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Role slizniční imunity a střevní mikroflóry při vývoji zánětlivých onemocnění

The role of mucosal immunity and gut microbiota in the inflammatory diseases

Diplomová práce

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Prohlášení:

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Podpis:

ABSTRAKT

Střevní mikroflóra zásadně ovlivňuje fungování lidského organismu. V okamžiku, kdy je narušena rovnováha v jejím složení, může dojít k indukci nebo prodloužení trvání relapsu již existujících chronických zánětlivých onemocnění, mezi něž patří i idiopatické střevní záněty (IBD). Mechanismus, jímž by bylo možné odlišit prospěšné mikroby od škodlivých, stále není znám. Cílem této práce bylo zkoumat interakce imunitního systému s mikroby, kteří jsou různým způsobem spojeny s patogenezí IBD.

Escherichia coli je převládajícím aerobním mikroorganismem lidského trávicího traktu. Tento druh zahrnuje nejen mikroby zapojené v indukci IBD, ale i ty které napomáhají s jejich léčbou. Pro další experimenty byly vybrány 4 kmeny *E. coli*, které mají různý vztah k patogenezi IBD: *E. coli* Nissle 1917 (EcN; je úspěšně používána při léčbě IBD), *E. coli* kmeny LF82 a p19A (patrně hrají roli při patogenezi IBD), *E. coli* kmen K6 (není používán v léčbě a není známo, že by hrál roli při patogenezi IBD).

K pokusům byly používány jak živé, tak inaktivované bakterie. Různé způsoby inaktivace bakterií (1% formaldehyd, teplo, UV záření) mohou měnit jejich antigenní strukturu, a proto jsme se zaměřili na sledování změn ve schopnosti inaktivovaných mikrobů vyvolat imunitní odpověď.

Nejprve jsme pomocí nepřímé imunoenzymatické metody (ELISA) analyzovali *E. coli*-specifické sérové IgA a IgG u pacientů s IBD a u zdravých kontrol. Různé způsoby inaktivace neměly vliv na protilátkami zprostředkovanou sérovou reaktivitu proti žádnému ze sledovaných kmenů *E. coli*. Taktéž jsme nepozorovali žádné rozdíly v protilátkové odpovědi mezi testovanými skupinami, kromě zvýšení koncentrace IgA protilátek proti patogennímu kmenu *E. coli* p19A u pacientů s IBD.

Dále jsme spolu s inaktivovanými bakteriemi kultivovali splenocyty nebo buňky izolované z mezenteriálních uzlin zdravých myší či myší s akutním střevním zánětem. Následně jsme měřili časnou aktivaci těchto buněk (exprese CD69) průtokovou cytometrií. Také jsme inaktivovanými bakteriemi stimulovali myší makrofágovou buněčnou linii (RAW 264.7) a stanovovali jsme aktivaci těchto buněk pomocí Griessovy reakce (produkce oxidu dusnatého) a průtokové cytometrie (exprese CD40). V žádném ze zmíněných pokusů nebyly pozorovány signifikantní rozdíly mezi jednotlivými stimuly.

Vzhledem k tomu, že porušení epiteliální buněčné vrstvy je významnou součástí patogeneze IBD, sledovali jsme pomocí průtokové cytometrie, kolik střevních epitelových buněk (myší MODE-k nebo lidské Caco-2) se uvolní ze souvislé buněčné vrstvy po

čtyřhodinové kultivaci s živými bakteriemi *E. coli*. U obou buněčných linií kmen p19A uvolnil většinu buněk, zatímco kmen EcN souvislou vrstvu vůbec nenarušil. Ve všech případech byly téměř všechny uvolněné buňky buď mrtvé (Hoechst+), nebo ve stavu apoptózy (Annexin V+).

Závěrem lze shrnout, že žádný způsob inaktivace signifikantně nezměnil imunogenicitu bakterií. Vazba protilátek na probiotické i patogenní mikroby byla velice podobná u pacientů s IBD i kontrol, lišila se pouze u p19A. Při sledování buněčné odpovědi na stimulaci různými kmeny *E. coli* jsme nepozorovali žádné významné rozdíly, ovšem oba patobionti *in vitro* poškozovali vrstvu epitelu. K největšímu poškození epitelialní vrstvy během kultivace došlo za přítomnosti kmene p19A. To naznačuje, že by tento mechanismus působeni kmene p19A mohl mít zásadní úlohu při vzniku a průběhu IBD.

Klíčová slova: idiopatické střevní záněty (IBD), *E. coli*, inaktivace, imunitní odpověď

ABSTRACT

Gut microbiota is important for our health and well-being, but when its composition is disrupted, it can induce or perpetuate several chronic inflammatory disorders, including inflammatory bowel diseases (IBD). The mechanisms which distinguish protective microbes from the deleterious or indifferent ones are largely unknown. The aim of this thesis was to study the interaction of the immune system with microbes that have different relationships to IBD pathogenesis.

Escherichia coli is a predominant aerobic microorganism of the gastrointestinal tract. This species includes microbes implicated in induction of IBD as well as in its therapy. Four *E. coli* strains with different relations to IBD were selected for our experiments: *E. coli* Nissle 1917 (EcN), which has been successfully used in IBD therapy, *E. coli* strains LF82 and p19A, which have been implicated in the pathogenesis of IBD, and *E. coli* strain K6, which has neither been implicated in pathogenesis nor in protection from this disease.

The experiments were performed both with living bacteria and inactivated ones. As the mode of inactivation may change the microbial antigenic structure, we measured how different methods of inactivation, i.e. 1% formaldehyde, exposure to heat or UV irradiation, influence the microbe's immunogenicity.

First, we analyzed the serum IgA and IgG against *E. coli* in sera of patients with IBD and healthy controls using indirect ELISA. The different mode of inactivation did not change the serum reactivity to any of the *E. coli* strains. There were no differences in the antibody responses among tested groups, except for the increase in IgA against the potentially pathogenic *E.coli* strain p19A in IBD patients.

Next, we cultivated spleen cells or cells isolated from mesenteric lymph nodes from either healthy mice or mice with active intestinal inflammation with inactivated bacteria, and measured the early cell activation (expression of CD69) by flow cytometry. In addition, we stimulated murine macrophage cell line (RAW264.7) with inactivated bacteria and measured the cell activation by Griess assay (nitrite production) and flow cytometry (CD40 expression). Overall, there were no significant differences among the stimuli.

Since the disruption of the epithelial cell layer is an important step in IBD pathogenesis, we measured the detachment of intestinal epithelial cells (murine MODE-K or human Caco-2) after their 4h cultivation with live *E. coli* by flow cytometry. In both cell lines, p19A detached the most epithelial cells, while EcN did not disrupt the cell monolayer at

all. In all cases, almost all detached cells were either dead (Hoechst+) or undergoing apoptosis (Annexin V+).

In conclusion, neither of the inactivation types induced significant changes in bacteria immunogenicity. The antibody avidity to both probiotic and pathogenic microbes was very similar in IBD patients and controls, except for p19A. We could not find any significant changes in cellular response to different *E. coli*, but both used pathobionts damaged the epithelial layer *in vitro*. Strain p19A caused the most extensive damage to epithelial cells, which suggests that this could be the major factor of virulence of this bacterium engaged in IBD pathogenesis.

Keywords: inflammatory bowel diseases, *E. coli*, inactivation, immune response

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ABBREVIATIONS

ACK	ammonium-chloride-potassium lysis buffer
AMP	antimicrobial peptide
PEROX	horseradish peroxidase
APC (dye)	allophycocyanin
APC	antigen presenting cell
ATB	antibiotic solution
BSA	bovine serum albumin
BV (dye)	brilliant violet
CD	cluster of differentiation number 4
CFU	colony-forming unit
CMIS	common mucosal immune system
CpG	cytosine-phosphate-guanine
DC	dendritic cell
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
dsRNA	double-stranded RNA
DSS	dextran sodium sulphate
E. coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FDC	follicular dendritic cell
FF	saline solution
FITC	fluorescein isothiocyanate
FVD	fixable viability dye
5-FU	5-fluoruracil
GALT	gut-associated lymphoid tissue
GIT	gastrointestinal tract
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTS	high throughput sampler
IBD	inflammatory bowel diseases

IBS	irritable bowel syndrome
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
IFN-γ	interferon γ
IgA	immunoglobulin A
IgG	immunoglobulin G
IL-10	interleukin-10
ILF	isolated lymphoid follicle
iTreg	induced regulatory T cell
LPS	lipopolysaccharide
LTA	lipoteichoic acid
M cell	microfold cell
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MLN	mesenterial lymph node
NEC	necrotizing enterocolitis
NFκB	nuclear factor-κB
NLR	nod-like receptor
NMS	normal mouse serum
nTreg	natural regulatory T cell
OD	optical density
PBS	phosphate buffered saline
PE (dye)	phycoerythrin
PMA/iono	phorbol 12-myristate 13-acetate / ionomycin
PMT	photomultiplier tube
PRR	pattern recognition receptor
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
rRNA	ribosomal ribonucleic acid
RT	room temperature
SCFA	short-chain fatty acids
sIgA	secretory immunoglobulin A
SPL	spleen

ssRNA	single-stranded RNA
TGF-β	transforming growth factor β
Th cell	helper T cell
Tc cell	cytotoxic T cell
TID	type I diabetes mellitus
TLR	toll-like receptor
TMB	3,3’,5,5’-tetramethylbenzidine
Tr1	type I regulatory T cell
Treg cell	regulatory T cells
UC	ulcerative colitis

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1 LITERATURE REVIEW

Every single day, one's immune system deals with a large amount of microbes. Some of them represent a serious threat, others live in harmony with the host organism, but generally they all influence the host's life in some way. The site of the main exposure to antigens are mucosea. A whole ecosystem of microbes associated with mucosae resides in the human organism – this ecosystem is called microbiota. Interaction between the host and a particular microbe can result in events, which are beneficial (probiotic), indifferent (commensal) or deleterious (pathogen) to the host. However, this is only an anthropocentric view on the problem and microbes can possess properties of both "probiotic" and "pathogenic" character. Results of the host-microbe interaction depend on the microbe (e.g. its adhesive properties, invasion ability) as well as on the host (particularly his health state). Despite the intensive research, mechanisms of these interactions are still largely unknown. Nevertheless, main factors influencing the result include: composition and function of the microbial community, state of the mucosal barrier, modulation of the immune response by microbes.

1.1 Mucosal immune system

The area of mucosal surfaces in an organism accounts for as much as 300m^2. The mucosae are an important site for the contact with many stimuli from the environment (e.g. microbiota, food, antigens from the air). Essential immunological functions of the organism are linked to the mucosal tissue; this is illustrated by the following facts. (i) Ninety percent of all infectious agents enter the organism through mucosal surfaces. (ii) Mechanisms of the innate immune system are strongly developed in the mucosae. (iii) Overall, eighty percent of immune cells are connected to the mucosal surfaces, which makes the mucosal lymphoid tissue the biggest immune organ in our body. (iv) A vast production of immunoglobulins, especially secretory IgA (sIgA) is a characteristic of every mucosal tissue. (v) The immune system associated with the mucosae has the ability to either induce reactivity or tolerance to recognized antigens. (Tlaskalová-Hogenová and Městecký, 2012)

All mucosae (i.e. mucosa of the oral cavity, nasal cavity, respiratory tract, digestive tract, urogenital tract, mucosa of the eye, internal ear and exocrine gland ducts) are connected to each other by cross-communication. This integrated network is termed common mucosal immune system (CMIS). The close relation of immune mechanisms in all possible mucosal

sites is the reason why intervention in the gut immune system may alter the immune response in other mucosal sites. (Iijima et al., 2001)

1.1.1 Mucosal immune system of the gut

The IgA secreted into the gut lumen seems to protect the tissue against viral and bacterial pathogens as well as to regulate homeostasis of the gut microbiota (Fagarasan and Honjo, 2003). The presence of a great number of lymphocytes in the gut mucosa brought Cheroutre (2004) and others to the idea that the adaptive immune system may have arisen from the mucosal immune system.

1.1.1.1 Structure and main organization

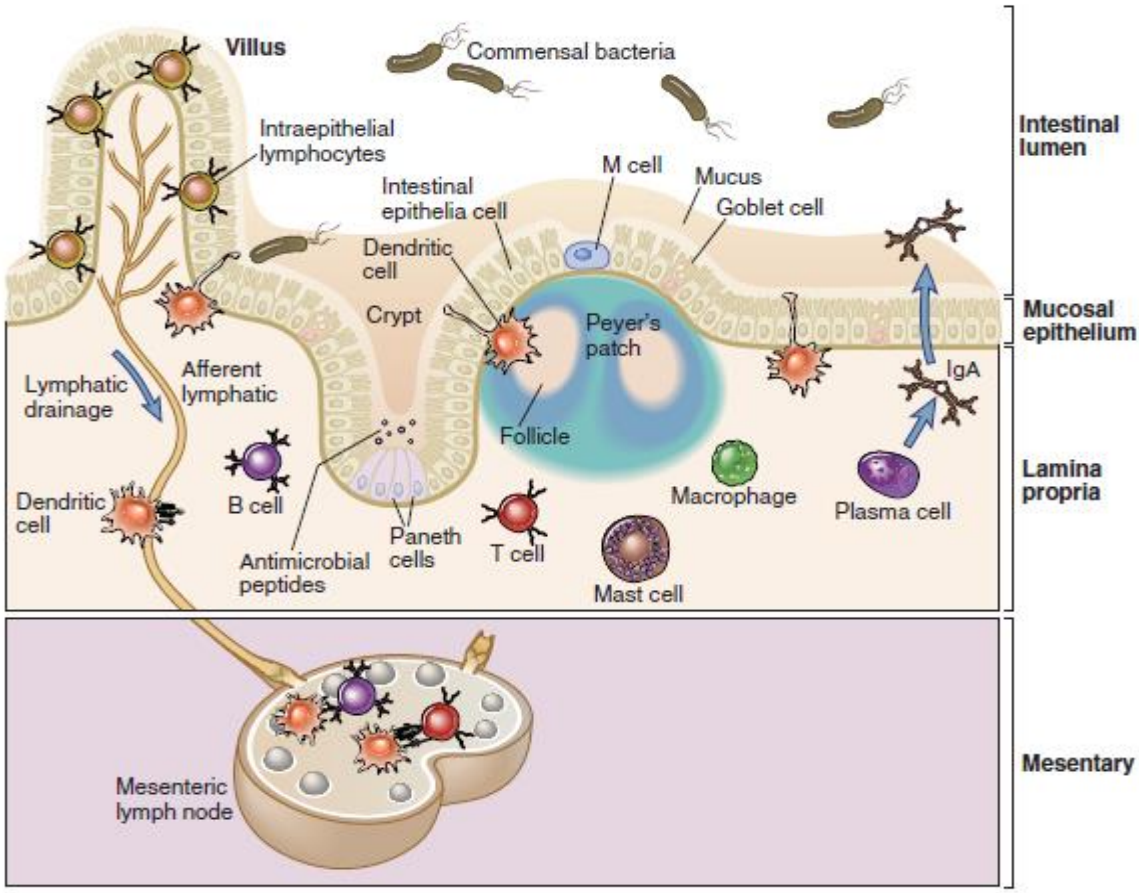
The GALT (gut-associated lymphoid tissue) which is the main site of the mucosal immune system has two functionally different parts: the inductive and the effector sites. An immune response is frequently initiated in the organized lymphoid tissue of the gut – in Peyer's patches or isolated lymphoid follicles (ILFs). Each Peyer's patch consists of a large number of B cells surrounding follicular dendritic cells (FDCs) and a smaller number of associated T cells. The route by which an antigen enters the Peyer's patch leads through specialized cells present in the epithelium called microfold cells (M cells). In a process called transcytosis, the M cells take up antigens from the mucosal surface by endocytosis and phagocytosis and release them at the basal surface where DCs and T cells are waiting. When looking at ILFs, a very similar morphological architecture to the Peyer's patches could be seen. (Murphy and Janeway, 2008; Wittig and Zeitz, 2003)

When transcytosed, antigens are caught upon DCs which process them and present them to the naive T cells in the Peyer's patches causing these T cells to proliferate and differentiate into effector cells. The intestinal T cells with regulatory properties, induced by intestinal epithelial cells (Artis, 2008), then produce cytokines (mainly TGF-β and IL-10) while causing the B cells class-switch to IgA. Following affinity maturation, B cells migrate to the mesenteric lymph nodes and finally to the lamina propria, where the last step – differentiation into plasma cells – is taken. (Murphy and Janeway, 2008)

Proliferation and differentiation of lymphocytes does not take place only in Peyer's patches and isolated lymphoid follicles. Effector DCs sampling antigens through the gut epithelial layer can interact with naive T and B cells surrounding them, or they can migrate to the mesenteric lymph nodes and interact with the lymphocytes there. Equipped with "homing molecules", effector lymphocytes enter the blood circulation and home back to the mucosal tissue into the lamina propria. (Abbas et al., 2012)

Lamina propria is the main effector site of all the gut immune responses. It is a residence to T and B lymphocytes, macrophages, dendritic cells (mainly of regulatory phenotype), neutrophils and mast cells. At this location, IgA is produced by plasma cells and transcytosed to the lumen of the gut through epithelial cells. The vast majority of T cells of the lamina propria express CD4 and are known to produce cytokines or regulate immune responses against food proteins and commensals. Oral tolerance, a term related to this T cell function, is defined as systemic unresponsiveness to the antigen administered previously orally. It was experimentally proved by Titus and Chiller (1981). There are different mechanisms leading to oral tolerance, but the most important is the induction of peripheral tolerance, which is based on Treg cells. Although CD8+ T cells are quite rare in the lamina propria, they often reside directly in the epithelium, and thus have been named intraepithelial lymphocytes (IELs). The IELs function as very effective cytotoxic cells by killing infected and stressed cells via the perforin/grandzyme or Fas-dependent pathways. (Abbas et al., 2012; Murphy and Janeway, 2008; Wittig and Zeitz, 2003)

Fig. 1 – Mucosal immune system of the gut



(Abbas et al., 2012)

The picture of immune mechanisms related to the gut tissue would not be complete without the most natural, innate immune mechanisms. Apart from regulatory DCs and inhibitory macrophages dampening inflammatory reactions in the lamina propria, epithelial cells also dispose of indispensable functions. Secretion of mucins by epithelial cells and production of antimicrobial peptides (AMPs) by specialized Paneth cells protect the GIT cells from direct contact with pathogens from the lumen. Epithelial cells can modulate the immune response by secreting cytokines and even expressing MHC class II molecules; however, the question whether these cells can act as APCs or not is yet to be answered. (Abbas et al., 2012; Forsum et al., 1979)

The proinflammatory responses of immune cells are, by all means, limited towards commensal microbes. This limitation is obvious from the distribution of PRR receptors (mostly TLRs and NLRs) among and within epithelial cells. Most of these innate immune receptors are expressed on the basal side (Rhee et al., 2005) or in the cytoplasm of epithelial cells ensuring restriction of reactivity to invasive microorganisms. (Abreu, 2010)

1.2 Microbes in health and disease

One of the crucial environmental pressures resides in infections (Bach, 2005). Microbes (infectious agents) can trigger autoimmune diseases by several different mechanisms. A microbe carrying a peptide similar to some kind of self-peptide can cause a shift of the immune response from the nonself microbe to a self-structure in an individual (a phenomenon called “molecular mimicry”) (Fujinami and Oldstone, 1989). By activating numerous APCs, microbes can indirectly activate autoreactive T cells (an event called “bystander activation”) (Fujinami et al., 2006). Production of superantigens by the microbes can lead to polyclonal activation of lymphocytes (Herman et al., 1991) and reactivity towards autoantigens. (Bach, 2005; Kverka and Tlaskalova-Hogenova, 2013)

Apart from triggering an autoimmune disease, microbes have been also implicated in protection of host by competing with pathogens at mucosal surfaces and regulating the immune responses (Kivity, 2009). The interaction between microbes and IECs, mediated by PRR receptors, can lead to enhancement of tight junctions and production of AMPs resulting in contribution to the maintenance of the mucosal barrier function (Abreu, 2010). Generation of IgA producing cells is also promoted by microbes as shown in comparison of the number of IgA- producing plasma cells in germ-free mice versus specific-pathogen free mice (Macpherson et al., 2001). Additionally, microbes initiate a signal transmission through IECs

to immune cells underneath them causing IECs to produce TGF-β and other cytokines. TGF-β can convert DCs into regulatory DCs and therefore promotes generation of Treg cells (Iliev et al., 2009). Treg cells are known to produce IL-10, an efficient regulatory cytokine, as well as to control the activity of constitutively activated Th_{17} (Chaudhry et al., 2011) and γδT cells (Park et al., 2010) in the gut.

1.3 Gut microbiota

Microbes living among many parts of the human body form a very diverse ecosystem called microbiota. The amount of microbial cells is 10 times higher (10^{14}) than the amount of all eukaryotic cells (of the human body itself), and microbiota is composed of at least 1000 different bacterial species. Furthermore, the bacterial genome contains at least 450 times more genes than the human genome itself (Li et al., 2014). Now that we know the extent of this unique ecosystem residing in our bodies, finding out that it holds many various indispensable features does not come as a surprise. (Tlaskalova-Hogenova et al., 2011)

Microbiota reaches highest numbers in the distal gut. To show the importance of gut microbiota, gnotobiological techniques are often used. Animals without natural microbiota in their bodies, breaded in germ-free conditions, are used to demonstrate what could happen if there were no bacteria inside an animal body (Herbst et al., 2011; Neufeld et al., 2011; Rodriguez et al., 2011; Schwarzer et al., 2013; Stepankova et al., 2010). It has been shown that colonization of germ-free animals has a solemn effect on the development of the immune system and on the preservation of the intestinal homeostasis (Cebra, 1999).

1.3.1 Composition

A large-scale comparative analysis of 16S rRNA sequences from colonic mucosa and feces of healthy subjects revealed that two main phyla dominate in the gut microbiota: *Firmucutes* and *Bacteroidetes* (Eckburg et al., 2005). Although the bacterial profile of human microbiota shows certain resemblance, every individual appears to have its own unique microbiota composition. During life, microbiota composition is influenced by many factors. These include birth conditions, breast feeding (weaning), nutrition, antibiotic treatment and age. Although microbiota of an individual may change, the changes are in general only temporary and a stable composition is maintained throughout the individual's life. (Ottman et al., 2012)

Interestingly, to classify humans into groups that would presumably respond differently to diet or drug intake, a metagenomic study was done and as a result, three different enterotypes within the human population were identified (Arumugam et al., 2011).

Escherichia coli from the phyla *Proteobacteria*, one of the best characterized bacterial species, is also present in the gut microbiota. It is a predominant aerobic microorganism of the GIT and it colonizes our intestine very early during infancy (Bezirtzoglou, 1997). However commensally behaving may *E. coli* seem, numerous host and environmental factors can cause conversion of this bacterium into a "pathogen" (Tenaillon et al., 2010).

1.3.2 Bioactive molecules of microbial origin

Molecular biologists put great effort into uncovering molecular core of many immunological processes, interaction between the immune system and microbiota being one of them. Microbes generally dispose of many molecules that cause a specific immune (or generally organismal) response and thus we term them bioactive. For some experiments, the whole microbe needs to be used, but commonly the same effect can be achieved by only a bacterial lysate. For many of these bioactive molecules, recognition receptors (PRRs) are found in an organism. Some of the extracellular molecules (structures) and their recognition receptors are mentioned here: lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, can be recognized by TLR4; peptidoglycan and lipoteichoic acid (LTA) of Gram-positive bacteria are recognized by TLR2; the flagellin protein from bacterial flagella is recognized by TLR5. On the other hand, recognition of double-stranded RNA (dsRNA) and numerous other signs of viral infection is performed by TLR3 and induces production of type I interferon as well as inflammatory cytokines. Similarly, TLR7 recognizes single-stranded RNA (ssRNA) derived from RNA viruses. Specific CpG (DNA) motifs, frequently found in bacterial and viral DNA (but not in mammalian DNA), can be recognized by TLR9. (Kawai and Akira, 2010)

Products of microbial metabolism which make an essential source of energy for ruminants (cows, sheep) are short-chain fatty acids (SCFAs). Although SCFAs have been proved to supply the host with energy from digestion of dietary fiber, it is not their only characteristics. According to Høverstad (1986), SCFAs have the ability to stimulate the absorption of chloride, sodium and water in the colon environment. Additionally, inflammation and atrophy of the colon mucosa can be caused by the absence of SCFAs. Interestingly, these microbial metabolites have been also shown to regulate the size and function of the colonic pool of Tregs. (Brody, 1998; Smith et al., 2013)

1.4 Interaction between microbiota and mucosal immune system of the gut

The relationship between two distinct compartments of the body, the gut-associated immune system and the gut microbiota, needs to be very well structured and balanced. Immune cells of the gut are in constant contact with bacterial antigens and thus any step towards enhanced reactivity to microbiota could lead to a rapid development of inflammation and destruction of the mucosal barrier. Some immunopathologies are believed to be associated with altered immune response to microbiota (e.g. Fava and Danese, 2011).

Although immune response is tolerogenic towards commensal microbes, response to pathogens must be adequately strong and result in elimination of the causative agent. The mechanisms of distinguishing between commensal and pathogenic bacteria is still not completely understood. However, some mechanisms how commensal/probiotic bacteria maintain the homeostasis are known: **1) the influence on TLR (toll-like receptor) signaling** - Profound research has shown that gram-positive and gram-negative bacteria, as well as commensal (including probiotic) and pathogenic bacteria, affect the expression of TLRs on IECs differently (Miettinen et al., 2008; Trevisi et al., 2008; Voltan et al., 2007). Additionally, commensal bacteria have the ability to induce expression of negative regulators of TLR signaling (Kelly et al., 2004; Otte et al., 2004) or interfere with the NFκB (Neish et al., 2000) and MAPK (Lin et al., 2008) signaling pathways. **2) the influence on cytokine induction** - Probiotic and commensal strains of bacteria modulate production of cytokines in the gut in both inflammatory (e.g. Hoffmann et al., 2008) and anti-inflammatory fashion. Induction of regulatory and anti-inflammatory cytokine production, particularly IL-10 and TGF-β, by probiotic and commensal bacterial strains prevents excessive inflammation in the gut (Niers et al., 2005; Zeuthen et al., 2007). **3) the influence on lymphocyte differentiation** – The cytokine environment plays a crucial role in lymphocyte differentiation. Therefore, induction of specific cytokine production by commensal/pathogenic bacteria (O'Mahony et al., 2006) may determine into which subset the lymphocyte will develop. Commensal and some probiotic bacteria induce regulatory T cell (Treg) development (O'Mahony et al., 2008; Smits et al., 2005). Two types of Tregs have been defined according to location of induction – while natural Tregs (nTregs) develop in the thymus, induced Tregs (iTregs) develop post-thymically in the presence of cytokine TGF-β (Murai et al., 2010). Despite the fact that nTregs and iTregs interact (Zheng et al., 2004) and create a suppressive environment in the gut, commensal bacteria are linked only to iTreg induction (Round and Mazmanian, 2010).

Among iTregs, Tr1 and Th3 are known to mediate the mucosal immune tolerance in the gut (Hořejší and Bartůňková, 2009). As for B cell differentiation, commensal and some probiotic bacteria activate local APCs to increase sIgA production in B cells (Majamaa et al., 1995).

1.5 The link between microbiota and diseases

Altered microbiota and gut barrier failure are the key players in many inflammatory and autoimmune diseases (Tlaskalová-Hogenová et al., 2011). These events together with the dysregulation of mucosal immune response can lead to intensified penetration of microbial components into the mucosa and in consequence lead to exaggerated immune response and inflammation. As an example, the role of microbiota in inflammatory bowel diseases is described below:

Inflammatory bowel diseases (IBD) such as the Crohn's disease and ulcerative colitis are chronic, relapsing, immunologically mediated disorders caused by aberrant immune response to commensal gut microbiota (Sartor, 2006). Experiments with germfree animals revealed that, in the absence of microbiota, intestinal inflammation is reduced or actually fails to develop (Hudcovic et al., 2001; Taurog et al, 1994). Furthermore, the fact that "lesions in IBD predominate in areas of the highest bacterial exposure" (Seksik et al., 2006) also points to the involvement of microbiota in the disease pathogenesis. Therefore, oral administration of probiotics may be successful in treatment of IBD patients.

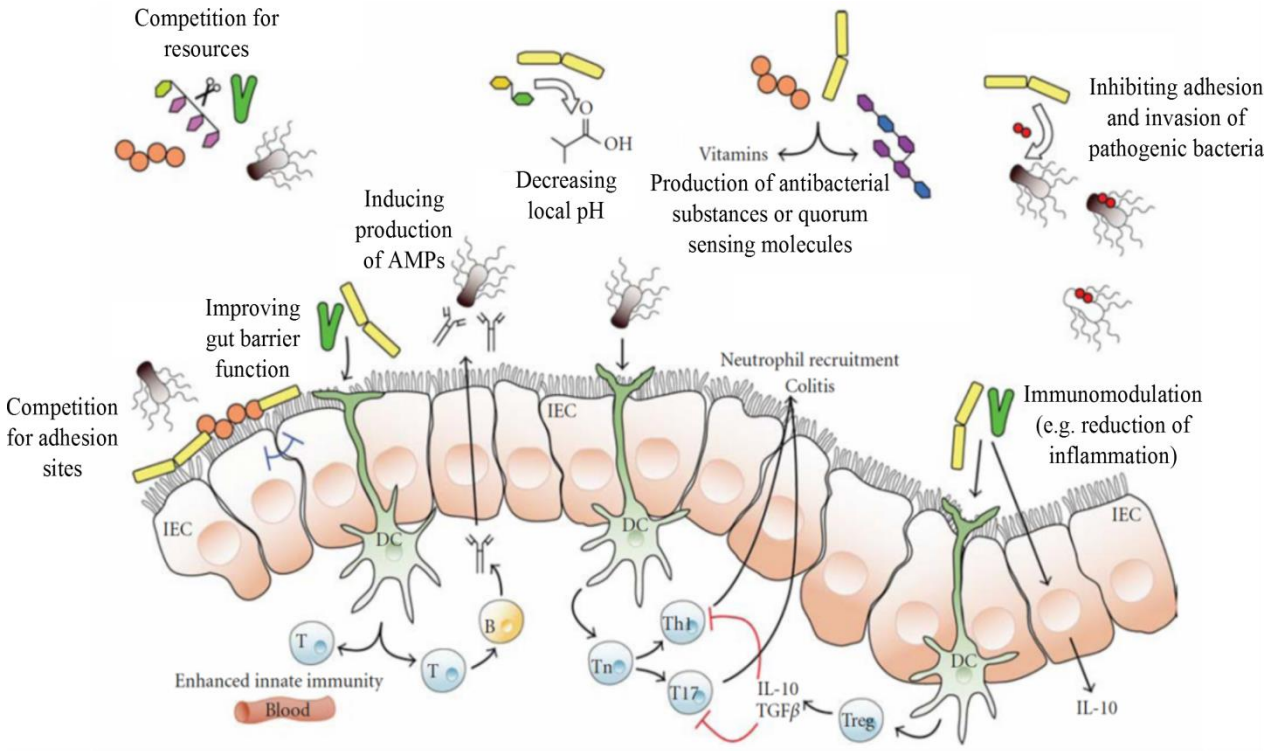
Probiotics are defined as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host" (Guarner and Schaafsma, 1998). However, this definition is very nondescriptive. After all, many microbes residing in the gut of every individual may possess properties, which could be described as "probiotic".

1.5.1 Mechanisms of action of probiotics

Probiotics (used as food supplements) can dampen or even prevent gut tissue inflammation by three main mechanisms. First, they may shape the gut ecosystem by decreasing the local pH by organic acid production, competing for limited resources and sites of adhesion and producing specific antibacterial substances (Stecher and Hardt, 2008) or quorum sensing modifiers which signal the need of growth adjustment to neighboring bacteria (Boyer and Wisniewski-Dyé, 2009). Additionally, certain probiotics can directly induce the production of antimicrobial peptides (AMPs) in the host (Schlee et al., 2008). Secondly, probiotics can ameliorate the gut barrier function and thereby protect it from pathogenic

invasion. They can inhibit the adhesion and invasion of pathogenic bacteria (Boudeau et al., 2003; Ingrassia et al., 2005) or even convert the adherent bacteria into nonadherent (Medellin-Peña et al., 2007). Finally, probiotics possess immunomodulatory properties such as downregulation of pro-inflammatory cytokine production, PRR expression and NFκB signaling (Grabig et al., 2006; Matsumoto et al., 2005; Sougioultzis et al., 2006).

Fig. 2 – The mechanisms of action of probiotics



(http://www.customprobiotics.com/about_probiotics_continued.htm, downloaded: 2.1.2014, modified by: Jana Málková)

1.5.2 Probiotics in the therapy of human diseases

Health promoting effect of the administration of probiotics has been confirmed in patients suffering from numerous diseases and it is not restricted to the gastrointestinal tract. In fact, disorders that can be attenuated by probiotics are of huge variability. Nevertheless, exact properties affecting the therapy have not yet been established.

Claiming that probiotics bring a health benefit to the patients with inflammatory diseases of the gut tissue comes as no surprise. When treating the most common disease of the GIT – **diarrhea** (acute infectious, traveler's or antibiotic-associated) by probiotics, the risk and average duration are often reduced (Corrêa et al., 2011; McFarland, 2007; Szajewska et

al., 2006). Another disease of the GIT, which occurs in premature infants, is the **necrotizing enterocolitis** (NEC). A meta-analysis by Deshpande et al. (2010) showed that probiotic supplements significantly reduce the risk of NEC and death of preterm neonates suffering from NEC. A group of intestinal diseases that affect an increasing number of patients is called **inflammatory bowel diseases** (IBD). This group consists of ulcerative colitis (UC), which is manifested by chronic inflammation in the distal parts of the gut (rectum and colon), and Crohn's disease affecting the gastrointestinal wall particularly in the terminal part of the ileum. Treatment of children suffering from UC by a specific probiotic preparation resulted in a remission of the disease in the majority of patients; moreover, endoscopic and histological scores were significantly lower than in the placebo group and a potential relapse of the disease was postponed (Miele et al., 2009). In total, a meta-analysis done by Shen et al. (2014) comparing 23 randomized trials confirmed that probiotics significantly increase the remission rates in patients (both children and adults) with active UC. Although studies that show efficacy of probiotics in treatment of Crohn's disease exist (Fujimori et al., 2007), the beneficial effect is still very much debatable (Doherty et al., 2010; Rahimi et al., 2008). One of the other diseases influencing the gut is a functional disorder characterized by abdominal pain and defecation discomfort or a general change in bowel habits. This functional gastrointestinal disorder is called the **irritable bowel syndrome** (IBS). The question of efficiency of probiotic treatment of IBS is still controversial (Hoveyda et al., 2009). Although not common in the population, **celiac disease** is a well explored autoimmune disorder. It is the only autoimmune disease, where the triggering agent is known – it is dietary gluten. In vitro studies and animal models brought great hope for celiac disease patients (D'Arienzo et al., 2011; Lindfors et al., 2008), but only a few clinical trials with probiotic treatment have been performed to date. For example, Smecuol et al. (2013) have recently demonstrated that *Bifidobacterium infantis* can alleviate the symptoms in untreated celiac disease. There are many other treatment approaches to celiac disease, aside from administration of probiotics, for example a peptide-based therapeutic vaccine and usage of genetically modified gluten in a patient's diet (Bakshi et al., 2012). Outside of the gut, diseases of other parts of the GIT have been investigated for the role played by microbiota. Inflammation in the oral cavity (e.g. in periodontitis or halitosis) can be prevented or treated by probiotics. In periodontitis, the mechanisms of action are the decrease of pH in the oral cavity and/or production of antioxidants (Shiva manjunath, 2011); in halitosis, it is competition with bacteria implicated in the disease (Burton et al., 2006).

Prevention of allergy by a probiotic *E. coli* (Lodinova-Zadnikova et al., 2003) has raised interest in this kind of approach towards allergies. Later, various allergic diseases have been investigated for efficacy of probiotic therapy. As for **asthma** and allergic rhinitis, the clinical symptom scores decreased in probiotically (*Lactobacillus gasseri* A5) treated children (Chen et al., 2010). Even though clinical trials show a limited effectivity of probiotics in the treatment of asthma, animal models bring great hope for future prevention and treatment (Jang et al., 2012). In **allergic rhinitis**, the ability of probiotics to alleviate nasal symptoms and prevent the pollen-induced infiltration of eosinophils into the nasal mucosa was reported (Ouwehand et al., 2009). A very common allergic disease which affects around one fifth of the world's population during their lifetime (Thomsen, 2014), **atopic dermatitis**, can be prevented by administration of probiotics (Drago et al., 2011; Lee et al., 2008). For example, *Lactobacillus rhamnosus* strain GG had a preventive effect on atopic eczema in children at risk and a 4-year follow-up confirmed the effect shown in the randomized placebo-controlled study (Kalliomäki et al, 2001; Kalliomäki et al., 2003). On the other hand, in a clinical trial regarding **food allergy,** oral supplementation with combination of probiotics did not accelerate the tolerance to cow's milk in infants with cow's milk allergy (Hol et al., 2008). A meta-analysis by Osborn and Sinn (2007) showed that the evidence supporting addition of probiotics to infant feeds to prevent food allergy and other allergic diseases was not sufficient. Thus, further research is needed to assess recommendations for probiotic treatment of allergic patients.

Type I diabetes mellitus (TID, also known as the insulin-dependent diabetes or juvenile-onset diabetes) is an organ-specific autoimmune disease, where T lymphocytes selectively destroy pancreatic β cells and thus disable the production of insulin (Paik et al., 1980). Although probiotics proved to be efficient in preventing animal models of TID (Calcinaro et al., 2005; Valladares et al., 2010), the evidence supporting probiotic usage in children at high risk of disease development is not sufficient (Ljungberg et al., 2006). Studies on **multiple sclerosis** (Fleming et al., 2011), **rheumatoid arthritis** (Mandel et al., 2010; de los Angeles Pineda et al., 2011) and **psoriasis** (Groeger et al., 2013) bring promising results. **Type II diabetes** is a metabolic disorder which affects approximately 10% of European population according to the World Health Organization (data from the year 2010). After administration of probiotics repression of oxidative stress (Ejtahed et al., 2012), reduction of total cholesterol levels (Ejtahed et al., 2011) and moderation of systemic inflammation (Alokail et al., 2013) were observed. In addition, beneficial effects of probiotics are studied

for future treatment or prevention of liver diseases (Cesaro et al., 2011) and atherosclerosis (Hlivak et al., 2004).

2 HYPOTHESIS AND AIMS

Our study is built on the hypothesis that bacterial strains of the same species, but with different relationships to IBD pathogenesis, could induce a different immune response in the host. As the methodological approach in most used experiments does not allow the use of living bacteria, we inactivated the microbes. Here, we hypothesized that the mode of inactivation may influence the microbe's immunogenicity. To confirm or disprove this assumption, we inactivated the microbes differently and compared the inactivation protocols in subsequent experiments.

This thesis had four main aims:

- To analyze if the mode of inactivation (formaldehyde, heat or UV irradiation) influences the immune response to given microbes.
- To compare the antibody response to *E. coli* strains between IBD patients and healthy controls.
- To compare the immune response to probiotic, commensal and pathogenic bacterium of the same species (*Escherichia coli*).
- To analyze how different live *E. coli* strains interact with the intestinal epithelial cells *in vitro*.

3 MATERIALS AND METHODS

3.1 Microbes and inactivation

Four different strains of *E. coli* (Nissle 1917, K6, LF82 and p19A) were used to study the differences between immune response to these microbes. The non-pathogenic strain *E. coli* Nissle 1917, frequently described as a probiotic, may be useful in treatment of IBD, as shown in several clinical trials (Kruis et al., 1997; Matthes et al., 2010; Rembacken et al., 1999). The adherent-invasive *E. coli* strain LF82 is considered as a possible agent responsible for the onset and development of IBD (Darfeuille-Mischaud et al., 2004). The pathobiont, *E. coli* strain p19A, was isolated from a patient with active ulcerative colitis and therefore linked to IBD (Petersen et al., 2009). The commensal *E. coli* strain K6 has not been implicated in pathogenesis or in protection from IBD.

3.1.1 Microbes

The non-pathogenic *E.coli* Nissle 1917 (EcN) isolated from human intestinal flora was kindly provided by Ardeypharm (Germany). The *E.coli* K6 was taken from the collection of bacteria at the Institute of Microbiology, Academy of Sciences of the Czech Republic. The *E.coli* LF82 was isolated from a chronic ileal lesion of a patient with Crohn's disease (Boudeau et al., 1999). The *E. coli* strain p19A was isolated from a patient with active ulcerative colitis (Petersen et al., 2009).

All bacterial strains were cultivated aerobically either on Nutrient agar No.2 (HIMEDIA) or in Lysogeny broth (HIMEDIA) for 24h at 37°C.

After harvesting, concentrations of the bacterial suspensions were assessed by measurement of optical density (OD_{600}) using a cell density meter Ultrospec 10 (GE Healthcare Bio-Sciences AB). All bacterial suspensions were brought to the same OD_{600} value.

3.1.2 Inactivation methods

The need to inactivate *E.coli* strains using different modes of inactivation emerged from assumption that the mode of microbe inactivation may influence its immunogenicity. For this purpose, three commonly used methods were chosen: addition of formaldehyde (3h, room temperature, 1% solution; for details see **protocol n.1**), heat inactivation (60 min, 70°C;

for details see **protocol n.2**), and UV irradiation (75 min under UV-C lamp; for details see **protocol n.3**).

3.1.2.1 Solutions and chemicals

PBS (phosphate buffered saline)

- 1,2g $Na_2HPO_4.12H_2O$ (Lachema) + 0,2g $NaH_2PO_4.2H_2O$ (Lachema) + 9,0g NaCl (Lachema) + relevant volume of distilled water to gain 1l of solution
- pH value is set to 7,35 using 4M NaOH
- sterilization using an autoclave (121°C, 30 min)

Formaldehyde

- 1% solution prepared from the 36-38% formaldehyde (Lach-Ner)

Protocol n.1 – Inactivation by formaldehyde

1) Prepare a 1% formaldehyde solution in PBS.
2) Centrifuge 1 ml of the bacterial culture (3000 x g, 10 min, 4°C) and discard the supernatant.
3) Add 10 ml of the formaldehyde solution and incubate 3h at room temperature.
4) Centrifuge (3000 x g, 10 min, 4°C). Wash 3 times with sterile PBS.
5) Resuspend in PBS.
6) Verify the effectivity of inactivation by 24h cultivation of bacteria on agar Petri dishes (Nutrient agar No.2).

Protocol n.2 - Heat inactivation

1) Transfer the bacterial suspension in an Erlenmeyer's flask and plug it with a cotton wool.
2) Immerse the flask into a 70°C water bath (the suspension must be under water).
3) Inactivate the bacteria for 60 min.
4) Wash with sterile PBS, centrifuge (3000xg, 10min, 10°C) and discard the supernatant - repeat 3 times.
5) Resuspend in PBS.
6) Verify the effectivity of inactivation by 24h cultivation of bacteria on agar Petri dishes (Nutrient agar No.2).

Protocol n.3 - Inactivation by UV irradiation

1) Irradiate 5 ml of sample in a 15 ml tube for 20 min – at 10 cm distance from the UV-C lamp.
2) Wash with sterile PBS, centrifuge (3000 x g, 10min, 4°C) and discard the supernatant - repeat 3 times.
3) Resuspend in PBS.
4) Verify the effectivity of inactivation by 24h cultivation of bacteria on agar Petri dishes (Nutrient agar No.2).

3.2 Primary murine cells and cell lines

To study the cell response to distinct bacterial strains, both primary cells and cell lines were used. Primary murine cells from the mesenterial lymph nodes and spleen were chosen for the analysis of the T cell activation levels. The appropriate cells were isolated from mice with active intestinal inflammation and healthy mice to assess the difference between these distinct environments.

In order to analyze the activation of macrophages, a murine monocyte/macrophage cell line RAW264.7 was used. This cell line was established from a tumor induced by Abelson murine leukemia virus in BALB/c mice (Raschke et al., 1978).

For the epithelial detachment assay, both human and murine epithelial cell lines were used, because various human diseases are studied in a mouse model. The human epithelial cell line Caco-2 was originally isolated from a colorectal adenocarcinoma (Fogh et al., 1977) and the murine epithelial cell line MODE-K is derived from the small intestine of healthy germ-free C3H/HeJ mice; these cells were subsequently transfected by a defective virus and thus immortalized (Vidal et al., 1993).

3.2.1 Isolation and cultivation of primary murine cells

Cell from the mesenterial lymph nodes (MLN) and spleen (SPL) were isolated (for details, see **protocol n.4**) from healthy mice and mice with active intestinal inflammation. Female BALB/c mice used in these experiments were obtained from a breeding colony at the Institute of Physiology, Academy of Sciences of the Czech Republic. The active intestinal inflammation (acute colitis) in mice was induced by 3% dextran sodium sulphate (DSS) dissolved in tap water for 7 days *ad libitum* (Wirtz et al., 2007).

Murine SPL and MLN cells were cultivated in a 96-well cultivation plate in Roswell Park Memorial Institute (RPMI) medium supplemented with fetal bovine serum (FBS) and antibiotic mixture (ATB). Flow cytometry analysis of murine SPL and MLN cells was

preceded by 12-hour cultivation with stimuli and Brefeldin A which allowed the subsequent measurement of intracellular production of interferon γ (IFN-γ).

In any step of centrifugation, the mid bench centrifuge Universal 32R (Hettichlab zentrifugen) was used. For work with organs, cell suspension and cells, we employed the laminar flow cabinet Steril-Antares 72 (Foester Wheeler divison, Steril Factory).

3.2.1.1 Solutions and media

FF (saline solution)

- 0,9% solution of sodium chloride in water for injection (Ardeapharma a.s.)

ACK lysing buffer

- 8,3 g NH_4Cl + 1 g $KHCO_3$ + 200 µl 0,5M EDTA + 800 ml dH_2O
- pH was set to 7,2 – 7,4

Complete RPMI-1640 medium

- RPMI 1640 (Sigma) + 1% ATB (antibiotic antimycotic solution; Sigma) + 2mM L-glutamine (Sigma) + 10% FBS (BIOCHROM)

Protocol n.4 – Isolation of primary murine cells

1) After sacrificing the mice, isolate at least three mesenterial lymph nodes and spleen from each mouse and put the organs quickly into a tube with sterile FF.

Keep the isolated organs on ice!

2) Mash spleen into the single-cell suspension in FF using sterile syringe, needles and Petri dishes and pass the cells through a 70µm nylon mesh into 50ml tubes. Mash MLN into single-cell suspension in FF using two microscope slides and pass the cells through a 70µm nylon mesh into 50ml tubes.

One filter can be used per organ per group.

3) Centrifuge (300 x g, 4° C, 5 min) and discard supernatant.

4) Add 5 ml of ACK lysing buffer to the pellet of splenocytes and incubate 5 min at room temperature. Mix gently a few times. Centrifuge (300 x g, 4° C, 5 min) and discard supernatant.

5) Calculate cells and bring them to a concentration of 2 x 10^6 cells/ml in complete RPMI-10 medium.

3.2.2 Cell lines and cultivation

RAW264.7 (ATCC TIB-71), Caco-2 (ATCC HTB-37) and MODE-K (INSERM Unit 851, Lyon, France) cell lines were used for the analysis of cell response to *E.coli* strains described above. Before analysis, the cell lines were cultivated for several days in complete DMEM medium (Sigma-Aldrich) at 37°C and 5% CO_2 in a CO_2 incubator (SANYO Electric CO.). For any work with cell lines, we employed the laminar flow cabinet Steril-Antares 72 (Foester Wheeler divison, Steril Factory).

3.2.2.1 Media

Complete DMEM (Dulbecco's Modified Eagle's Medium)

- High glucose DMEM (Sigma)+ 1% ATB (antibiotic antimycotic solution; Sigma) + 2mM L-glutamine (Sigma) + 10% FBS (BIOCHROM)

3.3 Analysis of antibody response

To detect the serum antibodies (of IgA and IgG isotype) against different strains of *E.coli* in IBD patients and controls, indirect enzyme-linked immunosorbent assay (ELISA) was used. Patient sera were collected at Hepato-gastroenterology clinic of IKEM.

3.3.1 Indirect ELISA

ELISA was performed with sera samples from ten IBD patients and ten controls for both IgA and IgG detection. Three independent experiments were done; the detailed steps of procedure are in **protocol n.5**, which was previously optimized for performance of our experiments. In the procedure, a microplate washer (TriContinent MultiWash II) was used for washing wells. Measurement of reaction was performed on ELISA reader (Multiskan Ascent Plate Reader) and captured by Ascent™ Software 2.4.1 (Thermo Scientific).

3.3.1.1 Solutions and chemicals

PBS

- 1,2g $Na_2HPO_4.12H_2O$ (Lachema) + 0,2 g $NaH_2PO_4.2H_2O$ (Lachema) + 9,0 g NaCl (Lachema) + relevant volume of distilled water to gain 1l of solution
- pH value is set to 7,35 using 4M NaOH

Wash buffer

- 0,05 % solution of Tween20 (Sigma) in PBS

1% BSA (bovine serum albumin)

- 1 g of BSA (Sigma) dissolved in 99 ml of PBS

TMB (3,3',5,5'-tetramethylbenzidine)

- 40 mg TMB (Sigma) + 27 ml DMF (Lachema) + 73 ml distilled water

Citrate buffer

- 2,94 g Trisodium citrate (Lachema) + 100 ml distilled water
- pH value is set to 4,2 using Citric acid

Substrate solution

- 3 ml TMB + 3 ml Citrate buffer + 1,2 μl H_2O_2 (Chemapol)

H_2SO_4 (sulfuric acid)

- 96% solution of H_2SO_4 (Lach-Ner)

3.3.1.2 Antibodies

anti- human IgA-PEROX (alpha chain) (AFF) (THE BINDING SITE)

anti-human IgG-PEROX (gamma chain) (THE BINDING SITE)

Protocol n.5 – Indirect ELISA with sera samples

1) COAT: Dilute the bacteria to 10^5 at 50 μl/well. Prepare all solutions (if needed). Leave overnight at 4°C.

2) ASPIRATE and WASH: Aspirate and wash 1x with wash buffer.

3) BLOCK: Block each well with 300 μl of 1% BSA in PBS, incubate 1-2 h at room temperature.

Prepare the samples in the meantime using FACS tubes.

4) ASPIRATE and WASH: Aspirate and wash 1x with wash buffer.

5) SAMPLES: Add relevant serum samples diluted 1:100 in 1% BSA 50 μl/well, and incubate 2h at RT. *Prepare detection antibodies in the meantime.*

6) ASPIRATE and WASH: Aspirate and wash 5x with wash buffer (>400 μl/well).

This is a critical step for low background!

7) DETECTION ANTIBODY: Incubate for 2h at room temperature in the dark with 50 μl/well of detection antibody:

anti-human IgG-PEROX diluted 1:2000 in 1% BSA

anti- human IgA-PEROX diluted 1:3000 in 1% BSA

8) ASPIRATE and WASH: Aspirate and wash 5x with wash buffer (>400 μl/well).

This is a critical step for low background.

9) SUBSTRATE: Add 50 μl/well of substrate solution and incubate for *approx. 10-30 min at RT in the DARK!*

10) STOP: Stop the reaction with 50 µl/well of 2M H_2SO_4. *Work fast!*
11) MEASURE: within 20 minutes, measure the absorbance at 450 nm (sample) and 650 nm (correction – not essential).

3.4 Analysis of cellular response

3.4.1 Response in murine RAW264.7 cell line

In order to assess the differences between macrophage responses to different *E. coli* strains, murine monocyte/macrophage RAW264.7 cell line was cultivated with inactivated *E. coli*. Subsequently, CD40 expression and nitrite production were measured by flow cytometry and Griess assay, respectively.

3.4.1.1 Cultivation with stimuli

RAW264.7 cells were cultivated with the four inactivated *E. coli* strains or LPS for 24 hours at 37°C and 5% CO_2 in a CO_2 incubator (SANYO Electric CO.). All stimuli added to the cells were diluted in complete DMEM medium, LPS (lipopolysaccharides from E.coli O55:B5; Sigma) was used as a positive control (for details, see **protocol n.6**).

Protocol n.6 – Cultivation of RAW264.7 cells with stimuli
1) Scrape the RAW 264.7 cells off the cultivation flask with a cell scraper.
2) Calculate cells in the suspension and bring them to a concentration of 2 x 10^6 cells/ml. Now, pipette 50 µl of the cell suspension into all used wells of the cultivation plate.
3) Add 100 µl of the bacterial suspension (10^7 CFU/ml) to each well. Don't forget to add positive (LPS 1ng/ml) and negative (cells without stimuli) controls.
4) Cultivate 24h at 37°C, 5% CO2.

3.4.1.2 Flow cytometry

For flow cytometry analysis, macrophage cells were stained with Hoechst 33258 (Life Technologies) and PE-conjugated anti-CD40 (clone 1C10; eBioscience). In any step of centrifugation, the mid bench centrifuge Universal 32R (Hettichlab zentrifugen) was used (for detailes, see **protocol n.7**). Measurement of cell surface markers was performed on the Becton Dickinson LSR II Flow Cytometer equiped with HTS. The measurement includes 3 crucial steps:

1) Fine tuning of PMT voltage – Check some wells for compensation at speeds 0.5 µl/s (Sample: 15 µl; Mixing: 10 µl).

2) Sample measurement – Collect 200k in 60 µl in samples and 50k in 30 µl in controls (Flow rate: 1.5 µl/s; Sample: 65 µl; Mixing: 55 µl; Mixing speed: 140 µl/s; Mixes: 3x; Wash: 400 µl).
3) Cleaning – 4wells of bleach, 4 wells of dH_2O.

Data were captured by software BD FACS DIVA 6.1.3 and subsequently evaluated using FlowJo 7.2.5 software.

Protocol n.7 – Preparation for flow cytometry analysis of surface markers.

Before transferring cells, take away the supernatant for further analysis (Griess reaction), then resuspend cells in FF or PBS.

1) Transfer cells to the 96-well U plate.

Add 100 µl of each dublet from cultivation, the final volume in the wells should be 200 µl. Mix the leftovers to get cells for singlestains.)

2) Centrifuge (300 x g, 4°C, 5 min), decant the supernatant and gently tap the plate on paper towels.

3) Block each well with 20 µl of 10% NMS (diluted in PBS) and incubate 20 min at 4°C. – Mix the cells well by gentle vortexing.

In the meantime, prepare mixtures of antibodies for surface staining.

4) Add 180 µl of PBS to each well. Centrifuge (300 x g, 4°C, 5 min), decant the supernatant.

5) Mix the cells well by gentle vortexing. Then, add 10 µl of PE-conjugated anti-CD40 antibody (diluted in PBS) to all sample wells and to the CD40 singlestain well. Incubate for 30 min at 4°C in the dark.

6) Add 180 µl of PBS and centrifuge (300 x g, 4°C, 5 min), decant the supernatant.

7) Resuspend in 180 µl of FACS and centrifuge.

8) Resuspend in 100 µl of FACS.

9) Add 10 µl of Hoechst to all sample wells and to the Hoechst singlestain well.

10) Now, you are prepared to measure your samples with flow cytometry.

3.4.1.3 Griess assay

Nitrite production of macrophages was assessed by Griess assay after 24-hour cultivation with stimuli (for details, see **protocol n.8**). Measurement of reaction was performed on ELISA reader (Multiskan Ascent Plate Reader) and captured by Ascent™ Software 2.4.1 (Thermo Scientific).

Protocol n.8 – Griess assay with supernatants from RAW264.7 cells.

1) Add 50 µl of supernatant to each used well of the 96-well F plate. Add 50 µl of standard $NaNO_2$. The standard is diluted to the concentrations from 125 µM to 1 µM by two-fold dilution in medium. Wells with 50 µl of medium serve as blank. The assay control is 50 µl of dH_2O.

2) Add 50 µl Griess reagent (Sigma-Aldrich) diluted in dH_2O (40 mg/ml) to each well.

3) Within a few minutes, measure the absorbance at 540 nm.

3.4.2 Response in primary murine cells from mesenterial lymph nodes and spleen

The early activation of T cells from the population of splenocytes (SPL) and cells from the mesenterial lymph nodes (MLN) was measured by flow cytometry after cultivation with inactivated *E. coli*. We also measured the intracellular production of IFN-γ in these cells.

3.4.2.1 Cultivation with stimuli

Primary murine cells (from SPL or MLN of healthy/DSS-treated mice) were isolated and brought to cell suspensions. Then, we added the inactivated bacteria and Brefeldin A (1000x Brefeldin A solution; eBioscience) and cultivated the cells for 12 hours at 37°C and 5% CO_2 in a CO_2 incubator (SANYO Electric CO.) (for details, see **protocol n.9**).

Protocol n.9 – Cultivation of MLN and SPL cells with stimuli

1) Pipette SPL and MLN cells from the suspension to the cultivation plates.

2) Add inactivated bacteria (10^7 CFU/ml) and Brefeldin A (20 µl/well) diluted in complete RPMI medium. Don't forget to add the positive (PMA/iono) and negative (cells without stimuli) controls.

3) Cultivate 12 hours at 37°C and 5% CO_2.

3.4.2.2 Flow cytometry

For flow cytometry analysis, SPL and MLN cells were stained with eFluor 780-conjugated FVD (eBioscience), FITC-conjugated anti-CD3 (clone 145-2C11; BD Bioscience), BV605-conjugated anti-CD4 (clone RM4-5; Life Technologies), APC-conjugated anti-CD8 (clone 53-6.7; eBioscience), BV510-cojugated anti-CD69 (clone H1.2F3; BD Horizon) and after permeabilization with PE-conjugated IFN-γ (clone XMG1.2; eBioscience). In any step of

centrifugation, the mid bench centrifuge Universal 32R (Hettichlab zentrifugen) was used (for detailes, see **protocol n.10**). Measurement of cell surface markers and intracellular cytokine was performed on the Becton Dickinson LSR II Flow Cytometer equiped with HTS. The measurement includes 3 crucial steps (see **chapter 4.4.1.2**). Data were captured by software BD FACS DIVA 6.1.3 and subsequently evaluated using FlowJo 7.2.5 software.

Protocol n.10 – Preparation for flow cytometry analysis for surface markers and intracellular cytokine

1) Prepare FACS+i (Brefeldin A diluted 1:1000 in PBS).

2) Transfer 200 µl of cells to the FACS plates.

Add 100 µl of each dublet from cultivation, the final volume in the wells should be 200 µl. Mix the leftovers to get cells for singlestains.)

3) Centrifuge (300 x g, 5 min, 4°C), decant the supernatant, turn and gently tap the plate on the paper towels.

4) Block each well with 20 µl of 10 % NMS (diluted in FACS+i), incubate 20 min at 4°C.

Prepare the mixtures of antibodies for surface staining in the meantime.

5) Centrifuge (300 x g, 5 min, 4°C), decant the supernatant.

6) Add 10 µl of antibody mixture in FACS+i (or only FACS+i or single stain mixture in FACS+i), and incubate for 30 min at 4°C in the DARK.

7) Add 160 µl of FACS+i, and centrifuge, decant the supernatant.

8) Resuspend in 180 µl of FACS+i, and centrifuge.

9) Permeabilize the cells with Fix/Perm solution (Fixation/Permeabilization diluent/concentrate 3:1; eBioscience) 180 µl/well for 30-45 min at RT in the dark (from here, the inhibitor is not needed).

10) Cetrifuge (300 x g, 5 min, 4°C) and decant the supernatant.

11) Wash 2 times with 150 µl of Permeabilization buffer 10x (eBioscience) diluted in dH_2O and centrifuge (350 x g, 5 min, 4°C).

12) Add 10 µl of anti-IFN-γ in PERM, and incubate for **30 min at 4°C in the DARK**.

13) Add 150 µl of PERM, and centrifuge.

14) Resuspend in 170 µl of PERM, and centrifuge.

15) Resuspend in 100 µl of PBS.

10) Now, you are prepared to measure your samples with flow cytometry.

3.5 Epithelium detachment assay

To analyze the differences between bacterial strains in the ability to disrupt the epithelial layer, we performed experiments with the cell lines MODE-K and Caco-2. After cultivation with live *E. coli*, we measured the detached cells with flow cytometry. The numbers of detached cells and the proportions of live/early apoptotic/late apoptotic/primarily necrotic cells were measured. After dissociation of adhered cells by trypsin-EDTA solution, the numbers and viability of these cells were determined.

3.5.1.1 Solutions and media

Trypsin/EDTA (Sigma)

HEPES (Sigma)

Complete DMEM (Dulbecco's Modified Eagle's Medium)

- High glucose DMEM (Sigma)+ 1% ATB (antibiotic antimycotic solution; Sigma) + 2mM L-glutamine (Sigma) + 10% FBS (BIOCHROM)

DMSO (Sigma)

5-FU

- 3,25 mg 5-FU, minimum 99% TLC (Sigma) + 5 ml DMSO

H_2O_2

- H_2O_2 (Chemapol) diluted in DMEM medium without ATB

3.5.2 Cultivation with stimuli

The MODE-K and Caco-2 cells were dissociated using trypsin/EDTA solution and then transferred to a cultivation plate. Then, we let the cells devide for several days until they established a confluent cell layer (for details, see **protocol n.11**). The cultivation at 37°C and 5% CO_2 took place in a CO_2 incubator (SANYO Electric CO.). When the cell layer was confluent, we added live *E. coli* to the cells and cultivated them for another 4 hours.

Protocol n.11 – Cultivation of MODE-K and Caco-2 cell lines in a cultivation plate

1) Dissociate the cells from the bottom of the cultivation flask with trypsin/EDTA solution. Dilute the cells to a suspension of 10^5 cells/ml.

2) Transfer 0,5 ml of the cell suspension to each well of a 48-well plate. Let the cells cultivate at 37°C, 5% CO_2 until the cell layer is confluent.

MODE-K cells need 2,5 day, while Caco-2 need 3 or more days of cultivation before other steps can be taken.

3) Wash cells with DMEM medium not including ATB. Then, add 1ml of stimuli - bacteria/ 5-FU (apoptosis control)/H_2O_2 (necrosis control). Incubate 4h at 37°C, 5% CO_2.

3.5.3 Flow cytometry

For flow cytometry analysis, both detached cells and adhered cells were used. The cells detached by *E. coli* were stained with Dyomics 647-conjugated Annexin V (BD Pharmingen) and Hoechst 33258 (Life Technologies) to determine apoptotic and early necrotic cells by flow cytometry. Cells, which stayed adhered to the bottom of the cultivation plate were stained with eFluor 780-conjugated FVD (eBioscience). After dissociation of these cells by trypsin/EDTA solution, they were measured by flow cytometry. From the adhered cells, dead and live cells were determined. (for detailes, see **protocol n.12**)
In any step of centrifugation, the mid bench centrifuge Universal 32R (Hettichlab zentrifugen) was used. Measurement of MODE-K and Caco-2 cells was performed on the Becton Dickinson LSR II Flow Cytometer equiped with HTS. The measurement includes 3 crucial steps (see **chapter 4.4.1.2**). Data were captured by software BD FACS DIVA 6.1.3 and subsequently evaluated using FlowJo 7.2.5 software. The gating strategy for detached cells is shown in **Fig.3** and for adhered cells in **Fig.4**.

Protocol n.12 – Preparation for flow cytometry analysis of detached and adhered MODE-K and Caco-2 cells

1) Very gently collect the supernatant from cells into 1,5 ml tubes.
All subsequent steps apply to the detached cells (supernatant from adhered cells).
2) Centrifuge (350 x g, 5min, 4°C) and discard the supernatant.
3) Resuspend in 100µl of HEPES buffer and transfer cells to 96-well U plate. Don't forget the apoptosis (5-FU) and necrosis(H_2O_2) controls.
4) Centrifuge (350 x g, 5min, 4°C). Discard the supernatant.
5) Resuspend in 200µl of HEPES buffer.
6) Centrifuge (350 x g, 5min, 4°C). Discard the supernatant.
7) Add 10µl of Annexin V diluted in HEPES buffer (1:100) to each well. Incubate 10 min at RT in the dark.
8) Add 90 µl of HEPES buffer per well.
9) Add 10 µl of Hoechst to all sample wells and to the Hoechst singlestain well. **Now, measurement of the detached cells will be done.**

10) Stain the adhered cells (that are left at the bottom of wells) with 100 µl of FVD (1:500). Incubate 30 min at RT in the dark.

11) Discard the supernatant and wash the cells with 500 µl PBS (*=add 500 µl PBS and discard the supernatant*).

12) Dissociate the cells from the bottom of the wells with 200 µl trypsin/EDTA per well.

13) Collect the cells into 1,5 ml tubes and centrifuge (350 x g, 5min, 4°C).

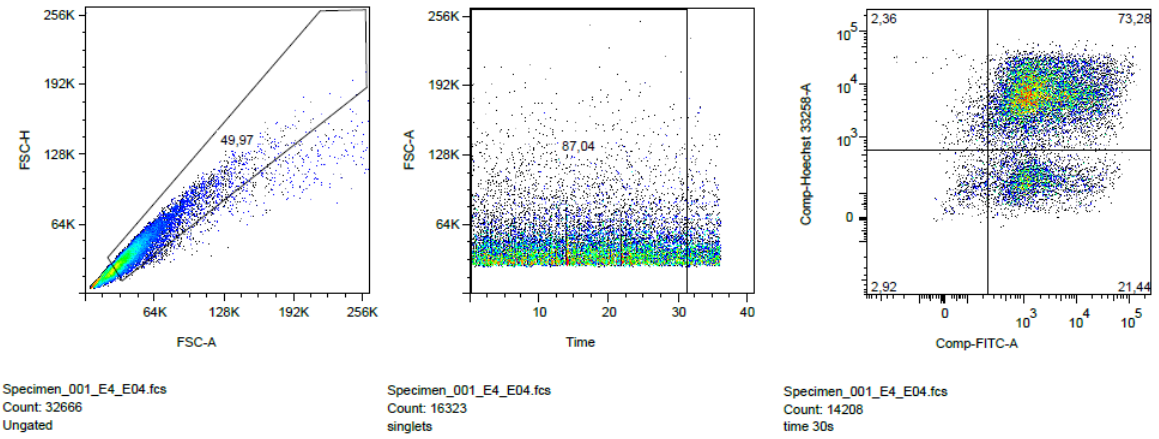
14) Discard the supernatant and transfer cells to the 96-well U plate in 100 µl of PBS. Centrifuge (350 x g, 5min, 4°C)

15) Add 200 µl of PBS and centrifuge (350xg, 5min, 4°C). Discard the supernatant.

16) Resuspend in 100 µl of FACS.

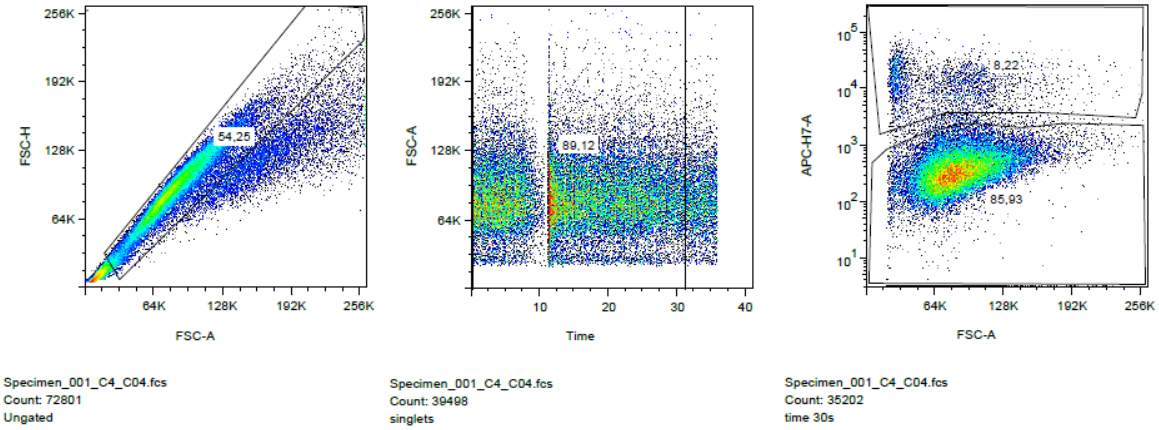
17) **Now, measurement of the adhered cells will be done.**

Fig.3 – Example of the gating strategy applied on the detached cells.



The gating strategy in steps: 1) Singlets are gated on the basis of a control well containing only bacteria. Therefore, this step substitutes also gating for cells. 2) A 30-second time section is gated (for subsequent calculation of flow rate). 3) Cells are devided into 4 subpopulations: live (FITC negative, Hoechst negative), early apoptotic (FITC positive, Hoechst negative), late apoptotic (FITC positive, Hoechst positive) and "primary necrotic" (FITC negative, Hoechst positive).

Fig.4 – Example of the gating strategy applied on the adhered cells.



The gating strategy in steps: 1) Singlets are gated on the basis of a control well containing only bacteria. Therefore, this step substitutes also gating for cells. 2) A 30-second time section is gated (for subsequent calculation of flow rate). 3) Cells are divided into live (APC-H7 negative) and dead (APC-H7 positive).

3.6 Statistical analysis

For statistical analysis of all experimental data, the GraphPad Prism 5.03 software was used. In the case of antibody measurement and epithelium detachment measurement, 3 repetitions of experiment were done. In the case of measurements of cell response towards inactivated bacteria, 5 repetitions of experiments were done. Only two types of tests were used: one-way ANOVA (analysis of variance) with Tukey's post test and unpaired t-test. For our experiments, we chose the significance level to be α=0,05.

4 RESULTS

4.1 Antibody response

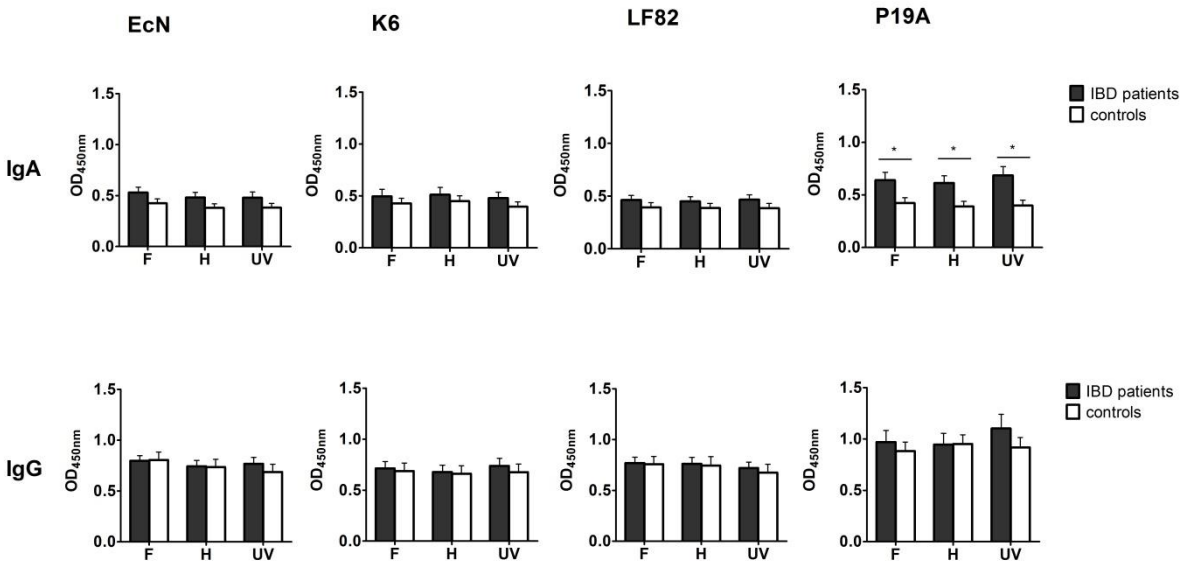
To analyze how different modes of inactivation and different bacterial strains influence the antibody response, indirect ELISA was performed. Avidity of IgA and IgG antibodies was measured. To analyze whether or not the chronic intestinal inflammation can change this response, we compared sera from ten IBD patients with ten healthy controls. The same default data were used to generate **Fig. 5** and **Fig. 6**, however, for better illustration of the conclusions, the two different figures were composed.

In general, the avidity of antibodies, both IgA and IgG, did not show any statistically significant difference between sera of control group of patients and sera of IBD group of patients (**Fig.5**). The only exception was significantly higher IgA response to p19A in IBD patients as compared to controls, regardless of the mode of microbe inactivation (**Fig.5**, on the top on the right). This result was consistent in all three independent experiments.

There were no statistically significant differences between the avidity of IgA/IgG antibodies to bacteria inactivated by different methods or between antibody-binding to different strains of *E. coli*.

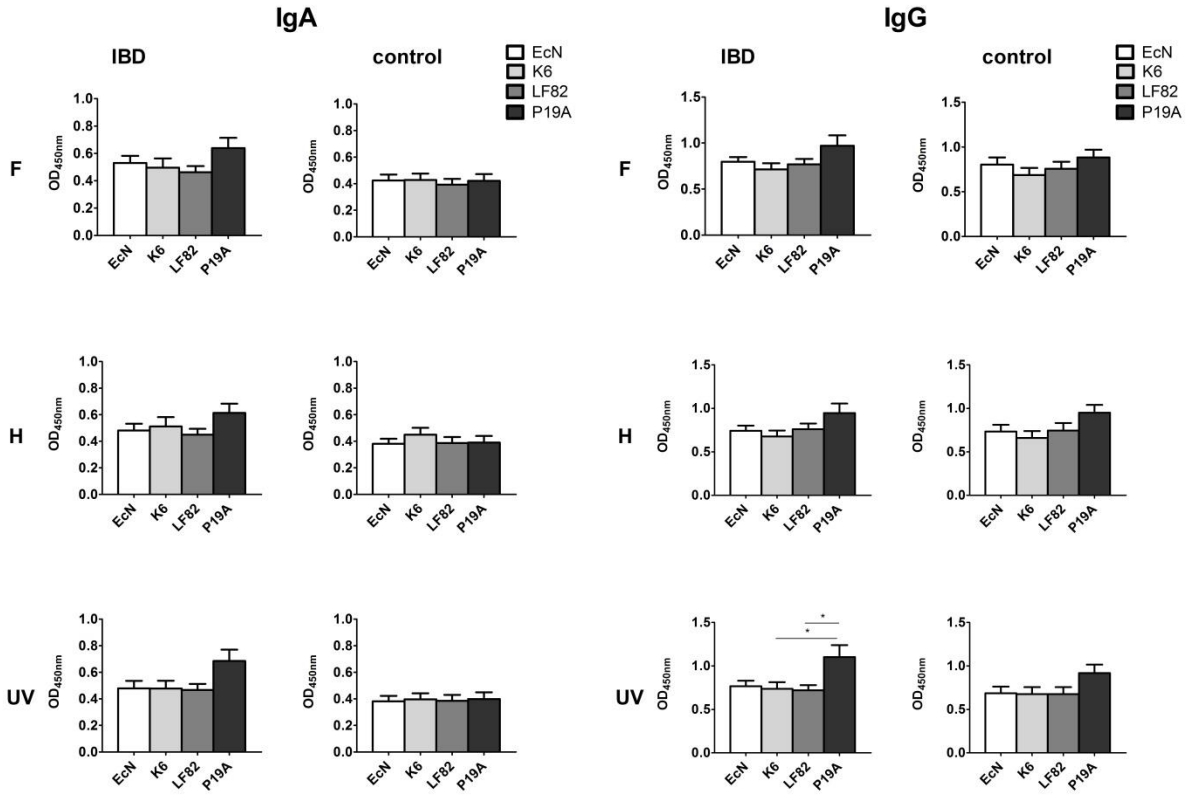
The IgG response to p19A has a tendency to be higher than to the other strains of *E. coli*, but this reached statistical significance only in case of UV-inactivated p19A in IBD patients (**Fig.6**).

Fig. 5 – The differences in antibody response to *E coli* between IBD patients and healthy controls as measured by indirect ELISA.



The graphs show the differences in IgA or IgG response to differently inactivated strains of *E. coli* in IBD patients and healthy controls. The results are a pool of 3 independent experiments; mean (bar) with SEM (whisker) are shown (n=10). Each inactivation of each bacterial strain were analyzed separately (unpaired t test).*P<0,05.

Fig. 6 – There is a significantly higher IgA response to p19A in IBD patients as compared to controls, regardless of the mode of microbe inactivation as measured by indirect ELISA.



The graphs show the differences in IgA or IgG response to differently inactivated strains of *E. coli* in IBD patients and healthy controls. The results are a pool of 3 independent experiments; mean (bar) with SEM (whisker) are shown (n=10). Each inactivation and each antibody were analyzed separately (unpaired t test).*P<0,05.

Collectively, these results clearly show that patients with IBD have higher IgA response against the pathobiont p19A regardless of the mode of inactivation. Generally, the *E. coli* strain p19A tends to bind more IgA and IgG antibodies, particularly in the IBD group of patients. The mode of inactivation does not change the magnitude of IgA or IgG response.

4.2 Cellular response to microbes *in vitro*

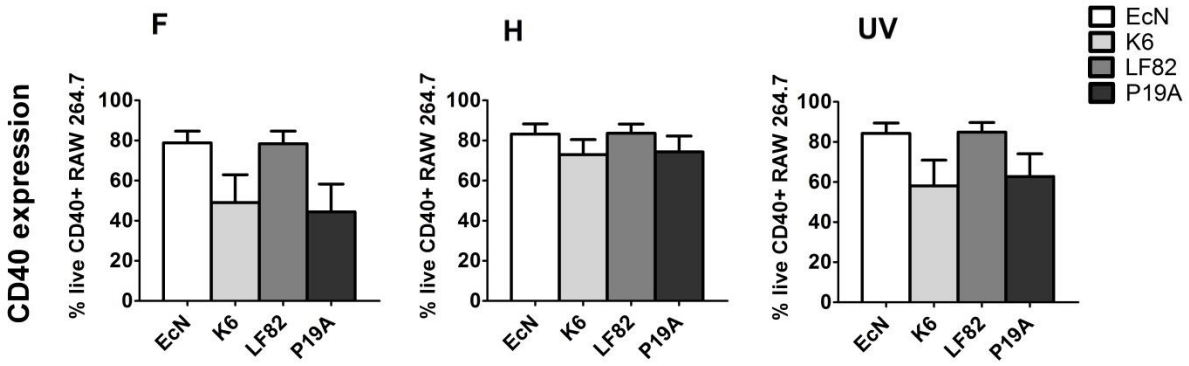
4.2.1 Response in murine RAW 264.7 cell line

To assess the differences between bacterial strains and modes of inactivation, the cell response (expression of CD40 and production of nitrite) of murine macrophage cell line RAW264.7 was measured by flow cytometry and Griess assay, respectively. The

cell/supernatant measurements were done after 24 hours of cultivation with inactivated *E. coli*.

We found no statistically significant differences (P<0,05) between the CD40 expression ratios of RAW 264.7 cells . Although not statistically significant (P=0,24), the strains EcN and LF82 have a tendency to activate (induce expression of CD40) the RAW264.7 cells more than strains K6 and p19A (**Fig.7**); this tendency appears less noticeable in the comparison of the heat-inactivated bacteria. All bacterial strains induce high expression of CD40 in RAW 264.7 cells compared to non-treated cells (P<0,05).

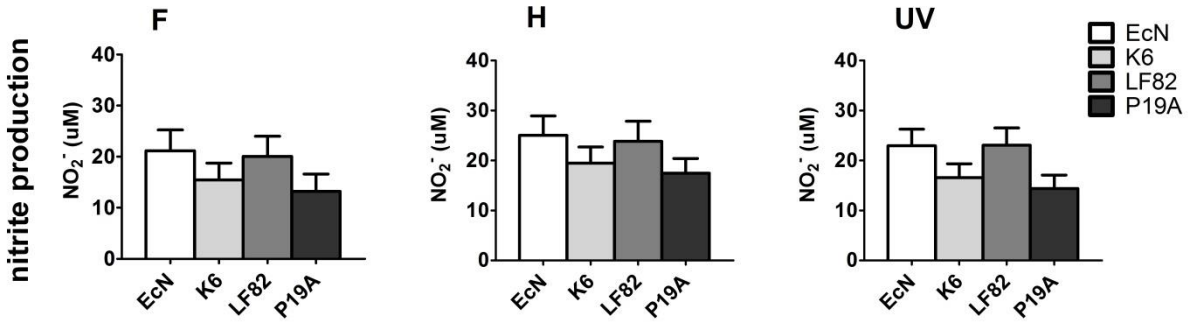
Fig. 7 – There are no differences between the CD40 expression ratios of RAW264.7 cells cultivated with different strains of *E. coli* as measured by flow cytometry.



The graphs show the differences in RAW264.7 activation (CD40 expression) in response to different inactivated strains of *E. coli*. The results are a pool of 5 independent experiments; mean (bar) with SEM (whisker) are shown (n=5). Each inactivation was analyzed separately (1-way ANOVA, Tukey's post test). Maximal activation of cells is 74,92% (cells + LPS). Negative control for activation of cells is 0,90% (cells).

There are no statistically significant differences between the bacterial strains or modes of inactivation in terms of nitrite production by RAW 264.7 cultivated with inactivated *E. coli* (**Fig.8**). Nevertheless, similarly as in case of CD40 expression, the strains EcN and LF82 tend to induce higher production of nitrite by RAW 264.7 cells than the strains K6 and p19A (P=0,32) as shown in **Fig.8**. All tested bacteria induced high production of nitrite in comparison with non-treated cells (P<0,05).

Fig. 8 – There are no differences between the amounts of produced nitrate by RAW264.7 cells cultivated with different strains of *E. coli* as measured by Griess assay.



The graphs show the differences in RAW264.7 activation (nitrite production) in response to different inactivated strains of *E. coli*. The results are a pool of 5 independent experiments; mean (bar) with SEM (whisker) are shown (n=5). Each inactivation was analyzed separately (1-way ANOVA, Tukey's post test). Maximal nitrite production by cells is 19,98 μM (cells + LPS). Negative control of nitrite production is 0,15 μM (cells).

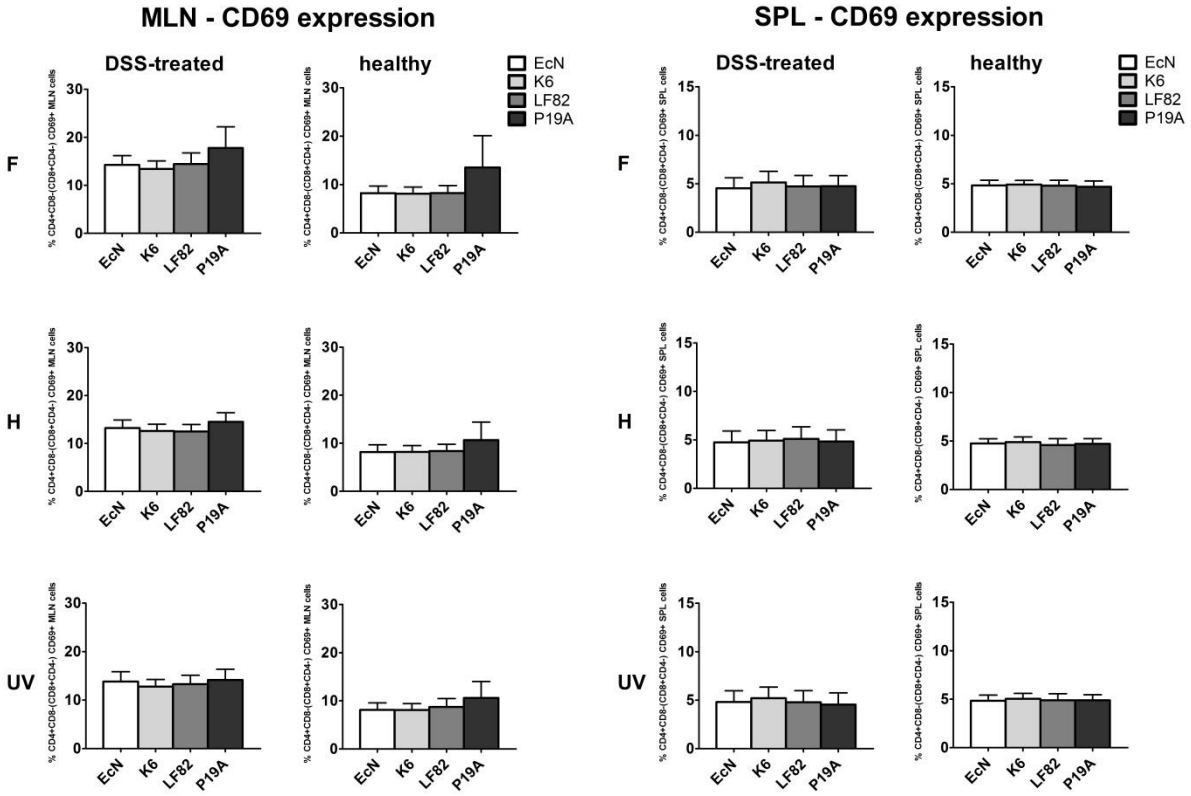
In conclusion, there are no differences in activation (CD40 expression or nitrite production) of murine macrophages RAW 264.7 cultivated with different strains of differently inactivated microbes. As compared to non-treated cells (negative control), all microbes induce high expression of CD40 and strong production of nitrite.

4.2.2 Response in primary murine cells from mesenterial lymph nodes and spleen

To assess the influence of mode of inactivation and type of bacterial strain on the early activation of primary murine cells, flow cytometry was used. The viability and CD69 expression were measured in murine cells isolated from the spleen and mesenterial lymph nodes after 12-hour cultivation with inactivated *E. coli*. In order to study the changes in immune response during acute intestinal inflammation, differences between cell responses to microbes were studied in the non-treated vs. DSS-treated groups of mice.

Statistical analysis did not reveal any significant differences ($P<0,05$) between bacterial strains or modes of inactivation in terms of induction of early activation (CD69 expression) in MLN or SPL cells. Similarly, no statistically significant differences were found between the early activation of MLN or SPL cells from non-treated and DSS-treated mice. However, the MLN cells from DSS-treated mice tend to ($P=0,16$) express slightly higher amounts of CD69 than MLN cells from non-treated mice (**Fig.9**).

Fig. 9 – There are no differences between the CD69 expression ratios of MLN or SPL cells cultivated with inactivated *E. coli* as measured by flow cytometry.



The graphs show the differences in MLN or SPL cell (isolated from DSS-treated or healthy mice) early activation in response to different inactivated strains of *E. coli*. The results are a pool of 5 independent experiments; mean (bar) with SEM (whisker) are shown (n=5). Each inactivation in each group of cells (from DSS-treated or non-treated mice) were analyzed separately (1-way ANOVA, Tukey's post test). Maximal activation of MLN cells is 45,47% and SPL cells is 20,43% (cells + PMA/Ionomycin). Negative control for activation of MLN cells is 9,06% and SPL cells is 4,23% (cells).

In conclusion, the bacterial strain, as well as the mode of inactivation, do not affect the cell response (early activation) of MLN or SPL cells. Nevertheless, MLN cells from mice with active intestinal inflammation (DSS-treated) tend to express higher amounts of CD69 than MLN cells from healthy mice (not statistically significant).

4.3 Epithelium detachment

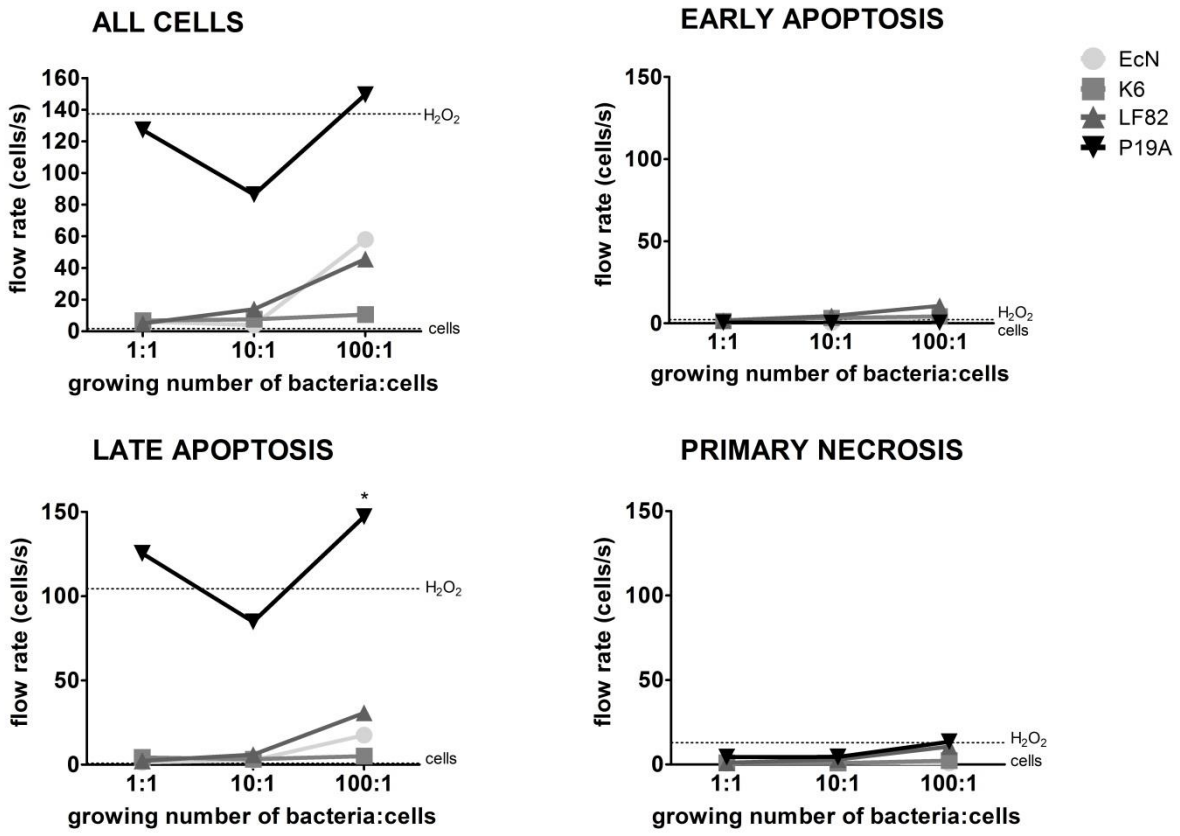
To analyze the differences between bacterial strains in the ability to disrupt the epithelial layer, experiments with the murine (MODE-K) or human (Caco-2) intestinal epithelial cell lines were done. First, the epithelial cells were cultivated for four hours with the live *E.coli* strains, and then flow cytometry was performed with detached cells that were

gently washed out from the cultivation wells. These cells were than stained with Hoechst 33258 and Annexin V to measure the proportions of live/early apoptotic/late apoptotic/primarily necrotic cells among the detached cells. Next, the adhered cells were stained for viability with fixable viability dye and then dissociated by trypsin-EDTA solution. The numbers and viability of these cells were determined.

4.3.1 MODE-K

No statistically significant differences (P<0,05) were found between bacterial strains in the ability to detach MODE-K cells from the cell layer **(Fig.10**; all cells). However, the numbers of detached epithelial cells in the phase of late apoptosis were significantly higher (P<0,05) in the case of *E. coli* strain p19A in comparison to the negative control (**Fig.10**; late apoptosis) – this applies only to the highest number of bacterial cells per epithelial cell (100:1). Here, comparison of p19A with the strains EcN and K6 also revealed statistically significant differences (P<0,05). Comparisons of the numbers of early apoptotic/necrotic cells detached by distinct bacterial strains did not reveal any statistically significant differences (P<0,05).

Fig. 10 – The highest concentration (100:1; bacteria:cells) of strain p19A detached a higher number of MODE-K than strains EcN, K6 and negative control as measured by flow cytometry.

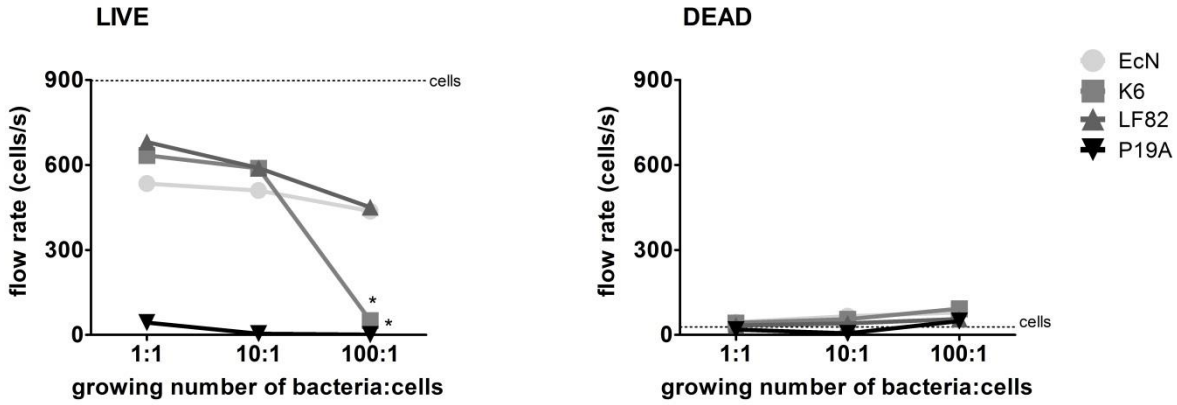


The graphs show flow rate of detached MODE-K cells after cultivation with different *E. coli* strains. The results are a pool of 3 independent experiments.. *P<0,05 (in comparison to negative control). Each concentration of bacteria was analyzed separately (1-way ANOVA, Tukey's post test). Negative control (cells) and necrosis control (H_2O_2) are shown in each graph. Apoptosis control (5-FU) is not shown.

In the analysis of adhered cells, the number of not-detached (adhered) live cells was significantly lower (P<0,05) in the case of strains p19A and K6 in comparison to the negative control (**Fig. 11**; live) – this applies only to the highest number of bacterial cells per epithelial cell (100:1). Among adherent cells, dead cells form only a minority and there were no statistically significant differences (P<0,05) between the differently treated cells (**Fig.11**; dead).

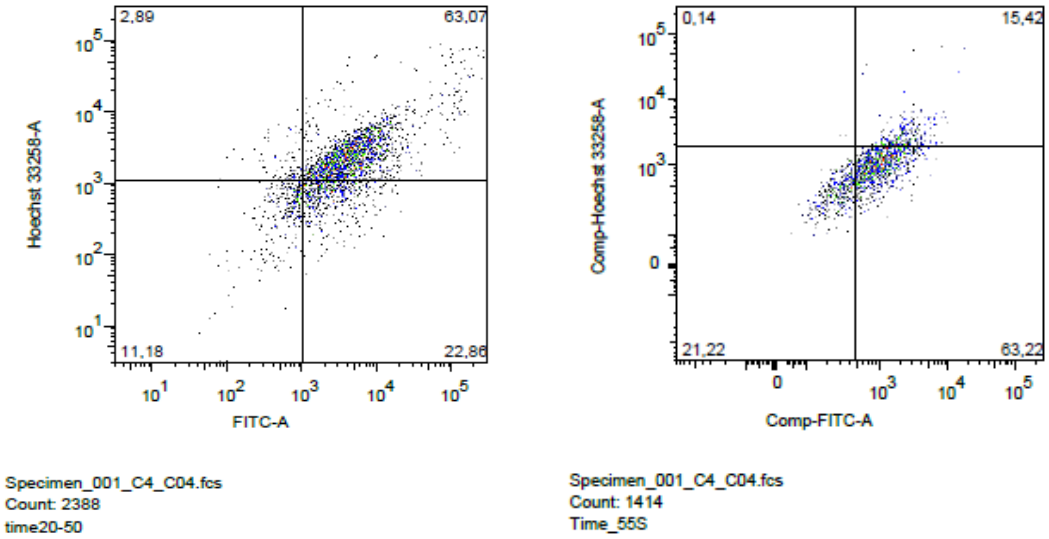
Data from the analysis related to the strain LF82 are disputable as cells cultivated with this strain act irregularly in the Annexin V/Hoechst quadrants (**Fig.12**; on the left).

Fig. 11 – Lower numbers of live cells stayed adhered after the cultivation with bacterial strains p19A and K6 in comparison with negative control. This applies only to the highest concentration of bacteria (100:1; bacteria:cells).



The graphs show flow rate of adhered MODE-K cells after cultivation with different *E. coli* strains. The results are a pool of 3 independent experiments. *P<0,05 (in comparison to negative control). Each concentration of bacteria was analyzed separately (1-way ANOVA, Tukey's post test). Negative control (cells) is shown in each graph.

Fig. 12 – Detached MODE-K and Caco-2 cells cultivated with *E. coli* strain LF82 act irregularly in the Annexin V/Hoechst quadrants.



Bacterial strain LF82 causes specific appearance of cell distribution in the live/early apoptotic/late apoptotic/primary necrotic quadrants. This pattern was seen consistently in all three independent experiments. MODE-K cells (on the left) appear as if they moved diagonally from the third quadrant to the second quadrant. The same accounts for Caco-2 cells (on the right).

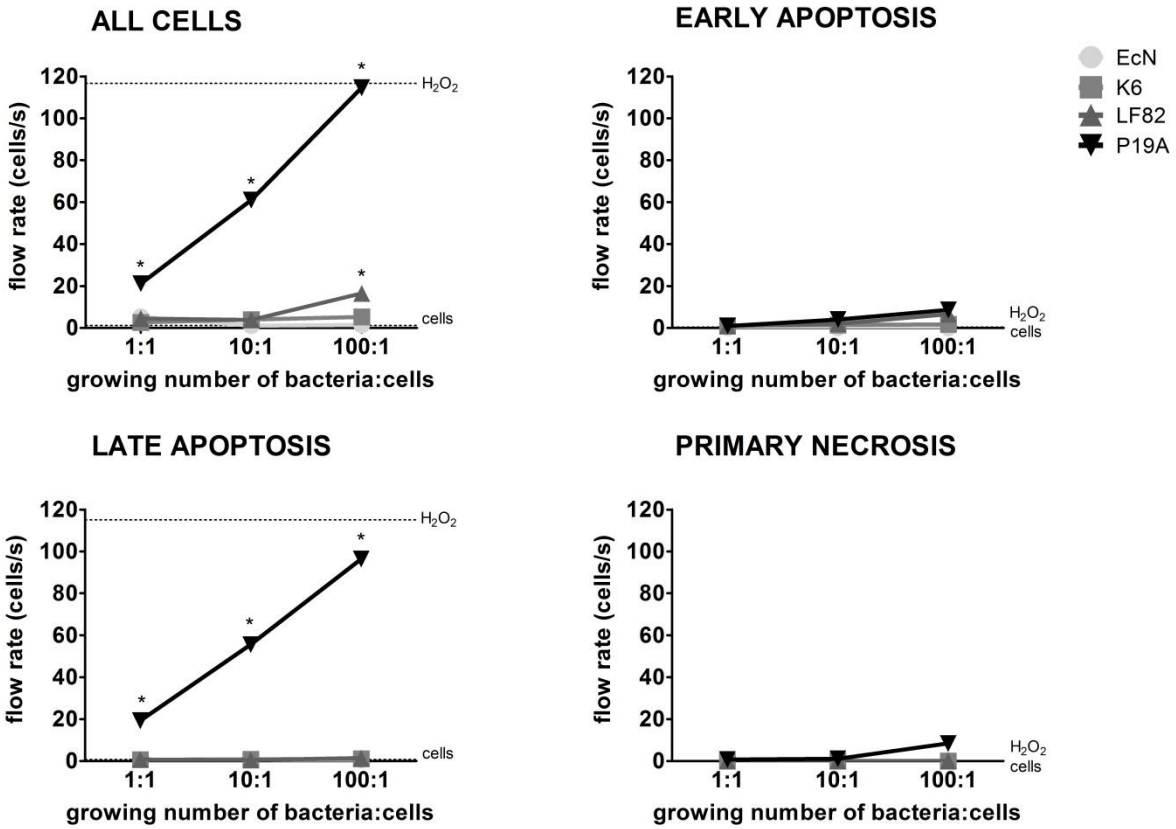
4.3.2 Caco-2

The *E. coli* strain p19A detaches significantly higher numbers (P<0,05) of Caco-2 cells than negative control in all used concentrations of bacteria per cells (**Fig.13**; all cells). Furthermore, in the two higher concentrations, p19A detached significantly more (P<0,05) epithelial cells than all the other bacterial strains. The cells detached by p19A are mostly in the phase of late apoptosis (**Fig.13**; late apoptosis). Comparison of the numbers of late apoptotic cells showed significant differences (P<0,05) between the bacterial strain p19A and negative control/all the other strains.

The highest concentration (100:1; bacteria:cells) of bacterial strain LF82 detached significantly more (P<0,05) Caco-2 cells from the cell layer than strain EcN and negative control. However, similarly to the experiments with MODE-K cells, data from the analysis related to the strain LF82 are disputable as cells cultivated with this strain act irregularly in the apoptotic quadrants (**Fig.12**; on the right).

The total amounts of epithelial cells detached by EcN and K6 during the 4-hour cultivation are not significantly different (P<0,05) from the negative control. This applies to all used concentrations of bacteria.

Fig. 13 – The bacterial strain p19A detached the highest numbers of Caco-2 cells, most of these cells were in the phase of late apoptosis.

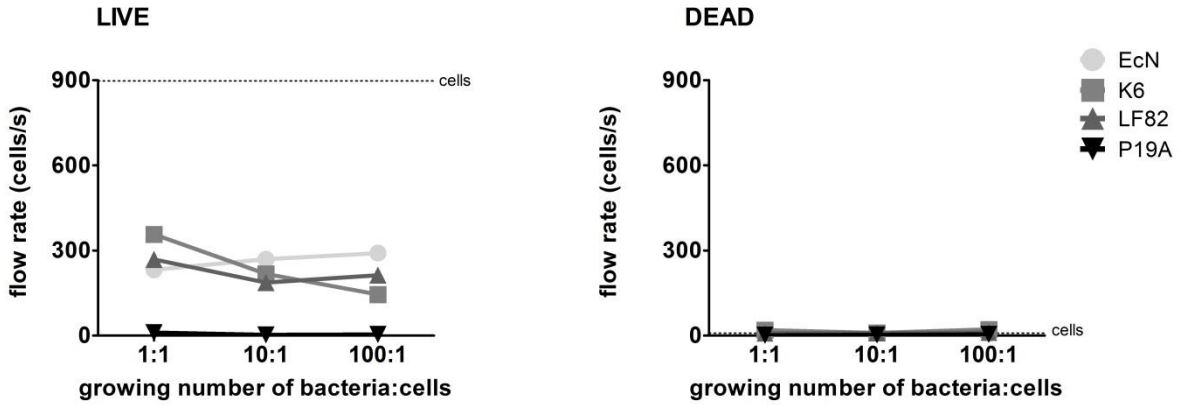


The graphs show flow rate of detached Caco-2 cells after cultivation with different *E. coli* strains. The results are a pool of 3 independent experiments. *P<0,05 (in comparison to negative control). Each concentration of bacteria was analyzed separately (1-way ANOVA, Tukey's post test). Negative control (cells) and necrosis control (H_2O_2) are shown in each graph. Apoptosis control (5-FU) is not shown.

In the analysis of adhered cells, the number of not-detached (adhered) live cells was significantly lower (P<0,05) in the case of strain p19A in comparison with strains K6 and EcN (**Fig. 14**; live) – this applies only to the lowest number (1:1) of K6 and highest number (100:1) of EcN per epithelial cell.

No statistically significant differences (P<0,05) were found between the numbers of not-detached (adhered) dead cells (**Fig.14**; dead).

Fig. 14 – Lower numbers of live cells stayed adhered after the cultivation with bacterial strain p19A in comparison with strains EcN and K6. This applies only to the lowest concentration (1:1) of K6 and highest concentration (100:1) of EcN.



The graphs show flow rate of adhered Caco-2 cells after cultivation with different *E. coli* strains. The results are a pool of 3 independent experiments. Each concentration of bacteria was analyzed separately (1-way ANOVA, Tukey's post test). Negative control (cells) is shown in each graph.

In conclusion, both epithelial cell lines, murine MODE-K and human Caco-2, show similar tendencies in the epithelium-detachment experiments. However, statistical analysis proved significant differences ($P<0,05$) mostly in the Caco-2 cell line. The potential pathobiont *E. coli* p19A was shown to detach the highest numbers of Caco-2 cells; these detached cells appear to be in the phase of late apoptosis. Probiotic strain EcN and commensal strain K6 are generally shown to detach similar amounts of cells ($P<0,05$) as the negative control.

5 DISCUSSION

Microbes in general dispose of many features allowing them to induce as well as protect from inflammatory diseases. Research studying the inflammatory bowel diseases showed several potential pathobionts (Carvalho et al., 2009; Momotani et al., 2012; Thomson et al., 2011) and probiotics (Fujimori et al., 2007; Hudcovic et al., 2007; Miele et al., 2009) influencing the pathology of these diseases. The main aim of this thesis was to study the interaction of the immune system with microbes that, despite of their closely related phylogenesis, have a different relationship to IBD pathogenesis.

Escherichia coli is a predominant aerobic microorganism of the gastrointestinal tract (Bezirtzoglou, 1997). Studies investigating the role of this bacterium in the pathogenesis of IBD appear quite often (de Souza, et al., 2012; Vejborg et al., 2011). In a comparative genomic study, Vejborg et al. (2011) showed that *E. coli* strains isolated from IBD patients represent a heterogeneous population with a wide variety of gene regions identified as possible virulence factors; whether these *E. coli* strains are responsible for intestinal inflammation or are simply a product of the inflammatory environment, remains unclear. In this thesis, we tried to answer the question, whether closely related microbes with different biological activities induce a different immune response. Therefore, we selected four strains of *E. coli* with different relation to IBD (EcN, K6, LF82 and p19A).

Measurement of the antibody response against commensal microbes in IBD patients can be an auxiliary diagnostic tool for determination of Crohn's disease (Adams et al., 2008). This fact brought us to the idea to measure the antibody response to *E. coli* strains. Serum samples were collected from IBD patients and controls, because antibody response to microbiota is changed in patients with IBD (Macpherson et al., 1996). We found that patients with IBD have a higher IgA response against the pathobiont *E. coli* p19A, isolated from patients with IBD (Petersen et al., 2009). Interestingly, the antibody response to p19A in both IgA and IgG tends to be higher than similar response to other strains, especially in IBD patients. However, this difference is not statistically significant. It must be taken into account, that the group of patients was relatively small (10 individuals in each group). The observed tendency in antibody response of the IBD patients is in agreement with the study of Macpherson et al. (1996), where IBD patients have an exaggerated immune response towards their own microbiota. In our experiments, very few statistically significant differences were found in the analysis of antibody response. This may be due to the resemblance between bacterial strains, as all of them are of the same species, so we expect the majority of their

antigens to be the same. Although anti-*E.coli* antibodies are a common finding both in IBD patients and healthy individuals, only a small portion of the protein fraction is usually used as an antigen for ELISA (Bernstein et al., 2011; Hevia et al., 2014; Petersen et al., 2011). In contrast to the authors mentioned above, we were interested in the differences between pathobionts and probiotic *E. coli* and not in antigens common to various bacteria which could be used for disease diagnosis. Additional experiments are to be performed (e.g. Western blot or 2-D electrophoresis) in order to characterize the microbial antigens to which given antibodies bind. This is also the reason why mode of inactivation, which may change the antibody binding, was an important part of our study. We found that the mode of inactivation does not change the ability of the antigens to bind IgA or IgG, which suggests that whichever inactivation protocol (described in the section **Materials and methods**) may be used for subsequent measurement of sera antibodies against microbes by ELISA.

The course of the immune reaction to a particular microbe is often determined by its first interaction with the innate immune system, which forms the first line of defense in the gut mucosa. From the innate immune cells we selected macrophages, because they play an important role in intestinal inflammation (Dieleman et al., 1994; Murano et al., 2000). Measurements of the CD40 expression and production of nitrite were done to determine the activation state of macrophages (RAW 264.7) and LPS-stimulated and non-treated cells were used as the positive and negative control, respectively. Using flow cytometry, we found that there are no differences between the magnitude of macrophage activation in response to probiotic, commensal and potentially pathogenic *E. coli*. The mentioned results could be ascribed to the resemblance between bacterial strains, as all of them are of the same species. Another explanation may be the high content of LPS in the outer-membrane of all used bacteria, which could cause the effect of other minority antigens (on macrophage activation) to be imperceptible. CD40 expression and production of nitrite should be tested also in different cell lines as RAW264.7 is derived from a murine tumor and thus the biological features of this cell line might be “non-physiological”. In the future, we would like to measure cytokines from the supernatant gained in our experiments (for example TNFα, IL-1β or IL-6) as these cytokines have an essential role in the pathogenesis of IBD (Mahida et al., 2000). Some might object to the usefulness of experiments with cell lines as they are an artificial substitute for cells or tissues present in the complex organism. Bearing this in mind, we decided to perform experiments with primary cells. From the innate immune cells, we moved to the adaptive immunity, as it represents the next step in an immune reaction. Cells from the

mesenterial lymph nodes and spleen were isolated to see the difference between two distinct compartments of the adaptive immune system, the systemic immunity (represented by spleen) and the mucosal compartment (represented by mesenterial lymph nodes). In addition, we were interested in the difference between immune responses of cells from an organism with acute inflammation in the gut vs. healthy organism. This is why we isolated MLN cells and SPL cells from mice with DSS-induced colitis (Wirtz et al., 2007), a chemically induced mouse model of IBD, and from healthy mice. Activation of the primary murine cells (in particular CD69 expression and production of IFN-γ) was measured by flow cymetry. Similarly to the study of Håkansson et al. (2014) or Hall et al. (2011), the proportions of early activated (CD69+) T cells from MLN and SPL were determined. In accordance with the results of Hall et al. (2011), the MLN cells from mice with active intestinal inflammation (DSS-treated) tend to express higher amounts of CD69 than MLN cells from healthy mice (not statistically significant) – however, this does not apply to SPL cells. This result comes as no surprise, as the acute inflammation of gut tissue is linked mainly to the mucosal department of the immune system. The relative lowness of early activation ratios in cells from DSS-treated mice in comparison to cells from non-treated mice could be ascribed to the short time of cultivation with stimuli. Splenic T cells are probably not activated by APCs in such a short period of time. Apart from the measurement of early activation in T cells, Håkansson et al. (2014) measured the levels of specific cytokines in serum of DSS-treated and non-treated mice, which is an interesting approach we could integrate into our research. As for the production of intracellular IFN-γ, Hall et al. (2011) found significantly higher amounts of IFN-γ producing cells in both MLN and SPL cells in the DSS-treated group (on day 8 of DSS treatment). To the contrary, the numbers of cells intracellulary producing IFN-γ in our experiments were very low and no differences between the cells from DSS-treated and non-treated mice were found in this regard. This could be explained by a presumably low amount of antigen-specific T cells (Hommel et al., 2004), which were identified by the IFN-γ production and are responsive to the given *E. coli*. The main aim the performed experiments, however, was to determine the difference between T cell response to distinct bacterial strains. We can conclude that the bacterial strain did not affect the early activation of T cells in MLN or SPL cells.

For technical reasons, we wanted to assess whether the mode of inactivation can change the response to *E. coli* strains. Need for this kind of determination was patterned on the fact that different types of inactivation treatments have an effect on the bacterial structure

and components (Ananta and Knorr, 2009). In all the mentioned experiments so far, the mode of inactivation did not change the response to *E.coli* strains. Inactivation of microbes in mainly studied in the case of subsequent vaccination usage (Datta et al., 2006), which does not correspond with our aims.

The damage of epithelial cells occurs in the inflammatory bowel diseases and it is an important step in the IBD pathogenesis (Boudeau et al., 2003; Matalka et al., 2013; Parlato and Yeretssian, 2014; Roda et al., 2010). Mucosa-associated strains of *E. coli* are augmented in the gut of IBD patients (De Souza et al., 2012). However, subsequent research in this group of *E. coli* did not specify any pathogenic features that would provide them with intracellular access to the epithelial cells (Elliott et al., 2013). To assess the effect of different *E. coli* strains on epithelia, we measured the capacity of bacteria to detach intestinal epithelial cells *in vitro*. For this purpose, murine MODE-K or human Caco-2 cell lines were cultivated in the presence of bacterial strains for several hours and subsequently measured by flow cytometry. Annexin V/Hoechst staining was used to distinguish live, early apoptotic, late apoptotic and primarily necrotic populations within detached epithelial cells. This method is similar to the frequently used method of staining which uses the Annexin V/PI (propidium iodide) combination for apoptosis detection (Pan et al., 2014; Roshan et al., 2014). The pathobiont, *E. coli* strain p19A, detached the highest numbers of Caco-2 cells and most of the detached cells were in the phase of late apoptosis. The epithelial cells cultivated with the adherent-invasive strain LF82 acted irregularly in the apoptotic-quadrants; this effect could be ascribed to the adhesive characteristic of the mentioned *E.coli* strain (Darfeuille-Michaud, 2002) and is a subject of our current investigations. Probiotic strain EcN and commensal strain K6 were generally shown to detach similar amounts of cells to the negative control. In MODE-K cells, similar, but not significant effects of microbes on the epithelial layer were observed. This could be ascribed to substantial differences between data obtained from every repetition of experiment. With the increasing concentration of EcN bacterial cells, we could see a slight increase in the viability ratios of Caco-2 cells that stayed adhered to the cultivation plate. This phenomenon could be explained by the probiotic characteristics of EcN and would make sense as other probiotics have been shown to improve the gut epithelial barrier function (Sokol et al., 2008; Okada et al., 2006). Analysis of adhered (not detached) cells in our experiments did not show any significant differences between distinct bacterial strains as compared to negative control. The question is, whether the trypsin/EDTA dissociation of cells used in the protocol was successful in all performed experiments and in both cell lines.

6 CONCLUSIONS

On the base of the experiments performed in the diploma thesis, we can conclude, that:

- The mode of inactivation (formaldehyde, heat or UV irradiation) did not influence the immune response to tested *Escherichia coli* strains.
- The antibodies from IBD patients and controls show similar reactivity to both probiotic microbes and pathobionts, except for higher reactivity to the strain p19A.
- There were no significant changes in the cell response to distinct *Escherichia coli* strains.
- Unlike the probiotic bacterium, co-cultivation of either pathobiont with the intestinal epithelial cells led to significant damage of epithelial layer *in vitro*.

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