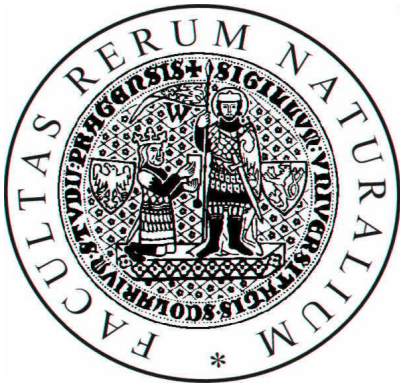
CHARLES UNIVERSITY IN PRAGUE

Faculty of Science

Study program: Immunology



Summary of dissertation

The effects of bacterial lysates on the gut barrier function and microbiota composition

RNDr. Zuzana Zákostelská

2012

Doctoral studies in biomedicine

Charles University in Prague and Academy of Sciences of the Czech republic

Field: Immunology

Chairmann of the Supervisory Committee: Doc. RNDr. Vladimír Holáň, DrSc.

Supervising Body: Department of Immunology and Gnotobiology
Institute of Microbiology
Vídeňská 1083, 14220, Prague 4 Krč

Author: RNDr. Zuzana Zákostelská

Supervisor: Prof. MUDr. Helena Tlaskalová-Hogenová, DrSc.

The summary of Dissertation was distributed on …….

The Defense of the Dissertation will be held on……..at…….in the Conference Room of the Institute of Microbiology ASCR, Vídeňská 1083, 14220, Prague 4 Krč

The dissertation is available at the library of Faculty of Science at the Charles University in Prague.

TABLE OF CONTENTS

Table of contents iii

Abbrevitations iv

Abstract v

1. Introduction 1

2. Hypothesis and aims 2

3. Material and methods 3

   3.1 Preparation of bacteria and bacterial components 3
   3.2 Bacterial inoculation 4
   3.3 Mice 4
   3.4 Study design and DSS colitis 4
   3.5 Experimental schedule of colorectal carcinoma 4
   3.6 Evaluation of intestinal barrier function 5
   3.7 Production of cytokines 5
   LPS stimulation of MLN lymphocytes 6
   3.8 Determination of specific antibodies 6
   3.9 β-glucuronidase determination 6
   3.10 Flow cytometry 6
   3.11 Evaluation of the anti-inflammatory properties of bacterial lysates in vitro 7
   3.12 Evaluation of microbiota changes by pyrosequencing 7
   3.13 Statistical analysis 7

4. Results 8

5. Discussion 11

   5.1 Microbial exposure during early life has persistent effect on future immune response of the host 11
   5.2 Impact of host-microbiota interaction on intestinal inflammation and colon cancer development 12
   5.3 Low microbial burden protects from colitis-associated cancer 15

6. Conclusions 16

7. References 17

8. Publications 20

ABBREVITATIONS

AOM	azoxymethane
ATB	antibiotic
CAC	colitis-associated cancer
CD	Crohn's disease
DC	dendritic cells
DSS	dextran-sulphate sodium
ELISA	Enzyme-linked immunosorbent Assay
FITC	fluorescein-iso-thiocyanate
GF	germ free
IBD	inflammatory bowel diseases
IFN-γ	interferon gamma
IRAK-M	IL-1 receptor associated kinase M
Lc	lysate of *Lactobacillus casei* DN-114 001
mPd	menbranous fraction of *Parabacteroides distasonis* lysate
M2	macrophages subset 2
MØ	macrophages
MLN	mesenteric lymph nodes
NF-κB	nuclear factor kappa B
NOD	nucleotide oligomerization domain
PBS	phosphate- buffered saline
PP	Peyers patches
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
SCID	severe combined immunodeficiency
SPF	specific pathogen free
TGF-β	transforming growth factor betta
TLRs	Toll-like receptors
TNF-α	tumour necrosis factor alpha
Tregs	regulatory T cells
UC	ulcerative colitis
ZO-1	zonula occludens

ABSTRACT

Dynamic molecular interactions between the microbiota and the intestinal mucosa play an important role in the establishment and maintenance of mucosal homeostasis. Aberrant host-microbiota interaction could lead to many diseases such as inflammatory bowel disease. The aim of our study was to evaluate the commensal and probiotic bacteria activities and their ability to induce pathological or exert beneficial effects.

The most important trigger for immune system development is an exposure to microbial components. Here, we show that there is a time window at about three weeks of age, which enables the artificial colonization of germ free mice by a single oral dose of cecal content. The delayed colonization by either inoculation or co-housing causes permanent changes in immune system reactivity, which may downgrade the results of experiments performed on first generation of colonized animals.

In this thesis we report that even non-living commensal bacteria such as *Parabacteroides distasonis* (mPd) or well known probiotics such as *L. casei* DN-114 001 (Lc) possess anti-inflammatory effects in experimental model of colitis. The mechanisms that this effect is achieved by the lysate of *L. casei* DN-114 001 comprise: a) improvement in the gut barrier function, b) correction of the dysbiosis, and c) modulation of the mucosal immune response. Unlike the oral treatment with Lc, mPd leads to increase of specific antibodies in serum.

These complex immunomodulatory properties of bacterial lysates may lead to the development of new therapeutic approaches for treatment of chronic intestinal inflammation and also highlight the importance of individualizing and characterizing the potential capacity of bacteria as immunomodulatory agents. Moreover, oral administration of sterile bacteria, in contrast to living bacteria, may be safer in severely ill or immunocompromised patients.

Next, we demonstrated that metabolic activity of certain commensal microbes substantially influences the process of colitis associated cancer. We showed that antibiotic treatment changes the microbiota composition, and that this change is responsible for the beneficial effect on tumorigenesis.

Therefore, understanding this host-microbiota crosstalk could bring new strategies in therapy and prevention of specific disorders associated with intestinal dysbiosis and disruption of mucosal homeostasis.

1. INTRODUCTION

The majority of epithelial surfaces of our body (skin, mucosae) are colonized by a vast number of microorganisms representing the so-called normal microflora, microbiota. Microbiota comprises mainly bacteria, but viruses, fungi and protozoans are also present. Our microbiota contains trillions of bacterial cells, 10 times more cells that the number of cells constituting our body. Most of commensal bacteria are symbiotic, however, after translocation through mucosa or under specific conditions (e.g. immunodeficiency) commensal bacteria could cause pathology. Considerable part, i.e. about 70%, of this microbial cosmos inside our body is constituted by bacteria that cannot be cultivated by current microbiological methods.

The close symbiosis of microbiota and human or animal hosts is the result of a long evolution and mutual adaptation of both inseparable partners, which define our ability to adapt to the ambient environment and defend ourselves against diseases. The period in which the human host is most sensitively influenced by microbiota is the postnatal period, i.e. the time span during which the germ-free neonate arrives from the sterile environment of the mother's uterus into the world full of microorganisms and his/her mucosal and skin surfaces are gradually colonized. The composition of main bacterial populations is not stabilized until after the first years of life. This is the period when the microbiota gradually colonizes mucosal and skin surfaces of the neonate and exerts the greatest effect on the development of the immune system (Adlerberth and Wold, 2009). The effect of colonization with microbiota on innate immunity cells was documented in our studies concerning the development of phagocytes, dendritic cells and intestinal epithelial cells (Petnicki-Ocwieja et al., 2009; Williams et al., 2006). Interestingly, the repertoire of T cell receptors is also influenced by colonization with microbiota (Probert et al., 2007).

Inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis, are severe chronic inflammatory illnesses of the gastrointestinal tract. Although their etiology and pathogenesis are not fully understood, it is generally accepted, that the inflammation is a result of an aberrant immune response to antigens of resident gut microbiota in genetically susceptible individuals (Sartor, 2006). Moreover, dysbiosis, an imbalance in the intestinal bacterial ecosystem, has been found in IBD and linked to its pathogenesis (Packey and Sartor, 2009). It has been suggested that this microbial

imbalances and an aberrant immune response could be restored by oral administration of certain beneficial bacterial species, probiotics (Sheil et al., 2007).

When administered in adequate amounts, probiotics, defined as living microorganisms, confer a health benefit to the host (FAO/WHO, 2001), and have been successfully used in treatment of IBD (Bibiloni et al., 2005). Using animal models of IBD, three main mechanisms of how these beneficial microbes protect from intestinal inflammation have been described. A single probiotic bacterium could possess more than one mechanism depending on its unique specific metabolic activities and cellular structures (Lebeer et al., 2010). First, probiotics may exclude or inhibit the growth of certain pathogens (Servin, 2004); second, they may improve the gut barrier function (Gupta et al., 2000); and third, they can modulate mucosal and/or systemic immune response or metabolic functions (Reiff et al., 2009). The outcome of probiotic therapy also depends on the stage of the disease and the overall health status of the patient. Despite of the generally safe profile of the probiotic therapy, the use of living microorganisms may lead to severe infections, and therefore represents considerable risk especially in severely ill patients (Besselink et al., 2008).

Even oral treatment with lysed bacteria could influence the development of experimentally induced intestinal inflammation. We showed that the severity of dextran sulphate sodium induced intestinal inflammation in BALB/c mice is reduced by orally administered sonicate of microbiota containing anaerobic bacteria (Verdu et al., 2000). Furthermore, we found that this effect could be directed through the manipulation with gut microbiota and immunomodulation of mucosal as well as systemic immune response.(Kverka et al.) Thus, the mechanisms of this protective and therapeutic effect have to be elucidated more precisely and this novel approach may be used for development of potential vaccine.

2. HYPOTHESIS AND AIMS

The general aim of this thesis was to describe more precisely host-microbiota interactions in different conditions. The main analyses that were included addressed the influence of the microbial exposure on immune system development and the immunomodulatory potential of bacterial lysates isolated from commensal and probiotic bacteria in chemical model of colitis.

Specific aims:

-to clarify and interconnect the relations between the microbiota and the host in relation to functional studies and metagenomic approaches at the same time. Using animal models of human diseases reared under defined conditions we describe the straight connection between microbiota and disease development.

-to study the importance of microbial community for the maturation of intestinal immune system and establishment of intestinal immune homeostasis during conventionalization in early life.

- to study if a preventive oral administration of sterile bacterial components of commensal *Parabacteroides distasonis* can protect from development of acute colitis in the experimental mouse model.

-to understand more about the mechanisms and effects of bacterial components and to compare the function of commensal *Parabacteroides distasonis* with well defined probiotic bacteria *Lactobacillus casei* DN-114 001 in the same experimental conditions

-to investigate whether an antibiotic treatment changes the microbiota composition and whether it had an effect on the severity of colitis-associated cancer in wild type and IRAK-M deficient mice.

3. MATERIAL AND METHODS

3.1 PREPARATION OF BACTERIA AND BACTERIAL COMPONENTS

L. casei DN-114 001, *L. plantarum* CCDM 185, *P. distasonis* were grown in an anaerobic chamber in De Man, Rogosa, and Sharpe broth (Oxoid, Basingstoke, UK). All bacteria were harvested by centrifugation (4000 x g, 30 min) and washed twice with sterile phosphate-buffered saline (PBS). After the treatment with the French press, bacteria were freeze-dried and diluted to a working concentration of 30 g/l. In order to kill all remaining viable bacteria, the lysate was heated to 60°C for 30 min. The individual components were prepared from lysates of Pd. After cell distruption with the French press, the lysate

was separated by centrifugation into two fractions, membranous (insoluble) and cytoplasmatic (soluble).The bacterial lysates were tested for anti-inflammatory activity in an acute colitis model.

3.2 BACTERIAL INOCULATION

Caecal contents from SPF SW mice (Taconic) were extracted and aliquots were frozen at -40°C until further use for inoculation.The bacterial suspension was inoculated orally in o1-3 weeks old GF SW mice.

3.3 MICE

We used BALB/c mice (8-12 weeks old),severe combined immunodeficient mice BALB/cJHanHsd-SCID (SCID), germ-free mice or IRAK-M C57BL/6 deficient mice in our experiments.

3.4 STUDY DESIGN AND DSS COLITIS

We administered 1.5 mg of Lc in 50 µl of sterile PBS, i.e. 6 x 10^8 CFU of heat killed bacteria, by gavage.The administration of lysates was repeated every 7 days for a total number of 4 doses (on days 0, 7, 14 and 21). Acute colitis was induced 7 days later by 3% (wt/v) DSS (molecular weight 36–50 kDa; MP Biomedicals) dissolved in tap water for 7 days, and on the last day of the experiment the colitis was evaluated by using a clinical activity score, colon length, and the histological scoring system as described previously (Cooper et al., 1993). For chronic colitis, mice received four cycles of DSS as described previously (Okayasu et al., 1990). In some experiments,the level of haptoglobin in mouse sera was assessed by Human Haptoglobin ELISA Quantitation Kit (GenWay Biotech., Inc).

3.5 EXPERIMENTAL SCHEDULE OF COLORECTAL CARCINOMA

We initiated tumorigenesis using modified protocol published previously (Clapper et al., 2007). Briefly, the mice were given single subcutaneous injection of azoxymethane (AOM, 10 mg/kg; Sigma-Aldrich, St. Louis, MO). Starting one week after the AOM injection, mice received one cycle of 3% dextran sodium sulfate (DSS, MW 36–50 kDa; MP Bio-medicals, Illkirch, France) in their drinking water continuously for up to 4 days. To induce the intestinal microbiota alteration in conventionally-reared mice, we treated a group of animals with antibiotics (ATB): metronidazole (500 mg/L; B. Braun, Melsungen AG,

Germany) and ciprofloxacin (100 mg/L; Zentiva, a.s., Hlohovec, Slovak Republic) in their drinking water for the whole experimental period (50 days).

3.6 EVALUATION OF INTESTINAL BARRIER FUNCTION

Intestinal permeability in vivo

The intestinal permeability was measured by determining the amount of FITC-dextran in blood after it was orally administered as described previously (Wang et al., 2001). Briefly, each mouse received 440 mg/kg of body weight of FITC-dextran (molecular weight 4.4 kDa) by gavage. The concentration of FITC-dextran was determined in serum by spectrophotofluorometry with an excitation wavelength of 483 nm and an emission wavelength of 525 nm.

Immunohistology

Segments of colon and terminal ileum were frozen in liquid nitrogen immediately after removal and stored at -80°C until used. Frozen sections (6 μm) were incubated with the rabbit polyclonal anti-mouse ZO-1 or occludin antibodies. After washing, the sections were incubated either with Texas Red or with DyLight 488 fluorochrome. Nuclei were counterstained using DAPI stain. Finally, the sections were mounted and viewed with a fluorescence microscope Olympus AX-70.

Determination of ZO-1 mRNA expression in intestinal tissue

Intestinal mucosa from terminal ileum and colon was placed in RNAlater stabilization reagent and extracted by using the RNeasy Mini isolation kit.The total RNA concentration was estimated by spectrophotometric measurements at 260 nm assuming that 40 μg of RNA per mililitre equal one absorbance unit. Real time PCR was performed as described previously (Zanvit et al., 2010).The data was analyzed with Genex software version 4.3.8.

3.7 PRODUCTION OF CYTOKINES

Intestinal tissue culture and measurement of cytokines

Sections of Peyer's patches (PP), ileum, cecum, and colon were cultivated for 48 hours at 37°C and 5% CO_2 in RPMI medium.Then commercial ELISA sets were used to measure the levels of TNF-α, IFN-γ, TGF-β, IL-10and IL-6 in these supernatants. The supernatants were screened for cytokine production with RayBio^{TM} Mouse Cytokine

Array II (Raybiotech, Inc.) capable to detect 32 cytokines, chemokines and growth factors.

Determination of cytokine mRNA expression in intestinal tissue

The samples were processed as described above (see Determination of ZO-1 mRNA expression in intestinal tissue). Gene expression assays for IL-10, IL-6, TNF-α and β-actin were all purchased by Applied Biosystems.

LPS stimulation of MLN lymphocytes

Isolated MLN cells from SW mice were cultivated at a concentration of $1x10^6$ cells/mL. LPS from *Escherichia coli* O26:B6 (Sigma-Aldrich) was added and the cells were placed in a humidified 5% CO_2 incubator at 37°C for 24 hours. Cytokine analyses on MLN culture supernatants were performed using the mouse Th1/Th2 10plex FlowCytomix Multiplex (eBiosciences, San Diego, CA).

3.8 DETERMINATION OF SPECIFIC ANTIBODIES

Sera and small intestine washings were collected for specific antibody evaluation. Indirect ELISA, optimized in our laboratory was used to assess the specific antibody response against Lc, Pd in serum (IgG, IgM, and IgA) and gut washings (secretory IgA; SIgA).

3.9 B-GLUCURONIDASE DETERMINATION

The β-glucuronidase enzyme in the intestine was extracted from stool samples. Product fluorescence was measured on microplate reader (Tecan GmbH) using 388 nm as excitation and 480 nm as emission wavelength.

3.10 FLOW CYTOMETRY

Single-cell suspensions of spleens, MLNs and PPs were prepared and stained for T_{regs} using FoxP3 Staining Set (eBioscience) with fluorochrome-labeled anti-mouse mAbs: CD4-Qdot® 605, CD8-BD Horizon™ V500, CD3-FITC and FoxP3-Phycoerythrin according to the manufacturer's recommendation (BD Bioscience). RAW 264.7 cells were cultivated and stained for IL-7R-Alexa647, CD206-PE, CD-11c-NC625 and F4/80-APC780. Hoechst 33342 (Sigma-Aldrich) was used to determine cell viability. Flow cytometric analysis was performed on LSRII (BD Biosciences), and the data was analyzed using FlowJo software (Tree Star Inc).

3.11 EVALUATION OF THE ANTI-INFLAMMATORY PROPERTIES OF BACTERIAL LYSATES IN VITRO

The LPS-activated macrophage cell line (RAW 264.7; ATCC TIB-71) was cultivated in the presence of different concentrations of bacterial lysate.The cells were cultured for 24 hour at 37°C and 5% CO_2 in complete DMEM medium (Sigma-Aldrich).The concentration of TNF-α in the supernatant was measured with ELISA (Invitrogen). The nuclear proteins were extracted by a nuclear extract kit (Active Motif) and used to quantify of the p65 subunit using the TransAM NF-κB family transcription factor assay kit (Active Motif).

3.12 EVALUATION OF MICROBIOTA CHANGES BY PYROSEQUENCING

Total DNA from stool samples was then isolated with ZR Fecal DNA Kit™ (Zymo Research Corp.). PCR was performed and PCR product was subsequently purified using magnetic beads (AMPure beads, Beckman Coulter Genomics). Equimolar amounts were used for unidirectional 454 FLX amplicon pyrosequencing using LIB-L emPCR kits following the manufacturer's protocols (Roche Diagnostics) or stool samples were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) as described previously (Hufeldt et al., 2010).

3.13 STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) with Dennett's multiple comparison test was used to compare multiple experimental groups with the control group. Differences between two groups were evaluated using an unpaired two-tailed Student's t-test and deviation of values from hypothetical mean were calculated by one sample t-test. The data is presented as the mean ± standard deviation (SD) unless stated otherwise and differences were considered statistically significant at $P \leq 0.05$. GraphPad Prism statistical software (version 5.0, GraphPad Software, Inc., La Jolla, CA,USA) was used for analyses.

4. RESULTS

Patterns of early gut colonization shape future immune responses of the host. Hansen CH, Nielsen DS, Kverka M, **Zakostelska Z**, Klimesova K, Hudcovic T, Tlaskalova-Hogenova H, Hansen AK. PLoS One. 2012;7(3):e34043. Epub 2012 Mar 27.

In this study, we examined how differences in early gut colonization patterns change the composition of the resident microbiota and future immune system reactivity.

-We found that a single oral inoculation of GF mice at three weeks of age permanently changed the gut microbiota composition, which was not possible to achieve at one week of age.

- The ex-GF mice inoculated at three weeks of age were also the only mice with an increased pro-inflammatory immune response. In contrast, the composition of the gut microbiota of ex-GF mice that were co-housed with SPF mice at different time points was similar to the gut microbiota in the barrier maintained SPF mice.

- The existence of a short GF postnatal period permanently changed levels of systemic regulatory T cells, NK and NKT cells, and cytokine production.

These data suggest that time window exists and enables the artificial colonization of GF mice by a single oral dose of caecal content, which may modify the future immune phenotype of the host. Moreover, delayed microbial colonization of the gut causes permanent changes in the immune system.

Oral administration of Parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. Kverka M, **Zakostelska Z**, Klimesova K, Sokol D, Hudcovic T, Hrncir T, Rossmann P, Mrazek J, Kopecny J, Verdu EF, Tlaskalova-Hogenova H. Clin Exp Immunol. 2011 Feb;163(2):250-9. doi: 10.1111/j.1365-2249.2010.04286.x. Epub 2010 Nov 19.

In this study, we report that oral treatment of BALB/c mice with components from the commensal, *Parabacteroides distasonis*, significantly reduces the severity of intestinal inflammation in murine models of acute and chronic colitis induced by dextran sulphate sodium (DSS).

–Oral administration of *P. distasonis* components (mPd) protects from experimentally induced intestinal inflammation through several innate and adaptive

immunomodulatory mechanisms.

–Oral treatment with mPd promotes an increase in the level of mPd-specific antibodies and in the numbers of Tregs in MLN. This therapy is not effective in mice lacking adaptive immunity (SCID).

–Oral treatment with mPd, Pd inhibits TNF-α production in LPS-activated macrophages in vitro and stabilizes the intestinal microbial ecology.

–The protective effect of oral treatment with mPd could be transferred from orally treated immunocompetent mouse to naïve immunocompetent mouse with serum.

Our study suggest that specific bacterial components derived from the commensal bacterium, *P. distasonis*, may be useful in the development of new therapeutic strategies for chronic inflammatory disorders such as inflammatory bowel disease.

Lysate of probiotic Lactobacillus casei DN-114 001 ameliorates colitis by strengthening the gut barrier function and changing the gut microenvironment. Zakostelska Z, Kverka M, Klimesova K, Rossmann P, Mrazek J, Kopecny J, Hornova M, Srutkova D, Hudcovic T, Ridl J, Tlaskalova-Hogenova H. PLoS One. 2011;6(11):e27961. Epub 2011 Nov 22.

In this study, we investigate if lysate of probiotic bacterium *L. casei* DN-114 001 (Lc) could decrease the severity of intestinal inflammation in a murine model of IBD and to analyze its underlying mechanism.

-**We found that the preventive effect of oral administration of Lc significantly reduces the severity of acute dextran sulfate sodium (DSS) colitis in BALB/c but not in SCID mice.**

- Oral treatment with Lc leads to a significant protection against increased intestinal permeability and barrier dysfunction shown by preserved ZO-1 expression.

- Oral treatment with Lc increases the numbers of $CD4^+FoxP3^+$ regulatory T cells in MLN, decreases production of pro-inflammatory cytokines TNF-α and IFN-γ, and anti-inflammatory IL-10 in Peyer's patches and large intestine.

- Oral treatment with Lc significantly changes the composition of gut microbiota. *Lactobacillus* increased in abundance after exposure to DSS and the Lc treatment. This increase in abundance was not observed in the control PBS group.

-Lc but not Lp treatment prevents lipopolysaccharide-induced TNF-α expression in RAW 264.7 cell line by down-regulating the NF-κB signaling pathway. Lc treatment polarizes macrophages to M2 phenotype.

Our study provided evidence that even non-living probiotic bacteria can prevent the development of severe forms of intestinal inflammation by strengthening the integrity of intestinal barrier and modulation of gut microenvironment.Moreover, oral administration of sterile bacterial components, in contrast to live bacteria, may be safer in severely ill or immunocompromised patients.

The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Tlaskalová-Hogenová H, Stěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, Rossmann P, Hrnčíř T, Kverka M, **Zákostelská Z**, Klimešová K, Přibylová J, Bártová J, Sanchez D, Fundová P, Borovská D, Srůtková D, Zídek Z, Schwarzer M, Drastich P, Funda DP. Cell Mol Immunol. 2011 Mar;8(2):110-20. Epub 2011 Jan 31. Review.

In this chapter, we review the relations between the microbiota and the host in relation to functional studies and metagenomic approaches at the same time. Using animal models of human diseases reared under defined conditions we describe the straight connection between microbiota and disease development.

Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M deficient mice. Klimesova K, Kverka M, **Zakostelska Z**, Hudcovic T, Hrncir T, Stepankova R, Rossmann P, Ridl J, Kostovcik M, Mrazek J, Kopecny J, Kobayashi K S, Tlaskalova – Hogenova H, Submitted in IBD

In this study, we investigated the role of negative regulation of a TLR signaling and gut microbiota in the development of colitis-associated cancer in mouse model.

-ATB treatment of wild-type mice reduced the incidence and severity of tumors. As compared with non-treated mice, ATB-treated mice had significantly lower numbers

of regulatory T cells in colon, altered gut microbiota composition, and decreased β-glucuronidase activity.

-IRAK-M deficient mice not only developed invasive tumors, but ATB-induced decrease in β-glucuronidase activity did not rescue them from severe carcinogenesis phenotype. Furthermore, IRAK-M deficient mice had significantly increased levels of pro-inflammatory cytokines in the tumor tissue.

Our study show that gut microbiota promotes tumorigenesis by increasing the exposure of gut epithelium to carcinogens and that IRAK-M negative regulation is essential for colon cancer resistance even in the conditions of altered microbiota. Therefore, gut microbiota and its metabolic activity could be potential targets for colitis-associated cancer therapy.

5. DISCUSSION

5.1 MICROBIAL EXPOSURE DURING EARLY LIFE HAS PERSISTENT EFFECT ON FUTURE IMMUNE RESPONSE OF THE HOST

The great importance of commensal bacteria on immune development is clearly demonstrated in GF mice raised in the absence of any bacteria. As recently supported by many studies generated in humans and gnotobiological rodents, immune effects of early-life microbial exposure are durable, persist into later life and may improve the health of the host. These effects could be restorable and are also age dependent, because, e.g., neonatal but not adult germ-free mice with conventional microbiota exhibited a complete normalization of iNKT cells and the subsequent related pathology also did not occur (Olszak et al., 2012).

Our work described reports on the outcome of conventionalization studies presented on germ-free mouse model that describe how timing alters the ability of the microbes to colonize the host. The time window which enables the artificial colonization is at about three weeks of age and is probably caused by changes in the gut physiology such as increase in gut redox potential, changes in peristaltis and secretions, expression of certain pattern recognition receptors and diet (Mackie et al., 1999), or maybe by the protective effect of the weaning when single microbial inoculation is not enough to influence the host (Newburg and Walker, 2007). Like others (Ivanov et al., 2008), we also found that later colonization but not the way of colonization causes permanent changes in immune system reactivity, e.g. in the number of tolerogenic DC cells, Tregs,

invariant NKT cells and in the amounts of cytokines (Olszak et al., 2012). This seems reasonable as the infant gut is immature and in a state favoring the development of regulatory mechanisms in response to environmental stimuli (Mold et al., 2008; Sudo et al., 1997). These effects may strongly influence the results of experiments made on first generation of colonized animals.

Moreover, not only bacterial microbiota but also the content of microbial components in sterile diets and to a lesser extent also the LPS-rich sterile diet has a significant effect on the development and function of the immune system, especially the expansion of B and T cells in Peyer's patches and mesenteric lymph nodes (Hrncir et al., 2008). It is worth emphasizing that the quality of a diet and the presence or absence of certain strains of bacteria could also be important factors that may influence the host (Gaboriau-Routhiau et al., 2009; Stepankova et al., 2007).

5.2 IMPACT OF HOST-MICROBIOTA INTERACTION ON INTESTINAL INFLAMMATION AND COLON CANCER DEVELOPMENT

Altered microbiota composition and function in inflammatory bowel disease result in increased immune stimulation, epithelial dysfunction and enhanced intestinal permeability. Moreover, increased intestinal permeability plays also a role in many other diseases. Although the most important findings in this field are still to come, it is clear that our microbiota affect our lives more than previously assumed.

In order to find the answers to all these questions concerning the role of microbiota in health and in various diseases, it is quite important to combine the use of genetically manipulatable model organisms and gnotobiotic models. This approach has the potential to provide new and important information about how bacteria affect normal development, establishment and maintenance of the mucosa-associated immune system, and epithelial cell functions as described. In our opinion, a study on GF animals and knockout mice can help to provide new insights into the pathogenesis of infectious diseases as well as acute and chronic inflammatory disorders.

We reported on the lysate of the mouse-commensal bacterium *Parabacteroides distasonis* and its fractions for their ability to influence intestinal inflammation. Although bacteroides phylum is known to play a role in intestinal inflammation (Swidsinski et al., 2002), in our study the bacterium, and especially its membranous fraction (mPd), was chosen among many other bacterial candidates as the organism most sufficient in suppressing acute DSS colitis. The cytoplasmic fraction does not possess any protective

effect, suggesting that the effective component is ,,hidden“ in membranous fraction. Our aim is to find and characterize the particular molecule responsible for this effect. Next, here we have reported that the well-known probiotic *Lactobacillus casei* DN-114 001 or its lysate also exert a protective effect in suppressing acute DSS colitis but function in a slightly different way. This is in agreement with recent experimental evidence indicating that beneficial effects of bacteria are species and strain specific(Yan and Polk, 2010). We have to mention that the administration route is very important.Using both bacteria we were able to achieve the protective effect in preventive schedule before the induction of inflammation but not if the inflammation was already present, like in chronic colitis. This is in agreement with the known fact that the prevention of experimental inflammatory bowel disease is achieved more easily than the treatment of ongoing inflammation (Obermeier et al., 2003).

We also presented the finding demonstrating that not only living probiotic bacteria but also bacterial lysates are able to ameliorate intestinal inflammation. This idea is further supported by other studies which showed that the protective effect on gut homeostasis and barrier function could also be realized by bacterial metabolites.This clearly shows that bacterial components could exert all the beneficial properties of probiotics and might be a safer alternative to living probiotics.

For the first time we showed that treatment not just with living bacteria but also with bacterial lysate can modulate gut microbial diversity. Our findings also coincide with the results of a previous report demonstrating that probiotic bacteria alter the composition of the intestinal microbiota and these changes correlate with disease protection(Uronis et al., 2011). It seems that each bacterial lysate is acting in a different way. Lysate of mPd prevents the microbiota changes caused by DSS. Lc supported a substantial increase in *Lactobacillus* genus which indicates that treatment with Lc promotes, among others, also this genus. Therefore, we can suggest that these microbial changes lead to an improvement in the gut barrier function and a decrease of susceptibility to intestinal inflammation by producing active substances such as lactate and butyrate similarly as reported by studies describing the influence of *Propionibacterium freudenreichii* and *Faecalibacterium prausnitzii* (Okada et al., 2006; Sokol et al., 2008). In case of lysate of mPd the mechanism of the beneficial effect could be direct or it could be just a consequence of improvement in inflammation.

Here, we report that Lc lysate *in vitro* polarizes MØ to M2 phenotype, which has in general a high scavenging activity, activates the process of tissue repair, suppresses

adaptive immune responses and is believed to participate in the blockade of inflammatory We demonstrated that tested Lc and mPd bacterial lysates prevented LPS-induced production of TNF-α and activation of NF-*κ*B pathway in MØ, since the bacterial lysates reduced the cytoplasm-to-nucleus translocation of the p65 subunit.

Altered intestinal barrier function plays a pivotal role in IBD (Laukoetter et al., 2008), but it is not certain if it is a result of disease progression or if it is a primary event. In this thesis we report that the lysate of Lc protects against increased intestinal permeability by up-regulation of the expression of proteins of the tight junctions, namely of occludin and ZO-1. These results are in agreement with the study describing the effect of both heat-killed and living *L. rhamnosus* OLL2838 in DSS colitis model on intestinal barrier improvement.Above all, maintaining paracellular permeability in the gut at normal is a very important function that plays a role in many diseases and it will be very challenging to implement probiotic lysates in clinical trials.

Induction of oral tolerance to food and microbiota is crucial for keeping the homeostasis in the gut. This mechanism is mediated by the innate immunity, especially in early ontogeny phases, where the inhibitory regulatory pathways play an important role (Biswas et al., 2011). An equal importance in induction of oral tolerance is ascribed to adaptive immunity mechanisms (Barnes and Powrie, 2009), especially to regulatory T cells (Tregs) whose protective role has been clearly established (Hrncir et al., 2008; Singh et al., 2001).

There are many findings indicating that specific probiotics can function through induction of Tregs . A probiotic mixture called IRT5 containing lactobacilli induces a protective effect associated with enrichment of Tregs in the inflamed regions in experimental inflammatory bowel disease, atopic dermatitis and rheumatoid arthritis. The co-administration of living *L. casei* with collagen can potentiate the oral tolerance and leads to increase in Tregs in collagen induced arthritis model, while oral treatment with live *L. casei* alone cannot (So et al., 2008). Also therapeutic treatment with a probiotic mixture containing three strains of lactobacilli is able to reverse established experimental autoimmune encephalomyelitis through regulation of systemic IL-10 release and induction of functional Tregs in intestinal mesenteric lymph nodes and in the periphery (Lavasani et al., 2010). Since oral tolerance is easier to achieve with killed or inactivated microbes than with living ones (Rubin et al., 1981), we decided to measure the changes in Tregs numbers after the treatment with bacterial lysate of Lc. Our results not only confirm that treatment with dead bacteria and the microbiota-derived components results in an increase

of Tregs expression in MLN similarly as shown by previous studies (Hrncir et al., 2008) but we also report for the first time an increase in Tregs numbers after stimulation with non-living Lc-treated mice in a model of acute colitis. We have obtained a quite similar result when we used the common commensal bacterium *Parabacteroides distasonis*.

To investigate further the role of adaptive immunity in the protective activity of bacterial lysates, we studied severely immunodeficient (SCID) mice lacking T and B lymphocytes. Although the severity of DSS-induced acute inflammation in SCID mice was similar to that in immunocompetent mice (Dieleman et al., 1994; Hudcovic et al., 2001), we have not seen any preventive effect of either Lc or mPd treatment. One limitation of the comparison between BALB/c and SCID mice, except the deficiency in adaptive immunity, relates to differences in gut microbiota composition and/or innate immune cell activity between strains (Keilbaugh et al., 2005). As suggested by our *in vitro* experiments, we can not rule out the involvement of innate immune mechanisms in the initiation of mPd and Lc protection. However, the lack of protective effect in SCID mice and induction of Tregs in our *in vivo* experiments clearly shows that adaptive immune response is involved in this beneficial effect.

5.3 LOW MICROBIAL BURDEN PROTECTS FROM COLITIS-ASSOCIATED CANCER

Our experiments with colitis-associated cancer (CAC) showed that intestinal homeostasis and the process of CAC are substantially influenced by the presence of commensal bacteria and gut microbiota composition. In our experimental model of carcinogenesis we were able to change the microbiota composition (decrease the microbial load) and a subsequent tumor development through a decrease in the local production of β-glucuronidase caused by changed microbiota composition after antibiotic treatment, thus decreasing the exposure of gut epithelium to carcinogens. Here we report that a lower tumor incidence is connected with changes in the microbiota such as an increase in proportions of Bacteroidetes and decrease in Firmicutes and Proteobacteria. Moreover, impaired regulation increases the activation of mucosal as well as systemic immune response, which results in chronic pro-inflammatory stimulation and, together with potent immune suppression, leads to massive tumor growth. Interesting finding is that enhanced immune suppression through Tregs in tumor bearing IRAK-M knockout mice led to massive tumor progression advanced by inflammation, as supported by the study by

Berglund et al. 2010 {Berglund, 2010 #92}.Therefore, individually-targeted manipulation of gut microbiota could be a promising strategy in IBD and CAC therapy and prevention.

6. CONCLUSIONS

An increasing number of *in vitro* and *in vivo* animal and clinical studies demonstrate the protective therapeutic role of probiotics and commensal bacteria. However, more studies are needed because many factors are to be considered in each disease or ethnical group. Doses of bacteria and growth phase at the time of harvest are additional considerations in tandem with traditional methods of determining strain robustness or functional effect. Heat-killed bacteria or its components have the advantage of allowing a longer product shelf life, easier storage and transportation and will be also safer when administered to children, to low birth weight neonates with an immature digestive tract or to immuno-compromised patients. Also, as described in our study and many others, the influence of microbiota on developing immune system is the greatest at the early stage of postnatal life so not all immunological disorders arising from altered microbiota will be efficiently influenced once the host microbiota diversity is established. Strain specific effects must be also taken into account; however, once the molecule with the desired effect is described and used, this complication will disappear. The growth phase and viability of the probiotic cultures is also a factor that varies between reported experiments and expected results. Moreover, human studies must be well designed with careful strain selection because specific indications, timing, dosing and potential health risk factors have to be carefully considered to prevent a possible adverse outcome of microbiota administration (Besselink et al., 2008). While we have merely scratched the surface new functions of different species are continually being discovered. The two studies of bacterial lysates described in this thesis indicate how beneficial the effect of a single bacterial strain could be. In our future studies we are planning to explore the detailed mechanism of its function.

The interplay between the host and the microbiota and its influence on mucosal immune system was intensively described topic in this thesis. Important issues comprise the effects of early microbial colonization on the immune response of the host, interaction of host bacterial components with probiotics or commensals and its relevance to pathological conditions such as IBD, colon cancer, etc. Also, the probiotic lysate can be simply

used to restore disturbed intestinal microbial flora, e.g. after an exposure to a broad spectrum of antibiotics. All together it may be an easy, natural and a very cheap way to influence the host immune system, metabolism and health status in general without using pharmacological drugs with their contraindications.

7. REFERENCES

Adlerberth, I., and Wold, A. E. (2009). Establishment of the gut microbiota in Western infants. Acta Paediatr *98*, 229-238.

Barnes, M. J., and Powrie, F. (2009). Regulatory T cells reinforce intestinal homeostasis. Immunity *31*, 401-411.

Besselink, M. G., van Santvoort, H. C., Buskens, E., Boermeester, M. A., van Goor, H., Timmerman, H. M., Nieuwenhuijs, V. B., Bollen, T. L., van Ramshorst, B., Witteman, B. J., *et al.* (2008). Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. Lancet *371*, 651-659.

Bibiloni, R., Fedorak, R. N., Tannock, G. W., Madsen, K. L., Gionchetti, P., Campieri, M., De Simone, C., and Sartor, R. B. (2005). VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am J Gastroenterol *100*, 1539-1546.

Biswas, A., Wilmanski, J., Forsman, H., Hrncir, T., Hao, L., Tlaskalova-Hogenova, H., and Kobayashi, K. S. (2011). Negative regulation of Toll-like receptor signaling plays an essential role in homeostasis of the intestine. Eur J Immunol *41*, 182-194.

Clapper, M. L., Cooper, H. S., and Chang, W. C. (2007). Dextran sulfate sodium-induced colitis-associated neoplasia: a promising model for the development of chemopreventive interventions. Acta Pharmacol Sin *28*, 1450-1459.

Cooper, H. S., Murthy, S. N., Shah, R. S., and Sedergran, D. J. (1993). Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest *69*, 238-249.

Dieleman, L. A., Ridwan, B. U., Tennyson, G. S., Beagley, K. W., Bucy, R. P., and Elson, C. O. (1994). Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology *107*, 1643-1652.

FAO/WHO (2001). Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. In, (Cordoba (Argentina): Food and Agriculture Organization of the United Nations and World Health Organization), p. 34.

Gaboriau-Routhiau, V., Rakotobe, S., Lecuyer, E., Mulder, I., Lan, A., Bridonneau, C., Rochet, V., Pisi, A., De Paepe, M., Brandi, G., *et al.* (2009). The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity *31*, 677-689.

Gupta, P., Andrew, H., Kirschner, B. S., and Guandalini, S. (2000). Is lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. J Pediatr Gastroenterol Nutr *31*, 453-457.

Hrncir, T., Stepankova, R., Kozakova, H., Hudcovic, T., and Tlaskalova-Hogenova, H. (2008). Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice. BMC Immunol *9*, 65.

Hudcovic, T., Stepankova, R., Cebra, J., and Tlaskalova-Hogenova, H. (2001). The role of microflora in the development of intestinal inflammation: acute and chronic colitis induced by dextran sulfate in germ-free and conventionally reared immunocompetent and immunodeficient mice. Folia Microbiol (Praha) *46*, 565-572.

Hufeldt, M. R., Nielsen, D. S., Vogensen, F. K., Midtvedt, T., and Hansen, A. K. (2010). Variation in the gut microbiota of laboratory mice is related to both genetic and environmental factors. Comp Med *60*, 336-347.

Ivanov, II, Frutos Rde, L., Manel, N., Yoshinaga, K., Rifkin, D. B., Sartor, R. B., Finlay, B. B., and Littman, D. R. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe *4*, 337-349.

Keilbaugh, S. A., Shin, M. E., Banchereau, R. F., McVay, L. D., Boyko, N., Artis, D., Cebra, J. J., and Wu, G. D. (2005). Activation of RegIIIbeta/gamma and interferon gamma expression in the intestinal tract of SCID mice: an innate response to bacterial colonisation of the gut. Gut *54*, 623-629.

Kverka, M., Zakostelska, Z., Klimesova, K., Sokol, D., Hudcovic, T., Hrncir, T., Rossmann, P., Mrazek, J., Kopecny, J., Verdu, E. F., and Tlaskalova-Hogenova, H. Oral administration of *Parabacteroides distasonis* antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. . Clin Exp Immunol, in press.

Laukoetter, M. G., Nava, P., and Nusrat, A. (2008). Role of the intestinal barrier in inflammatory bowel disease. World J Gastroentero *14*, 401-407.

Lavasani, S., Dzhambazov, B., Nouri, M., Fak, F., Buske, S., Molin, G., Thorlacius, H., Alenfall, J., Jeppsson, B., and Westrom, B. (2010). A novel probiotic mixture exerts a therapeutic effect on experimental autoimmune encephalomyelitis mediated by IL-10 producing regulatory T cells. Plos One *5*, e9009.

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. (2010). Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol *8*, 171-184.

Mackie, R. I., Sghir, A., and Gaskins, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr *69*, 1035S-1045S.

Mold, J. E., Michaelsson, J., Burt, T. D., Muench, M. O., Beckerman, K. P., Busch, M. P., Lee, T. H., Nixon, D. F., and McCune, J. M. (2008). Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. Science *322*, 1562-1565.

Newburg, D. S., and Walker, W. A. (2007). Protection of the neonate by the innate immune system of developing gut and of human milk. Pediatr Res *61*, 2-8.

Obermeier, F., Dunger, N., Strauch, U. G., Grunwald, N., Herfarth, H., Scholmerich, J., and Falk, W. (2003). Contrasting activity of cytosin-guanosin dinucleotide oligonucleotides in mice with experimental colitis. Clin Exp Immunol *134*, 217-224.

Okada, Y., Tsuzuki, Y., Miyazaki, J., Matsuzaki, K., Hokari, R., Komoto, S., Kato, S., Kawaguchi, A., Nagao, S., Itoh, K., *et al.* (2006). Propionibacterium freudenreichii component 1.4-dihydroxy-2-naphthoic acid (DHNA) attenuates dextran sodium sulphate induced colitis by modulation of bacterial flora and lymphocyte homing. Gut *55*, 681-688.

Okayasu, I., Hatakeyama, S., Yamada, M., Ohkusa, T., Inagaki, Y., and Nakaya, R. (1990). A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology *98*, 694-702.

Olszak, T., An, D., Zeissig, S., Vera, M. P., Richter, J., Franke, A., Glickman, J. N., Siebert, R., Baron, R. M., Kasper, D. L., and Blumberg, R. S. (2012). Microbial exposure during early life has persistent effects on natural killer T cell function. Science *336*, 489-493.

Packey, C. D., and Sartor, R. B. (2009). Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Curr Opin Infect Dis *22*, 292-301.

Petnicki-Ocwieja, T., Hrncir, T., Liu, Y. J., Biswas, A., Hudcovic, T., Tlaskalova-Hogenova, H., and Kobayashi, K. S. (2009). Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci U S A *106*, 15813-15818.

Probert, C. S., Williams, A. M., Stepankova, R., Tlaskalova-Hogenova, H., Phillips, A., and Bland, P. W. (2007). The effect of weaning on the clonality of alpha beta T-cell receptor T cells in the intestine of GF and SPF mice. Dev Comp Immunol *31*, 606-617.

Reiff, C., Delday, M., Rucklidge, G., Reid, M., Duncan, G., Wohlgemuth, S., Hormannsperger, G., Loh, G., Blaut, M., Collie-Duguid, E., *et al.* (2009). Balancing inflammatory, lipid, and xenobiotic signaling pathways by VSL#3, a biotherapeutic agent, in the treatment of inflammatory bowel disease. Inflamm Bowel Dis *15*, 1721-1736.

Rubin, D., Weiner, H. L., Fields, B. N., and Greene, M. I. (1981). Immunologic tolerance after oral administration of reovirus: requirement for two viral gene products for tolerance induction. J Immunol *127*, 1697-1701.

Sartor, R. B. (2006). Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol *3*, 390-407.

Servin, A. L. (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev *28*, 405-440.

Sheil, B., Shanahan, F., and O'Mahony, L. (2007). Probiotic effects on inflammatory bowel disease. J Nutr *137*, 819S-824S.

Singh, B., Read, S., Asseman, C., Malmstrom, V., Mottet, C., Stephens, L. A., Stepankova, R., Tlaskalova, H., and Powrie, F. (2001). Control of intestinal inflammation by regulatory T cells. Immunol Rev *182*, 190-200.

So, J. S., Kwon, H. K., Lee, C. G., Yi, H. J., Park, J. A., Lim, S. Y., Hwang, K. C., Jeon, Y. H., and Im, S. H. (2008). Lactobacillus casei suppresses experimental arthritis by down-regulating T helper 1 effector functions. Mol Immunol *45*, 2690-2699.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L. G., Gratadoux, J. J., Blugeon, S., Bridonneau, C., Furet, J. P., Corthier, G., *et al.* (2008). Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A *105*, 16731-16736.

Stepankova, R., Powrie, F., Kofronova, O., Kozakova, H., Hudcovic, T., Hrncir, T., Uhlig, H., Read, S., Rehakova, Z., Benada, O., *et al.* (2007). Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. Inflamm Bowel Dis *13*, 1202-1211.

Sudo, N., Sawamura, S., Tanaka, K., Aiba, Y., Kubo, C., and Koga, Y. (1997). The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. J Immunol *159*, 1739-1745.

Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S., Loening-Baucke, V., Ortner, M., Weber, J., Hoffmann, U., Schreiber, S., Dietel, M., and Lochs, H. (2002). Mucosal flora in inflammatory bowel disease. Gastroenterology *122*, 44-54.

Uronis, J. M., Arthur, J. C., Keku, T., Fodor, A., Carroll, I. M., Cruz, M. L., Appleyard, C. B., and Jobin, C. (2011). Gut microbial diversity is reduced by the probiotic VSL#3 and correlates with decreased TNBS-induced colitis. Inflamm Bowel Dis *17*, 289-297.

Verdu, E. F., Bercik, P., Cukrowska, B., Farre-Castany, M. A., Bouzourene, H., Saraga, E., Blum, A. L., Corthesy-Theulaz, I., Tlaskalova-Hogenova, H., and Michetti, P. (2000). Oral administration of antigens from intestinal flora anaerobic bacteria reduces the severity of experimental acute colitis in BALB/c mice. Clin Exp Immunol *120*, 46-50.

Wang, Q., Fang, C. H., and Hasselgren, P. O. (2001). Intestinal permeability is reduced and IL-10 levels are increased in septic IL-6 knockout mice. Am J Physiol Regul Integr Comp Physiol *281*, R1013-1023.

Williams, A. M., Probert, C. S., Stepankova, R., Tlaskalova-Hogenova, H., Phillips, A., and Bland, P. W. (2006). Effects of microflora on the neonatal development of gut mucosal T cells and myeloid cells in the mouse. Immunology *119*, 470-478.

Yan, F., and Polk, D. B. (2010). Probiotics: progress toward novel therapies for intestinal diseases. Curr Opin Gastroenterol *26*, 95-101.

Zanvit, P., Tichopad, A., Havlickova, M., Novotna, O., Jirkovska, M., Kolostova, K., Cechova, D., Julak, J., Sterzl, I., and Prokesova, L. (2010). Adjuvant effect of Bacillus firmus on the expression of cytokines and toll-like receptors in mouse nasopharynx-associated lymphoid tissue (NALT) after intranasal immunization with inactivated influenza virus type A. Immunol Lett *134*, 26-34.

8. PUBLICATIONS

Publications *in extenso* related to the present disertation:

Klimesova K, Kverka M, **Zakostelska Z**, Hudcovic T, Hrncir T, Stepankova R, Rossmann P, Ridl J, Kostovcik M, Mrazek J, Kopecny J, Kobayashi K S, Tlaskalova – Hogenova H, Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M deficient mice. Submitted in IBD

Hansen CH, Nielsen DS, Kverka M, **Zakostelska Z**, Klimesova K, Hudcovic T, Tlaskalova-Hogenova H, Hansen AK. Patterns of early gut colonization shape future immune responses of the host. PLoS One. 2012;7(3):e34043. Epub 2012 Mar 27.
IF_{2011}= 4,092

Zakostelska Z, Kverka M, Klimesova K, Rossmann P, Mrazek J, Kopecny J, Hornova M, Srutkova D, Hudcovic T, Ridl J, Tlaskalova-Hogenova H. Lysate of probiotic Lactobacillus casei DN-114 001 ameliorates colitis by strengthening the gut barrier function and changing the gut microenvironment. PLoS One. 2011;6(11):e27961. Epub 2011 Nov 22.
IF_{2011}= 4,092

Tlaskalová-Hogenová H, Stěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, Rossmann P, Hrnčíř T, Kverka M, **Zákostelská Z**, Klimešová K, Přibylová J, Bártová J, Sanchez D, Fundová P, Borovská D, Srůtková D, Zídek Z, Schwarzer M, Drastich P, Funda DP. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. Cell Mol Immunol. 2011 Mar;8(2):110-20. Epub 2011 Jan 31. Review.
IF_{2011}= 2,992

Kverka M, **Zakostelska Z**, Klimesova K, Sokol D, Hudcovic T, Hrncir T, Rossmann P, Mrazek J, Kopecny J, Verdu EF, Tlaskalova-Hogenova H. Oral administration of Parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition.Clin Exp Immunol. 2011 Feb;163(2):250-9. doi:

10.1111/j.1365-2249.2010.04286.x. Epub 2010 Nov 19.
IF_{2011}= 3,360

Publications *in extenso* not related to the present disertation:

Kovar M, Tomala J, Chmelova H, Kovar L, Mrkvan T, Joskova R, **Zakostelska Z**, Etrych T, Strohalm J, Ulbrich K, Sirova M, Rihova B. Overcoming immunoescape mechanisms of BCL1 leukemia and induction of CD8+ T-cell-mediated BCL1-specific resistance in mice cured by targeted polymer-bound doxorubicin.Cancer Res. 2008 Dec1;68(23):9875-83.
IF_{2011}= 7,856