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Role of bacteria and mucosal immune system and their interaction in the pathogenesis of inflammatory bowel disease

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ABBREVIATION

AIEC	adherent and invasive *E. coli*
CD	Crohn's disease
CFU	colony forming unit
DGGE	denaturing gradient gel electrophoresis
DSS	dextran sulfate sodium
DMEM	Dulbecco's modified Eagle medium
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FID	flame ionization detector
FITC	fluorescein isothiocyanate
GF	germ-free
HMA	human microbiota-associated
Hly	hemolysin
IBD	inflammatory bowel diseases
IEC	intestinal epithelial cell
IFN-γ	interferon-γ
LB	Luria broth
MOI	multiplicity of infection
MPO	myeloperoxidase
NOD	nucleotide-binding oligomerization domain
OD	optical density
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RBC	red blood cell
PVDF	polyvinylidene fluoride
SCFA	short chain fatty acid
SCID	severe combined immunodeficiency
TER	transepithelial electric resistance
TNF-α	tumor necrosis factor-α
Treg	T regulatory cell
UC	ulcerative colitis
ZO	zonula occludin

ABSTRACT

Although the etiology and pathogenesis of inflammatory bowel disease (IBD) is not fully understood, it is generally accepted that the inflammation results from aberrant immune response to resident gut microbiota antigens in genetically susceptible individuals. Due to the complexity of the disease, multifaceted interactions between genetic factors, host immune response, gut microbiota and environment factors need to be taken into account.

In this thesis, the pathogenesis of IBD was first reviewed in respect with the four factors mentioned above We investigated the ability of mucosal-associated bacteria from IBD patients to induce spontaneous colitis in germ-free (GF) mice and the impact of those bacteria on development of dextran sulfate sodium (DSS)-induced colitis. Together with the analysis of colonized mice microbiota, we demonstrated the potential deleterious microbes were able to increase the susceptibility to DSS colitis. We revealed the mechanism of *E. coli* p19A strain which were reported to be more frequently present in IBD patients and damage the integrity of the intestinal epithelium by their hemolytic activity. We also elucidated the protective effect of a probiotic strain *Clostridium tyrobutyricum* against acute colitis by promoting the mucosal immune homeostasis and we found butyrate produced by the bacterium as a key component to elicit the anti-inflammatory capacity.

This thesis provides us better knowledge of bacteria interacting with the immune system and may bring new insights into treatment of IBD.

ABSTRAKT

Etiologie a patogeneze chronického zánětlivého onemocnění střevního traktu (IBD) není doposud zcela jasná. Je obecně přijato tvrzení, že zánět je důsledkem nepřiměřené imunitní reakce na antigeny rezidentní mikrobioty u geneticky citlivých jedinců. IBD je velmi komplexní onemocnění a pro pochopení jeho vzniku a rozvoje je nutné brát v úvahu také složité interakce mezi genetickými faktory, imunitním systémem, střevní mikrobiotou a životním prostředím. V předkládané dizertační práci jsme se zaměřili na interakci mezi střevní mikrobiotou spojenou s IBD a mukózním imunitním systémem. V gnotobiotickém myším modelu jsme zkoumali schopnost bakterií asociovaných s mukózou (MAB), které byly odebrány od pacientů s aktivním IBD, vyvolat spontánní kolitidu u bezmikrobních myších. Byl posuzován vliv těchto bakterií na vznik a vývoj experimentálně vyvolané kolitidy dextran sulfátem sodným (DSS). Objasnili jsme mechanismus působení *E. coli* p19A, která se vyskytuje často u IBD pacientů a která díky své hemolytické aktivitě poškozuje integritu střevního epitelu. Zaměřili jsme svou pozornost i na vliv nepatogenní bakterie s probiotickými účinky *Clostridium tyrobutyricum*. Tato bakterie, produkující butyrát, je klíčovou složkou při udržování homeostázy a zabránila rozvoji DSS kolitidy u myší.

Tato dizertační práce umožňuje lépe porozumět bakteriím komunikujícím s imunitním systémem a přináší nové terapeutické možnosti v léčbě IBD.

1. INTRODUCTION

Inflammatory bowel disease is the chronic remittent inflammatory disorder that can affect any part of the gastrointestinal tract and colonic mucosa. There are two major clinical defined forms of IBD, namely Crohn's disease (CD) and ulcerative colitis (UC) [1, 2]. The incidence rates of both types of IBD have been climbing rapidly. Till 2011, UC and CD together had affected over 3.7 million people around the world [3, 4].

IBD is considered a result of an aberrant immune response to antigens of resident gut microbiota [5, 6]. It has been proposed that either imbalances in intestinal microbiota (dysbiosis) or presence of commensal bacteria with increased virulence could both cause excessive immune response to microbiota by penetrating through the mucosal barrier and stimulating local and systemic immunity [7–9].

Innate immunity and adaptive immunity are deeply involved in the pathogenesis of IBD. Disruption of the integrity of the epithelium is one of the major issues in IBD patients, leading to the elevated bacterial translocation into the mucosa [10, 11]. Impaired regulation of inflammatory response caused by T effector cells resistant to apoptosis and immunosuppressive effects of Treg cells resulted in excessive inflammatory response also contributed to the pathogenesis and progression of chronic inflammatory diseases [12, 13]. Besides the deficiency in modulation of inflammatory response, decreased phagocytic activity of macrophages due to low level of expression of intracellular tumor necrosis factor (TNF)-α was reported in IBD patients by Smith et al. [14]. This impaired acute immune response could lead to insufficient bacterial clearance.

Gut microbiota is another major factor implicated in the pathogenesis of IBD. Decreased biodiversity or species richness or changes in microbial composition, such as reduction of mucosal *Faecalibacterium prausnitzii*, *Bifidobacterium* and *Lactobacillus* species and short-chain fatty acids

(SCFAs)-producing bacteria, along with increase in the abundance of pathogenic bacteria like adherent-invasive *E. coli* (AIEC) has been consistently reported in both CD and UC patients compared to healthy subjects [15-18]. Those changes have profound effect on intestinal inflammation since the “good” guys have protective effect against mucosal inflammation while the “bad” guys promote inflammation [19-23].

Other factors like genetic background and environmental factors are also involved in the pathogenesis of IBD. To understand how each factors participates in the pathogenesis of the disease and interact with one another, the more effective therapies may be created to benefit our patients.

2. HYPOTHESIS AND AIMS

This thesis provides insights into how mucosa- and IBD-associated bacteria contribute to the development of intestinal inflammation and impact on the local or systemic immune system and how our selected probiotics protect the host against inflammation through regulating the host immune system in our experimental IBD animal models. The thesis is mainly focused on 1) reviewing the pathogenesis of IBD in terms of environmental factors, genetic predisposition, gut microbiota and host immune response up-to-date; 2) enhancing our understanding of the role of microbiota playing in IBD and the interactions between host and microbiota.

Specific aims of the thesis:

- Investigating how mucosa-associated bacteria isolated from patients with active UC could affect the intestinal homeostasis of germ-free (GF) mice and the dynamics of gut microbiota after colonization.

- examining the pathogenicity of an *E. coli* B2 strain isolated from active UC patients and how the bacterium interacts with epithelial cells.
- Investigating how *Clostridium tyrobutyricum* could exert a protective effect on dextran sodium sulphate (DSS)-induced colitis in mice.

3. MATERIAL AND METHODS

3.1 Study material

Biopsy was taken from inflamed sites of colon descendens of three patients. The biopsies were transferred to sterile tubes pre-loaded with Schaedler anaerobe broth (Oxoid Ltd, Cambridge, UK) containing 0.05 % cysteine-HCl, 10 % glycerol and covered with the layer of paraffin to preserve anaerobes. **(Article 1)**

Stool samples were collected from healthy medical students and IBD patients, and cultured at Statens Serum Institute [24]. **(Article 2)**

Bacteria *C. tyrobutyricum* (DSM 2637) isolated from raw cow's milk were cultured in sterile Bryant Burkey bouillon with resazurin and lactate (Merck KGaA, Darmstadt, Germany) at 37°C under anaerobic conditions. Prior to administration to mice a fresh overnight culture of bacteria was adjusted to 10^9 colony-forming units (CFU)/ml in saline. **(Article 3)**

3.2 Mice

All mice used in experiments were 8–10 week-old unless indicated.

GF BALB/c mice were maintained under sterile conditions, supplied with sterile water and diet ST-1 (Velaz, Unetice, Czech Republic) *ad libitum*. The conventional (CV) BALB/c mice on the same diet were regularly checked for absence of potential pathogens according to an internationally established standard (FELASA). **(Article 1)**

BALB/c and severe combined immunodeficiency (SCID) mice were reared in specific pathogen-free conditions. The absence of T lymphocytes in SCID mice was proved with fluorescein isothiocyanate (FITC)-labelled monoclonal anti-CD3 antibody (Serotec, Oxford, UK) using fluorescence activated cell sorter (FACS) Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). **(Article 3)**

3.3 Experimental model of acute DSS-induced colitis

Each human biopsy was homogenized and oral administrated to 2-month old GF mice with a dose of 0.2 ml. Those mice were employed as Parental human microbiota-associated (HMA) mice. Colitis was induced in GF, HMA and CV mice by 7 days exposure to 2.5 % (weight/volume) dextran sulfate sodium (DSS; 36–50 kDa; ICN Biomedicals, Cleveland, OH, USA) in sterile drinking water. **(Article 1)**

The experimental groups (group1-3 BALB/c; group 4-6 SCID) received the following treatment: Group 1, 2, 4 and 5 received 2.5% DSS in drinking water *ad libitum* for 1 week. Group 3 and 6 received only drinking water. Group 1 and 4 received intrarectally (via tubing) a daily dose of 2×10^8 CFU of strain DSM 2637 in 0.2 ml saline for 7 days prior to DSS exposure and also during the 7 days exposure to DSS in the drinking water. Control group 3 and 6 received 0.2 ml saline [phosphate-buffered saline (PBS)]. **(Article 3)**

3.4 Evaluation of colitis

Clinical symptoms were measured: firmness of faeces, rectal prolapses, rectal bleeding and colon length. The colon descendens were cut and fixed in 4% buffered paraformaldehyde or Carnoy's fluid or frozen for histological and immunohistochemical analysis. **(Article 1, 3)**

The supernatant of processed colon descendens was used for myeloperoxidase (MPO) measurement. The MPO activity is expressed in units (U) per 1 gram of the tissue. **(Article 1)**

3.5 Histological and immunohistochemical evaluation

Paraffin-embedded sections of colon descendens were cut and stained with haematoxylin and eosin or Alcian Blue and post-stained with Nuclear FastRed (Vector, USA) for mucin production. The degree of damage of the surface epithelium, crypt distortion and mucin production were evaluated according to Cooper et al. [25] **(Article 1, 3)**

Cryosections of acetone-fixed colon were used for immunohistochemical determination of expression of CD11b, ZO-1 and MUC-2. **(Article 3)**

3.6 Microbiota analysis

Bacterial DNA was isolated from cecum contents of HMA mice using ZR fecal Kit™ (Zymo Research, Irvine, CA, USA). The identification of microbiota was carried out by biochemical testing using enzymatic tests (oxidative-fermentative test, enterotest, anaerotest, en-coccustest or PYRtest, all from PLIVA-Lachema Diagnostika, Brno, Czech Republic), TNW® software (PLIVA-Lachema Diagnostika, Brno, Czech Republic), and PCR-based methods. The PCR products were processed by denaturing gradient gel electrophoresis (DGGE) using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) on polyacrylamide gel. The fingerprinting profiles were analyzed with BioNumerics (version 7.1, Applied Maths, Sint-Martens-Latem, Beigium) and dendrogram was constructed using Pearson correlation coefficient and Shannon-Wiener index. PCR products from interested amplicons were subjected to sequencing using ABI PRISM® BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc, Foster City, CA, USA) and analyzed on 3100 Avant Genetic Analyser (Applied Biosystems Inc). PCR products using primer pair 341F and 806R was sequenced on Illumina MiSeq instrument and data were processed using QIIME 1.8.0 [26] and submitted to the Short Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/) under the study accession number

[SRP066136; http://www.ncbi.nlm.nih.gov/sra/SRP066136]. **(Article 1)**

3.7 Cellular immune responses

Spleens were prepared and cultivated in complete RPMI 1640 medium at 37 °C under 5 % for 48 h. The cell supernatant was then used for determination of IFN-γ, TNF-α and IL-10 by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems; Minneapolis, MN, USA). **(Article 1)**

Pre-weighed colonic fragments were cultured in complete RPMI 1640 medium for 48 h. Quantification of TNF-α level was performed by ELISA (R&D Systems, Minneapolis, MN, USA). Acetone-fixed sections of colon descendens were used for measurement of IL-8 expression by confocal fluorometry. **(Article 3)**

3.8 Evaluation of intestinal barrier function and permeability *in vitro*

Expression of occludin was detected by immunofluorescence microscopy [18] and western blotting. Caco-2 cell monolayers were infected with bacteria for 15 hours. Caco-2 cell monolayers were either fixed for immunofluorescence or lysed. The supernatant of cell lysates was used for western blotting. The permeability of Caco-2 monolayers after co-incubated with bacteria was measured by transepithelial electric resistance (TER) by using cellZscope apparatus (Nanoanalytics, Münster, DE). **(Article 2)**

3.9 Characterization of *E. coli* B2 strains

The presence of α-hemolysin was demonstrated on 5% sheep blood agar plates (SSI no. 31349 Statens Serum Institut, Diagnostica, DK). Bacterial pellet and the bacterial growth supernatant from overnight bacterial culture were tested for hemolytic activity by incubating with red blood cells (RBC) and measured at OD 562 nm. **(Article 2)**

Isogenic mutants of p19A were constructed by allelic exchange with antibiotic resistance encoding cassettes using the λ-Red recombinase method as previously described [27]. Quantification of hemolysin expression of *E. coli* B2

strains was done by using Quantitative-PCR using Takara SYBR Premix Ex Taq II (RR820A) on cDNA prepared by a First Strand cDNA synthesis kit (# K0702, Thermoscientific) from total RNA extracted from LB growing cultures. **(Article 2)**

Cytotoxicity of bacteria was measured by neutral red uptake assay. Caco-2 cells infected with *E. coli* were incubated with 50 µg/ml neutral red (N4638, Sigma-Aldrich, Brøndby, DK). Neutral red was extracted with 1% acetic acid-50% ethanol and quantified in a spectrometer (OD 450 nm). The amount of extracted neutral red is expressed as a percentage of the amount recovered from uninfected cells. **(Article 2)**

3.10 Measurement of short-chain fatty acids (SCFA)

Bacterial cultures and faecal samples of SCID mice were used for SCFAs measurement. Concentrations of acetic acid, propionic acid, n-butyric acid, iso-butyric acid, valeric acid, iso-valeric acid and 2-methylbutyric acid were measured by gas chromatography on a HP 5890 GC with flame ionization detector (FID) and nitrogen as carrier gas. The samples were analyzed on an Equity-1 column (30 m x 0.32 mm i.d., 1 µm film thickness; Supelco, Prague, Czech Republic). **(Article 3)**

3.11 Statistics

The software "GraphPad Prism 5" (version 5.03, GraphPad Software, USA) was used for statistical analysis. Differences between more experimental groups were analyzed by ANOVA with Dunnet's multiple comparison test. Differences between two groups were accessed by using an unpaired two-tailed Student's t test.

4. RESULTS

This thesis is based on 3 following articles:

Article 1: Development of gut inflammation in mice colonized with mucosa-associated bacteria from patients with ulcerative colitis.
Zhengyu Du, Tomas Hudcovic, Jakub Mrazek, Hana Kozakova, Dagmar Srutkova, Martin Schwarzer, Helena Tlaskalova-Hogenova, Martin Kostovcik and Miloslav Kverka. *Gut Pathogens 2015 7:32*

In this study, we investigated whether the mucosa-associated bacteria derived from UC patients could induce spontaneous colitis or render the mice more sensitive to DSS-induced colitis by creating 3 lines of human microbiota-associated (HMA) mice (a, b, c) and monitored the composition of bacterial community in cecum content of HMA mice for several generations to understand its dynamics with respect to colonization at adult age (parental generation) or neonatal mother-to-offspring (filial generations) mode of colonization.

- Next generation sequencing analysis revealed the diversity of microbiota which was decreased after the colonization.
- Micro- and macroscopic evaluation showed that colonization of GF mice did not lead to spontaneous colitis and aHMA mice exhibited an increase in DSS-colitis sensitivity whereas bHMA mice failed to develop colitis.
- Production of proinflammatory (TNF-α, INF-γ) and regulatory (IL-10) cytokines was increased in colitic F4 aHMA mice.
- Shannon-Wiener index showed that HMA mice exhibited higher biodiversity in intestinal bacterial community in the later generation.
- Colitis-associated *Clostridium sp*. was identified in cecum samples of aHMA mice but not in bHMA mice.

We showed that mucosa-associated bacteria from colonic biopsy of the patients with active UC can increase sensitivity to DSS-induced colitis, although not able to induce spontaneous one. The increase in DSS-induced colitis severity between earlier and later generations of aHMA, together with the appearance of *C. difficile* and disappearance of *C. symbiosum*, suggests that change in the relationship between these two particular microbes, rather than their presence or absence, is important for the sensitivity to colitis.

Article 2: Secretion of alpha-hemolysin by *Escherichia coli* disrupts tight junctions in ulcerative colitis patients.
Hengameh Chloé Mirsepasi-Lauridsen*, **Zhengyu Du***, Carsten Struve, Godefroid Charbon, Jurgen Karczewski, Karen Angeliki Krogfelt, Andreas Munk Petersen, Jerry M. Wells. *Clinical and Translational Gastroenterology 2016 Mar 3;7:e149* (*equally contributed)

In this study, we tested isolates of B2 phylogroup *E. coli* from IBD patients on permeability, tight junction stability and viability of human intestinal cell epithelial monolayers *in vitro*. We investigated the role of CNF1 and HlyA in causing epithelial damage by the construction and testing of genetic mutants in cellular assays.

- Data from TER revealed that hemolytic strains of *E. coli* isolated from IBD patients with active disease were able to disrupt the epithelial cell barrier integrity after 2 h with multiplicity of infection (MOI) 10.
- Rapid loss of epithelial integrity was caused by *hly* expression in IBD-associated strain p19A which contained *cnf1* and two *hly* gene clusters.

- *Hly* expression was linked to rapid dissolution of occludin from the tight junctions of epithelial cell monolayers rather than the cytotoxicity of p19A strain.

- B2 phylogroup *E. coli was* found to be more frequently present in IBD patients. These bacteria were able to disrupt the epithelial cell barrier integrity by their *hly* expression which was associated with the rapid degradation of occludin from the tight junctions of epithelial cell monolayers instead of with the cytotoxicity property.

Article 3: Protective effect of *Clostridium tyrobutyricum* in acute dextran sodium sulphate-induced colitis: differential regulation of tumour necrosis factor-α and interleukin-18 in BALB/c and severe combined immunodeficiency mice.
T Hudcovic, J Kolinska, J Klepetar, R Stepankova, T Rezanka, D Srutkova, M Schwarzer, V Erban, **Z Du**, J M Wells, T Hrncir, H Tlaskalova-Hogenova, H Kozakova. *Clinical Exp Immunology 2012 Feb;167(2):356-65*

We studied the effect of *C. tyrobutyricum* (DSM 2637) on the development of experimental acute colitis in immunocompetent (BALB/c) and immunodeficient (SCID) mice. Changes in intestinal mucins, barrier function of TJ and production of the inflammatory cytokines IL-18 and TNF-α were measured during colitis induction.

- Treatment of *C. tyrobutyricum* attenuated DSS-colitis in both BALB/c and SCID mice by reducing histological scores.
- Treatment of *C. tyrobutyricum* attenuated the loss of mucin and ZO-1 in DSS-induced colitis in both BALB/c and SCID mice.
- Massive infiltration of inflammatory cells in lamina propria occurred in DSS-treated SCID and BALB/c mice, while the colon of *C.*

tyrobutyricum-DSS-treated SCID mice and BALB/c mice did not exhibit infiltration of these cells.

- Treatment of *C. tyrobutyricum* decreased the expression of proinflammatory cytokine TNF-α in DSS-treated SCID mice but not in DSS-treated BALB/c mice.
- Treatment of *C. tyrobutyricum* decreased the expression of proinflammatory cytokine IL-18 in colonic segments of DSS-treated BALB/c mice but increased the expression of the cytokine in DSS-treated SCID mice.
- *C. tyrobutyricum* increased levels of propionic and butyric acids in SCID mice.

This study demonstrates that the severity of inflammatory symptoms depends largely but not exclusively on host immune functions in the DSS models. Thus, *C. tyrobutyricum* protection against destruction of mucosal barrier is equally effective in immunodeficient SCID mice and immunocompetent BALB/c mice. Manifestation of cytokines IL-18 and TNF-α in acute DSS-colitis depends largely on immune cell repertoire of the host mouse.

5. DISCUSSION

For many years, scientists are trying to track down any individual microbial pathogens as causative agents in IBD. So far no such microbe has been claimed to be responsible for triggering the disease. However, gut-resident microbes remain of interest due to their exposure to and subsequently breach of the epithelium in IBD [28] leading to a series immune responses which may promote intestinal inflammation.

5.1 Mucosal immune system of the intestine in IBD

In healthy individuals, intestinal epithelium is covered by two layers of mucus,

the inner layer of which is supposed to devoid of bacteria and be sterile. The separation between luminal microorganisms and epithelium by mucus protects epithelial cells from direct contact to any possible pathogens as well as commensal bacteria which may turn into pathobionts under particular circumstances driving the inflammation in gut [29]. Decreased numbers of goblet cells and reduced levels of MUC-2 protein measured in the inflamed colonic mucosa of DSS-treated BALB/c and SCID mice (which was similar to those observed in humans with active UC) may contribute to the development of ulcerative colitis [30,31]. Recently it has been observed that DSS in drinking water rapidly affects the biophysical structure of the inner mucus layer, making it permeable to bacteria within 12 h [32]. Thus the increased contact of bacteria with the epithelium is probably the trigger for the inflammatory reactions observed in colitis and would explain why DSS does not induce colitis in germ-free mice. **(Article 3)**

Disruption of the integrity of the epithelium is one of the major issues in IBD patients, which leads to the elevated bacterial translocation into the mucosa. Three of five UC-associated *E. coli* strains (p7, p19A and p22) isolated from patients with active UC, causing disruption of gut epithelial tight junctions, was indicated by inducing a rapid loss of transepithelial electric resistance (TER) at low MOI without any loss of cell viability. The IBD-associated strains causing loss of epithelial integrity were all of the phylotype B2 and showed an increased abundance of the phylotype B2 *E. coli* in UC and CD patients with active disease [33-35]. Lesions in tight junctions of intestinal epithelium from IBD patients with active disease have been associated with a reduction in several tight junction proteins including claudin 1 and 4, occludin and tricellulin [36]. The finding of *hly*-producing *E. coli* strains p7, p19A or p22 were able to cause degradation of occludin is consistent to these previous studies. And the loss of tight junction protein ZO-1 in the intestinal epithelium, as observed in our study, was interpreted as an early event in DSS-induced colitis and is

associated with increased permeability and intestinal inflammation [37]. **(Article 2, 3)**

Impaired barrier function in intestinal inflammation leads to activation of the systemic immunity and production of pro-inflammatory cytokines [38]. We showed significantly increased IL-18 colon content in DSS-induced colitis in BALB/c mice. In SCID mice, the severity of colitis was associated with limited production of biologically active form of IL-18. This finding led us to the assumption that SCID mice lack some components of inflammasome, pro-IL-18 could not be activated, and display an increased susceptibility to DSS-induced colitis associated with increased lethality, especially in the chronic phase of inflammation [38]. Lack of IL-18 secretion is compensated by increased secretion of inflammatory TNF-α from the colon found in organ cultures. As a matter of fact, the activation of pro-inflammatory cytokine production is less profound in the mucosal compartment, including mesenteric lymph nodes, than in systemic one, due to more active inhibitory mechanisms in the gut [39]. This effect is probably caused by the regulatory mechanisms of the mucosal immune system [40]. Therefore, an increased IL-10 production in DSS-treated F4 aHMA mice and DSS-treated CV mice maybe caused by negative-feedback loop, where immune system regulates the inflammation caused by gut barrier breach. **(Article 1, 3)**

5.2 Interaction between bacteria and host in IBD

Bacteria are deeply involved in maintaining the normal function of intestinal epithelial cells (IECs) and keeping intestinal homeostasis. Mucosa-associated bacteria are considered to be more likely involved in UC due to their close proximity to the host epithelium. Interestingly, none of the mucosa-associated bacteria isolated from patients with active UC was able to cause spontaneous colitis in GF mice though colonization of GF mice with bacteria from UC patient a (aHMA mice) increased CCS and MPO activity. CCS and MPO gradually decreased in subsequent generations supporting the notion that

lack of exposure to microorganism in the early life could interfere with the development of immune system and permanently alter important immune functions [41]. Therefore, the increase in MPO and presence of pasty stool in parental aHMA mice appears to be a result of the poorly regulated host-microbe interaction in the ex-GF mice. However, the increase in susceptibility to DSS-induced colitis between F1 and F4 aHMA mice suggests that potentially harmful microbes found suitable niche during natural colonization with co-housing. **(Article 1)**

Particular bacteria species were found either increased or decreased in IBD patients [15-18]. We found disappearance of *C. symbiosum* and appearance of *C. difficile* between F1 and F4 aHMA. *C. symbiosum* (member of the *Clostridium* cluster XIVa) is the most abundant bacterium found in human gut mucins, where it probably protects the mucosa by producing high levels of butyrate [42]. Disappearance of *C. symbiosum* during DSS treatment of F1 aHMA mice could be even partially responsible for the DSS-induced epithelium damage. *C. difficile*, on the other hand, may produce toxins that can damage colon mucosa of infected patients [43]. Indeed, there is a strong association between UC and colonization with this bacterium [44, 45]. The close association of *C. difficile* with colitis may be responsible for the marked increase in susceptibility to DSS-colitis between F1 and F4 generations. Since all these microbes could not be introduced in other way than with the original biopsy, their appearance on DGGE of F4 aHMA suggests that they found suitable niche and increased in numbers. Moreover, enhanced numbers of *E. coli* were measured in inflamed tissue in UC patients [46]. The role of *E. coli* pathobionts in the pathophysiology in IBD was attributed to their capability to adhere and invade epithelial cells and replicate in macrophages. All the UC-associated *E. coli* strains that caused loss of tight junctions in epithelial cell monolayers were hemolytic in our study. The *E. coli* α-hemolysin is known to be able to lyse many cell types [35, 47-50]. We showed that both *hlyA* gene

clusters in p19A contributed to the damaging effects on the epithelial integrity, suggesting that intestinal *E. coli* strains possessing more than one *hlyA* locus may have increased pathological consequences in intestinal inflammation. **(Article 2, 3)**

5.3 Probiotics promote homeostasis of our immunity in IBD

Large intestine is well-known to be the place of enzymatic degradation of indigestible fiber by its microbiota that subsequently provides host SCFAs which exert a protective effect on epithelial cells [51]. However, reduced production of SCFAs resulted from an alteration in the composition of intestinal microbiota has been frequently found in IBD patients [52-55]. In our study, a decrease in butyrate oxidation was found in the colonocytes of mice with DSS-induced colitis. Treatment of *C. tyrobutyricum* increased level of butyric acids in DSS-treated mice. Intrarectal administration of *C. tyrobutyricum* also prevented the reduction of MUC-2 protein and dissolution of ZO-1 in DSS-treated mice. Probiotics usually exerts their beneficial effect by initiating anti-inflammatory immune responses such as down-regulation of TNF-α expression [56-58]. Treatment of *C. tyrobutyricum* had a strongly attenuating effect on TNF-α production in SCID mice. **(Article 3)**

6. CONCLUSION

In conclusion, this thesis brings new insights on the interaction between host mucosal immune response and IBD-associated bacteria as well as potential probiotics. We demonstrated that mucosa-associated bacteria from patients with active UC were able to increase the susceptibility to DSS-colitis. We reveal a novel mechanism of an IBD-associated *E. coli* damaging intestinal epithelium, which may provide us new insights on treatment of hemolytic *E.coli* associated intestinal inflammation. The protective effect of *C. tyrobutyricum* was elucidated suggesting a role of butyrate in mucosal

immune responses and in immune modulation.

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8. CURRICULUM VITAE

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Education and Employment:

- 9.2002 - 6. 2006: Chemistry and Pharmaceutics, East China University of Sciences and Technology (ECUST), Shanghai, China - Bachelor of Science
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AWARDS and HONORS:

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Outstanding student leadership honor – ECUST, 2004

Publications:

Total: 3

Sum of citations: 7

H-index: 1 (WoS)

9. PUBLICATIONS

Du Z, Hudcovic T, Mrazek J, Kozakova H, Srutkova D, Schwarzer M, Tlaskalova-Hogenova H, Kostovcik M, Kverka M. *Development of gut inflammation in mice colonized with mucosa-associated bacteria from patients with ulcerative colitis.* Gut Pathog. 2015 Dec 21;7:32. doi:10.1186/s13099-015-0080-2. eCollection 2015

Mirsepasi-Lauridsen HC*, **Du Z***, Struve C, Charbon G, Karczewski J, Angeliki Krogfelt K, Petersen AM , Wells JM. *Secretion of alpha-hemolysin by Escherichia coli disrupts tight junctions in ulcerative colitis patients*. Clin Transl Gastroenterol. 2016 Mar 3;7:e149. doi: 10.1038/ctg.2016.3. (* equally contributed)

Hudcovic T, Kolinska J, Klepetar J, Stepankova R, Rezanka T, Srutkova D, Schwarzer M, Erban V**, Du Z**, Wells JM, Hrncir T, Tlaskalova-Hogenova H, Kozakova H. *Protective effect of Clostridium tyrobutyricum in acute dextran sodium sulphate-induced colitis: differential regulation of tumour necrosis factor-α and interleukin-18 in BALB/c and severe combined immunodeficiency mice*. Clin Exp Immunol. 2012 Feb;167(2):356-65. doi: 10.1111/j.1365-2249.2011.04498.x.