of industrial, medical and hygiene changes, people are becoming more sensitive to allergens in pollution,

food or pollen. International studies prove the relationship between children living on farms and lower allergy incidence in these children. Children living on farms have high and frequent exposure to allergens (prenatally and postnatally), lipopolysaccharides presented on bacteria. These children are less receptive to allergic disorders than the urban ones. Regular sensitization with antigens during infancy and youth can significantly decrease the probability of having an allergic disease in adulthood by promoting the balance of T-cells (Th_1 and Th_2, T regulatory cells (Tregs)). Furthermore, the development of vaccination and antibiotics reduced infectious diseases but also increased the presence of allergy. Administration of antibiotics in early age results in abnormal development of microbiota, leading to impaired setting tolerance to the compounds of microbiota, food and inhalation antigens/allergens [2, 3, 13].

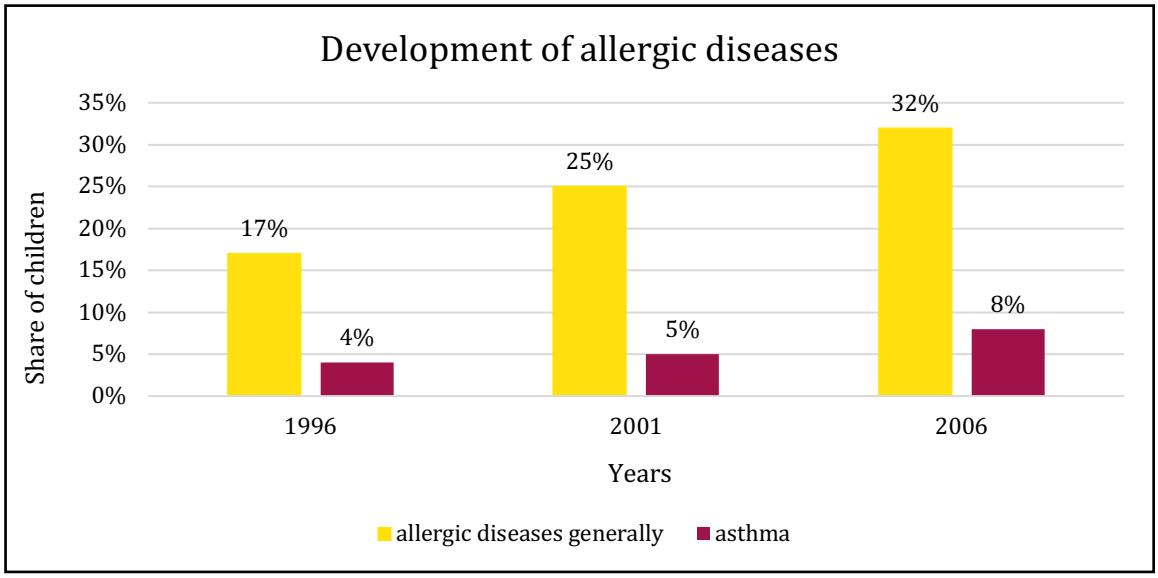


Figure 1 - The percentage of children with allergic diseases (allergic diseases in generally; asthma) in the population of the Czech Republic in years of research – 1996, 2001, 2006 [12, own source].

An allergen is a primary substance which induces sensitization and develops inflammation. The allergic symptoms depend on the biological properties of an allergen entering the body and which organ will be affected. Often allergens are called main allergens and commonly initiate IgE response. Some allergens can be contained in various items, e.g., profiling in apples, pears, peanuts, etc. [1, 3]. Peanut allergy affects 1% of children under five years old and food allergy, in general, afflicts 6-8% of children younger than those who are four years old [4]. Some substances can start an allergic reaction without the presence of IgE. They are

called histamine liberators, and these elements are included in medicine, food or contrast medium used in diagnosing imagining methods. Examples of food with histamine-releasing properties are citrus, peanuts, shellfish, and egg whites [1, 3]

2.1.1 Atopic triad

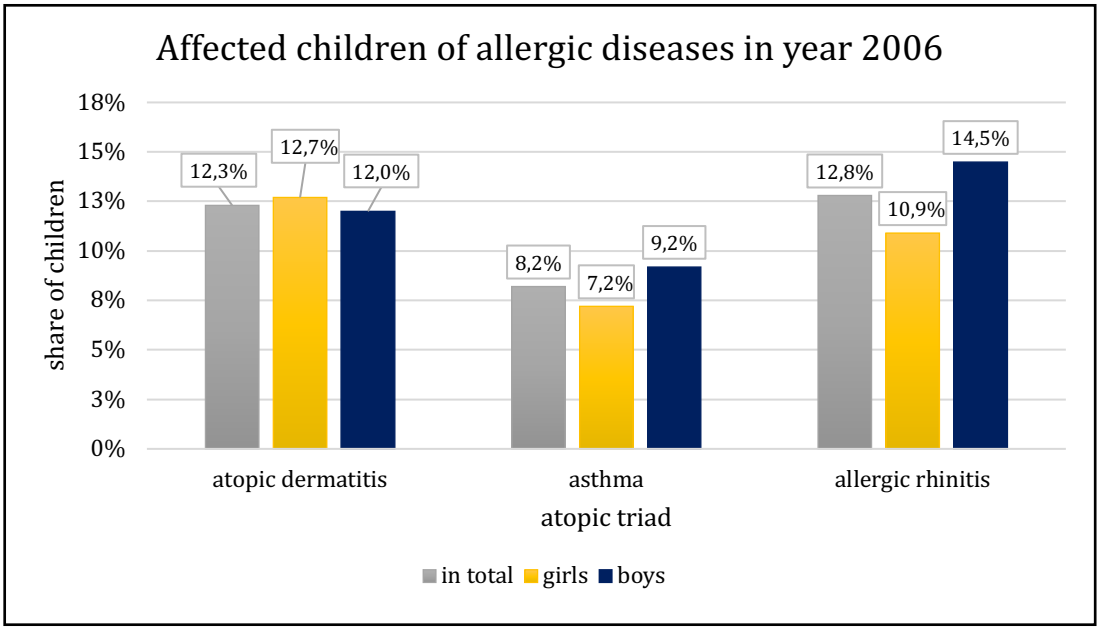


Figure 2 - The share of children with allergic diseases belonged to the atopic triad (atopic dermatitis, asthma, allergic rhinitis) in the Czech Republic in the year 2006 [12, own source].

The percentage of affected children with allergic disorders is presented in the Figure 2. Characterization is divided into three common atopic allergic diseases. Atopic dermatitis, asthma and allergic rhinitis create a group named atopic triad. The 12.3% of all children, 12.0% of boys and 12.7% of girls are afflicted by atopic dermatitis. This figure shows that asthma affects 8.2% of children in total, 7.2% girls and 9.2% of boys. The allergic rhinitis appears in 12.8% of children in total, 10.9% of girls and 14.5% of boys. The figure depicts that boys are more prone to suffer from asthma or allergic rhinitis than girls [14].

Atopic dermatitis (also known as eczema) is a skin disorder which predominantly affects infants and young children. Atopic dermatitis (AD) has not been identified by deep itching and repetitive eczematous lesions. The AD is not entirely clarified yet, but it is classified as one of the most common chronic diseases. Eczema affects 20% of children, and 60% is manifested during the first year of their

life. The first signs of the AD are coarseness and the lack of moisture. The AD is characterized by a defective epidermal barrier and causing cutaneous inflammatory. The absence of the epidermal barrier by the dysfunction of tight-junction proteins joins with keratinocyte in stratum granulosum. The Filaggrin null mutation is hereditary and creates a derangement of the skin barrier. Although mutation in fillagrin leading to its loss is predisposing for AD development early in life, in an extended period this disappearance has been considered as a secondary effect of the immunological mechanism. This disorganization lets environmental allergens to penetrate inside of the body and induces allergic inflammation [15, 16, 17, 18].

The AD can result in an acute or chronic form. The chronic AD is accompanied by Th1 cells secreting interferon-gamma (IFN-γ), Th2 cells and fewer Th22, and Th17 than acute lesions. The acute eczema is mediated by imbalance towards Th2 cells, Th22 cells and less Th17 cells. Th2 cytokines (IL-25, IL-33) polarize inflammatory dendritic epidermal cells and promote Th2 response. Likewise, the production of other Th2 cytokines IL-4, IL-5, IL-13, etc. [15, 16, 17, 18, 19].

The eczema lesions can affect any part of the body, but mostly the distribution of lesions is age-related. In infants, lesions are mainly located on the face, chest and the top of the thighs. Children between the age of one and two are affected particularly in flexural folds and adults at wrist, ankles, eyelids, neck, shoulders, etc. *Xerosis* is a universal sign of all phases, especially in winter when it is associated with water loss. Although the increased levels of IgE can be found in the sera of the patient, it is difficult to prove the association with the clinical symptoms [15, 16, 17, 18].

The epidermal barrier of the skin of patients with the AD is damaged and more prone to the secondary infections. The infection is commonly caused by *Staphylococcus aureus* and often associated with worsening of the disease. The *herpes simplex* virus is responsible for a skin infection called *eczema herpeticum*. Children and adolescents have a higher possibility of the infection caused by *Molluscum contagiosum*. Individuals with AD are more likely sensitized by fragrances, preservatives or antiseptics than healthy ones [15, 16, 17, 18].

The AD cannot be cured at present. The diagnosis of the AD is subordinate to clinical symptoms. The valuable artifact is an existence of eczematous lesions. These special locations of eczematous lesions can be sorted as acute, subacute and chronic lesions [17] The primary treatment of AD is to mitigate the signs and control the disease. One of the principles is to prevent dryness (losing water) of the skin by oil-based products; decreasing the symptoms are used antihistamines and corticosteroids as inhibitors of inflammatory [15, 17, 18]. The primary prevention of eczema is breastfeeding for at least four months. Secondary prevention is optimal skin care, avoidance of specific allergens such as food and aeroallergens. Following measures are to control household temperature and humidity, use natural cosmetic, bath in warm water once a day for 15 - 20 minutes, immediate application of emollients and do not wear clothes made up of synthetic fibers [18].

Atopic asthma (AA) is an allergic disease affecting the respiratory tract. AA can begin at any age, but traditionally first signs occur during childhood. This allergic disease affects 300 million people worldwide, particularly people living in the urban areas. Asthma incidence is 10% in infants, children or adolescents and 5% of adults are afflicted. Boys at the age between 10 and 12 are more frequently affected than girls at the same age. The reason why the atopic asthma in the last decades is increasing is unknown [3, 20, 21]. Partial impulse can be given by the viral respiratory infections. Severe *paramyxoviral* infections in the early age of life can increase the risk of suffering from asthma in later childhood. [20] Asthma is associated with the airway obstruction in bronchioles which is caused by hypersensitivity to allergen from the external environment and the chronic airway inflammation with a presence of eosinophils and mast cells. The eosinophils are stimulated and releasing their mediators (e.g., histamines, leukotrienes, prostaglandin, etc.). This put bronchioles in cramp, makes them swelling and increase mucus production, which causes severe to distribute air inside of lungs and back. Asthmatic patients can have an asthma exacerbation (attack) during which bronchus is, even more, narrowing, leading to the life-threatening condition [3, 21].

Patients with asthma are more sensitive to infection of *rhinovirus*. Changes in the composition of microbial communities have also been discovered. Whether

these microbial changes are caused after infection by *rhinovirus* or whether the lack of bacteria communities, settling the mucosa, provide the opportunity to *rhinovirus* settle down, is unknown [21].

It was shown that infections by helminth parasite, *Heligmosomoides polygyrus*, lead to the production of molecules which prevent the host from having an allergic reaction (type 2 immunity reaction), especially asthma. This protein *Heligmosomoides polygyrus* alarmin release inhibitor(HpARI), released by *Heligmosomoides polygyrus,* showed the ability to prevent binding of active IL-33 to the IL-33 receptor and suppress eosinophilic responses to allergen manifestation [22].

Atopic rhinitis (AR), as well as the disease mentioned earlier, belong to the atopic triad. AR is the prevalent allergic disorder in West Europe, Japan or the USA. In the USA is the most frequent allergic disease and the fifth most common chronical disorder overall. AR is significant with clinical symptoms such a sneezing, nasal drainage or nasal itching. AR is an IgE-mediated inflammatory disorder occurring in the nasal mucous membranes. This response is provoked by the exposure to the inhaled external allergen as pollen, dust mites or animal dander. Most often treatment of AR is represented by administration of antihistamines, corticosteroids or the other pharmacotherapy. If this is ineffective, patients may undergo allergen immunotherapy (AIT) [23, 24, 25].

2.1.2 Food allergy

Food allergy is a prevalent type of atopy on the rise, with food allergens inducing more anaphylactic responses than any other type of atopy. Approximately 4% of adults and 6-8% of infant and children are affected by any kind of food allergy. The most frequent food allergy is in milk of cow, eggs, soy, peanuts, wheat, fish, etc. The molecules inducing allergy are most commonly water-soluble glycoproteins. These substances are stable to heat, acid or enzymes. An allergic reaction is based on the same principle as the previous allergic process, i.e., to IgE on mast cells is banned allergen promoting degranulation. The release of histamine etc. leads to muscle contraction and dilatation of blood vessels. The permeability

of the membrane is increased, and allergen enters the blood system. Clinical signs can be vomiting and diarrhea. Patients with a food allergy can have some symptoms same as atopy such as asthmatic attack or skin manifestation [2].

Peanut allergy (*Arachis hypogaea*) is IgE-mediated disease which occurs in 1% of children and 0.6% of adults in the USA. Clinical symptoms appear within seconds, or up to 2 hours after ingestion of even a few milligrams of peanut glycoprotein *Ara h 1* (one peanut has about 300 mg of proteins). It has been identified that 11 types of peanut allergens exist (*Ara h 1-11*). One hypothesis says that graphically or ethnically different populations are sensitive to different peanut allergen. The clinical signs of eating peanuts are cutaneous, gastrointestinal, or respiratory disorders. The allergy caused by peanuts is one of the most frequent reasons of food-induced anaphylaxis. The following attribute for diagnosis of a peanut allergy is evidence of IgE [2, 4, 8].

2.2 Prevention and possible therapy of allergic diseases

The exact trigger of the allergy has not been identified yet. As mentioned earlier, allergic disease attains two basic predispositions – genetic factors and environmental including the exposure to the allergen [7].

It seems that environmental influences are more important for developing an allergy than the assumption of genetic. Due to the enormous growth of allergies after World War II, the change of lifestyle seems to be a better explanation of the rising of allergic diseases than genetic mutations [7]. This issue has been discussed more the section devoted to hygiene hypothesis.

One of the first considerable influences in the neonatal immune system is breastfeeding. The immune system of newborn is immature and with low capacity to induce adaptive immune responses, especially the production of antibodies is impaired. Formula fed children could develop inappropriate regulation responses supporting later allergy development. Major substances and cells present in breast milk are included, e.g., IgA, IFN-γ, tumor necrosis factor β (TGF-β), soluble molecules receptors, soluble adhesive molecules, macrophages, B-lymphocytes and T-lymphocytes (including Tregs) [26].

Taking these considerations, primary prevention of allergy could involve breastfeeding, and the most natural therapy is based on the reduction of contact with the allergen (if it is possible). As another alternative to the prevention is. the administration of probiotics in infants and to the treatment is immunotherapy or administration of medicaments.

2.2.1 Probiotics

The allergy can be mediated by the dysfunction of the human body barriers, e.g., skin or intestinal mucosa. The reaction of the immune system to antigen stimuli (which penetrated into the body through broken barrier) can lead to the development of allergic inflammation. The abilities of intestinal mucosa are directly influenced by colonization of microbiota. The settlement of newborn microbiome by bacteria is affected by the mode of the delivery. Bacterial colonization of vaginally delivered infants is primarily impacted by vaginal microbiota colonizing newborns during the passage through the birth canal. This is characterized by the presence of *Prevotella, Sneathia, Lactobacillus* and genus *Bacteroides* which affects the maturity of the immune system [27, 28, 29]. In contrast the microbiome of newborns delivered by Caesarean section (CS) is dominated by *Propionibacterium*, *Corynebacterium,* and *Streptococcus* from maternal skin or oral microbiota. Infants delivered by CS have a higher ratio of bacterial antibiotic-resistance genes than vaginally delivered infants. CS also increases the risk of long-term health problems such as celiac disease, obesity, and asthma [28, 30]. Microbiota consists of 400-500 bacterial species and represents 1-2 kg of the intestinal content. The number of genes of these microorganisms is hundred times bigger than the number of human genes. Microbiota is necessary for proper digesting, immunologic homeostasis, keeping the barrier integrity of inner surface, and for nutrition of organs and tissues. Favorable influences of the physiological microbiota are inhibition of microbial adhesion of pathogenic bacteria to the epithelial surface, inhibition of proliferation of pathogenic microbes (by a production of microcins and colicines), stimulation of immunity, synthesis of vitamins, stimulation of peristalsis of the bowel, etc. Some kinds of bacteria can control the immune system by limiting inflammation

responses. The microbiome is profoundly influenced by environment, age, type of diet, medication, stress, and lifestyle or state of health. These bacteria are also contained in live microbial food supplements called probiotics [26, 31, 32, 33].

A probiotic is live microorganism when administered in sufficient amount confers beneficial effect on host health. Probiotics support the balance of microbiota composition in the gut of host [34]. A probiotic has the benefit to change the settlement or metabolic activity of microbiota. A newborn is delivered relatively sterile; therefore, any changes in microbiota occurring after birth can rapidly influence immune reactions of the neonate (including breastfeeding) [26, 31, 32].

Ideally, probiotics could be used in combination with prebiotics. Prebiotics (formed, e.g., by oli- or poly-saccharides) are non-digestible supplements which selectively support the growth of bacteria contained in probiotic preparations. [31] Probiotics and prebiotics create complex called synbiotics which favorably affect intestinal microbiome [32, 35]. The claims for prebiotics are resistance to acids in stomach and enzymes in the intestine, not being absorbed in the upper part of the intestinal tract and be simply fermentable by beneficial microorganisms in the colon [36]. The function of prebiotics as inulin or pectin is a reduction of symptoms of diarrhea or colon inflammation preventing bowel cancer development [37].

New-generation probiotics such as psychobiotics have also been defined. Psychobiotics are living microorganisms that can have a positive effect on the patient's mental state after use. Common bacterial strains are *Escherichia, Bacillus, Candida, Streptococcus, Enterococcus* or *Lactobacillus acidophilus*. These strains can trigger production of neurotransmitters such as serotonin, norepinephrine or gamma-aminobutyric acid. The possible benefit might be in use for prevention or treatment of serious neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, dementia or depression [38].

Probiotics also have to accomplish some important criterions such as absence of pathogenic properties, lactic acid production, be of human origin (not always necessarily), genetic stability, short generation time, live culture, resistance to gastric juices and bile, safety, etc. [32, 5].

Dairy and fermented dairy products are good sources of beneficial microorganisms which are after isolation often used for the production of probiotic supplements. Although the content of the bacteria in breast milk was for a long time explained by a skin of fecal contamination, today is breast milk also considered to be a source of bacteria for probiotic supplements preparation. Breastfed infants are less affected by allergies and gastrointestinal infections than formula-fed infants probably due to the presence of beneficial bacteria in maternal milk. Bacterial stains contained in human breast milk are *staphylococci*, *streptococci*, *micrococci*, *lactobacilli*, *enterococci*, *lactococci*, and *bifidobacteria*. Researchers reported that the *Lactobacillus* strains activate the natural killers and T-cells and support the induction of Tregs which are beneficial in the reduction of the allergy manifestation [32, 35, 39].

Probiotics can also be produced by the isolation of microorganisms from the gut. One of the bacteria presented in the gut is *Lactobacillus fermentum*. This bacteria is profitable as a protection of the body against food-borne pathogens. The source of the probiotics is not limited only to the origin of the human. Some microorganisms can be also isolated from an animal intestinal track or separated from fruits or meat. To achieve satisfactory isolation and production of microorganism an emphasis must be put on selection criteria. [39, 39].

Preclinical evaluation of probiotics is based on *in vitro* testing or animal studies characterizing strain-specific immunomodulatory response. *In vitro* studies are used animal or human cell lines as a model of the gut (e.g., intestinal epithelial cell lines (IEC-6, IEC-1), epithelial colorectal adenocarcinoma cell line 2 (Caco-2), etc.). Currently used models (3D models) which are consisted of the intestinal epithelial cell line and the microporous

membrane (rule the polarization of cells). On the basolateral side are epithelial and immune cells (macrophages, dendritic cells (DCs)) which stimulate the mucosal lymphoid tissue. In animal studies, probiotics were used to confirm the immunomodulatory effects *in vivo* in various experimental models of allergy, autoimmune diseases or inflammatory bowel disease. Probiotics demonstrate the ability to decrease the production of pro-inflammatory cytokines, IgE, airway hyper-responsiveness or support of induction on regulatory mechanisms [39].

Many clinical studies have been trying to confirm the beneficial effect of probiotics, but unfortunately, most of the older studies showed the imperfect result in some way. The major flaw is represented mainly by the lack of the appropriate control group, with a small number of patients (or individual outcomes) or (and) outdated, insufficient data. The most suitable design of clinical study should be placebo controlled double blinded trial [39, 40].

One study showed that the consumption of probiotic yogurt containing *Lactobacillus acidophilus* and *Bifidobacterium animalis* daily for nine weeks reduced the expression of C-reactive protein but with no effect on tumor necrosis factor alpha (TNF-α) [39, 41]. Study from the year 2009 assessed the impact of probiotics used prenatally and postnatally for six months in infants with high risk for allergy development (both parents allergic). One group received twice time per day mixture of *Lactobacillus rbamnosus*, *Bifidobacterium breve*, and *Propionibacterium freudenreichii* twice per day. Similarly, the mixture was administrated to infants every day until the age of six months of the age and then followed prospectively until the age of two. At the age of six months the Hemoglobin (Hb) values of infants were significantly lower in comparison with the placebo group. At the age of two, Hb values were similar in both groups [42]. Other study described the effect of consumption of yogurt with probiotic strains in allergic children. The results of blood tests showed a significant decrease in the level of IgE in the plasma and increase in CD4+CD25 +Tregs and IgA. In 61% of the tested individuals, beneficial effect was demonstrated after preventive probiotics administration (not treatment of the disease) of atopic dermatitis in children.

Although the later study represented the higher ratio of using probiotics prenatally than postnatally [39, 43]. Use of Mutaflor (lyophilized bacteria *Escherichia coli* Nissle strain) as a probiotic showed improvement of specific antibodies in the preterm born infant stool, saliva and even in serum. The higher concentration of specific antibodies (IgA and IgM) against *E. coli* was after 14 days of trial significantly higher in the test group than in control group. The result demonstrated the beneficial effect of Mutaflor supplementation on the maturation of immune system in the preterm born infants [32, 34]. A systematic review and meta-analysis of randomized controlled trials from 2015 alerted the insufficiency of some clinical trials conducted in past years. Results of 29 selected tests (although used data are not fully compliant) represent advantageous influence on prevention of eczema (including AD) by supplementation of probiotics to pregnant women in last trimester; women after giving birth and directly to infants. The administration of probiotics does not exclude or confirm the reduction of the risk of allergy development in children [40].

The study from 2017 confirmed the convenient effect of bacteria particularly *Escherichia coli* O83:K24:H31 (hereinafter referred to as E. coli O83) on the immune system in infants. The probiotics strain E. coli O83 supported DC maturation by increasing activation markers (CD83). A higher presence of CD83 is connected with DC of newborns of allergic mothers and with the stimulation of DC by E. coli O83. Increased gene expression and secretion of IL-10 was noticeable in stimulated DC by E. coli O83 (higher in children of healthy mothers). The support of Th1 immune response in the naïve CD4+ T cells cocultured with E. coli O83 primed DC has not been proved. The trial observed increased levels of Th1 and Th2 cytokines in the mixed culture of CD4+ T cells of infants born to the allergic mothers and DC stimulated with E. coli O83. Generally, newborns of the allergic mothers have more increased activation markers together with decreased IL-10 expression in DC than infants of mothers without allergy [44].

Some of the well-known probiotic organisms are *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus acidophilus*-group, *Escherichia coli* strain Nissle

1917 or *Enterococcus faecium. Lactobacilli and Bifidus bacteria* represented short-term or no allergy protection, *Lactobacillus acidophilus* showed no influence on food allergies or asthma and *Bifidobacterium lactis* has no effect on the gut microbiota in preterm infants. These and more microorganisms are added into dairy products, individually or combined [35, 45]. Studies have shown a high efficiency with multi-strain mixtures [46].

Probiotics can have many functions such as anticarcinogenic or antimutagenic property, influence to reduction of serum cholesterol and preventing intestinal inflammations (e.g., *Helicobacter pylori* infection), etc. [47] Especially the important features of probiotics for our research are stimulation of immune system route postnatally by the maturation of DCs in newborns and reduction of the occurrence of allergy [26]. The process of stimulation immune system by probiotics is based on the improvement of intestinal barrier by supporting the intestinal IgA and controlling the intestinal permeability by non-immune gut defense [47]. The groups of structures on the surface of pathogens are recognized by specific receptors named pathogen recognition receptors including Toll-like receptors (TLRs), which are present on macrophages, DCs, B cells, phagocytes or epithelial cells. After the detection of the foreign molecules by TLRs is initiated response of DCs by the production of the cytokines (Th1-cytokines, transforming growth factor β (TGF-β)) [47].

Another eventual increase of the chance to manifest allergic inflammation is repeated ingestion of the broad-spectrum antibiotics. Recur intervention of broad-spectrum antibiotics interferes the microbiota which cannot be fully recovered (even by using probiotics or synbiotics). Even riskier is antibiotics treatment in pregnancy and in infants after birth. The increasing number of antibiotics treatment could support the increasing number of developing allergic diseases [26].

2.2.2 Medicaments

Effective and safe treatment of allergy is considerable challenge for public health. If we are not able to avoid the allergen, therapy by medicaments will be inevitable. Oral use of antihistamine drugs is one of the most common choices of treatment in clinical medicine. Allergic diseases occur in kids and seniors, and its treatment is more expensive than *diabetes mellitus*, infections of myocardial or coronary heart disease [48, 49]

Histamine has a particular influence on immunoregulation of inflammation. These substances react with receptors for histamine (H1, H2, H3, H4). "Classical" antihistamines drugs (first generation of anti-histamines) work without the specific selectivity. They can react with all four kinds of receptors (preclude histamine to bind) and also other receptors from different organs such as adrenoreceptors, dopaminergic receptors, muscarinic receptors, etc. Reaction to these receptors causes urinary, cardiovascular or gastrointestinal negative influence. However, the most dangerous problem is the result of high lipophilicity of histamine and easy crossing of the blood-brain barrier. The effect on CNS causes drowsiness, lower concentration, psychomotor efficiency, decreased activity of studying and memorizing. Another impact on the immune system by antihistamines is the reduction of expression of allergy supporting cytokines (IL-4, IL-6, IL-7, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF)), decrease expression of eosinophils, adhesive molecules or metalloproteinases, etc. [1, 48, 49].

Antihistamines targeting H1 receptors represent the secondary generation drugs. Most of the side effects (mentioned earlier) are bent from secondary generation drugs. Also, the turn-over the second generation drugs is longer and allows administration once per 12-24 hours. However, it can be connected with other problems as a body mass gain, potentially life-threating cardiotoxicity, etc. These drugs are effective in decreasing allergic symptoms of AR or urticaria. The antihistamines treatment is recommended for all adults or children, but unfortunately, this treatment is only symptomatic [1, 48, 49].

Corticoids (mainly corticosteroids, glucocorticoids) are used for their ability to stop production of histamine. Short-term use blocks mainly late allergic response, i.e., downregulates development of inflammation in affected tissues. Long-term use has the potential to function even an early response to the allergy. Their use is frequent in a cure for asthma, in ointments for skin disorders, eye drops and nasal spray for curing rhinitis. Corticoids lower the symptoms of a delayed allergic reaction [1, 47].

In the case of the systematic anaphylaxis is the first choice of treatment in the injection of epinephrine. The function of epinephrine is to block vasodilatation and prevent the loss of fluid from capillary, decrease symptoms of shock, etc. [1].

Metabolites of arachidonic acid are important in the early and also the late phase response. Their effect on bronchoconstriction is higher than histamine one. In consideration of this, developing antileukotrienes drugs is beneficial for the treatment of allergy [48].

2.2.3 Immunotherapy

Long-term stimulation of immune system by administration of the causal antigen and achieving clinical tolerance is a major principle of immunotherapy. In the first phase gradually increased the concentration of antigen (allergen) is administrated. Intervals between applications are short and regular. In the second phase high but a well-tolerated dose of allergen is applied. Intervals are more distant from each other. Allergen is given by the injection or through sublingual route. Average therapy takes about three years, in insect venom allergy the immunotherapy duration reaches even about six years. This therapy is expensive and time-consuming [48, 49, 50].

It is still not fully elucidated how immunotherapy works but in most of the trials activation of Th1 and downregulation of Th2 together with the decrease of IgE were described. Immunotherapy is also accompanied by induction of Tregs (especially Tr1). This therapy takes longer to develop, but when it is installed it means long-term relax from symptoms for patients.

Immunotherapy is useful in those patients who do not respond well to standard therapy by medicaments. The contraindications might be in the use of immunosuppressive drugs, patients with autoimmune disorders, tuberculosis and also in patients with the AD who are not recommended for immunotherapy [48, 49, 50].

2.3 Escherichia coli

The *Escherichia coli* is a gram-negative bacteria from *Enterobacteriaceae* family. More than 700 antigenic types (serovars) of *E. coli* are described according to their O-somatic, H-flagellar, K-capsular and F-fimbrial antigens. At present, approximately 170 types of O-antigen (lipopolysaccharides) of *E. coli* are described. It makes *E. coli* one of the most thoroughly described bacteria. *E. coli* is responsible for the production of vitamin K, which is essential for the function of coagulation, mineralization of bone and metabolism of wall blood vessels protein [51].

E. coli is a microorganism with a wide range of properties. *E. coli* can obtain a specific combination of mobile genetic elements and become from harmless commensal to the genuinely adapted pathogen and occur either in human or animals causing a wide range of diseases. Eight pathovars of *E. coli* are classified and some mechanisms of pathogenesis and virulence as well. These eight pathovars can be divided into two groups as diarrhoeagenic *E. coli* or extraintestinal *E. coli* (ExPEC). The diarrhoaegenic *E. coli* includes enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC). The EPEC is characterized by two pathovars of *E. coli* such as uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) [52].

The EPEC is an enteropathogen which causes inflammation (mainly in infants and seniors). A life threating risk for patients is dehydration. The familiar strains of EPEC are, e.g., O26, O55, O111. The ETEC is settled in tropes and sub-tropes. The common strains are O6 and O8 and cause cholera-like disease. The EIEC affects mostly adults (e.g., O6 or O15). Another pathovar

is enterohaemorrhagic *E. coli* significate haemolytic-uretic syndrome. Common strains of EHEC are, e.g., O157:H7 [53].

The presence of *E. coli* in food products or water is an indicator of fecal contamination. The incubation period can range 9-12 hours (EPEC, ETEC), 10-18 hours (EIEC) to 3-8 days (EHEC). The source of *E. coli* bacteria can be contaminated food (especially bovine origin), water, unpasteurized milk, infected animals, also person-to-person transmission or by the fecal-oral route [53].

Pathogenic *E. coli* has a lot of virulence factors and strategies. All pathogens except EIEC have the ability of adhesion to the host cells. These adhesive organelles such as fimbriae or pili are basic factors of virulence. Each pathovar has its own characteristic mechanism of contacting and abusing host cells, although they often attach the same host system [52].

Researchers showed that the colonization by *E. coli* (in infants) has decreased in the last decades. The reason for this adjustment might change in the hospital and the human lifestyle. The environmental factors such as having siblings, pet or specific feeding, did not change the time of colonization by *E. coli* after birth. Fifty years ago, *E. coli* was usually found in the developing microbiota of neonates shortly after the birth. Nowadays, 42% of infants are colonized by *E. coli* within three days after the birth. 50% of infants and 61% of children were colonized by *E. coli* after two weeks and two months, respectively. *Staphylococcus aureus* has become the major colonizing bacteria of the infant intestine (gut). It is caused by the lower presence of *E. coli*, therefore, reduction of defense against gut microorganisms (via bacteriocins) [54].

2.3.1 Shiga toxin-producing *Escherichia coli*

EHEC serotype can be also called Shiga toxin-producing *Escherichia coli* (STEC). Not all of STEC (over 160 different serogroups) are pathogenic for human. One of the most common, virulent strain O157:H7. STEC O157 is. the originator of many illnesses such as edema disease in piglets, diarrhea in

calves, hemorrhagic colitis, hemolytic-uremic syndrome (HUS) of human and in some cases can even cause death [53, 55].

The coding of hemolysin genes (hlyA, ehxA, and sheA) are one of the potential virulence strategies. Genes such as ehxA and hlyA are connected with generating illnesses in human. Association with sheA is unknown. Researchers represented that expression of sheA and ehxA decrease or totally inhibit expression of hlyA. The ehxA is represented by six subtypes: A, D (eae-negative); and B, C, E, F (eae positive). These subtypes variate with changes of sources (e.g., type A is commonly present in food) or with types of serogroups (e.g., subtype A with O104, O113; B with O157; and C with O26, O111, etc.) [56].

Shiga toxin in two variants (stx1 and stx2), the intimin protein (encoded by aea allele) or hemolysin genes are parts of virulence factors. The intimin is involved in attaching the organism and damaging the gut mucosal cells. Until now four types of hemolysins have been identified (a water-soluble polypeptide of relative molecular mass 107.000), the alpha-hemolysin (hlyA), plasmid-and phage-carried enterohemolysin (ehxA, e-hlyA) and silent hemolysin (sheA). EhxA and hlyA are widespread among gram-negative pathogens and have the ability to lyses. Iron is released into external environment and influences immune system via cytotoxicity for leukocytes. These are two basic pathogenic functions of hemolysin. The hlyA is also associated with urinary tract infections and ehxA cause diarrhea disease and hemolytic-uremic syndrome [54, 55, 56].

2.3.2 Urinary tract infections and influence of hlyA

In the USA, 60% of women were affected by urinary tract infection (hereinafter referred to as UTI) at least once during their life [57]. In most of the cases (over 80%) is UTIs induced by uropathogenic *Escherichia coli* (UPEC) [58]. UPEC is one of the microorganisms which physiologically colonize the gut. Urinal tract is commonly infected via fecal contamination [59]. UTIs symptoms can variate from asymptomatic bacteriuria to infections of the bladder with specific symptoms and serious kidney infections [60].

UPEC can use a number of virulence factors necessary for colonization and survival in the urinary tract. For example, type l pili have the ability to adhere to bladder cells, p pili adhere to cells in kidney and also express toxins, including hlyA [61, 62, 63].

UPEC also interacts with the immune system. Urinary tract expresses TLR 4 and 5. Contact between lipopolysaccharide and TLR initiate inflammation of urinary tract [60, 64]. Activation of TLRs triggers secretion of factor NF-κB, inducing transcription of antimicrobial cytokine IL-6 and chemokine IL-8. Interleukin 8 influences migration of neutrophils from blood to bladder. However, UPEC surmounts these innate immune response and cause UTIs by inhibiting the secretion of NF-κB [60].

The hlyA is a calcium-binding, pore-forming toxin, which is secreted by type 1 secretion system. Pro-hlyA is encoded by the hlyCBAD operon, maturation is generated by acyltransferase and ATPase, and inner membrane protein with outer protein secreted the toxin. HlyA has the capability to insert into the cellular membrane, increase their permeability ending by the cell lysis. The hlyA is also typical virulence factor of E. coli O83 [60].

2.4 Dendritic cells

Dendritic cells are diverse collective of haematopoietic cell types and the most effective antigen presenting cells (APCs). DCs as the main APCs, connect innate immunity and adaptive immunity. In organism are presented immature or activated forms of DCs [1, 65, 66].

With DCs are connected expressions of the pattern recognition receptors (PRRs), the toll-like receptors, the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), etc. Other characteristic molecules are C-type lectins which have the skill bind typical molecules of pathogens or molecules associated with skin harm [65, 66].

The classical ontogenesis of DC is deduced from the mouse studies. In bone marrow are present muramyl dipeptides (MDPs) which are macrophage and DC precursors and give rise to the monocytes and DCs. The MDPs probably rise from lymphoid-primed multipotent progenitor (LLMP). MDPs are formed in DC precursors (CDPs). CDPs can be after that transformed into two DC precursors, i.e., the plasmacytoid DCs (pDCs) precursor and the controventional DCs (cDCs) precursor. The precursors pDCs/cDCs leave the bone marrow and migrate to the organs where are finally transformed in DCs [67].

Both mice MDPs and CDPs express CD115, CD135. They can be only recognized from each other by the fact that CDPs express lower levels of CD117 than MDPs. Another distinguishing marker is NK lectin group receptor-1 (DNRG-1) marker cells similar to CDPs, not MDPs. DNRG-1+ CDPs express CD115 and generate cDCs i.e., CDPs CD115 negative forms pDCs [67].

FLT3L (CD135) is a marker of the DC lineage and is strongly expressed on pDCs and cDCs *in vivo*. Deficiency of CD135 affects the number of MDPs and preDCs [67].

2.4.1 Antigen recognition and processing

The non-activated DCs have a strategic location in tissues which gives DCs the capacity to be in close contact with external and internal environment. The most occupied tissues are a skin tissue, a respiratory tissue and an intestinal tissue. DCs transmit their spurs between epithelial cells and gather samples of Ag (contained in a food or air). DCs are enlarged into almost all organs or tissues, and dynamically migrate between blood and lymph. Migration of DC to the secondary lymphoid organs is influenced by chemokine CCL-19 and CCL-21. On DCs surface are specific receptors CCR-7 which correlate with these chemokines. In the period of no infection DC are resting and DC able to devour dead cells from healthy tissues, process them and exhibit the fragments on the MHC complex on their surface. Specific T-lymphocytes which recognized these normal autoantigens are not activated

by contact with resting DCs. The T-lymphocytes are fully inhibited or transformed into Tregs which suppress immune reactions against these autoantigens. It follows that resting DCs have influence on tolerance against own tissue [1, 65, 67].

2.4.2 Activation and maturation of dendritic cells

When immatured DC recognize a potentially dangerous impulse (pathogenic microorganism or own cells died by necrosis) is activated and becomes mature DC. The dangerous stimuli are especially pathogen-associated molecular patterns (PAMPs) which are recognized by specific receptors. The activation evoked by these receptors leads to maturation of DC which is also connected with changes in properties of DC. The maturating DC migrate into the lymph node or the secondary lymph organ, lose the ability of phagocytosis and become an effective APC. DC increase the expression of MHC class I or II proteins, costimulatory molecules (CD80/86), adhesive molecules and production of cytokines (e.g., IL-1, IL-6, TNF-α, IL-12, prostaglandins E2). These cytokines are important for an optimal stimulation and maturation of antigen-specific effector T-cells. Only matured DCs can activate T-lymphocytes. DC maturate in 48 hours, and this process is irreversible. Matured DC lives between 2 and 3 days and after this period die by apoptosis. The effective specific immune response is usually mediated on 4 - 7 days after pathogen penetration. New naïve DCs are produced in bone marrow as a bipotent progenitor called macrophage and DC progenitor (MDP). In case of inflammation process can be DC transformed from blood monocytes [1, 66, 67, 68].

2.4.3 Subtypes of dendritic cells

It has been classified at least three types of DCs. DCs described below (naive/matured) are marked as a conventional/classical DCs. The conventional/classical DCs can be divided in two more subtypes as a conventional dendritic cells 1 and conventional dendritic cells 2. Except

conventional DCs are morphologically and functionally different plasmacytoid dendritic cells and monocyte-derived DCs [1, 66].

The cDC express most of the TLR, and their major function is to induce antigen-specific stimulation (in the matured state) of naïve T-lymphocytes [1, 66].

The plasmacytoid DC express TLR specific mainly for nucleoid acids receptors TLR-7 and TLR-9. These receptors effectively recognize RNA/DNA viruses explaining the fact that after pDCs produce a high concentration of IFN-α after virus recognition. Production of this interferon alpha is also needed for activation of natural killers which can significantly contribute to the protection against viruses [1, 66].

The monocyte-derived DCs are the result of inflammation or infection. Lymphoid and non-lymphoid organs generate DCs from monocyte infiltrates and they are termed as monocyte-derived DCs (moDCs) or inflammatory DCs. MoDCs are very close to cDCs because of similarities in expression of MHC II, CD11b, and CD11c. On the other hand, moDCs express CD64 and FcγRI. The DCs can be generated from human blood monocytes in the presence of GM-CSF and IL-4 [67].

3 AIM OF THESIS

The main purpose of bachelor's thesis is a comparison of the immunomodulatory capacity of Escherichia coli O83:K24:H31 with hemolysin included in Colinfant Newborn and Escherichia coli O83:K24:H31 mutant without hemolysin on dendritic cells. The results of this work can be beneficial for further researches focusing characterization of potential mechanisms of an action of a non-pathogenic probiotic strain of bacteria in prevention of allergic and other diseases development.

The theoretical part was focused on a description of the allergy, including the characterization of some of the common types of allergy (atopic dermatitis, atopic rhinitis, asthma and food allergy). The next point was devoted to the prevention and the possible therapy of allergic diseases with a particular focus on the probiotics which shows. a positive contribution to the stimulation of the newborns immune system.

The purpose of the theoretical part of bachelor's thesis was also to describe Escherichia coli, subtypes of Escherichia coli and the possible impact of pathogenic Escherichia coli subtypes and their toxins with the focus on hemolysin. In the terminal section of the theoretical part, dendritic cells and their role in the immune system were described together with the antigen recognition and processing, activation and maturation of dendritic cells and subtypes of dendritic cells.

The practical part of bachelor's thesis was initially aimed on isolating and cultivating monocyte-derived dendritic cells from the umbilical cord blood of the newborns. After cultivation of appropriate amount of dendritic cells from precursor, dendritic cells were stimulated by Escherichia coli O83:K24:H31 containing hemolysin and bacteria without hemolysin. The main object of conclusion was the observation of the immunomodulatory properties of Escherichia coli O83:K24:H31 with and without hemolysin by labeling and measuring specific markers using flow cytometry.

4 METHODOLOGY

Umbilical cord blood samples were provided by the Institute of Care for Mother and Child in Podolí (Prague, Czech Republic). The bacterial samples of *E. coli* O83 HLY$^-$ were obtained as a gift from prof. Peter Šebo.

4.1 Isolation and cultivation of the monocyte-derived DCs from the umbilical cord blood

Due to the aim of this work, firstly I had to isolate monocytes from the cord blood (CB) then cultivate dendritic cells (monocyte-derived DCs) from them.

4.1.1 Introduction

Approximately 20-30 ml of the CB was drawn into a sterile glass flask containing heparin (10 U/ml), which is presented as an anticoagulant agent. The cord connects the fetus and placenta and provides a high level of stem cells.

After quick and appropriate transport (avoiding high-temperature fluctuations, closed dark transporting box, transported within four hours after collection, etc.) into the laboratory was needed to isolate monocytes (and other bigger cells like basophils, eosinophils, T lymphocytes, etc.) from nuclear-free cells (erythrocytes), small cells (granulocytes) and cell fragments. This isolation was done by a solution called histopaque. This solution histopaque (commercially produced solution Ficoll-Paque could be also used) has a higher density than water and enable formation of ring containing mononuclear cells (after centrifugation) over the fluid with erythrocytes, granulocytes and other small molecules as characterized in Figure 3. Over these two layers (fluid with erythrocytes, granulocytes, histopaque with leukocytes) is plasma.

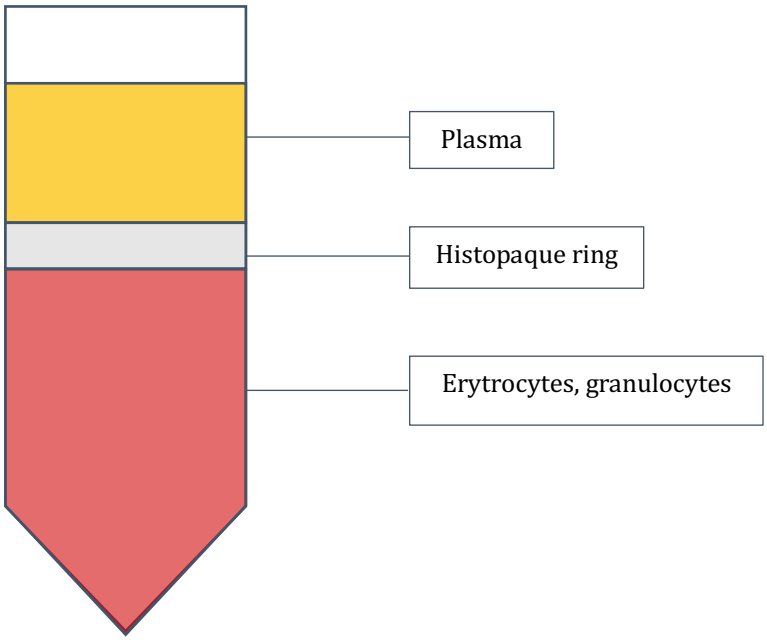


Figure 3 - The illustration of tube containing cord blood and histopaque after centrifugation [own source].

Türk's solution lyses erythrocytes and prepare solution for counting only lymphocytes in Burker's chamber.

After the isolation of leukocytes monocyte fraction was obtained by the adherence to the plastic contained in cultivating flask. The principle of the cell adhesion to the flask bottom is due to the different properties of DCs and monocytes and it is represented in Figure 4. The monocytes are supported in growth and differentiation in monocyte-derived dendritic cells (moDC) by supplementing recombinant human IL-4 and recombinant human GM-CSF in culture medium named RPMI. For desired cultivation of monocytes (moDC) cannot be present more than 60 million cells on 1 ml of the solution in the flask. A higher number of cells could avoid the right adherence of monocytes to the flasks wall.

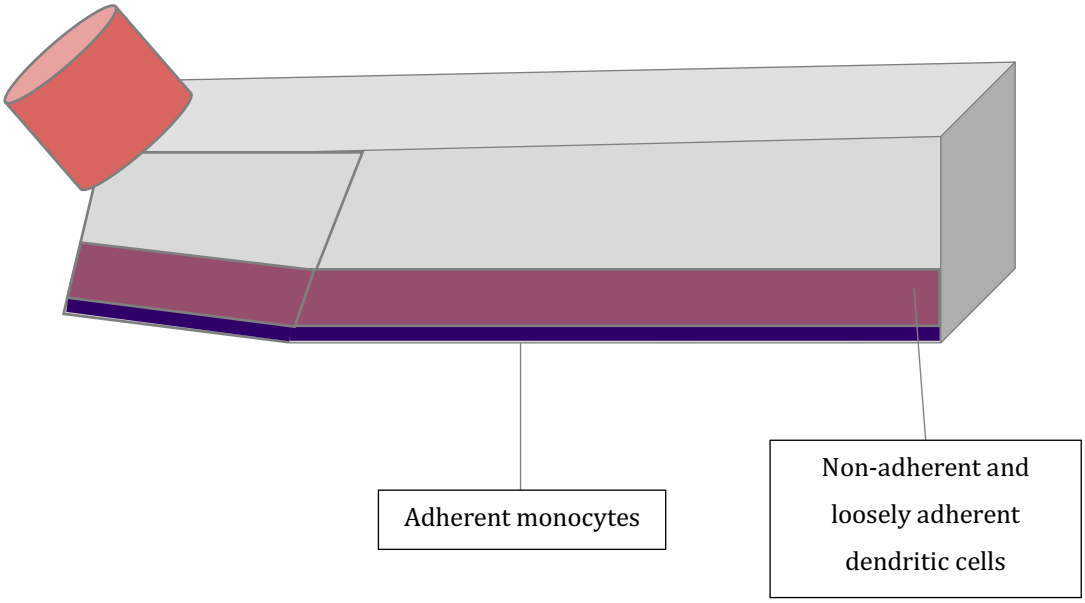


Figure 4 - Scheme of adhesion of monocytes to the bottom of the cultivation flask [own source].

4.1.2 Materials

- Umbilical cord blood
- Histopaque® 1077 by Sigma-Aldrich
- Türk's solution five times diluted in water
- RPMI 1640 Medium without L-Glutamine by Lonza
    - Fetal Bovine Serum
    - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
    - Gentamicin
- Supplemented RPMI 1640 Medium with L-Glutamine by Sigma-Aldrich
- Recombinant human IL-4 (rhIL-4) by Peprotech
- Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) by Leucomax
- The culture flask Nunc by Sigma-Aldrich

4.1.3 Procedure

- The cord blood was pipetted into the laboratory glass bottle with heparin, transported into the laboratory and diluted with the physiological saline in the ration 1:1.

- 3 ml of histopaque was transported into each of eight tubes.
- Histopaque was slowly overlayed with the cord blood and physiological saline.
- Tubes were centrifuged for 30 minutes (20°C, relative centrifugal force (RCF) 250g).
- Histopaque with present leucocytes was drained into four tubes and completed by the physiological saline.
- Tubes were centrifuged for 10 minutes (20°C, 575g).
- The supernatant was removed, the pellet was resuspended in the physiological saline, and four tubes were reduced into two tubes.
- Tubes were centrifuged for 10 minutes (20°C, 300g).
- The supernatant was removed, the pellet was resuspended in the physiological saline.
- Tubes were centrifuged for 10 minutes (20°C, 300g).
- 5 ml of culture medium (commercially named as RPMI) was added.
- Preparation cell suspension for counting: Into the new tube was pipetted 50 μl of cell suspension and 950 μl of Türk's solution and waited 10 minutes.
- In Burker's chamber were counted two times 50 squares and made an average of the number of cells (the amount of cells had to be lower than 60 million cells/ml).
- All mixture was poured into the cultivating flask placed for 60 minutes (37°C in a 5% CO_2 atmosphere) into the incubator.
- After the incubation, cells which had not adhered to the flask were carefully cleaned by 10 ml of physiological saline. The presence of adhered monocytes in the flask was controlled under the microscope.
- 15 ml of supplemented RPMI medium for DCs (together with 2,7 μl rhIL-4 and 10 μl rhGM-CSF) was added to adherent cells into the flask.
- Flask with cells was placed into the incubator (37 °C in a 5% CO_2 atmosphere) for six days to differentiate.

4.2 Stimulation of the monocyte-derived DCs

For evaluating the immunomodulatory capacity of DCs is needed to include a positive control. In our experimental design, LPS (lipopolysaccharide) was used as a positive control.

4.2.1 Introduction

The cultivated colony of monocyte-derived DCs was stimulated by lipopolysaccharide (LPS) represents a positive control, *Escherichia coli* O83:K24:H31 with hemolysin and *Escherichia coli* O83:K24:H31 without hemolysin. The concentration of *E. coli* 083 HLY^+/HLY^- was in ratio 10:1 DC and concentration of LPS 5 μg/ml. One well contains approximately one million of DCs.

4.2.2 Materials

- 12-well plate Nuclon Delta Surface by Thermo Fisher Scientific
- *E. coli* 083 HLY^+
- *E. coli* 083 HLY^-
- Lipopolysaccharide

4.2.3 Procedure

- Cells (moDCs) were carefully pipetted from the flask into the tube.
- Flask was cleaned twice with 10 ml of physiological saline.
- Tube was centrifuged for 10 minutes (20 °C, 300g).
- The supernatant was removed.
- Cells were resuspended in 2 ml of supplemented RPMI medium for DCs and counted in Burker's chamber. Concentration was modified into million cells per one well.
- Cells were divided into a well of the plate Nuclon (containing 12 wells) labeled as a control, LPS, HLY^+ and HLY^-.
- Cells were stimulated with *E. coli* 083 HLY^+, *E. coli* 083 HLY^- and with LPS.
- Stimulated cells were stored in the incubator for 24 hours (37°C in a 5% CO_2 atmosphere).

4.3 Labeling and flow cytometry analysis

Flow cytometry is a method with the possibility to measure size (forward scatter) and complexity/structure (side scatter) of cells. In addition to that, the usage of monoclonal antibodies conjugated with fluorochrome enables us to tract the changes in the presence of cell surface or intracellular markers.

4.3.1 Introduction

The evaluation of the test was allowed to do by using specific labeled antibodies which are displayed by the flow cytometry. Antibodies (specific for markers/antigens on DCs surface) are commercially produced and conjugated with characteristic fluorochromes listed in Table 1. Purchased markers specific for DCs were CD11c, CD80, CD86, CD83, CD40 and HLA-A2. With the sensitive antibodies is necessary to manipulate on melting ice.

4.3.2 Materials

- Phosphate-buffered saline (PBS) composed of NaCl, KCl and Na_2HPO_4.

Table 1 - The list of used flow cytometry antibodies [69, 70]. (CD11c) cluster of differentation 11c. (CD40) cluster of differentation 40. (CD80) cluster of differentation 80. (CD86) cluster of differentation 86. (HLA-A2) Human leukocyte antigen α2 domain. (PE-Cy7) Phycoerythrin-Cyanine7. (FITC) Fluorescein isothiocyanate. (PerCP) Peridin chlorophyll protein complex. (APC) Allophycocyanin. (APC-Cy7) Allophycocyanin-Cyanine7.

Name of the antibody	Clone	Conjugate	Reactivity	Manufacturer
CD11c	BU15	PE-Cy7	Anti-human	Exbio, plc
CD40	HI40a	FITC	Anti-human	Exbio, plc
CD80	MEM-233	PerCP	Anti-human	Exbio, plc
CD83	BU 63	APC	Anti-human	Exbio, plc
CD86	HB15c	PE	Anti-human	Exbio, plc
HLA-A2	BB7.2	APC-Cy7	Anti-human	BioLegend

4.3.3 Procedure

- The DCs were removed from the plate and centrifuged for 10 minutes (4°C, 300g).
- The supernatant was carefully removed from the tube.
- The DCs were resuspended in 100 μl of PBS buffer labeled with the primary surface antibodies shown in Figure 4 for 15 minutes (4°C, in darkness).
- Cells were centrifuged for 10 minutes (4°C, 300g) and then washed with 2 ml of PBS buffer (this step was repeated twice).
- Cells were measured by BD FACS CantoTM II Flow Cytometer BD using FACS Diva version 6.1.2 software by BD Biosciences (processing of analyses was done by using FlowJo 7.6.5 software by Tree Star).

5 RESULTS

The immunomodulatory capacity of *E. coli O83* mutant without hemolysin on DCs of the newborns was evaluated *in vitro* on stem cells contained in the umbilical cord blood. The moDCs were isolated from the adherent fraction of the cord blood mononuclear cell after six days of cultivation. Cultivation was supported by a supplemented medium with rhIL-4 and rhGM-CSF. The moDCs were stimulated by *E. coli O83* HLY$^+$, *E. coli O83* HLY$^-$ and LPS and followed up for expression of the typical molecules on DCs surface.

5.1 Gating strategy

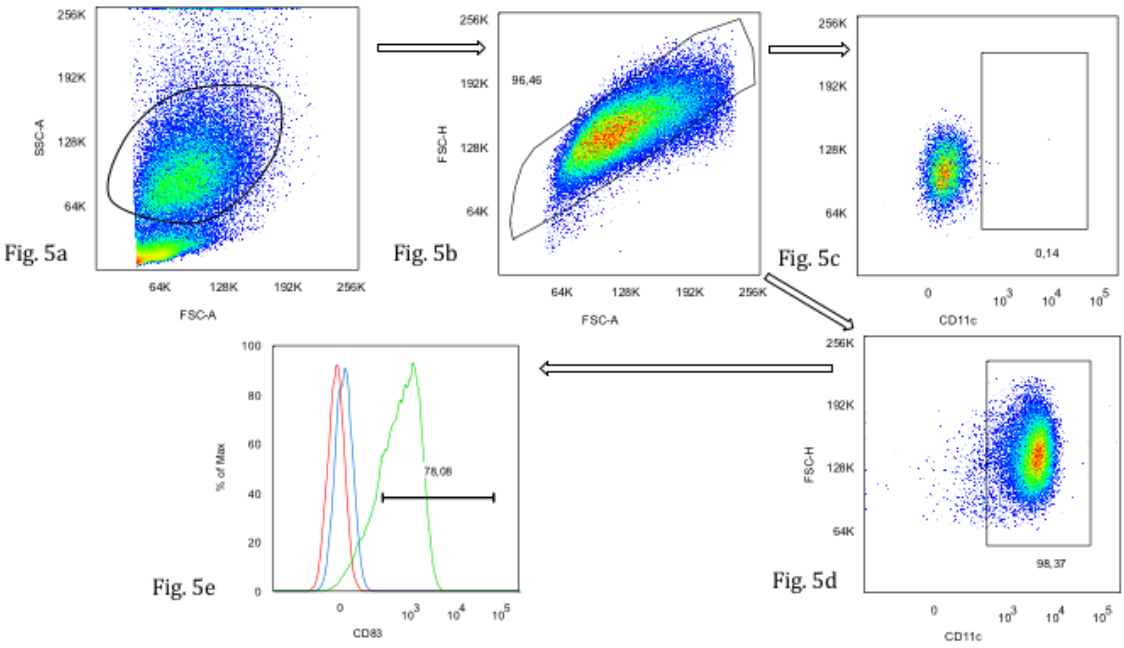


Figure 5 - Flow cytometry gating strategy for moDCs. (5a) Selection of intended population of cells using FSC and SSC. (5b) Removing of doublets using A x H. (5c) Non-labeled control for setting a gate. (5d) Selection of CD11c positive cells (DCs). (5e) Histogram of CD83+ cells. (A) Peak area. (H) Peak height. (Red peak) Not labeled control. (Blue peak) Fluorescence minus one (FMO) control. (Green peak) Labeled markers.

The strategy of gating is firstly based on selecting intended population of cells (moDCs) and removing cellular debris (Fig. 5a). In the next step, doublets (under the curve) are removed, (Fig. 5b). Finally, CD11c positive cells are selected according to fluorescein isothiocyanate (FITC) labelled monoclonal antibody against CD11c (Fig. 5d). The negative control without FITC labeling (unstained cells) is represented in Fig. 5c. The histogram of matured DCs by tracking a specific CD83 marker is showed in Fig. 5e.

5.2 Analysis of dendritic cells

The data was acquired using BD FACS Diva 6 followed by analyses using the FlowJo program and compensated to eliminate spectral overlaps. In the initial parts of the measurement were used six following specific extern proteins (marks) also shown in Table 1 – CD11c, CD40, CD80, CD83, CD86, HLA-A2. From these markers, CD83 was selected as the most appropriate marker. CD83 is a protein expressed on maturated DCs. As a positive control has been used stimulation of cells by LPS which is presented on all gram-negative bacteria as a part of cells membrane. Unstimulated CD11c positive cells (DCs) were used as a control to recognize mechanical damage of cells, etc. The same gate strategy demonstrated in Figure 5 was used for all samples and controls of the $CD11c^+$ cell population.

Illustration of the result is shown using histograms demonstrated in Figure 6. Increased expression of CD83 after stimulation of LPS is shown in Fig. 6a as a positive control (LPS) to confirm the ability of DCs to be stimulated and matured. In Fig. 6b is illustrated a low expression of CD83 of $CD11c^+$ cells without any stimulator as a control (C). Increased expression of CD83 after stimulation by *E. coli* O83 HLY^+ was displayed in Fig. 6c. A similar trend in expression of CD83 as an *E. coli* O83 HLY^+ can be observed in the case of *E. coli* O83 HLY^- and it is shown in Fig. 6d. The expression of $CD83^+$ cells in control sample was 19.63% of $CD11c^+$ cells, in LPS sample 78.06%, in *E. coli* O83 HLY^+ 53.88% and in *E. coli* O83 HLY^- 47.96%.

The data was processed and evaluated in GraphPad 7. The differences between groups were compared using a parametric t-test, where the zero hypothesis assumes the equation of the mean values. The T-test was chosen based on normal data distribution. The result of statistical processing is shown in Figure 7. The statistical tests are used for comparison whether the results of one measurements of one group differ significantly from those measured in the second group. For statistical tests was chosen the significance level $p = 0.05$. The data are shown as a median of mean values (columns) with SEM included. The p value of control and LPS is $p = 0.0002$, of control and *E. coli* O83 HLY^+ is $p = 0.0103$, of control and *E. coli* O83 HLY^- is $p = 0.061$, of *E. coli* O83 HLY^+ and LPS is $p = 0.0024$ and of *E. coli* O83 HLY^- and LPS is $p = 0.0007$. All p values reject the zero hypothesis. The median of mean values of mean fluorescence

intensity (MFI) of control, LPS, *E. coli* O83 HLY$^+$ and *E. coli* O83 HLY$^-$ stimulation is represented in Figure 8. The p value of MFI of control and LPS is p = 0.0002, of control and *E. coli* O83 HLY$^+$ is p = 0.0235, of control and *E. coli* O83 HLY$^-$ is p = 0.0272, of *E. coli* O83 HLY$^+$ and LPS is p = 0.0030 and of *E. coli* O83 HLY$^-$ and LPS is p = 0.0005. These values correspond to the values of CD83+ cells.

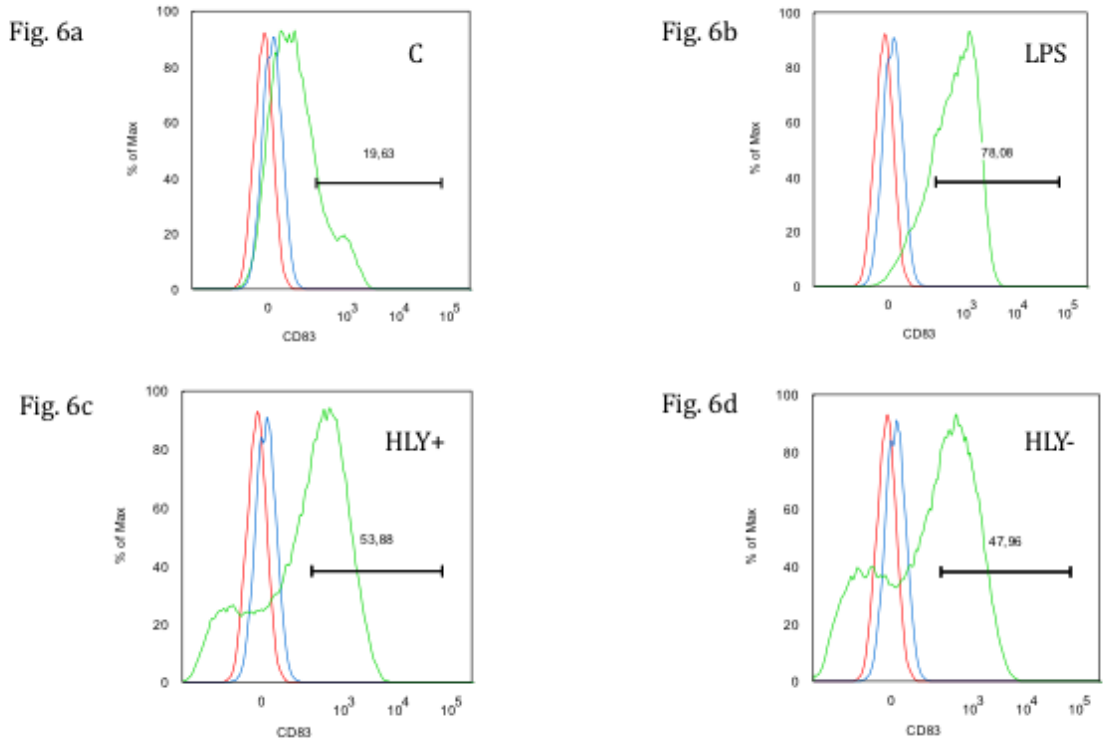


Figure 6 - Histogram of non-labeled control (red peak), fluorescence minus one (FMO) control (blue peak) and labeled markers (green peak). (6a) Histogram of non-stimulated control. (6b) Histogram of positive control (lipopolysaccharide). (6c) Histogram of the followed cluster of differentiation 83 (CD83) after stimulation of E. coli O83 HLY$^+$. (6d) Histogram of followed CD83+ after stimulation of E. coli O83 HLY$^-$.

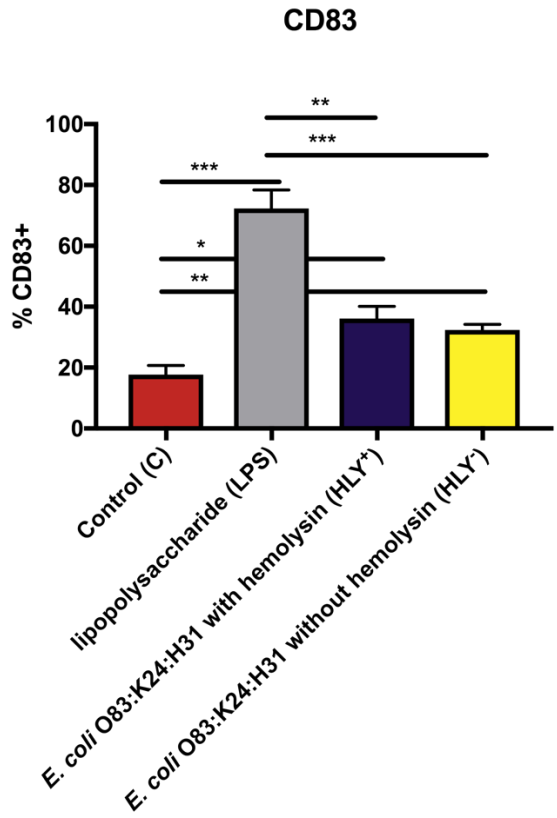


Figure 7 - The percentage of the cluster of differentiation 83 (CD83) positive cells. (Red) Non-stimulated control. (Grey) Positive control (lipopolysaccharide). (Purple) MoDCs after stimulation of E. coli O83 HLY+. (Yellow) MoDCs after stimulation of E. coli O83 HLY-.

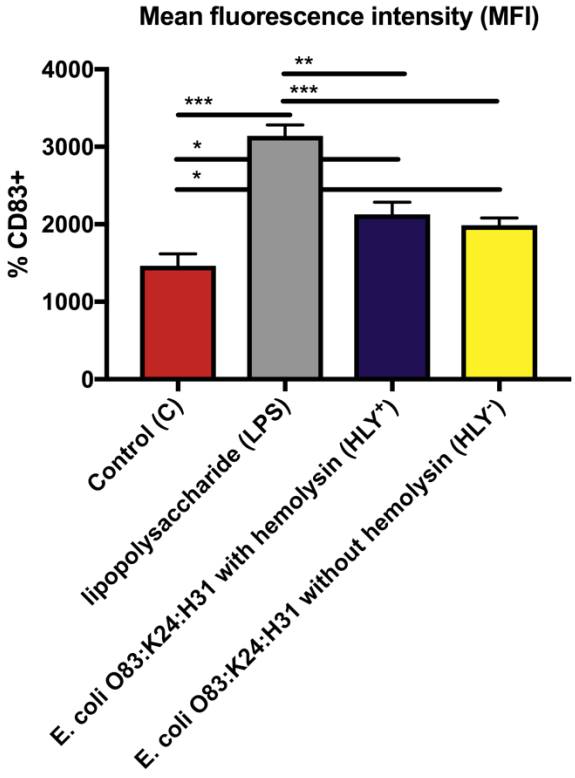


Figure 8 – Representation of mean fluorescence intensity (MFI).

6 DISCUSSION

In the view of the evaluation of gathered information and results of our test is possible to conclude that *E. coli* O83 HLY$^-$ and *E. coli* O83 HLY$^+$ have the same immunomodulatory capacity on induction of the maturation of moDCs derived from umbilical cord blood of the newborns. Immunomodulatory capacity was assessed by following specific inner membrane protein (marker) for matured DCs – CD83$^+$. The importance of immunomodulatory capacity of *E. coli* O83 HLY$^-$ could be significant for production of specific and non-specific antibodies in the intestine, mucous membranes and in the blood system. By stimulation some of naturally presented bacteria in the intestine is induced Th1 response instead of Th2 response which can cause predominance for development of allergy. The promotion of the newborn immune system has beneficial property such as disability to the settlement of pathogenic gut microorganisms. Other ability of promotion of newborn immune system includes prevention of development of several diseases such as diabetes mellitus, Alzheimer's disease, dementia or autism and also allergy [71].

One of the characteristic features of aging is reduced the variability of gut bacteria, which is likely to result in neurological pathological conditions such as depression, Alzheimer's disease or Parkinson's disease. One of the most well-known ways of combining brain and gut is through the *nervus vagus*, metabolites that have been produced by the intestinal bacteria, the influence of bacteria on the function of the intestine, the immune system and the metabolism [71].

Some studies have shown that bacteria colonizing the human organism are involved in almost all physiological and pathological reactions in the human body [72]. The examples of these reactions include immunomodulation of immune cells, vitamin biosynthesis or energy requirements during the day. The microbiome structure also affects the development of obesity, *diabetes mellitus* type II or cardiovascular disease [73]. Microbiota is involved in influencing the synthesis of various neurotransmitters such as dopamine, serotonin or norepinephrine [74]. It has been shown that germ-free mice had higher production of these three neurotransmitters compared to mice without pathological colonization of the intestines. Particularly in newborn and early aged mice, serotonin has been shown to be susceptible to colonization by microorganisms [71].

Some molecules produced by the metabolism of bacteria can cause inflammation of the nervous system and graft in severe nerve diseases. Adult intestinal microbiota is influenced by the genetics, eating habits, using certain drugs (especially antibiotics), exercises and moreover, greatly influencing circadian rhythms. Aging individuals may have imbalanced circadian rhythms and that have a major influence on the intestinal microbial activity. In addition, an aging population of mice has been shown to increase the permeability of the intestinal wall associated with an increase in peripheral proinflammatory cytokines [71]. The psychobiotic is term including both probiotics and prebiotics as well as other external influences such as the above-mentioned exercises, medications, etc. that affects the brain and its function. Early infection of the immature immune system is one of the most important influences in the development of the adult's phenotype [74].

In Alzheimer's disease was observed a reduced microbial diversity with a low presence of *Bifidobacteria* and on the contrary, an increased presence of *Bacteroides* which appears to be characteristic bacteria presents in patients suffering from Alzheimer's disease. An increased amount of proinflammatory bacteria such as pathogenic *E. coli* or *Shigella* and a concurrent reduction of anti-inflammatory bacteria such as *Eubacterium rectale* lead to an increase in inflammatory mediators. Reduced amount of *Akkermansia muciniphila* was presented in obese mice and mice with *diabetes mellitus* type II, which belongs to two potential risk factors for the development of Alzheimer's disease. The results of mice studies suggest that modulation of intestinal microbiota by probiotics can create such features as to prevent/suppress the manifestation of the neurodegenerative disease. However, the authors of the study agree that additional research and obtaining more detailed information are needed to confirm the possible prevention/treatment of AD by modulating the intestinal microbiota [71].

E. coli O83 HLY$^+$ is bacteria contained in probiotic medicament Colinfant New Born (colinfant). Colinfant is intended for newborns and infants under the age of one, for preterm infants and especially for non-lactated infants. Colinfant is mainly used as a prevention of nosocomial infections, gastric and intestinal infections, disorders of the intestinal microbiota composition especially after antibiotic treatment and as a prevention of carriers of enteropathogenic strains causing intestinal diseases [75].

E. coli O83 was previously used as a preventive and therapeutic agent for the proper colonization of the intestines of preterm and term born infants. The proper colonization of the intestines helps (not only) to prevent nosocomial infections by protecting against colonization of pathogenic bacteria in newborns intestines [45]. Earlier studies confirmed the protective properties of *E. coli* O83 against the colonization by pathogenic *E. coli* strains – antibiotic-resistant, colicin-sensitive and enterotoxigenic strains [76].

E. coli O83 as a non-pathogenic strain has been used for decades in the Czech Republic as a probiotic for the newborns as a live oral vaccine. In practice, the use of *E. coli* O83, hemolysin did not manifest the probability of harmfulness of hemolysin in the administration of probiotic strain to the newborns. Conversely, ability of *E.* coli O83 to colonize the intestine was confirmed. For a risky group of the newborns (premature newborns, low birth weight the newborns, caesarean delivered newborns, etc.) is *E. coli* O83 used as a prevention of bacterial and nosocomial infections [77]. The intestinal stimulation of *E. coli* O83 has been shown to reduce the incidence of possible allergic diseases in childhood and after re-application also at later age [45].

The study by Súkeníková et al. 2017 demonstrated the induction on the maturation of moDCs derived from precursors of umbilical cord blood and stimulated by *E. coli* O83. Research confirmed the ability of moDCs stimulated by *E. coli* O83 to express pro-inflammatory cytokine IL-10. Owing to this induction of maturation, were moDCs able to activate T regulatory lymphocytes and induce production of more IL-10 and IFN-γ which are cytokines supporting Th1 immune response. Considering these properties, *E. coli* O83 could be able to primarily respond to the immune system of a newborn by promotion T regulatory cells. This could increase the development of Th1 immune response and affect IgE-producing cells in markdown production of IgE in newborns. In this case, the allergen may not develop an allergic reaction. However, the study does not confirm the prevention of allergy in reducing the Th2 response. The trial also showed the increased reactivity of moDCs in newborns blood of allergic mothers. Stimulation of the Th17 immune response could be an intermediate step balancing the Th2 and Th1 immune response in newborns. Because of the lack of suitable studies, the induction of Th17 by *E. coli* O83 has not been established yet [44].

The study of the genome of a non-pathogenic strain of *E. coli* O83 showed that *E. coli* O83 is similar in most genes with pathogenic UPECs strains in particular, the hemolysin A virulent factor gene [78].

In the study by Shesko et al. 2006, the effect of *E. coli* O83 with hemolysin and *E. coli* O83 without hemolysin was tested *in vivo* on the intestine of conventional and gnotobiotic piglets. *E. coli* O83 HLY$^+$ was cultured on medium supplemented with sheep erythrocytes. After the detection of genomic sequences of *E. coli* O83 HLY$^+$ by the Sanger sequencing method, the hlyA gene was deleted. The selection of new genome enabled rise of *E. coli* O83 HLY$^-$ [79].

The results of the analysis showed that after inoculation of the newborn piglets by *E. coli* O83 HLY$^+$ and *E. coli* O83 HLY$^-$, their intestines were strongly colonized by *E. coli* O83 within 24 hours. 28 days before the end of the test, *E. coli* O83 was still presented in the faeces. These results confirmed that the absence of hlyA has no effect on colonization of the newborn's piglets intestines [79].

The effect of *E. coli* O83 HLY$^+$ was compared with *E. coli* O83 HLY$^-$ on germ-free, colostrum-deprived newborn piglets that do not have developed humoral immunity and they are highly susceptible to any bacterial infection. The *E. coli* O83 HLY$^+$ and *E. coli* O83 HLY$^-$ were given to piglets after in three days after birth when is intestinal barrier still open and after three days of birth. Until three days most of the piglets have experienced sepsis or hemorrhagic syndrome with the final death of the animal. In the case of *E. coli* O83 HLY$^+$ it was until 24 hours (all animals), in the case of *E. coli* O83 HLY$^-$ it was until 48 hours (three piglets survived). The survival rate is shown in Figure 9 (A). *E. coli* O83 was also administered to the newborn piglets three days after. the birth. The first half of piglets inoculated by *E. coli* O83 HLY$^+$ died and second half inoculated with the same strain survived. Piglets with administered *E. coli* O83 HLY$^-$ all survived and lived until the end of the experiment (28 days). The characterization is demonstrated in Figure 9 (C). This experiment confirmed the higher safety of *E. coli* O83 HLY$^-$ in one of the most sensitive animal strains [79].

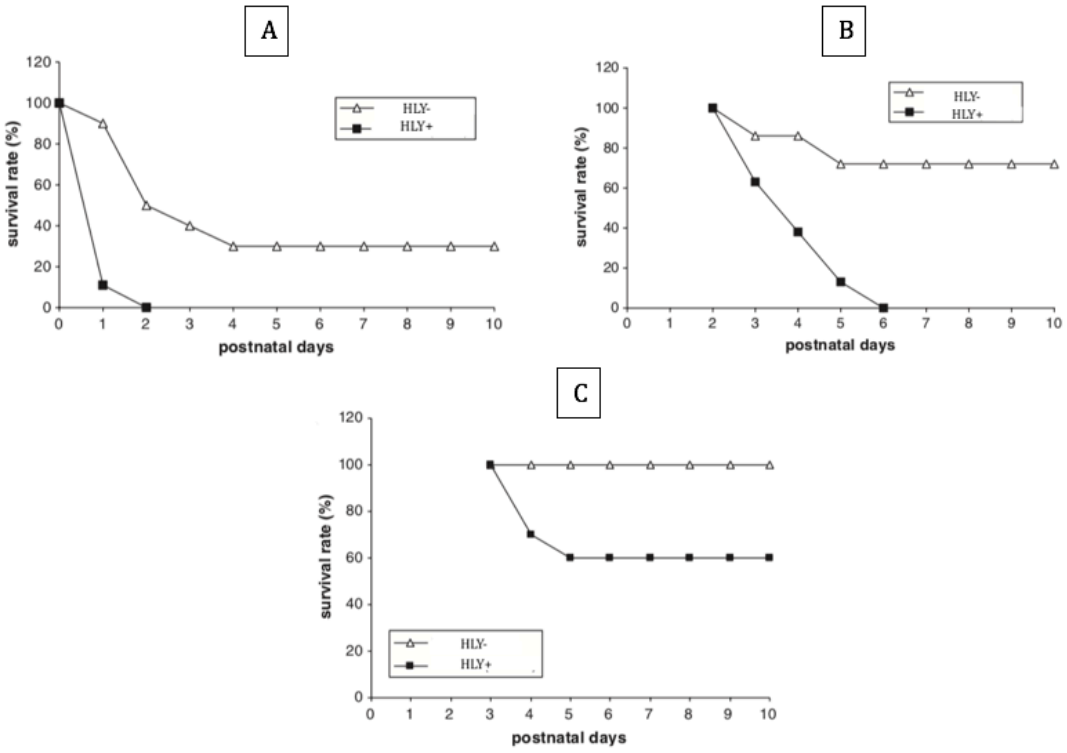


Figure 9 – The percentage of the survival rate of mice after administration of E. coli O83 HLY⁺ and E. coli O83 HLY⁻ [79]. (A) The administration of E. coli O83 HLY⁺ and HLY⁻ in day 0. (B) The administration of E. coli O83 HLY⁺ and HLY⁻ in day 2. (C) The administration of E. coli O83 HLY⁺ and HLY⁻ in day 3.

The results of researchers from the following two articles could have a major impact on the results of our analysis and influence on the interpretation of the results. Studies have been conducted *in vitro* on bone marrow mice samples [80].

DCs can differentiate under the influence of different hormones from several precursors. Generally, DCs contain the main significant markers such as CD11c or MHC II which is important for the processing and exposure of antigens, followed by stimulation of T lymphocytes. Bone marrow-derived dendritic cells (BMDCs), expressing MHCII and CD11c are derived from bone marrow cells being stimulated with GM-CSF. BMDCs can be differentiated into cDCs or into monocyte-derived macrophages. Both of these subgroups may be matured after the stimulation by lipopolysaccharide but each of them exert different properties. Although monocyte-derived macrophages and cDCs show different properties, there is a connection between DCs and macrophages such as occasional MHC II macrophage expression [80].

General knowledge of DC and macrophages derived from blood and cultivated in presence of GM-CSF distinguish free-adhering BMDCs expressing MHC II and CD11c and

fully adherent macrophages without expression of MHC II and CD11c. It was assumed that the non-adherent components (BMDCs/moDCs) are homogeneous population with differences between cells related to the maturation state of cells (moDCs). MoDCs supposed to be separated by initial washing and removing the procedure from fully adherent macrophages [80].

The results of the first study reflected rather the differences between cells in the state of maturation, the occurrence of more than one subtype of cells; a heterogeneous cells population. One of the loosely adherent population express a higher amount of MHC II and CD11c which resembling more matured cells similar to DCs; on contrary, cells with lower expression of MHC II and CD11c are disrupted to the immature population of macrophages. A subpopulation of DCs of CD11c positive and MHC II high cells expressed molecules such as CD24, DEC205, PD-L2, IRF4, CD135, CCR7, CD115 or CD117. A subpopulation of MHC II intermediate and CD11c negative cells expressed molecules CD11b, SIRPa, CD135, CD64 or CD14. The graphs representing the expression of specific markers for subpopulations of monocytes are attached in Figure 10 (A), (B) and (C). In the three-day cultivation of moDCs in the presence of GM-CSF was the expression of CD135/CD115 markers was not significantly preferred and most of the cells were negative for both markers. After prolonged culture for three more days, cells started expressing CD135 but not CD115. Cells positive for CD135 correspond to DCs and cells negative for CD135 resembles to macrophages. After cultivation in supplemented medium with GM-CSF and IL-4, cells with higher expression of CD11b were expressing CD115. The use of IL-4 has confirmed that IL-4 does not inhibit the development of monocyte-derived macrophages and again that moDCs are heterogeneous subpopulation [80].

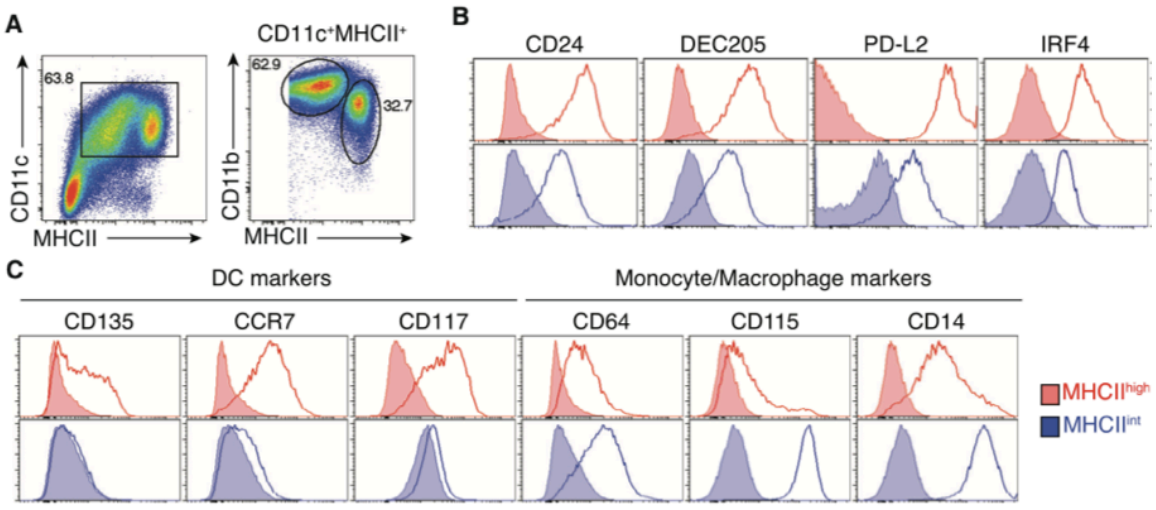
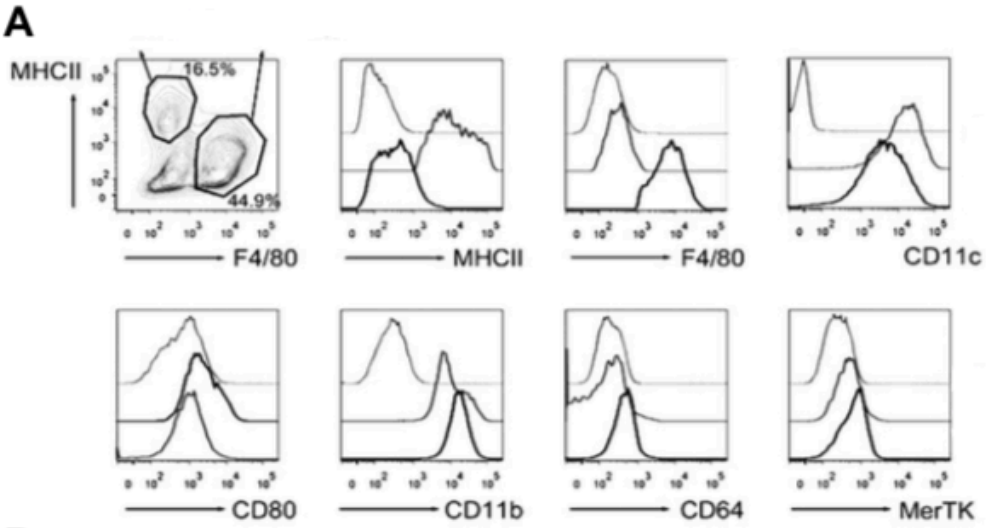


Figure 10 - The expression of specific markers of moDCs in first study [80]. (A) The presence of positive MHC II, CD11c, and CD11b markers. (B) The presence of positive CD24, DEC205, PD-L2, and IRF4 markers of moDCs and macrophages. (C) The presence of positive CD135, CCR7, CD117, CD64, CD115, and CD14 markers of moDCs and macrophages. (CD) Cluster of differentation. (CCR7) C-C chemokine receptor type 7. (PD-L2) Programmed cell death ligand 2. (IRF4) Interferon regulatory factor 4.

In the second study, the cells obtained from the bone marrow of the mice were moDCs cultured for seven days with GM-CSF addition and particularly markers such as MHC II (DCs marker) and F4/80 (Macrophages marker) were observed. The following markers that were tracked are CD11c and CD11b. The expression of both kinds of markers was increased in moDCs and expression of CD11b in macrophages population. CD11c expression in macrophages was low. The expression of specific markers is shown in Figure 11 [81].

In both studies was not only tested the phenotype of cells in the response to their markers and cytokines, but there was also analyzed the gene transcript of both cells subtypes – moDCs and macrophages [80, 81]. In the view of these findings, it would be advisable to take this resulted information, into account in continuing *in vitro* studies.



Marker	moDCs	macrophages
MHCII	22,865±12011	361±150
F4/80	331±48	7,458±4,087
CD11c	20,300±436	7,394±102
CD80	3,148±36	-
CD11b	16967±2,603	18933±289
CD64	-	458±47
MerTK	549±95	628±75

Figure 11 - The expression of specific markers of moDCs in the second study [81]. (A) The expression of MHC II, F4/80, CD11c, CD80, CD11b, CD64 and MerTK markers of moDCs and macrophages. (B) The table with mean fluorescent intensity values (MFI). (CD) Cluster of differentation. (MerTK) Proto-oncogene tyrosine-protein kinase Mer. (MHC II) Class II major histocompability complex.

Nevertheless, we can assume that our experiments outlined the positive effect of probiotic *E. coli* O83 without hemolysin on the immune system of the newborns with a potential to be used in further studies of allergy prevention as well as possible use of *E. coli* O83 HLY$^-$ for testing of beneficial properties as a prevention of other diseases.

7 CONCLUSION

The aim of our work was to compare the immunomodulatory capacity of *Escherichia coli* O83:K24:H31 containing hemolysin used as probiotic with an immunomodulatory capacity of *Escherichia coli* O83:K24:H31 mutant with a missing hemolysin gene. The proof of properties was applied to monocyte-derived dendritic cells obtained from umbilical cord blood of newborns.

Escherichia coli O83:K24:H31 HLY^- demonstrated the ability to increase the CD83 maturation markers of CD11c positive monocyte-derived dendritic cells as well as *Escherichia coli* O83:K24:H31 HLY^+.

The result of our work could contribute to the development of further studies and researchers to identify the *Escherichia coli* O83:K24:H31 HLY^- immunomodulatory capacity in experiments using animal. The use of *Escherichia coli* O83:K24:H31 HLY^- could prove to be a more acceptable variant of the probiotic used to colonize intestine of newborns in the prevention of allergy but also to prevent further recurrent diseases whose development may be dependent on the bacterial colonization of the intestine in the newborns.

8 LIST OF ABBREVIATIONS

Ab	Antibody
AA	Atopic asthma
AD	Atopic dermatitis
AIT	Allergy immunotherapy
APC	Antigen presenting cell
APC	Allophycocyanin
AR	Atopic rhinitis
BMDC	Bone marrow derived dendritic cell
CaCO-2	Epithelial colorectal adenocarcinoma cell line 2
CB	Cord blood
CCL-19	C-C motif chemokine ligand 19
CCL-21	C-C motif chemokine ligand 21
CCR-7	C-C motif chemokine receptor 7
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CNS	Central nervous system
Colinfant	Colinfant New Born
CRP	C-reactive protein
CS	Caesarean section
DAEC	Diffusely adherent Escherichia coli
DC	Dendritic cell
DNRG-1	Natural killer lectin group receptor-1
E. coli O83	Escherichia coli O83:K24:H31
eae	Allele encoding protein intimin
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
ehxA	Enterohemolysin gene
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extraintestinal *Escherichia coli*

FcϵRI	Low-affinity receptor for the Fc region
FcϵRII	High-affinity receptor for the Fc region
FITC	Fluorescein isothiocyanate
FLG	Filaggrin
FLT3L	FMS-like tyrosine kinase 3 ligand
FMO	Fluorescence minus one
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
hlyA	α-hemolysin gene
HLY$^+$	With hemolysin
HLY$^-$	Without hemolysin
HpARI	Heligmosomoides polygyrus alarmin release inhibitor
HUS	Hemolytic-uremic syndrome
IEC-1	Intestinal epithelial cell line 1
IEC-6	Intestinal epithelial cell line 6
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IFN-γ	Interferon γ
LLMP	Lymphoid-primed multipotent progenitor
LPS	Lipopolysaccharide
MDP	Muramyl dipeptide
MerTK	Proto-oncogene tyrosine-protein kinase Mer
MHC I	Class I major histocompatibility complex
MHC II	Class II major histocompatibility complex
MoDC	Monocyte-derived dendritic cell
NF-κB	Nuclear factor KB
NLR	Nucleotide-binding oligomerization domain-like receptor
NMEC	Neonatal meningitis *Escherichia coli*

NOD	Nucleotide-binding oligomerization domain
PAF	Platelet-activating factor
PAMP	Pathogen-associated molecular patterns
pDC	Plasmacytoid dendritic cell
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein complex
PRR	Pattern recognition receptor
RCF	Relative centrifugal force
rhGM-CSF	Recombinant human granulocyte-macrophage colony-stimulating factor
rhIL-4	Recombinant human interleukin 4
sheA	Silent hemolysin gene
SNP	Single nucleotide polymorphism
STEC	Shiga toxin-producing *Escherichia coli*
TCR	T cell receptor
TGF-β	transforming growth factor β
Th	Helper Th lymphocyte
Th	Helper Th lymphocyte
TLR	Toll like receptor
TNF-α	Tumor necrosis factor α
TNF-β	Tumor necrosis factor β
Treg	T regulatory cells
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection

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10 LIST OF FIGURES

11 LIST OF TABLES