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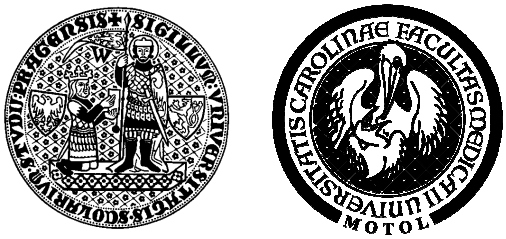

Dissertation

Role of microbiota and gut inflammation in the pathogenesis of experimental colorectal cancer

by

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Key words: *Microbiota, Mucosal immune system, Probiotics, Inflammatory bowel diseases, Colorectal carcinoma, Intestinal stem cells, Animal model, DSS colitis, AOM/DSS, Colitis-associated colon cancer.*

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The work contained in this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of my knowledge and believe, the thesis contains no material previously published or written by another person except where due references are made.

Prague 27th September 2012

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Thesis summary

Mucosal surface of the gut is in continuous contact with foreign compounds derived from diet as well as from commensal or pathogenic microorganisms. Thousands of years of symbiosis resulted in tight cooperation between the host and its microbiota. Microbiota composition and metabolism actively influence host's physiological as well as pathological processes. Chronic inflammation is characterized by prolonged active inflammatory response associated with tissue damage. This status results from accumulation of defects in various factors including gut barrier functions as well as mechanisms of innate and adaptive immunity. It’s commonly accepted that chronic inflammatory diseases of the gastrointestinal tract – inflammatory bowel diseases, are associated with an increased risk of colitis-associated cancer development.

Two publications related to this thesis deal with modulatory effects of peroral administration of components of commensal and probiotic bacteria on intestinal inflammation. Using acute or chronic model of dextran sulfate sodium colitis, we demonstrated that oral treatment of BALB/c mice with membranous fraction of the commensal, *Parabacteroides distasonis*, as well as with lysate of probiotic bacterium *Lactobacillus casei* DN-114 001 significantly reduces the severity of intestinal inflammation. Moreover, the treatment was associated with reduction of local pro-inflammatory cytokines and with improvement of barrier function, and led to stabilization of the intestinal microbial community.

Sequentially, we studied the role of negative regulator of TLR signaling – IRAK-M and gut microbiota in the development of colitis-associated colorectal cancer, and related molecular mechanisms. In the associated study, we investigated the role of the Wnt/β-catenin signaling pathway in gut epithelium homeostasis and during malignant transformation. We found that gut microbiota promotes tumorigenesis by increasing the exposure of gut epithelium to carcinogens and that negative regulation of TLR is essential for colon cancer resistance. Moreover, we identified a negative modulator of the Wnt pathway – Troy, whose expression was significantly upregulated in neoplastic tissues of both mice and humans.

In conclusion, influencing gut microenvironment by components of commensal bacteria or probiotics leads to intestinal inflammation improvement and thus brings new safe strategies for colitis and also for colitis-associated cancer prevention. Moreover, we tested

two regulatory molecules involved in maintaining the intestinal homeostasis, which can contribute to better understanding of the basic mechanisms of tumorigenesis.

Key words: *Microbiota, Mucosal immune system, Probiotics, Inflammatory bowel diseases, Colorectal carcinoma, Intestinal stem cells, Animal model, DSS colitis, AOM/DSS, Colitis-associated colon cancer.*

Souhrn práce v češtině

Slizniční povrch střeva je v neustálém kontaktu s cizorodými látkami pocházejícími jak z potravy tak z komensalních nebo patogenních mikroorganismů. Tisíciletá symbióza vyústila ve velmi úzkou spolupráci mezi hostitelem a jeho mikroflórou, jejíž složení a metabolizmus aktivně ovlivňují fyziologické i patologické procesy hostitele. Chronický střevní zánět je charakterizován dlouhodobou zánětlivou odpovědí spojenou s poškozením tkání, kterému napomáhá poškození bariérové funkce střeva podporované prozánětlivou reakcí vrozené i adaptivní imunity. Je prokázáno, že chronická zánětlivá onemocnění gastrointestinálního traktu (např. nespecifické střevní záněty) zvyšují riziko vzniku kolorektálního karcinomu.

První dvě publikace se zabývají ovlivněním experimentálního střevního zánětu pomocí perorálního podávání složek komensalních nebo probiotických bakterií. V modelu akutního a chronického střevního zánětu jsme prokázali protizánětlivý účinek podávání membránové frakce komensalní bakterie *Parabacteroides distasonis* i lyzátu probiotické bakterie *Lactobacillus casei* DN-114 001. Navíc jsme popsali snížení lokální produkce prozánětlivých cytokinů, zlepšení bariérové funkce střeva a stabilizaci složení střevní mikroflóry spojené s podáváním bakteriálních lyzátů.

Dále jsme studovali roli negativního regulátoru TLR signalizace (IRAK-M) a střevní mikroflóry při vzniku experimentálního kolorektálního karcinomu. V přidružené studii jsme se zabývali Wnt signalizační kaskádou a její rolí ve střevní homeostáze a maligní transformaci. Zjistili jsme, že střevní mikroflóra podporuje nádorovou přeměnu tím, že svým metabolismem zvyšuje koncentraci karcinogenů ve střevě. Navíc úspěšná prevence vzniku nádoru byla spojena s přítomností negativního regulátoru IRAK-M. V myší i lidské nádorové tkáni jsme identifikovali signifikantně zvýšenou expresi modulátoru Wnt signalizace – Troy.

Využití složek komensalních nebo probiotických bakterií k ovlivnění střevního homeostázy a prevenci vzniku střevního zánětu znamená posun ve vývoji nových bezpečných přístupů v prevenci jak střevního zánětu tak se zánětem spojeného nádoru. Navíc studium molekul regulujících střevní homeostázu přineslo nové poznatky o základních mechanismech nádorové přeměny a jijího ovlivnění.

Klíčová slova: *Mikroflóra, slizniční imunitní systém, probiotika, nespecifické střevní záněty, kolorektální karcinom, střevní kmenové buňky, zvířecí model, DSS kolitida, AOM/DSS, zánětem indukovaný nádor tlustého střeva.*

1 General introduction

1.1 Host side of gut homeostasis

Mucosal surface in the gut is in continuous contact with foreign compounds derived from diet as well as from commensal or pathogenic microorganisms. Therefore maintaining balance between the inner and outer milieu is the hallmark of whole mucosal system. Many different cell types and their products are involved in this complex dialogue, like epithelial and immune cells, cells of supporting tissues, antimicrobial peptides, growth factors, cytokines and other mediators.

1.1.1 Mucosal barrier

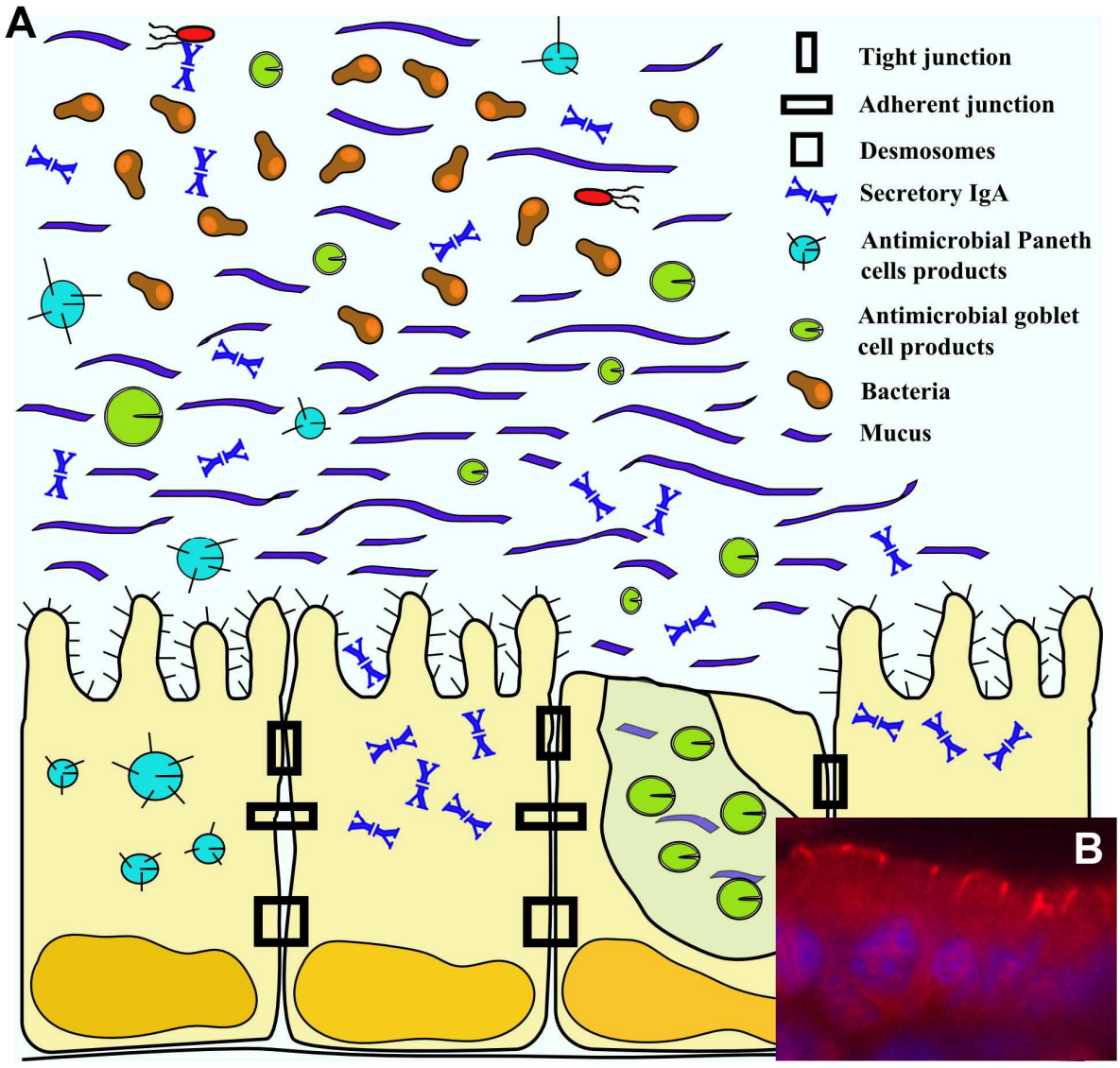
Cells, functions, products – mucus, antibacterial peptides, SIgA, intercellular junctions

Epithelial cell monolayer, which separates luminal space from host itself, is made of columnar-shaped enterocytes in small intestine and colonocytes in large intestine. Besides the common epithelial cells, there are other epithelial cells like goblet, Paneth, or neuroendocrine cells specialized in many different functions. Barrier is made of the intact layer of cells, which are in the apical part tightly sealed by intercellular junctions limiting paracellular transport from lumen. There are three types of different protein structures – tight and adherent junctions, and desmosomes (Figure 1) (Turner 2009).

Tight junction complex, the first one from the apex, is composed of transmembrane proteins, scaffolding proteins and regulatory molecules. Main transmembrane proteins – claudins and occludin form filamentous structures sealing tightly neighboring cells. Claudins and occludin are via scaffolding proteins zonula occludens 1 (ZO-1) and ZO-2 anchored to the actin and myosin cytoskeleton. Activity of these proteins regulates the paracellular passage of ions and soluble antigens. As tight junctions are important for permeability regulation, the other intercellular junctions are crucial for epithelial barrier architecture. Second type of junction located just below the tight junction is adherent junction. A protein complex, which strongly binds adjacent cells, is composed of transmembrane cadherins (primarily E-cadherin) linked to intracellular catenins (α-catenin and β-catenin), which are connected to actin/myosin cytoskeleton. Function of adherent junction is life important for cellular contact with other cells and intercellular matrix and for epithelial cells polarization,

differentiation and apoptosis (Turner 2009). The third junctional complex – desmosome, plays an important role in the maintaining cell-cell adhesion and thus epithelial integrity. It's formed by transmembranous proteins that are connected to keratin intermediate filaments in the cytoplasm on one side and to adhesion proteins of neighboring cells on the outer side (Mestecky et al. 2005).

Figure 1. Mucus morphology and components of intestinal epithelial surface. (A) Mucus in large intestine forms two layers with different properties; the inner layer is dense and impenetrable for most of bacteria, and the outer layer, which flows with intestinal content. Mucus is enriched with distinct products of intestinal epithelial cells, like antimicrobial peptides and secretory IgA, which play an important role in protection of gut mucosa against pathogenic bacteria invasion or excessive inflammatory response to commensals. To ensure impermeability, epithelial cells are on their apical site sealed by proteins forming apical junctional complexes. Modified from (Turner 2009; Kim and Ho 2010). (B) ZO-1 protein (red) localization in healthy terminal ileum visualized by immunofluorescence method (cells nuclei in blue).



Mucus is the first line of defense of the mucosa and the whole host, respectively. One or two layers of heavily glycosylated proteins – mucins cover the simple layer of epithelium in the intestines. Besides epithelial cells, the main producers of mucins throughout the intestines are goblet cells. Two different groups of mucins are known, the first is made up by the gel-forming polymers (mainly MUC2) and the second by transmembrane mucins (e.g. MUC3, 12, 13, 17) forming a structure called glycocalyx (Figure 1). The inner layer of the mucus in the distal colon, which is about 50 – 100 μm thick, is barely permeable to bacteria, thus limits the mucosa-antigen contact and is important for keeping balanced state in health. On the other hand mucus layer in the small intestine, even composed of the same mucin – MUC2, is more liquid and therefore bacteria can easier penetrate through and affect the mucosal immune system (Johansson et al. 2011). The *in vivo* experiments documented the important role of MUC2 in the protection of healthy state of individual. Mice deficient in *Muc2* gene showed increased gut permeability and susceptibility to spontaneous colitis and colitis-associated tumorigenesis accompanied by bacterial translocation into crypts and even further into epithelial cells (Velcich et al. 2002). Intestinal trefoil factor 3 is a peptide secreted together with MUC2 by goblet cells increasing the viscosity of mucus. Beside this, it promotes epithelial cells regeneration, migration and immune response via toll-like receptors (TLR) and blocks apoptosis. Its deficiency leads to mucus layer alteration with tendency to intestinal damage and inflammation (Mashimo et al. 1996). Goblet cells secrete many others factors, like resistin-like molecule (RELM) β and Fc-γ binding protein (Fcgbp), which promote mucin functions and thus protect intestinal epithelial cells. All together they improve the gut homeostasis.

Cells specialized in the production of broad spectrum of antimicrobial peptides are Paneth cells, which reside the base of Lieberkühn's crypts of the small intestine. Antimicrobial peptides are cationic molecules that contribute to the mucosal barrier by inactivating microorganisms in the gut lumen by various mechanisms, e.g. disruption of membrane integrity or interaction with cell wall synthesis. These peptides include defensins, lysozyme C, phospholipases and C-type lectins. Their antimicrobial effect simultaneously shapes the microbiota composition and control the microbial population (Bevins and Salzman 2011).

The most abundant antimicrobial peptides in the intestine are defensins, which show wide antimicrobial potential against bacteria, fungi and enveloped viruses. According to the molecular structure, defensins can by divided into two subgroups – α- and β-defensins.

Precursors of α-defensins are constitutively expressed by Paneth cells, natural killer (NK) cells and neutrophils and their activation is associated with proteolytic processing by trypsin or matrix metalloproteinase 7 (Shirafuji et al. 2003). Beside the antimicrobial function, α-defensins possess many other activities as chemoattractants or agents neutralizing bacterial exotoxins (Yang et al. 1999; Lehrer et al. 2009). Mouse Paneth cells produce peptides similar to human α-defensins called cryptdin-related sequence (CRS) peptides, which have the same role in mucosa defense (Bevins and Salzman 2011). Expression of β-defensins is inducible and takes place in the epithelial cells throughout the entire intestine. Like α-defensins, β-defensins also have immunomodulating functions, as they work as an adjuvant and trigger the histamine and prostaglandin D2 release from mast cells and so regulate inflammatory immune response (Maldonado-Contreras and McCormick 2011).

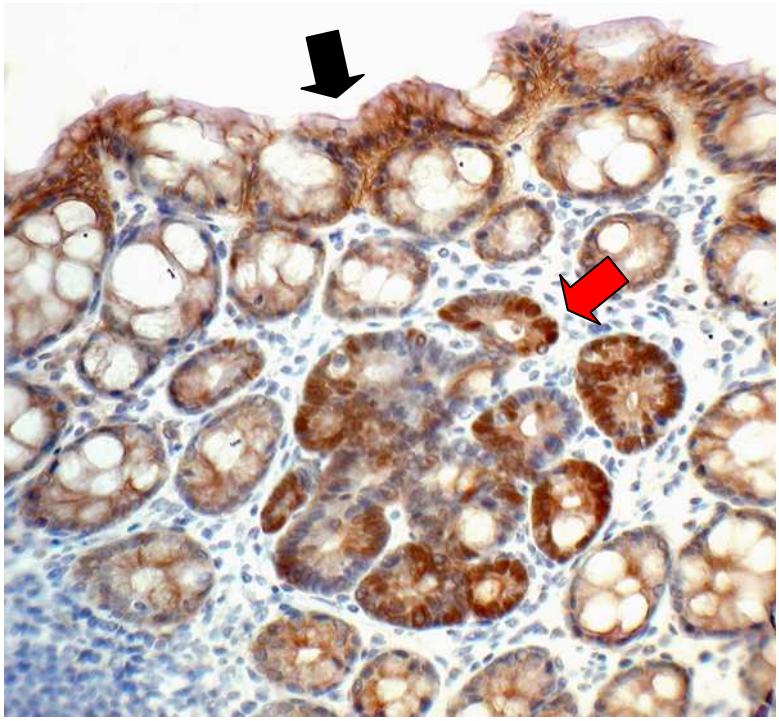
Lyzozymes are enzymes abundant in mucosal secretions causing hydrolysis of glycosidic bond in peptidoglycan thus protecting host against Gram (+) bacteria infection. Similar function fulfills secretory phospolipases, which are targeted against phospholipids in bacterial membranes. Cathelicidins are produced after stimulation by bacteria, viruses and fungi at various types of cells, including epithelial cells, neutrophils and macrophages not only in the gastrointestinal tract. These polypeptides protect the mucosa from colonization by luminal bacteria but also mediate epithelium regeneration and angiogenesis (Bevins and Salzman 2011; Maldonado-Contreras and McCormick 2011).

Immunoglobulin (Ig) A is the most abundant immunoglobulin in the body and particularly, the secretory IgA (SIgA) forms an important part of mucosal immune system promoting defense against pathogens invasion and induction of tolerance to commensals. Microbial stimuli are critical for SIgA production; microfold cells (M cells) localized in the epithelial layer on the surface of Peyer's patches deliver luminal antigens to the dendritic cells (DCs), which via direct presentation and cytokine tuning initiate Th2 immune response. Follicular B cells interact with the antigen-specific T cells and with TGF-β, which stimulate class-switch DNA recombination into IgA and somatic hypermutation. These activated B cells undergo recirculation through lymphatic and blood vessels to home again in the intestinal lamina propria as IgA-producing plasma cells. SIgA antibodies are transported through the epithelial cells bound to a polymeric Ig receptor (pIgR) and secreted on the apical site into the gut lumen as dimeric molecules containing J chain and stabilized by secretory component. By this T cell-dependent process are produced high-affinity antibodies, which protect the mucosa from pathogens' invasion. Low-affinity antibodies are secreted by plasma

cells, which developed by T cell-independent way from B cells in lamina propria. These antibodies react with antigens derived from diet and commensal microbes thus limit the immune system stimulation by process known as “immune exclusion” (Brandtzaeg 1996; Cerutti and Rescigno 2008; Macpherson et al. 2012).

Barrier transport of luminal compounds is affected by condition of the epithelial layer. Therefore impaired barrier function, so called “leaky gut”, is associated with many inflammatory and autoimmune diseases as well as allergies. Barrier continuity can be affected by various exogenous and endogenous factors, like pathogenic bacteria and their products (toxins) or pro-inflammatory stimuli by immune cells in the mucosa. For example pro-inflammatory cytokines of both Th1 (IFN-γ, TNF-α) and Th2 (IL-4, IL-13) types of immune response can trigger pathways resulting in tight junction proteins redistribution with consequent increase in gut barrier permeability (Berin et al. 1999; Wang et al. 2005). Impaired cell-cell contact, as an important part of tumorigenesis, was described in colon cancer samples where membranous localization of β-catenin is changed into nuclear (Figure

Figure 2. Membranous and nuclear localization of β-catenin. Nuclear translocation of β-catenin is associated with colorectal carcinogenesis (red arrow). An early dysplastic lesion of intestinal epithelium was found in AOM/DSS mouse model of colitis-associated cancer. Membranous staining is preserved in healthy tissue on the mucosa surface (black arrow).



2). Furthermore, nuclear β-catenin has totally different functions (Morin et al. 1997). As part of Wnt signaling pathway, it mediates transcription of various factors affecting cell cycle and growth or apoptosis during embryonic development and in cancer in adulthood (Jamieson et al. 2012).

1.1.2 Mucosal immune system

GALT, pattern-recognition receptors, dendritic cells, mucosal T cells subsets, cytokines

Mucosa-associated lymphoid tissue (MALT), or specifically gut-associated lymphoid tissue (GALT) in the gastrointestinal tract, represents the densest accumulation of immune cells in the body (TlaskalovaHogenova et al. 1995), which maintain different immune functions when put the microbiota into context. Primarily the mucosa is the main place of pathogen recognition, tolerance induction and generation of IgA antibody response to luminal antigens. According to the structure, there are two types of GALT: 1st organized lymphoid tissue like Peyer's patches (PPs), isolated lymphoid follicles (ILFs), and then cryptopathes in mouse, spread throughout the entire intestines with segregated areas of DCs, T and B cells, and 2nd a net of immune cells diffusely distributed in the mucosa, including some special types of T cells.

PPs are aggregated lymphoid follicles distributed throughout the small intestine with the highest density in the distal ileum. Surface of PPs is covered with a single layer of unique follicles-associated epithelium (FAE) harboring M cells. These cells take up luminal antigens, which pass the mucus layers and reach the apical membrane, by endocytosis or by the internalization of ligand-receptor complex. These antigens are delivered directly to follicular dendritic cells (FDCs) in the subepithelial dome where are further presented to B and T cells. Active adaptive immune response takes place in germinal centers containing proliferating B-lymphocytes and in T cell areas, respectively (Mestecky 2005; Jung et al. 2010; Tezuka and Ohteki 2010). The development of PPs depends on the presence of intestinal microbiota as it's known that germ-free animals have only few small PPs primarily in jejunum probably induced by food antigens (Mestecky 2005). Smaller lymphoid aggregates scattered throughout the small intestine are known as isolated lymphoid follicles that have the same structure and functions as PPs (induction of antigen specific response and SIgA production by maturated B cells) (Hamada et al. 2002). Beside these, mice have cryptopatches, small groups

of lymphoid cells that are randomly distributed in the intestinal lamina propria and are hypothesized to be the sites of extrathymic T cell development (Saito et al. 1998).

Microbe-associated molecular patterns (MAMPs) recognition is one of the most important features of mucosal immune system. Various components of microbiota can differentially trigger cellular pathways that shape local as well as systemic immune response and physiological functions. These receptors are known as pattern-recognition receptors (PRR) and can be divided into families of retinoic acid inducible gene I (RIG-I)-like receptors (RLR), nod-like receptors (NLR), and TLRs (Table 1). RLR are expressed ubiquitously in cellular cytoplasm and recognize viral RNAs with consequent induction of innate inflammatory response. NLRs are represented by two main cytoplasmic receptors – NOD1 (nucleotide-binding oligomerization domain) and NOD2, which recognize bacterial peptidoglycans. These receptors differs in cellular distribution, thus NOD1 is expressed by all epithelial cells whereas NOD2 only by monocytes, macrophages, DCs and Paneth cells. NOD1 receptor has role in the development of isolated lymphoid follicles as a result of the interaction of mucosal immune system with luminal microbiota and is also important for distinguishing commensals from pathogens (Bouskra et al. 2008). NOD2 stimulation promoting the expression of antimicrobial peptides can also result in NF-κB activation, which progresses into pro-inflammatory response. Disruption of NOD2 signaling alters mucosal immunity as polymorphisms in *nod2* gene are associated with inflammatory bowel diseases (IBD), especially ileal form of Crohn's disease (Wehkamp et al. 2004). Till now, 12 TLRs have been discovered in mice and 10 in human and their distribution and function differ throughout the intestines. TLR2, 3, 4, 5 and 9 were found on the epithelial cells in both small and large intestines having specific spatial distribution (apical, basolateral and intracellular) according to their function. Activated receptors interact with many downstream kinases and protein complexes (like MyD88, TRIF, TRAF6, TAK1, IRAKs) transferring the signals to the nucleus where they regulate the activity of nuclear factors (e.g. NF-κB, AP-1, IRF3) with subsequent modulations of cell cycle and metabolism. Recognition of commensals on the apical site or pathogens on the basolateral membrane or inside the cell induces appropriate immune response, which means, in fact, induction of unresponsiveness or inflammation, respectively. Moreover, TLRs are important for stimulation of gut epithelium growth and barrier integrity as well as production of mucus, SIgA, antimicrobial peptides and chemokines (Abreu 2010). Generally, the expression of *tlr*s in the epithelium is low in the steady state but increases during inflammation. Recently, we described the increase in TLR2 expression in

Table 1. Pattern-recognition receptors characteristics. Modified from (Wells et al. 2011; Lee et al. 2012; Oldenburg et al. 2012).

Receptor	***Subcellular localization***	***Ligand***
TLR2	Cell surface	Lipoprotein/lipopeptides of various pathogens Gram (+) bacteria lipoteichoic acid Viral hemoagglutinin protein Glycosyl-phosphatidylinositols of parasites
TLR2/TLR1	Cell surface	Bacterial triacyl lipopeptides
TLR2/TLR6	Cell surface	Mycobacterial diacyl lipopeptides Fungal zymosan
TLR3	Cellular compartment	Viral dsDNA
TLR4	Cell surface	Gram (-) bacteria lipopolysaccharide Viral envelope proteins Glycosyl-phosphatidylinositols of parasites
TLR5	Cell surface	Bacterial flagellin
TLR7/8	Cellular compartment	Viral ssRNA
TLR9	Cell surface/ Cellular compartment	Bacterial and viral CpG containing DNA
TLR2/TLR10 (human)	Cell surface	Bacterialand fungal antigens
TLR11 (mouse)	Cell surface	Uropathogenic bacteria components Profilin of parasites
TLR12 (mouse)		Not determined
TLR13 (mouse)	Cellular compartment	Bacterial RNA
NOD1	Cell cytoplasm	Meso-diaminopimelic acid from peptidoglycan of mainly Gram (-) bacteria
NOD2	Cell cytoplasm	Muramyl dipeptid from peptidoglycan of mainly Gram (+) bacteria
RIG-I	Cell cytoplasm	Viral 5'-triphosphate-bearing RNAs

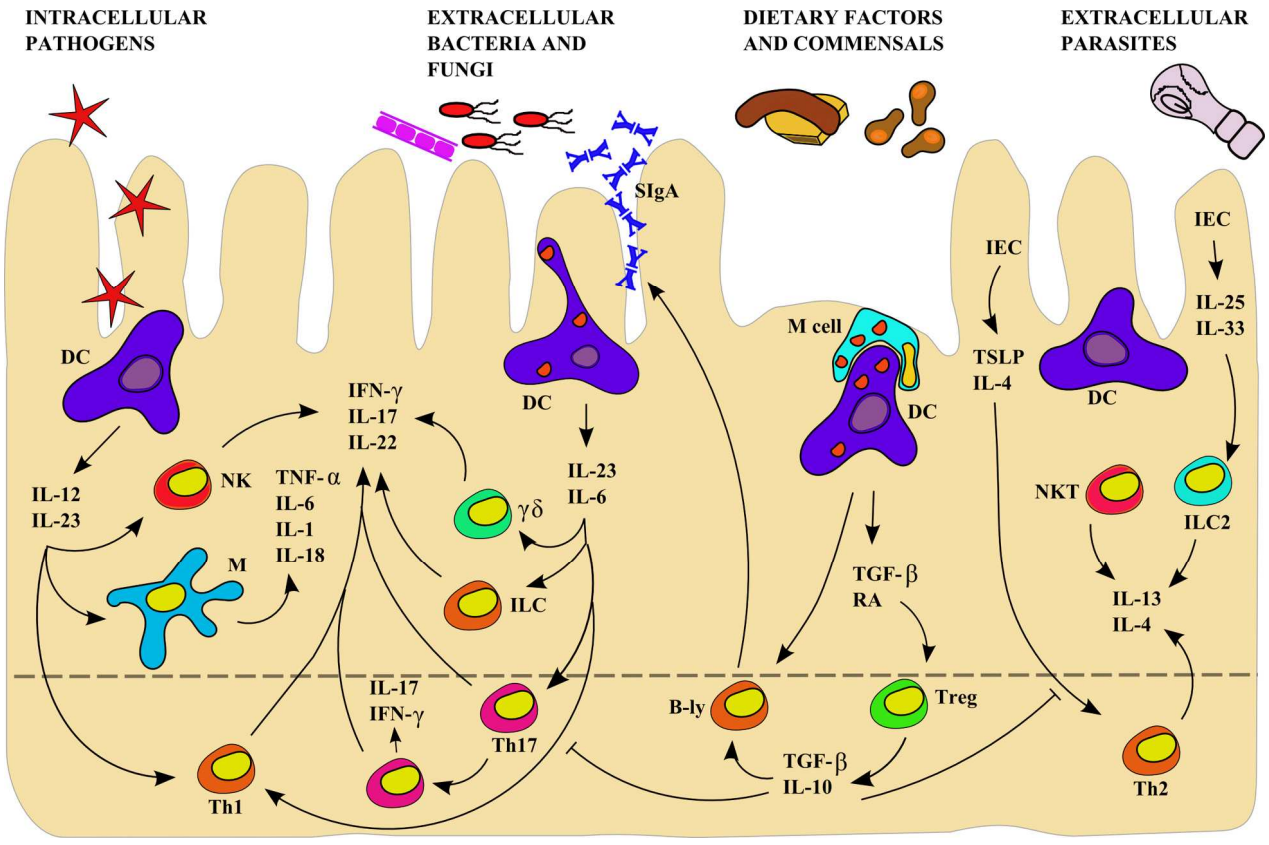
terminal ileum of patients with ulcerative colitis, and interestingly, also in cases of periproctal inflammation (Frolova et al. 2008). Besides the epithelium, the TLRs are expressed on antigen presenting cells (APC) like DCs and macrophages, which play important role in pathogens recognition and alarming local immune system. Their activation leads to production of pro-inflammatory cytokines and chemokines and thus ensures the first and effective response to microbial invasion. Although these receptors are dominant for functions of innate immune cells, they were also found on Treg cells and thus can directly modulate adaptive immune response (Liu et al. 2010).

PRR signalization having strong pro-inflammatory potential is effectively controlled by molecules interfering with down-stream pathways proteins. Besides the regulatory cells and molecules, which are discussed later (e.g. T regulatory cells, Th0, IL-10, retinoic acid), the main intracellular negative regulators, found in the gastrointestinal tract, are PPARγ, A20, IRAK-M and Tollip. These regulators prevent prolonged and excessive activation of pro-inflammatory factors transcription in both epithelial and immune cells and thus limit the development of chronic inflammation (Shibolet and Podolsky 2007). Their deficiency is frequently associated with excessive NF-κB activation and subsequent increased inflammatory response not only in the intestine (Hammer et al. 2011). We documented their significance in studies on IRAK-M molecule whose deficiency increased the severity of experimental colitis as well as colitis-associated cancer (Berglund et al. 2010; Klimesova et al. 2012). IRAK-M negatively regulates TLR/IL-1R signaling by binding the MyD88 adaptor complex resulting in decreased expression of pro-inflammatory cytokines. IRAK-M is expressed in many types of cells, including monocytes, macrophages and epithelium in response to exogenous (e.g. LPS) or endogenous ligands. Recently, it's studied in relation to chronic inflammation (colitis, asthma) and cancer in mice and humans (Balaci et al. 2007; Xie et al. 2007; Biswas et al. 2011; Standiford et al. 2011).

Mainly mucosal DCs are active APC with crucial role in the induction of Th2 antigen-specific immune response, regulatory $Foxp3^+$ T cells and SIgA production during steady-state (Figure 3). On the other hand, when needed, processing and presentation of components from pathogens leads to development of effector immune cells and inflammation (in the process the epithelial cells become the APC, too) (Mestecky 2005; Tezuka and Ohteki 2010). Considering the surface markers expression, DCs in PPs can be $CD11b^+CD8\alpha^-$, $CD11b^-CD8\alpha^+$ or $CD11b^-CD8\alpha^-$. Immature DCs from PPs immediately after receiving the antigen from M cells migrate to local lymph nodes, mature to APC ($MHCII^+CD40^+CD80^+CD86^+$) and promote the

development of T cells with gut-homing properties, like regulatory T cells by production of retinoic acid (RA) and TGF-β. CD11b+ DCs in the PPs produce mediators as RA, IL-10, TGF- β that stimulate T cell-dependent isotype switching of IgM-producing B cells into IgA (B cells priming and migration was mentioned above). DCs in lamina propria mostly express $CX_3CR1^+CD70^+$ and can sample antigen directly by projections (dendrites) passing through the epithelium and reaching intestinal lumen. These cells do not migrate to lymph nodes and induce the development of pro-inflammatory Th17 cells from naive T cell population in the lamina propria by bacterial ATP-dependent manner (Rescigno and Di Sabatino 2009; Tezuka and Ohteki 2010).

Figure 3. Innate and adaptive mucosal immune cells and cytokines response to luminal antigens in the gut. Modified from (Maloy and Powrie 2011).



Mucosal T cells mature in local organized lymphoid follicles (PPs and MLNs) receiving gut-homing phenotype, which, in a simplified way, means the expression of $\alpha_4\beta_7$ integrin and chemokines CCR9 (for small intestine) and CCR10 (for large intestine). Intraepithelial lymphocytes are a population of effector memory cells residing within epithelial layer that maintains intestinal environment via early recognition of pathogens, support of epithelium integrity and growth, and regulation of immune response. According to

their surface molecules T cells belong to TCRαβ$^+$CD8αβ$^+$, TCRαβ$^+$CD8αα$^+$, or TCRγδ$^+$CD8αα$^+$ T cells subsets, and in few cases to TCRαβ$^+$CD4$^+$ T cells or NKT cells. These cells can produce broad spectrum of mediators (as pro-inflammatory IL-17, IFN-γ, anti-inflammatory IL-10, or cytolytic granzyme B) and thus regulate intestinal immune response in homeostasis and disease (van Wijk and Cheroutre 2009; Sheridan and Lefrancois 2010).

Lamina propria lymphocytes mainly consist of CD4$^+$ helper T cells, including Th1, Th2, Th3, Tr1, Th17 and regulatory Foxp3$^+$ (Treg) subsets. Close presence of such an antigen-rich environment like intestinal lumen needs to be sensitively controlled. Keeping the tolerance (immunological unresponsiveness) to luminal antigens – commensals and food compounds is crucial for mucosal homeostasis and so the main task for Treg cells. Treg cells achieve their phenotype either in thymus or in the periphery in the PPs and MLNs after the contact with APC (mainly CD103$^+$ DCs) and then preferentially migrate to the gut mucosa. Silencing milieu is ensured by production of regulatory cytokines and by expression of inhibitory receptors, like IL-10, TGF-β and CTLA-4. RA was found as a key regulator of TGF-β-induced pro- and anti-inflammatory immune response (Mucida et al. 2007) as well as of the expression of gut-homing molecules on T cells after priming by DCs (Iwata et al. 2004). TGF-β, produced by epithelial cells, APCs and other Th subsets, is important cytokine involved in Treg induction and immune suppression but when secreted together with pro-inflammatory IL-6 and IL-23 it promotes the induction of Th17 cells (van Wijk and Cheroutre 2009). Th17 cells are characterized by the expression of transcription factor Rorγt and their development is associated with the presence of luminal bacteria, especially popular, in these days, are segmented filamentous bacteria (SFB) (Ivanov et al. 2009). Although Th17 cells produce pro-inflammatory cytokines (IL-17A, IL-22, IL-17F) having a role in the pathogenesis of chronic inflammatory diseases, they seem to coexist in the mucosa together with Treg cells during steady state promoting the protection of mucosa against extracellular bacterial and fungal pathogens (Curtis and Way 2009).

1.2 Intestinal microbiota

Gut microbiota is a complex ecosystem composed of bacteria, viruses and fungi. Thousands of years of symbiosis resulted in tight cooperation between the host and his microbiota. Its composition and metabolism actively influence host's physiological as well as pathological processes. On the other hand, host's metabolites and immunity significantly shape the microbial community. Therefore, the host/human is now often taken as a superorganism containing both prokaryotic and eukaryotic cells.

1.2.1 Composition of the gut microbiota

Commensals, pathogens, influencing factors

Human gastrointestinal tract (GIT) harbors about 500 to 1000 different species of bacteria (predominantly the phyla *Bacteroidetes* and *Firmicutes*), four species of fungi (genus *Candida, Saccharomyces, Penicillium*), few *Archaea* (the methanogenic strains) and about 1200 variants of viruses and bacteriophages. Bacteria, which, in their quantity, 10-times exceed the total number of host's cells, form complex ecosystem with 150-times more genes then is in human genome (Arumugam et al. 2011). From the host point of view, the relationship with microbes can be taken as commensal when one side benefits from the symbiosis, or as pathogenic when the bacteria produce harmful substances and cause diseases (Gill et al. 2006).

Intestinal microbiota differs within the GIT according to the localization. Although the bigger part of microbiota lives in the lumen passing through the lower GIT, there is a smaller community of microbes inhabiting the mucus layers, tightly communicating with cells in the mucosa and submucosa (Green et al. 2006). Changes in composition were firstly observed when comparing various parts of GIT as a whole like small intestine, caecum, colon and stool samples (Table 2). Later detailed investigation aimed on local differences in abundance of particular species and their presence in the health and disease, which became a topic number one (Gill et al. 2006; Green et al. 2006; Greer and O'Keefe 2011; Nava and Stappenbeck 2011; Tlaskalova-Hogenova et al. 2011).

Huge variability in the composition of gut microbiota within the population leads to search for a core microbiom, the group of microbes common for typical group of healthy or diseased individuals. Recent study by Arumugam and colleagues classified only three types –

enterotypes of gut microbiota in the healthy humans based on reevaluation of metagenomic datasets from previous studies. The enterotypes are characterized by significantly increased abundance of certain genus either *Bacteroides*, or *Prevotella*, or *Ruminococcus* (Arumugam et al. 2011).

The factors influencing the composition of gut microbiota can be of host (diet, age, diseases, genetic and medicinal drugs intake), microbiological (competition for nutrients, adhesion, metabolic cooperation) and environmental (geography, pH, redox potential) origin (Macfarlane and Macfarlane 2012). Many of host's products secreted into gut lumen like antibacterial peptides, SIgA, mucin, or neuromediators, can shape the bacterial community. Secretion of all these factors is influenced by host genome polymorphisms and their expression usually depends on microbial stimulation of host's cells, thus forming positive feedback loop (Marchesi 2011).

Table 2. Composition of human microbiota through the GIT. Modified from (Bik et al. 2006; Robinson et al. 2010; Claesson et al. 2011; Nava and Stappenbeck 2011).

Location	***Cell counts***	***Predominant microbiota (genus)***
Oral cavity	10^{10}cfu/mL	*Streptococcus, Eikenella, Lautropia, Syngeristes, Bacteroides, Haemophilus, Actinobacillus, Gemella, Neisseria, Prevotella, Megasphaera, Stomatococcus, Veillonella*
Esophagus	10^{3} – 10^{4}cfu/mL	*Streptococcus, Actinomyces, Prevotella, Gemella*
Stomach	$<10^{2}$cfu/mL	*Caulobacter, Actinobacillus, Corynebacterium, Rothia, Gemella, Leptotrichia, Porphyromonas, Capnocytophaga,* TM7, *Flexistipes, Deinococcus, Lactobacillus*
Small intestine	10^{2} – 10^{8}cfu/mL	*Streptococcus, Escherichia, Veillonella, Clostridium* cluster I, *Lactobacillus*
Colon and feces	10^{11} – 10^{12}cfu/g	*Bacteroides, Alistipes, Parabacteroides, Lachnospiraceae, Sporobacter, Faecalibacterium, Ruminococcus, Roseburia, Anaerophagy, Prevotella, Erysipelotrichaceae, Coprococcus, Peptostreptococcaceae, Subdoligranulum, Dorea*

Microbiota is relatively conserved within individual during whole life, but varies in composition between individuals even in close relationship. Newborns sterile gastrointestinal tract is gradually colonized immediately after birth and about 2-3 years are needed to establish

the stable community. Recent studies showed that there are significant differences between gut microbiota of naturally and cesarean section delivered children (Biasucci et al. 2010). This seems to be an important fact when given in association with e.g. autoimmune disease and allergy onset as microbiota in the gut promote the early immune system development. Thus microbiota-mediated tuning of immune response in childhood could affect diseases trait in adulthood (Greer and O'Keefe 2011; Guarino et al. 2012).

Another factor influencing the composition of the gut microbiota is diet. There are differences in microbiota composition of breast fed and formula fed children, particularly the abundance of *Bifidobacteria* (Favier et al. 2003). Various components of diet can shift the microbiota composition e.g. diet high in resistant starch increase the abundance of bacteria metabolizing non-digestible polysaccharides (Walker et al. 2011). One of the studies comparing microbiota pattern of children from Africa (Burkina Faso) and western countries (EU) found interesting shift in Bacteriodetes/Firmicutes ratio based on different kind of food habits. They showed an increase in short chain fatty acid (SCFA) producing bacteria in African children, which can potentially affect the later onset of various illnesses like allergies, IBD or cancer (Greer and O'Keefe 2011). The effects of SCFA are discussed later.

Many of xenobiotics like medicinal drugs and supplements have adverse effects on microbiota composition (Arthur and Jobin 2011). Administration of antimicrobials causes reversible alteration of microbial ecosystem. Both broad-spectrum and specific antibiotics target sensitive populations of the gut microbiota and reduce their communities with consequent overgrowth of surviving bacteria. Therefore, frequent complication of antibiotic use is diarrhea, which develops either after the reduction of commensal bacteria metabolizing carbohydrates and bile acids (osmotic diarrhea), or after the disruption of colonization resistance (e.g. pseudomembraneous colitis and diarrhea caused by *Clostridium difficile*) (Beaugerie and Petit 2004).

Colonization resistance is an important feature of microbiota, which protects host against pathogens by microbiota own guns. Microbiota constitutes a competitive barrier to pathogenic microbes by active struggle for existence, fighting for nutrients and space. Moreover, the expression of antimicrobial effector molecules (bacteriocins) by commensals represents an effective tool for community shaping by endogenous microbiota. The third mechanism is indirect and includes constitutive stimulation of mucosal immune system by commensal microbes triggering host’s defense system leading to reduction of pathogens translocation (Robinson et al. 2010; Stecher and Hardt 2011).

Lots of recent studies concern with the link between host genotype and microbiota composition. Suspected genes include those of mucosal immune system and some related to the host metabolism. The linkage was found for murine genes like *MyD88*, *Nod2*, *ApoA1*, *RELMB*, or genes coding the IgA and defensin proteins, recently reviewed by Spor et al. (Spor et al. 2011). So far the only human gene polymorphisms linked with significant change in the microbiota composition are the mutations in *MEFV*, which lead to the auto-inflammatory disorder, known as familial Mediterranean fever (Khachatryan et al. 2008).

1.2.2 Metabolism of gut bacteria

Short-chain fatty acids, H_2S, enzymes

Gut microbiota possesses various catabolic and anabolic pathways, which enable it to utilize a broad spectrum of substrates, which are not absorbed in the small intestine. These pathways interact with the metabolism of xenobiotics and micronutrients bioavailability, lead to production of essential vitamins and degradation of fibers, and influence the secretion of neuroactive molecules affecting mood and behavior (Arthur and Jobin 2011). Recent studies found a link between gut microbiota composition and obesity in both mice and humans, so the metabolism of certain bacteria modifies energy harvest and fat storage, as well as satiety (Ley et al. 2006; Turnbaugh et al. 2006).

Predominant diet of human is formed by plant polysaccharides but our digestion pathways lack the enzymes for the degradation of non-digestible ones, like resistant starch and dietary fiber. On the other hand, distal gut microbiom encodes about 81 different families of glycoside hydrolases (bacterial polysaccharidases, glycosidases), which are not present in the human genome (Gill et al. 2006). Thus microbiota significantly contributes to the utilization of starch, primary fiber, host-derived secretions (mucus glycans), sucrose, and monosaccharides, like glucose, galactose, fructose, and arabinose. Subsequent fermentation of depolymerized substrates leads to the production of SCFA, mainly acetate, propionate, and butyrate. Gut microbiome is significantly enriched with the genes involved in the pathways generating SCFA when compared with other microbiomes in gene libraries (Gill et al. 2006). There are two main producers of butyrate in the intestine – *Feacalibacterium prausnitzii* and *Eubacterium rectale/Roseburia* group (Louis et al. 2010). SCFA are one of the most important sources of energy not only for intestinal epithelial cells but also for muscles, kidneys, heart and brain. Their physiologic production influences transport and metabolism

through epithelium as well as epithelial cells renewal and differentiation. Moreover, SCFA largely influence the immune system, colonic functions and carcinogenesis. Besides these, various forms of SCFA have broad spectrum of different effects on the host organism. Production of acetate also interacts with adipogenesis, propionate levels influence cholesterol metabolism, satiety and neurological functions, and butyrate production modifies barrier integrity, insulin sensitivity, satiety, oxidative stress and inflammation (Macfarlane and Macfarlane 2012).

Another enrichment was found in bacterial genes for the synthesis of amino acids and vitamins essential for human health (Gill et al. 2006). Mainly those involved in the production of deoxyxylulose 5-phosphate and isopenteryl pyrophosphate, which are the precursors of vitamins B1 (thiamin) and B6 (pyridoxal form), and carotenoids and cholesterol, respectively (Wang and Ohnuma 2000; Rodriguez-Concepcion and Boronat 2002). On the other hand, degradation of dietary proteins, peptides, and amino acids by bacterial fermentation leads to production of by-products, like phenols, indoles, ammonia, amines, thiols and HS^-, which are all somehow harmful to the host as being co-carcinogens, mutagens and cellular toxins (Macfarlane and Macfarlane 2012). Hydrogen released as the end-product of fermentation is processed by methanogenic species of *Archaea* (e.g. *Methanobrevibacter*) to methane, which regulates local conditions (redox potential and pH) and thus biochemical pathways courses (Gill et al. 2006).

Although many substances important for host are produced by bacterial enzymatic pathways as vitamins and micronutrients, metabolism of bile acids or xenobiotic can result in the generation of potentially harmful products (Arthur and Jobin 2011). Gut microbiom encodes many enzymes, like β-glucosidase, β-rhamnosidase, esterase, 7α-dehydroxylase, and β-glucuronidase, modifying both exogenous and endogenous substrates and therefore changing their biological activity. The metabolism of xenobiotics can, therefore, result in various effects as changed bioavailability of medicinal drugs or increased susceptibility to cancer (Gill et al. 2006).

1.2.3 Microbiota influence on host immunity

Germ-free status, immune response

During the million years of co-evolution, the microbes acquire a crucial role in the regulation of development of innate as well as adaptive immunity (Clemente et al. 2012). Most of the interactions were mentioned in the first chapter, except some specific.

As was shown in germ-free animals, the lack of microbial stimulation on mucosal surfaces leads to increased susceptibility to infection, and reduced vascularity, digestive enzyme activity, cytokine and SIgA production, muscle wall thickness, and defects in GALT development with smaller PPs (Stepankova et al. 1998; Shanahan 2002). First experiments in germ-free animals showed the importance of bacterial components for induction of immune response (Tlaskalova et al. 1970; Tlaskalova-Hogenova et al. 1983). Although the colonization of germ-free mice restores the mucosa function and immune system response, significant differences in immune response among groups of early and lately conventionalized germ-free mice and specific pathogen free mice were recently described (Hansen et al. 2012).

PRR (described above) are the sites of activation of several downstream pathways as an answer to the microbial antigens. Bacterial components influence the development of host's immune system from the first contact early after birth, primarily, by triggering MALT organization. Microbiota composition is given, in part, by the way of delivery (naturally versus cesarean section) and also by the form of nutrition (breast feeding versus formula). Passive intake of mother's antibodies in milk is important for newborn protection from pathogens, because of immature mucosal immune system of the newborn (Hanson 2007). Components of intestinal content interact and modulate immune cells, which are present in the mucosal surfaces. This process leads to induction of mucosa-derived tolerance, which is crucial for unresponsiveness of mucosal immune system to food and commensal microbiota antigens. Several T cells subsets development (maturation) is associated with presence of certain bacteria or their compounds. Thus germ-free mice fed by diet with different LPS content showed significant variations in lymphocytes counts in MLN and PP (Hrncir et al. 2008). Recent studies showed that IgA secretion as well as Th17 cells are induced by SFB (Talham et al. 1999; Ivanov et al. 2009), and IL-10 secretion and Treg cells are promoted by mixture of *Clostridia* cluster IV and XIVa or by *Bacteriodes fragilis* component – polysaccharide A (Atarashi et al. 2011; Round et al. 2011).

Various models of inflammatory, autoimmune and allergic diseases showed the important role of microbiota in modulation of systemic immune response. Thus presence of microbiota or microbial antigens (e.g. LPS, flagellin) can influence host susceptibility to disease induction in animal models. Recent studies showed elimination of diseases like experimental colitis (Hudcovic et al. 2001), arthritis (Wu et al. 2010), and autoimmune encephalomyelitis (Lee et al. 2011) in germ-free condition. On the other hand, there are diseases where the presence of microbiota in the gut plays protective role. Type I diabetes (Funda 2007) or atherosclerosis (Stepankova et al. 2010) showed increased morbidity and lesion severity, when induced in germ-free mice. Fine tuning of the mucosal immune system by the microbiota is therefore complex process driving both local gut homeostasis as well as systemic immune response.

1.3 Intestinal inflammation and cancer

Chronic inflammation is characterized by prolonged active inflammatory response associated with tissue damage. This status results from accumulation of defects in various factors including gut barrier functions as well as mechanisms of innate and adaptive immunity. It's commonly accepted that chronic inflammatory diseases are associated with an increased risk of cancer development due to continuous damage of cells and defective immune response. Same situation is in chronic disorders of the gastrointestinal tract – inflammatory bowel diseases, which increase the eventuality of colitis-associated cancer development.

1.3.1 Inflammatory bowel diseases and colitis-associated cancer

Crohn's disease, ulcerative colitis, colorectal cancer

Inflammatory bowel diseases (IBD) are chronic idiopatic disorders of gastrointestinal tract with a relatively high prevalence range of 10-200 cases per 100,000 individuals in developed countries. Although the exact mechanisms are not known, it's widely accepted that IBD pathogenesis is tightly associated with the aberrant immune reaction to commensal mucosal microbiota, in part, supported by individual gene polymorphisms. Familial aggregation, aggregation in twins, susceptible genes, lifestyle, geographical variability, intestinal immune system malfunction are the influencing factors frequently discussed with these diseases (Baumgart and Carding 2007).

In Crohn's disease (CD) transmural inflammation affects any part of the gastrointestinal tract, but the lower portion, including terminal ileum, cecum, colon and rectum is more often the affected area. On the other hand, ulcerative colitis (UC) is characterized by non-transmural inflammation limited to the colon (Baumgart and Sandborn 2007). Interestingly, patients with IBD produce higher numbers of various antibodies against microbial antigens as *Saccharomyces cerevisieae* (ASCA), *Escherichia coli* (Omp-C), Pseudomonas (I2) and bacterial flagellin (cBIR), which are partially used as IBD serological markers mainly for CD (Lewis 2011). UC is associated with presence of perinuclear anti-neutrophil antibodies (pANCA), which are also used in differential diagnosis for distinguishing between CD and UC (Ruemmele et al. 1998).

Colorectal cancer is a disease with one of the highest incidences almost all over the world. Furthermore, for a long time, the Czech Republic belongs to the countries with the highest incidence and mortality caused by colorectal cancer (WHO, http://globocan.iarc.fr). Although the most of the cases rank to sporadic colorectal cancers (85-95 %), there are particular categories of conditions increasing the risk of colorectal cancer development. Among these high-risk conditions belong hereditary non-polyposis-associated colorectal carcinoma (HNPCC, Lynch syndrome; 3 %) and other hereditary syndromes as familial adenomatous polyposis (FAP, about 1 %), and chronic inflammation-related tumors like in IBD (2-3 %).

Possible association of intestinal inflammation with colorectal cancer was firstly mentioned by Crohn and Rosenberg in 1925 (Crohn and Rosenberg 1925). And as was specified later, the development of carcinoma in IBD patients is associated with inflammation-dysplasia-cancer sequence. For colitis-associated cancer (CAC) is the chronic inflammation a key predisposing factor, which is cumulative in time and with severity of the disease (Eaden et al. 2001; Itzkowitz and Yio 2004). The probability of cancer development in patients with UC is 2% after 10 years and 18% after 30 years of disease (Eaden et al. 2001). CD increases the risk of cancer from 2.9% after 10 years to 8.3% after 30 years of disease (Canavan et al. 2006). Even though studies about an engagement of genetic factors in the pathogenesis of CAC (genome-wide association studies, GWAS) found many suspected genes, their applicability in preventive screening is not yet clear. It's proved that positive family history of colorectal cancer two-fold increases the risk of cancer development in IBD patients (Askling et al. 2001).

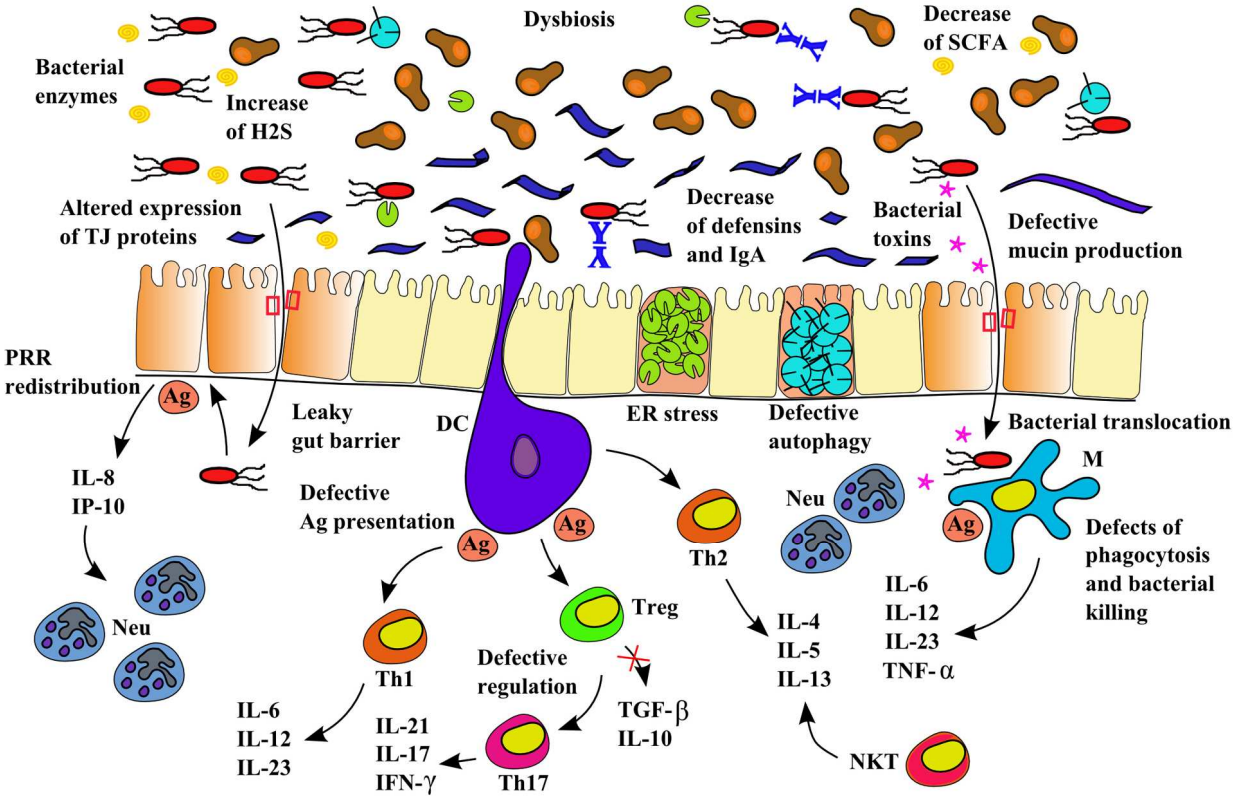
1.3.2 Intestinal inflammation

Immuno-pathological mechanisms

Innate immune cells stimulate the mucosal immune response and particularly, T cell-mediated immunity, which plays an important role in the pathogenesis of IBD. Development of mucosal inflammation in animal models often interacts with either deficient T cell regulation or with excessive effector T cell function (Bouma and Strober 2003). Moreover, alteration of epithelial cell layer integrity that accompanies the gut inflammation helps microbial translocation and thus progression of the disease (Figure 4).

Defect in any part of mucosal homeostasis leads to enhanced local microbial stimulation of the immune system. Impaired epithelial barrier, so called “leaky gut”, facilitates the translocation of gut microbes and antigens to the submucosa where they trigger the pro-inflammatory immune response (Sartor 2008). Permeability can be increased as a consequence of changed gene expression. Suspected genes encode tight junction proteins or cytokines, which secondarily affect the barrier functions, like down-regulation or redistribution of claudin 5 and 8, or up-regulation of claudin 2 forming pores (by TNF and IL-13) (Zeissig et al. 2007). Mucosal integrity is also disrupted by factors influencing apoptosis, mucus layer (*Muc2*), clearance of bacteria and bacterial toxins (defensins, *Mdr-1*, *IBD5*) as recently reviewed by Sartor (Sartor 2008).

Figure 4. Immuno-pathological mechanisms of intestinal inflammation. Modified from (Fava and Danese 2011).



Increased susceptibility to inflammation can be caused by defects in autophagy gene allele *ATG16L1*. Polymorphisms affect mainly the Paneth cells where impaired autophagy leads to accumulation of non-functional lysosomal complexes – autophagosomes, restricting the physiological production of granules (Deretic and Levine 2009). Accumulated unfolded or misfolded proteins in the endoplasmatic reticulum (ER) affect the secretory cells like Paneth

cells and goblet cells. Defects in the unfolded protein response (degradation of unfolded proteins) result in ER stress, which increases the susceptibility of intestinal inflammation development (Kaser et al. 2008).

Many studies in both humans and mice confirmed the redistribution and changed expression of PRRs during intestinal inflammation. PRRs have crucial role in maintaining mucosal homeostasis, therefore impaired signalization leads to pro-inflammatory response. Increase in TLR2 and TLR4 on the basolateral site of the epithelium, TLR5 activation by flagellin passing through the inflamed mucosa, and TLR9 by DNA result in IL-8 secretion and attraction of granulocytes (Sartor 2008). Polymorphisms of TLR1, TLR2 and TLR6 are associated with severe colitis (Pierik et al. 2006). PRR signaling via adaptor protein MyD88 triggers the downstream pathway to transcription factor NF-κB, which interacts with the production of pro-inflammatory cytokines, cell cycle proteins and apoptosis, leading to chronic inflammation. Mutations in *CARD15* gene, which are associated with alteration in NOD2 antigen perception and further signalization, also interact with mucosal pro-inflammatory response in mice and humans. Autophagy, antigen presentation and TLR signalization are all processes influenced by NOD2 (Bouma and Strober 2003). Recent studies have identified single nucleotide polymorphisms in other NLRs (mainly *NLRP3*), which lead to either dampened or excessive activation of inflammasome with subsequent decreased or increased IL-1 and IL-18 production promoting the mucosal inflammation by both ways (Siegmund 2010).

Antigen recognition and processing by APCs in the mucosa plays an important role in the induction of inappropriate inflammatory response. Local DCs, macrophages, and epithelial cells induce prolonged stimulation of overreactive T cells thus promoting chronic inflammation (Baumgart and Carding 2007). These T cells can be overreactive or autoreactive because of ineffective clearance in the thymus due to failure in tolerance induction. Animal experiments showed that defects in IL-2 resulted in colitis development with increase in aggressive T cells released from thymus (Ludviksson et al. 1997). Regulatory T cells, which are primed in the mucosa, induce anergy or deletion of self-antigen-specific T cells, including those responding to mucosal antigens. This process is known as oral tolerance. Failure in the induction of tolerance results in increase in effector T cells promoting inflammation. This was recently confirmed, as the ability to proliferate and produce cytokines is increased in IBD patients' T cells cultivated with samples of their own microbiota when compared with healthy individuals (Duchmann et al. 1995). Also deficiency in the production or function of

regulatory cytokines IL-10 or TGF-β leads to inflammatory phenotype in mice (Kuhn et al. 1993; Gorelik and Flavell 2000).

Balance of regulatory and effector T cell response is deeply impaired in chronic inflammation. Local environment is the main trigger of different immune reaction associated with the disease. CD is more likely caused by activation of Th1 and Th17 cells producing IFN-γ, IL-17 and IL-21, further boosted by TNF-α, IL-6, IL-12, and IL-23 cytokines promoting mucosal inflammation. On the other hand, UC is associated with an aberrant Th2 immune response with high levels of IL-5 and IL-13 (Sartor 2008).

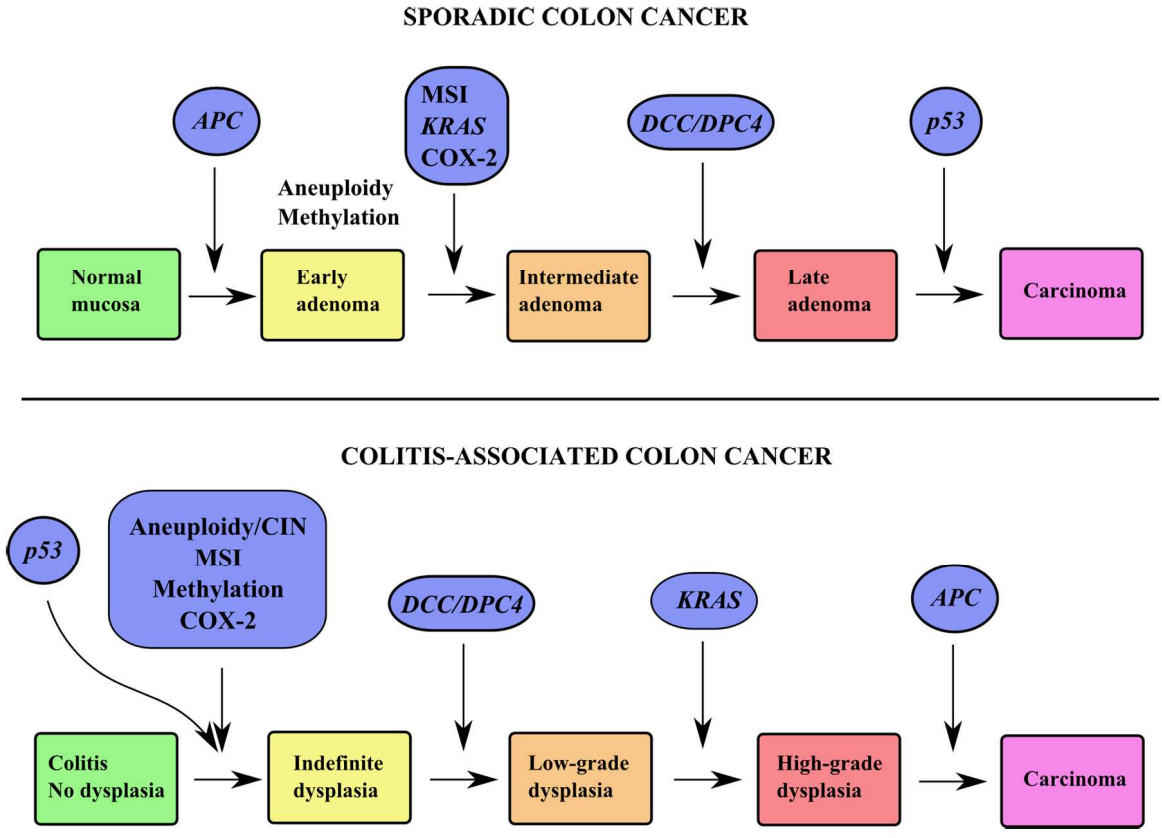
1.3.3 Colorectal cancer

Immuno-pathological mechanisms, tumor microenvironment

Chronic inflammation leads to massive accumulation of activated immune cells and their mediators (cytokines and chemokines), residues of damaged cells, and large amounts of oxygen and nitrogen reactive species. Recurrent cycle of epithelial cells damage and renewal is associated with oncogene mutations, inactivation of tumor-suppressor genes, loss of heterozygosity, and chromosomal and microsatellite instability. Prolonged inflammation also potentiates the CpG islands methylation and histone modification. Predominant type of genomic instability in CAC is the chromosomal one (85% of cases), which is characterized by abnormal counts of chromosomes or their content associated with deletions, translocations, and amplifications. The rest of cases is linked with microsatellite instability caused by impaired DNA repair mechanisms. Loss of function of tumor-suppressor genes is typical for early stages of CAC, including genes like *p53,* and *DCC* (*deleted in colorectal cancer*), whereas tumor progression is associated with mutations in another tumor-suppressor gene – *APC* (*adenomatous polyposis coli*), and protooncogenes like *KRAS* and *Bcl-2* (Goel et al. 2011). Isolated dysplastic cells are further modified by the local microenvironment as pro-inflammatory immune cells and cytokines promote the dysplasia progression into carcinoma. Therefore, the sequence (timing and frequency) of changes is different in sporadic and inflammation-related cancer (Figure 5). Moreover, lesions forming sporadic colorectal cancer are usually limited to one or two monocellular focuses, whereas colitis-associated lesions are multifocal and affecting larger area (Itzkowitz and Yio 2004; Kraus and Arber 2009; Rizzo et al. 2011).

Activated neutrophils and macrophages produce large amounts of reactive oxygen and nitrogen species that cause DNA and cellular damage during chronic inflammation. The mutation of *p53* leads to impaired reaction to oxidative stress present at the inflamed mucosa resulting in cell proliferation and resistance to apoptosis. Another key molecule is COX-2, which is an inducible enzyme and is increased in inflamed tissue, synthesizing the derivates of arachidonic acid like prostaglandins. COX-2 expression is promoted by pro-inflammatory cytokines like IL-1 and TNF-α and, in reverse, prostaglandins enhance inflammation and subsequent cancer development (Goel et al. 2011).

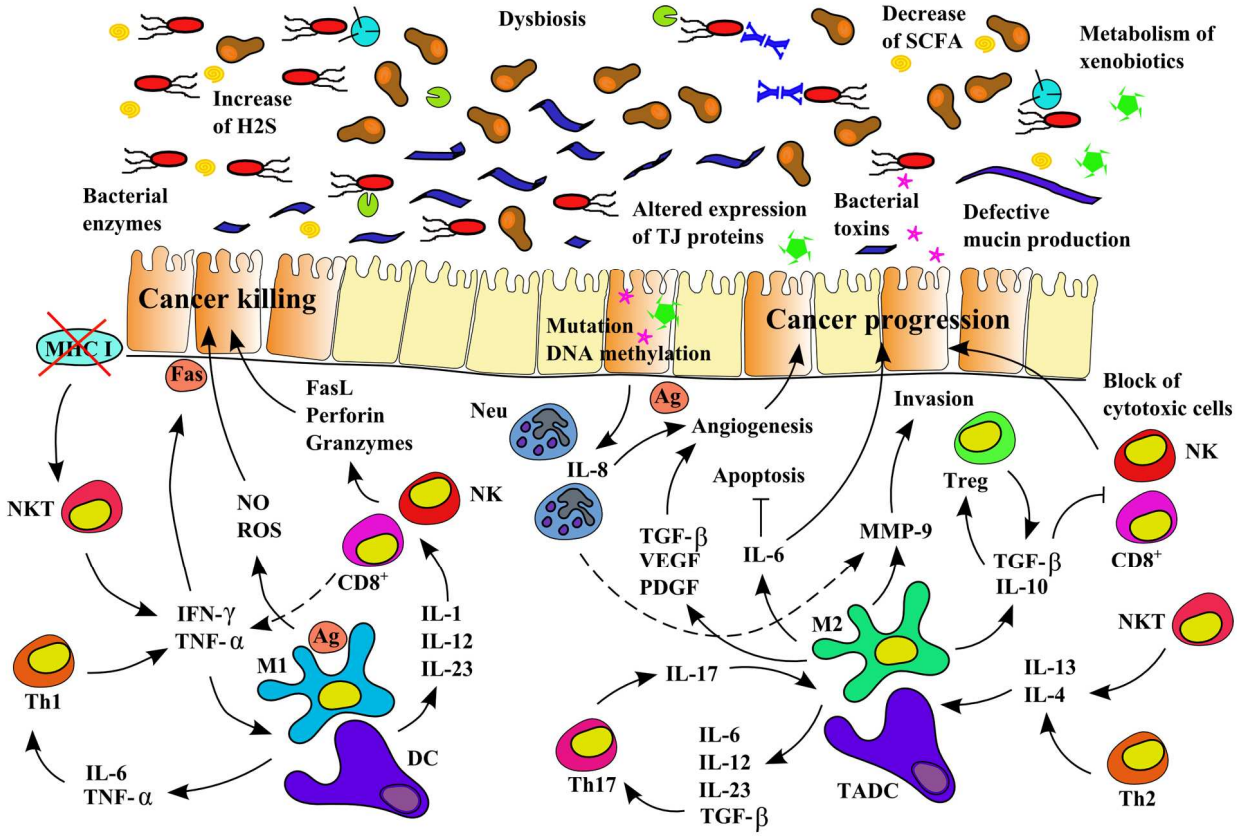
Figure 5. Sporadic and colitis-associated cancer – sequence of tumorigenesis. Adopted from (Itzkowitz and Yio 2004).



Innate immune cells and their products are tightly linked to inflammation-related tumorigenesis. PRR signalization in the milieu of chronic inflammation leads to activation of MyD88-dependent pathways promoting pro-inflammatory cytokine release and tumor progression. Lack of microbial stimuli, like in germ-free conditions, reduced the cancer incidence in mice and rats (Vannucci et al. 2008). Subsequent experiments showed decrease of tumor incidence in mouse deficient in TLR4 when compared with wild-type mice (Abreu

2010). Similarly, the absence of MyD88 prevented colorectal tumor development in non-inflammatory condition (Rakoff-Nahoum and Medzhitov 2007; Uronis et al. 2009), whereas led to aggressive tumorigenesis when associated with inflammation (Salcedo et al. 2010). Recent studies also suggested the association of TLR polymorphisms with the progression of colitis into cancer as TLR expression is changed during inflammation (Frolova et al. 2008).

Figure 6. Colitis-associated cancer microenvironment. Cells involved in anti- and pro-tumor immunity. Modified from (Rizzo et al. 2011).



Immune cells and cytokines important for tumorigenesis are summarized on the Figure 6. In brief, TNF-α is a key molecule regulating inflammation and host defense, chronically increased levels of TNF-α are associated with tumor growth, invasion and metastasis (Popivanova et al. 2008). IL-6 is one of the most important cytokines promoting transition of colitis into cancer as increased levels of IL-6 was found in patients with UC, that further progressed into cancer (Li et al. 2010). Both these cytokines activates intracellular signaling pathways including STAT3 and NF-κB, which are associated with the expression of pro-inflammatory factors that support tumorigenesis. NF-κB activation leads to increased cell

proliferation, inhibition of cell death, and promotion of angiogenesis, cell invasion and metastasis. Although IL-10 mainly acts as anti-inflammatory cytokine and IL-10 deficient mice spontaneously develop severe colitis (Kuhn et al. 1993), together with TGF-β are important immunosuppressive cytokines. They control functions of Treg cells and tumor-specific CD8+ T cells and thus might favor tumor growth and progression (Chen et al. 2005). Subunit p40 associates IL-12 and IL-23 cytokines thus influencing Th1 and Th17 polarization, respectively. Th17 cells producing IL-17 influence the expression of pro-inflammatory factors like TNF-α, IL-1, IL-6, iNOS, metalloproteinases and chemokines further promoting CAC development (Fouser et al. 2008).

Tumor-associated macrophages (TAM) develop from circulating monocytes, which are attracted into tumor microenvironment by chemotactic factors. Cytokines and chemokines produced by tumor cells (e.g. M-CSF) influence the final phenotype and activity of macrophages, as we can distinguish two types of them – M1 and M2 (Pollard 2004). TAM, accumulated in the hypoxic areas of the tumors, express various factors promoting tumor growth like VEGF (neovascularization), TNF-α and EGF (proliferation), IL-1β (metastasis), and metalloproteases, PDGF, t-PA and IL-6 (extracellular matrix degradation) (Allavena et al. 2008). The important function of TAM is suppression of anti-tumor immunity due to low expression of IL-12 and high IL-10. Moreover, such milieu reduces maturation and antigen presenting function of DC forming, so called, tumor-associated DC (TADC) and thus resulting in T cell unresponsiveness (Bronte and Zanovello 2005).

Studies in mice as well as observations in humans showed important role of Th subsets in tumor pathogenesis. Basically, Th2 type of immune response leads to tumor progression, whereas Th1-mediated response supports anti-tumor immunity (Osawa et al. 2006). In accord, patients with UC are more endangered by CAC when compared with CD patients. Tumor-infiltrating lymphocytes are located in the peritumoral stroma of solid tumors and include Treg cells, CD8+ T cells, NK, and NKT cells. Their proportions influence local anti- and pro-tumor immune response. Although immunosuppressive effect of Treg cells is an important part of anti-inflammatory response during colitis, active participation of these cells in tumor-associated immune reaction is unfavorable. Treg cellular subset and its cytokines (IL-10 and TGF-β) suppress effector CD8+ and NK cells and thus reduce effective anti-tumor immunity (Rizzo et al. 2011).

CD8+ T cells, NK and NKT cells play an important role in the anti-tumor immunity. Activated CD8+ T cells produce various mediators limiting tumor cells growth by different

mechanisms. IFN-γ and FasL, highly secreted by CD8+ T cells, leads to the expression of Fas receptor on dysplastic cells, subsequent interaction of Fas with FasL induces apoptosis. Pores-forming proteins (perforin, granzyme A and B) directly kill dysplastic cells by permeabilization of the cell membranes (Hoves et al. 2010). NK cells are activated by lack of molecules on dysplastic/tumor cells, which are often short of MHC class I molecules. Ligands like NKG2D are expressed by tumor cells and activate NK cells, which then produce high levels of IFN- γ, perforin, and granzymes leading to target cell destruction (Hoves et al. 2010). IL-21 was shown to activate NK cells but its role in cancer is still unclear as it induces pro-inflammatory response with Th17 cells generation in IBD on one side but on the other enhances cytotoxic activity of NK cells (Liu et al. 2009). Interestingly, NKT cells can induce both responses – Th1 and Th2 and thus promote tumor suppression and progression according to the cytokine microenvironment.

1.3.4 Role of bacteria in colitis and colitis-associated cancer pathogenesis

Dysbiosis, suspected pathogens, toxins, mechanisms

Although the role of bacteria in both colitis and CAC was proposed and generally accepted, there are many evidences that the pathogenesis is not limited to only one particular strain but rather to a specific composition and function of whole gut microbiota. The lower ability to induce colitis and colorectal cancer in germ-free animals confirmed the important role of bacteria in the pathogenesis of these diseases. Microbes actively interact with the local microenvironment responding to nutrients, mucosal cells and secretions. And, indeed, lack of microbial presence decreases the mucosal inflammation (Hudcovic et al. 2001) as well as tumor incidence (Vannucci et al. 2008). Our further studies confirmed this finding in BALB/c mice, on the other hand, the results in C57BL/6 mice was not convincing, so genetic background seems to be another strong co-factor (unpublished data). Basically, there are two approaches how to link microbes with diseases. First is via host PRR and immune response (inflammation) and the second involves the microbiota itself. Metabolic capacity of microbiota (metabolome) was found as crucial feature influencing tumor development in animal models (Arthur and Jobin 2011).

CD is, for a long period, given to link with the infection with an intracellular pathogen *Mycobacterium avium*, subspecies *paratuberculosis* (MAP). The MAP causes intestinal

inflammation (paratuberculosis) in ruminants known as Johne's disease and via milk or water is thought to infect humans. Although the polymorphisms of NOD2 are not associated with the MAP infection in CD patients (Sechi et al. 2006), it's assumed that MAP preferentially infects individuals with defects in intracellular killing such as those with polymorphisms of *ATG16L1*, or *NCF4*. The heterogeneity in the results of MAP presence in the gut mucosa and in the serum antibody response thus needs more research. However, the suspicion remains even if refer to a small group of CD patients (Sartor 2008).

New approaches in the metagenomic screening of complex ecosystems bring the advantage of finding the differences among gut microbiota in healthy and diseased status. The variation in the microbiota composition during disease, differing from healthy, is called dysbiosis (Sartor 2008). IBD-associated shifts in microbiota composition include decrease of *Clostridium* XIVa and IV groups and *Bacteroides*, and an increase in *Peptostreptococcus, Eubacteria, Coprococcus* and *Enterobacteriaceae* such as *Escherichia coli*. Most of the studies confirmed the *E. coli* increase in inflamed mucosa of patients with CD suggesting its role in pathogenesis (Swidsinski et al. 2002). Adherent invasive *E. coli* (AIEC) was isolated from inflamed mucosa of CD patients. The bacterium has specific type 1 pilus-mediated mechanism of adherence, which leads to predominant infection of ileal epithelial cells and subsequent induction of TNF secretion (Boudeau et al. 2001). On the contrary, potentially probiotic bacterium *Faecalibacterium prausnitzii* was decreased in IBD patients (specifically in CD), pointing out the beneficial effects of SCFA producers in the gut health (Sokol et al. 2008). Sulfate-reducing bacteria overgrowth, which was found in UC patients, leads to the decreased utilization of butyrate assuming altered epithelial nutrition as crucial factor in UC pathogenesis (Roediger et al. 1993).

Commensal bacteria protect the host against opportunistic pathogens. Therefore, alterations in gut microbiota composition can lead to the increased expression of microbial toxins. Their role in the IBD as well as identification of particular bacterial source is investigated. Microbial toxins are also produced by gut commensals like enterotoxigenic *Bacteroides fragilis, Clostridium difficile,* and *Enterococcus feacalis* (Sartor 2008).

Bacteria adhered to mucosa differ between healthy mucosa and adenoma/carcinoma. Local microenvironment associated with cancer has changed nutritional conditions as there is decrease in glucose and pyruvate, and an increase in lactate, amino acids, lipids and fatty acids. This leads to decreased pH and changes in redox potential and consequently to change in mucosal microbiota composition (Hirayama et al. 2009). Recently, the microbiota

associated with colorectal cancer was identified by deep parallel pyrosequencing. Comparison of adenoma with healthy mucosa showed decrease in *Bacteroides* and *Coprococcus* and increase in *Dorea, Shigella, Faecalibacterium, Oscillospira, Clostridium, Eubacterium, Ruminococcus,* and *Akkermansia* (Shen et al. 2010). Another similar study broadens the rows of bacteria associated with colorectal cancer about *Slackia, Collonsella, Roseburia, Coriobacterium* and *Fusobacterium*. Surprisingly, many of these bacteria are known as SCFA producers, thus bacteria with beneficial effects on host health, but their presence in colorectal cancer is not jet fully elucidated (Marchesi et al. 2011). Role of particular bacteria as possible causative agents is summarized in Table 3.

Table 3 Bacteria associated with colorectal carcinogenesis. Modified from (Arthur and Jobin 2011; Castellarin et al. 2012). (A, animal; H, human)

Bacteria	*****	***Evidence***
Helicobacter hepaticus	A	Augments AOM-induced, and spontaneous colorectal cancer in *Smad3*$^{-/-}$, *Rag2*$^{-/-}$ and *Apc*$^{Min/+}$ mice
H. hepaticus + *H. bilis*	A	Dual infection induces colorectal cancer in *Mdr1a*$^{-/-}$ mice
H. typhlonius + *H. rodentium*	A	Dual infection in neonates induces colorectal cancer in *Il10*$^{-/-}$ mice
Streptococcus bovis	**H**	*S. bovis* bacteremia and endocarditis associated with colorectal cancer
	A	Augments AOM-induced cancer in rats
Bacteroides fragilis	A	Enterotoxigenic *B. fragilis* augments spontaneous (*Apc*$^{Min/+}$) colorectal cancer in mice
	H	Increased prevalence of enterotoxigenic *B. fragilis* in colorectal cancer
B. vulgatus	A	Induces mild AOM-induced colorectal cancer in *Il10*$^{-/-}$ mice
Escherichia coli	**H**	Increased mucosa-associated *E. coli* in Crohn's and colorectal cancer
Citrobacter rodentium and C. freundii	A	Etiologic agent of transmissible murine colonic hyperplasia Augments spontaneous (*Apc*$^{Min/+}$) and DMH-induced colorectal cancer in mice
Fusobacterium nucleatum	**H**	Increased abundance in colorectal cancer tissue samples

Metabolic activity of gut bacteria leads to the production of huge amounts of various biologically active molecules. Activation or inhibition of potential carcinogens is one of the most important features of microbiota having crucial role in gut tumorigenesis. Bacterial enzymes can convert pro-carcinogens from diet or bile acids conjugates. Among the enzymes increasing the exposure to the carcinogens belong β-glucuronidase, β-glucosidase, nitroreductase, azoreductase, mucinase, catalase, and 7α-dehydroxylase (Arthur and Jobin 2011). Animal experiments confirmed the interaction of bacterial β-glucuronidase with genotoxic chemical – azoxymethane, which is commonly used for the colorectal cancer induction (Fiala 1975). Recently, various substances reducing the enzyme activity are tested in animals with promising results in preventing colorectal cancer development (Kanauchi et al. 2008; Vergara-Castaneda et al. 2010).

Similarly as in inflammation, commensal bacteria can produce toxic substances, which can interfere with the pathogenesis of colorectal cancer. Toxins like *Bacteroides fragilis* enterotoxin and *Helicobacter pylori* cytotoxin-associated antigen A influence epithelial cells' cycle, barrier permeability, or inflammatory response, which promotes tumorigenesis (Arthur and Jobin 2011).

1.3.5 Bacteria in colorectal cancer prevention

Probiotics, prebiotics, synbiotics

Probiotics were defined by FAO and WHO as "live microorganisms, which confer a health benefit on the host when administered in adequate amounts" (FAO/WHO 2001). Among the most common bacteria used as probiotic belong lactic acid bacteria, *Bifidobacteria*, bacilli, and few types of yeasts. Although the use of probiotics in cancer prevention and therapy has been the aim of many experiments and clinical studies, the results are not clearly convincing.

In vitro experiments revealed multiple mechanisms, by which probiotics can inhibit colon cancer. Cultivation of immune and cancer cells with living bacteria showed changes in cell cycle, apoptosis, proliferation, production of reactive oxygen species, and metabolism of eukaryotic cells, as well as in the metabolism and enzyme activity of bacteria. Kim and colleagues found broad anti-cancer properties of *Bifidobacterium adolescentis SPM0212*, which inhibited the proliferation of human colon cancer cell lines accompanied with decreased TNF-α expression. Moreover, this strain can reduce the activity of harmful bacterial enzymes like β-glucuronidase, β-glucosidase, tryptophanase, and urease (Kim et al. 2008).

Another studies found the important role of probiotics in stimulation of production of defensins, and cytokines activating innate immunity (*Lactobacillus plantarum, L. casei* and *L. fermentum*), or in inhibition of tumor cell growth (*Bacillus polyfermenticus SCD* and *Lactobacillus GG*) (Shida et al. 2006; Paolillo et al. 2009).

Probiotics contribute to the development and reactivity of mucosal immune system by promoting epithelial cells, DC, and T cells response. Moreover, they have stabilizing effect on gut microbiota during administration with potential to reduce pro-inflammatory response. Administration of various single or multiple species formulations showed enhancement of anti-tumor immunity (increase in IFN-γ, TNF-α, or decrease in reactive oxygen species) associated with reduced tumor incidence. Most beneficial effects were linked with administration of living probiotic bacteria. Colitis and cancer development was reduced and delayed in IL-10 deficient mice treated with *L. salivarius*, subspecies *salivarius UCC118* (O'Mahony et al. 2001). Favored multiple species formulation is VLS#3, which is composed of four strains of lactobacilli (*L. casei, L. plantarum, L. bulgaricus*, and *L. acidophilus*), three strains of bifidobacteria (*B. longum, B. breve, and B. infantis*) and *Streptococcus thermophilus*. Recent study in azoxymethane/dextran sulfate sodium model of CAC showed that oral treatment with VLS#3 probiotic decreased chronic inflammatory response and subsequent tumorigenesis. Beneficial effect was associated with changed ratios of Treg and Th17 cells in MLN and colonic lamina propria (Bassaganya-Riera et al. 2012).

Although the experimental data show positive results of probiotics effect on inflammation and tumorigenesis, studies in humans are not as clear. There are many aspects of treatment that need to be considered in clinical trials as disease status (prevention versus therapy), which and how many strains are used, storage of the formulation, viability, dosage etc... Therefore the few studies that were done are not easily comparable (Table 4). All in all, supportive therapy with probiotics is accepted as helpful in cancer prevention, but not curative (Capurso et al. 2006).

Revealing the complexity of the gut ecosystem leads to development of new approaches in the way of boosting the viability of probiotics. Prebiotics are non-digestible polysaccharides, which are fermented by bacteria and thus promote the growth of probiotic ones, conferring benefits upon host well-being and health (Gibson et al. 2004). Various types of inulin, galacto-oligosaccharides or short-chain fructo-oligosaccharides belong to the most used prebiotics. Synbiotics is a term for simultaneous use of probiotics with prebiotics, which takes the advantage of combination of benefits. Synergistic effects of combined formulation

seem to be more effective in colorectal cancer prevention and include alteration of gut microbiota (Worthley et al. 2009), and improvement of epithelial barrier function, increase of IFN-γ, and prevention of dysplasia (Ishikawa et al. 2005; Rafter et al. 2007) (Table 4).

Table 4. Clinical studies on prevention of colorectal cancer by microbiota treatment. Modified from (Azcarate-Peril et al. 2011; Zhu et al. 2011).

Model	***Treatment***	***Results***
20 volunteers. R/DB/PC/C.	Resistant starch (RS) containing high-amylose maize starch +/- *B.lactis*	Increase in the fecal counts of *Lachnospiraceae* spp. No significant effects on epithelial proliferation and fecal SCFA concentrations (Worthley et al. 2009).
38 healthy men. R/DB/PC/C.	*L.rhamnosus* LC705 (LC705) + *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS)	Increase in the fecal counts of lactobacilli and propionibacteria and decrease in the activity of β-glucosidase (Hatakka et al. 2008).
150 patients diagnosed with CRC.	*Lactobacillus* GG	Reduction of frequency of severe diarrhea and abdominal discomfort related to 5-FU-based chemotherapy (Osterlund et al. 2007).
398 patients free from CRC with CRC history.	Wheat bran and/or *L.casei*	Prevention of high-grade atypia of colorectal tumors (Ishikawa et al. 2005).
31 patients with CRC.	*B.longum* (BB536) and *L.johnsonii* (La1)	La1, but not BB536, reduces the concentration of pathogens and modulates local immune response (Gianotti et al. 2010).
37 patients with CRC and 43 polypectomized patients. R/DB/PC.	Oligofructose-enriched inulin (SYN1) + *L.rhamnosus* GG (LGG) and *B.lactis* Bb12 (BB12)	Numbers of Bifidobacteria and Lactobacilli increased, and *C.perfringens* decreased. Reduction of CRC proliferation and the capacity of fecal water to induce necrosis of colonocytes, and improvement of epithelial barrier function (Rafter et al. 2007).
100 patients with CRC scheduled for radical colorectomy.	*L.plantarum* (CGMCC No. 1258, 1011 CFU/g), *L.acidophilus* (LA-11, 1010 CFU/g), and *B.longum* (BL-88, 1010 CFU/ g)	The treatment improved the integrity of the gut mucosal barrier and balance of the gut microbiota and decreased rate of post surgical infection (Liu et al. 2011).
45 241 volunteers	*S.thermophilus* and *L.delbrueckii* ssp. *bulgaricus* as yogurt	Treatment during 12 years decreased CRC risk (Pala et al. 2011).

1.4 Mouse models of colitis and colitis-associated colon cancer

In vivo models are used for better understanding of the pathogenesis of various human diseases. Models used can be divided, according to the approach, into two main groups: models based on genetic modification or polymorphism, and chemically-induced models. Recent data support the idea that all animal models are strongly influenced by composition of local microbiota, as well as chow, water, and bedding, which complicates the comparability and reproducibility of the experiments.

1.4.1 Mouse models of chemically-induced intestinal inflammation

TNBS, oxazolone and DSS colitis

Exogenous agents are often used for the induction of inflammation, especially in the intestines, to mimic the pathogenesis of human IBD. These models are commonly used to follow molecular and cellular immuno-pathological mechanisms of the diseases and to test new preventive and therapeutic strategies. Among the most frequently used models belong sensitization and subsequent exposure to the haptenating chemicals like 2,4,6-trinitrobenzenesulfonic acid (TNBS) and oxazolone, inducing T cell mediated pro-inflammatory immune response, as we can see in both CD and UC. Thirdly, oral administration of dextran sodium sulfate (DSS) cause epithelial layer damage linked with increased mucosal permeability and mainly innate immune response similar to UC (Bouma and Strober 2003; Wirtz et al. 2007).

TNBS can induce either Th1 or Th2 type of immune response considering the mouse stain. As CD4+ T cells play central role in this model, it's often used in the studies concerning with the adaptive immune response in the intestinal inflammation. The experimental procedure includes initial skin sensitization and subsequent direct intrarectal application of TNBS dissolved in ethanol. Alcohol cause the intestinal barrier damage and the hapten induce local inflammatory immune response (Morris et al. 1989).

Oxazolone-mediated colitis is another type of hapten-induced experimental model of intestinal inflammation. Immune reaction results into Th2 immune tuning with increased secretion of IL-4, which rather corresponds to UC (Boirivant et al. 1998). NK-T cells producing Th2 cytokines like IL-4 and IL-13 induce severe inflammation after intrarectal

instillation of oxazolone/ethanol solution. The model is also dose and strain sensitive and is used in research of Th2 response in the pathogenesis of gut inflammation (Heller et al. 2002).

The most commonly used animal model of colitis involves oral treatment with DSS solution. Several days of treatment result in the induction of acute colitis with diarrhea and rectal bleeding. Chronic inflammation develops after repeated administration of DSS (e.g. one week) alternating with tap water (e.g. two weeks) (Wirtz et al. 2007). Animal strain and microbiota both influence the sensitivity to colitis, therefore concentration of DSS and duration of the treatment needs to be established in every animal facility. Pathogenesis includes redistribution of tight junction proteins and subsequent induction of mucosal inflammation with accumulated granulocytes and macrophages producing pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α. Chronic DSS colitis is driven by the activation of CD4+ T cells secreting IFN-γ or IL-4 resulting in either Th1 or Th2 type of immune response, respectively (Dieleman et al. 1998).

1.4.2 Mouse models of colorectal cancer

Spontaneous and chemically-induced cancer

Apc$^{Min/+}$ mice have naturally lost or mutated variant of *Apc* gene, which is also the cause of sporadic colon carcinogenesis called familial adenomatous polyposis (FAP) in humans. Encoding protein – APC (adenomatous polyposis coli) – regulate Wnt signalization by preventing β-catenin translocation to nucleus. APC and other proteins of Wnt cascade (e.g. AXIN, GSK-3β) bind β-catenin and mediate his phosphorylation and degradation by a proteasome. *Apc* mutation predisposes not only to intestinal but also to mammary adenomas formation in females (Moser et al. 1995; Morin et al. 1997). In most cases the mutations of *Apc* gene result in the synthesis of truncated APC protein incapable of proper regulation. Wnt pathway is life important during embryonic development but its activation in the adult epithelium is fatal (Morin et al. 1997). Cell cycle defects, including cell proliferation and apoptosis due to microsatellite instability, lead to reduced crypt to villus migration and differentiation with final multiple microadenoma formation or multiple intestinal neoplasia (Min) in mice. Although the *Apc* mutations were found in human colorectal carcinomas, there are some limiting facts as different locality – small intestine in mice versus colon in human, and tendency to progression – no adenocarcinomas development and very little or no invasion in mice (Kanneganti et al. 2011). Therefore a modified protocol for *Apc*$^{Min/+}$ mice was

established using DSS to shift the inflammation and tumorigenesis into the colon (Tanaka et al. 2006).

Chemically-induced models of CAC comprises of parenteral (s.c. or i.p.) injection of azoxymethane (AOM) or 1,2-dimethylhydrazin (DMH) with subsequent administration of DSS solution (Tanaka et al. 2003; Neufert et al. 2007; Kanneganti et al. 2011). AOM and DMH are pro-carcinogens and their utilization in liver leads to secretion of methylazoxymethanol-glucuronic acid conjugates, which are further dissociated in the gut by bacterial β-glucuronidase. Released methylazoxymethanol is the main genotoxic compound causing DNA methylation (Fiala 1975). Combination of these chemicals therefore leads to CAC formation in the distal gut and rectum. By using different strains of mice in the experiments, it was shown that not only microbiota composition but also genetic background induces variability in the tumor development (Suzuki et al. 2006). Susceptibility to tumor induction as well as tumor counts and morphology are similar in inbred mice (C57BL/6J) when compared with outbred ones (Swiss Webster) (Clapper et al. 2008). Nuclear translocation of β-catenin is also observed during tumorigenesis after AOM/DSS treatment (Kohno et al. 2005). β-catenin mutations and altered distribution are consistent with findings in human colitis-associated colorectal cancer (Aust et al. 2001).

1.5 References

Abreu, M. T. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol (2010) **10** (2): 131-144.

Allavena, P., Garlanda, C., Borrello, M. G., Sica, A. and Mantovani, A. Pathways connecting inflammation and cancer. Curr Opin Genet Dev (2008) **18** (1): 3-10.

Arthur, J. C. and Jobin, C. The struggle within: microbial influences on colorectal cancer. Inflamm Bowel Dis (2011) **17** (1): 396-409.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H. B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E. G., Wang, J., Guarner, F., Pedersen, O., de Vos, W. M., Brunak, S., Dore, J., Antolin, M., Artiguenave, F., Blottiere, H. M., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K. U., Friss, C., van de Guchte, M., Guedon, E., Haimet, F., Huber, W., van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Merieux, A., Melo Minardi, R., M'Rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S. D. and Bork, P. Enterotypes of the human gut microbiome. Nature (2011) **473** (7346): 174-180.

Askling, J., Dickman, P. W., Karlen, P., Brostrom, O., Lapidus, A., Lofberg, R. and Ekbom, A. Family history as a risk factor for colorectal cancer in inflammatory bowel disease. Gastroenterology (2001) **120** (6): 1356-1362.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., Taniguchi, T., Takeda, K., Hori, S., Ivanov, II, Umesaki, Y., Itoh, K. and Honda, K. Induction of colonic regulatory T cells by indigenous Clostridium species. Science (2011) **331** (6015): 337-341.

Aust, D. E., Terdiman, J. P., Willenbucher, R. F., Chew, K., Ferrell, L., Florendo, C., Molinaro-Clark, A., Baretton, G. B., Lohrs, U. and Waldman, F. M. Altered distribution of beta-catenin, and its binding proteins E-cadherin and APC, in ulcerative colitis-related colorectal cancers. Mod Pathol (2001) **14** (1): 29-39.

Azcarate-Peril, M. A., Sikes, M. and Bruno-Barcena, J. M. The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer? Am J Physiol Gastrointest Liver Physiol (2011) **301** (3): G401-424.

Balaci, L., Spada, M. C., Olla, N., Sole, G., Loddo, L., Anedda, F., Naitza, S., Zuncheddu, M. A., Maschio, A., Altea, D., Uda, M., Pilia, S., Sanna, S., Masala, M., Crisponi, L., Fattori, M., Devoto, M., Doratiotto, S., Rassu, S., Mereu, S., Giua, E., Cadeddu, N. G., Atzeni, R., Pelosi, U., Corrias, A., Perra, R., Torrazza, P. L., Pirina, P., Ginesu, F., Marcias, S., Schintu, M. G., Del Giacco, G. S., Manconi, P. E., Malerba, G., Bisognin, A., Trabetti, E., Boner, A., Pescollderungg, L., Pignatti, P. F., Schlessinger, D., Cao, A. and Pilia, G. IRAK-M is involved in the pathogenesis of early-onset persistent asthma. Am J Hum Genet (2007) **80** (6): 1103-1114.

Bassaganya-Riera, J., Viladomiu, M., Pedragosa, M., De Simone, C. and Hontecillas, R. Immunoregulatory mechanisms underlying prevention of colitis-associated colorectal cancer by probiotic bacteria. PLoS One (2012) **7** (4): e34676.

Baumgart, D. C. and Sandborn, W. J. Inflammatory bowel disease: clinical aspects and established and evolving therapies. Lancet (2007) **369** (9573): 1641-1657.

Baumgart, D. C. and Carding, S. R. Inflammatory bowel disease: cause and immunobiology. Lancet (2007) **369** (9573): 1627-1640.

Beaugerie, L. and Petit, J. C. Microbial-gut interactions in health and disease. Antibiotic-associated diarrhoea. Best Pract Res Clin Gastroenterol (2004) **18** (2): 337-352.

Berglund, M., Melgar, S., Kobayashi, K. S., Flavell, R. A., Hornquist, E. H. and Hultgren, O. H. IL-1 receptor-associated kinase M downregulates DSS-induced colitis. Inflamm Bowel Dis (2010) **16** (10): 1778-1786.

Berin, M. C., Yang, P. C., Ciok, L., Waserman, S. and Perdue, M. H. Role for IL-4 in macromolecular transport across human intestinal epithelium. Am J Physiol (1999) **276** (5 Pt 1): C1046-1052.

Bevins, C. L. and Salzman, N. H. Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis. Nat Rev Microbiol (2011) **9** (5): 356-368.

Biasucci, G., Rubini, M., Riboni, S., Morelli, L., Bessi, E. and Retetangos, C. Mode of delivery affects the bacterial community in the newborn gut. Early Hum Dev (2010) **86 Suppl 1**: 13-15.

Bik, E. M., Eckburg, P. B., Gill, S. R., Nelson, K. E., Purdom, E. A., Francois, F., Perez-Perez, G., Blaser, M. J. and Relman, D. A. Molecular analysis of the bacterial microbiota in the human stomach. Proc Natl Acad Sci U S A (2006) **103** (3): 732-737.

Biswas, A., Wilmanski, J., Forsman, H., Hrncir, T., Hao, L., Tlaskalova-Hogenova, H. and Kobayashi, K. S. Negative regulation of Toll-like receptor signaling plays an essential role in homeostasis of the intestine. Eur J Immunol (2011) **41** (1): 182-194.

Boirivant, M., Fuss, I. J., Chu, A. and Strober, W. Oxazolone colitis: A murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. J Exp Med (1998) **188** (10): 1929-1939.

Boudeau, J., Barnich, N. and Darfeuille-Michaud, A. Type 1 pili-mediated adherence of Escherichia coli strain LF82 isolated from Crohn's disease is involved in bacterial invasion of intestinal epithelial cells. Mol Microbiol (2001) **39** (5): 1272-1284.

Bouma, G. and Strober, W. The immunological and genetic basis of inflammatory bowel disease. Nat Rev Immunol (2003) **3** (7): 521-533.

Bouskra, D., Brezillon, C., Berard, M., Werts, C., Varona, R., Boneca, I. G. and Eberl, G. Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature (2008) **456** (7221): 507-510.

Brandtzaeg, P. History of oral tolerance and mucosal immunity. Ann N Y Acad Sci (1996) **778**: 1-27.

Bronte, V. and Zanovello, P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol (2005) **5** (8): 641-654.

Canavan, C., Abrams, K. R. and Mayberry, J. Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. Aliment Pharmacol Ther (2006) **23** (8): 1097-1104.

Capurso, G., Marignani, M. and Delle Fave, G. Probiotics and the incidence of colorectal cancer: when evidence is not evident. Dig Liver Dis (2006) **38 Suppl 2**: S277-282.

Castellarin, M., Warren, R. L., Freeman, J. D., Dreolini, L., Krzywinski, M., Strauss, J., Barnes, R., Watson, P., Allen-Vercoe, E., Moore, R. A. and Holt, R. A. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. Genome Res (2012) **22** (2): 299-306.

Cerutti, A. and Rescigno, M. The biology of intestinal immunoglobulin A responses. Immunity (2008) **28** (6): 740-750.

Claesson, M. J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., de Weerd, H., Flannery, E., Marchesi, J. R., Falush, D., Dinan, T., Fitzgerald, G., Stanton, C., van Sinderen, D., O'Connor, M., Harnedy, N., O'Connor, K., Henry, C., O'Mahony, D., Fitzgerald, A. P., Shanahan, F., Twomey, C., Hill, C., Ross, R. P. and O'Toole, P. W. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. Proc Natl Acad Sci U S A (2011) **108 Suppl 1**: 4586-4591.

Clapper, M. L., Gary, M. A., Coudry, R. A., Litwin, S., Chang, W. C., Devarajan, K., Lubet, R. A. and Cooper, H. S. 5-aminosalicylic acid inhibits colitis-associated colorectal dysplasias in the mouse model of azoxymethane/dextran sulfate sodium-induced colitis. Inflamm Bowel Dis (2008) **14** (10): 1341-1347.

Clemente, J. C., Ursell, L. K., Parfrey, L. W. and Knight, R. The impact of the gut microbiota on human health: an integrative view. Cell (2012) **148** (6): 1258-1270.

Crohn, B. B. and Rosenberg, H. The sigmoidoscopic picture of chronic ulcerative colitis (non- specific). American Journal of Medical Science (1925) **170**: 220-228.

Cubeddu, R. J., Cruz-Gonzalez, I., Kiernan, T. J., Truong, Q. A., Rosenfield, K., Leinbach, R. C., Cadigan, M. E., Pomerantsev, E. V. and Palacios, I. F. ST-elevation myocardial infarction mortality in a major academic center "on-" versus "off-" hours. J Invasive Cardiol (2009) **21** (10): 518-523.

Curtis, M. M. and Way, S. S. Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens. Immunology (2009) **126** (2): 177-185.

Deretic, V. and Levine, B. Autophagy, immunity, and microbial adaptations. Cell Host Microbe (2009) **5** (6): 527-549.

Dieleman, L. A., Palmen, M. J., Akol, H., Bloemena, E., Pena, A. S., Meuwissen, S. G. and Van Rees, E. P. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. Clin Exp Immunol (1998) **114** (3): 385-391.

Duchmann, R., Kaiser, I., Hermann, E., Mayet, W., Ewe, K. and Meyer zum Buschenfelde, K. H. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol (1995) **102** (3): 448-455.

Eaden, J. A., Abrams, K. R. and Mayberry, J. F. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut (2001) **48** (4): 526-535.

FAO/WHO (2001). Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. <http://www.who.int/entity/foodsafety/publications/fs_management/en/probiotics.pdf>.

Fava, F. and Danese, S. Intestinal microbiota in inflammatory bowel disease: friend of foe? World J Gastroenterol (2011) **17** (5): 557-566.
Favier, C. F., de Vos, W. M. and Akkermans, A. D. Development of bacterial and bifidobacterial communities in feces of newborn babies. Anaerobe (2003) **9** (5): 219-229.
Fiala, E. Investigations into the metabolism and mode of action of the colon carcinogen 1, 2-dimethylhydrazine. Cancer (1975) **36** (6 Suppl): 2407-2412.
Forbes, S. A., Tang, G., Bindal, N., Bamford, S., Dawson, E., Cole, C., Kok, C. Y., Jia, M., Ewing, R., Menzies, A., Teague, J. W., Stratton, M. R. and Futreal, P. A. COSMIC (the Catalogue of Somatic Mutations in Cancer): a resource to investigate acquired mutations in human cancer. Nucleic Acids Res (2010) **38** (Database issue): D652-657.
Fouser, L. A., Wright, J. F., Dunussi-Joannopoulos, K. and Collins, M. Th17 cytokines and their emerging roles in inflammation and autoimmunity. Immunol Rev (2008) **226**: 87-102.
Frolova, L., Drastich, P., Rossmann, P., Klimesova, K. and Tlaskalova-Hogenova, H. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. J Histochem Cytochem (2008) **56** (3): 267-274.
Funda, D., Fundova, P., Harrison, L. Microflora-dependency of selected diabetes-preventive diets: germ-free and ex-germ-free monocolonized NOD mice as models for studying environmental factors in type 1 diabetes. 13th International Congress of Immunology, MS-11.4 16 (Brazilian Society for Immunology, Rio de Janeiro). (2007).
Gianotti, L., Morelli, L., Galbiati, F., Rocchetti, S., Coppola, S., Beneduce, A., Gilardini, C., Zonenschain, D., Nespoli, A. and Braga, M. A randomized double-blind trial on perioperative administration of probiotics in colorectal cancer patients. World J Gastroenterol (2010) **16** (2): 167-175.
Gibson, G. R., Probert, H. M., Loo, J. V., Rastall, R. A. and Roberfroid, M. B. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev (2004) **17** (2): 259-275.
Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M. and Nelson, K. E. Metagenomic analysis of the human distal gut microbiome. Science (2006) **312** (5778): 1355-1359.
Goel, G. A., Kandiel, A., Achkar, J. P. and Lashner, B. Molecular pathways underlying IBD-associated colorectal neoplasia: therapeutic implications. Am J Gastroenterol (2011) **106** (4): 719-730.
Gorelik, L. and Flavell, R. A. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity (2000) **12** (2): 171-181.
Green, G. L., Brostoff, J., Hudspith, B., Michael, M., Mylonaki, M., Rayment, N., Staines, N., Sanderson, J., Rampton, D. S. and Bruce, K. D. Molecular characterization of the bacteria adherent to human colorectal mucosa. J Appl Microbiol (2006) **100** (3): 460-469.
Greer, J. B. and O'Keefe, S. J. Microbial induction of immunity, inflammation, and cancer. Front Physiol (2011) **1**: 168.
Guarino, A., Wudy, A., Basile, F., Ruberto, E. and Buccigrossi, V. Composition and roles of intestinal microbiota in children. J Matern Fetal Neonatal Med (2012) **25 Suppl 1**: 63-66.
Hamada, H., Hiroi, T., Nishiyama, Y., Takahashi, H., Masunaga, Y., Hachimura, S., Kaminogawa, S., Takahashi-Iwanaga, H., Iwanaga, T., Kiyono, H., Yamamoto, H. and Ishikawa, H. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. J Immunol (2002) **168** (1): 57-64.
Hammer, G. E., Turer, E. E., Taylor, K. E., Fang, C. J., Advincula, R., Oshima, S., Barrera, J., Huang, E. J., Hou, B., Malynn, B. A., Reizis, B., DeFranco, A., Criswell, L. A., Nakamura, M. C. and Ma, A. Expression of A20 by dendritic cells preserves immune homeostasis and prevents colitis and spondyloarthritis. Nat Immunol (2011) **12** (12): 1184-1193.
Hansen, C. H., Nielsen, D. S., Kverka, M., Zakostelska, Z., Klimesova, K., Hudcovic, T., Tlaskalova-Hogenova, H. and Hansen, A. K. Patterns of early gut colonization shape future immune responses of the host. PLoS One (2012) **7** (3): e34043.
Hanson, L. A. Session 1: Feeding and infant development breast-feeding and immune function. Proc Nutr Soc (2007) **66** (3): 384-396.
Hatakka, K., Holma, R., El-Nezami, H., Suomalainen, T., Kuisma, M., Saxelin, M., Poussa, T., Mykkanen, H. and Korpela, R. The influence of Lactobacillus rhamnosus LC705 together with Propionibacterium freudenreichii ssp. shermanii JS on potentially carcinogenic bacterial activity in human colon. Int J Food Microbiol (2008) **128** (2): 406-410.
Heller, F., Fuss, I. J., Nieuwenhuis, E. E., Blumberg, R. S. and Strober, W. Oxazolone colitis, a Th2 colitis model resembling ulcerative colitis, is mediated by IL-13-producing NK-T cells. Immunity (2002) **17** (5): 629-638.

Hirayama, A., Kami, K., Sugimoto, M., Sugawara, M., Toki, N., Onozuka, H., Kinoshita, T., Saito, N., Ochiai, A., Tomita, M., Esumi, H. and Soga, T. Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry. Cancer Res (2009) **69** (11): 4918-4925.

Hoves, S., Trapani, J. A. and Voskoboinik, I. The battlefield of perforin/granzyme cell death pathways. J Leukoc Biol (2010) **87** (2): 237-243.

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

Hudcovic, T., Stepankova, R., Cebra, J. and Tlaskalova-Hogenova, H. 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) (2001) **46** (6): 565-572.

Chen, M. L., Pittet, M. J., Gorelik, L., Flavell, R. A., Weissleder, R., von Boehmer, H. and Khazaie, K. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. Proc Natl Acad Sci U S A (2005) **102** (2): 419-424.

Ishikawa, H., Akedo, I., Otani, T., Suzuki, T., Nakamura, T., Takeyama, I., Ishiguro, S., Miyaoka, E., Sobue, T. and Kakizoe, T. Randomized trial of dietary fiber and Lactobacillus casei administration for prevention of colorectal tumors. Int J Cancer (2005) **116** (5): 762-767.

Itzkowitz, S. H. and Yio, X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. Am J Physiol Gastrointest Liver Physiol (2004) **287** (1): G7-17.

Ivanov, II, Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K. C., Santee, C. A., Lynch, S. V., Tanoue, T., Imaoka, A., Itoh, K., Takeda, K., Umesaki, Y., Honda, K. and Littman, D. R. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell (2009) **139** (3): 485-498.

Iwata, M., Hirakiyama, A., Eshima, Y., Kagechika, H., Kato, C. and Song, S. Y. Retinoic acid imprints gut-homing specificity on T cells. Immunity (2004) **21** (4): 527-538.

Jamieson, C., Sharma, M. and Henderson, B. R. Wnt signaling from membrane to nucleus: beta-catenin caught in a loop. Int J Biochem Cell Biol (2012).

Johansson, M. E., Ambort, D., Pelaseyed, T., Schutte, A., Gustafsson, J. K., Ermund, A., Subramani, D. B., Holmen-Larsson, J. M., Thomsson, K. A., Bergstrom, J. H., van der Post, S., Rodriguez-Pineiro, A. M., Sjovall, H., Backstrom, M. and Hansson, G. C. Composition and functional role of the mucus layers in the intestine. Cell Mol Life Sci (2011) **68** (22): 3635-3641.

Jung, C., Hugot, J. P. and Barreau, F. Peyer's Patches: The Immune Sensors of the Intestine. Int J Inflam (2010) **2010**: 823710.

Kanauchi, O., Mitsuyama, K., Andoh, A. and Iwanaga, T. Modulation of intestinal environment by prebiotic germinated barley foodstuff prevents chemo-induced colonic carcinogenesis in rats. Oncol Rep (2008) **20** (4): 793-801.

Kanneganti, M., Mino-Kenudson, M. and Mizoguchi, E. Animal models of colitis-associated carcinogenesis. J Biomed Biotechnol (2011) **2011**: 342637.

Kaser, A., Lee, A. H., Franke, A., Glickman, J. N., Zeissig, S., Tilg, H., Nieuwenhuis, E. E., Higgins, D. E., Schreiber, S., Glimcher, L. H. and Blumberg, R. S. XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. Cell (2008) **134** (5): 743-756.

Khachatryan, Z. A., Ktsoyan, Z. A., Manukyan, G. P., Kelly, D., Ghazaryan, K. A. and Aminov, R. I. Predominant role of host genetics in controlling the composition of gut microbiota. PLoS One (2008) **3** (8): e3064.

Kim, Y., Lee, D., Kim, D., Cho, J., Yang, J., Chung, M., Kim, K. and Ha, N. Inhibition of proliferation in colon cancer cell lines and harmful enzyme activity of colon bacteria by Bifidobacterium adolescentis SPM0212. Arch Pharm Res (2008) **31** (4): 468-473.

Kim, Y. S. and Ho, S. B. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep (2010) **12** (5): 319-330.

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. Inflamm Bowel Dis (2012); in press.

Kohno, H., Suzuki, R., Sugie, S. and Tanaka, T. Beta-Catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. Cancer Sci (2005) **96** (2): 69-76.

Kolligs, F. T., Nieman, M. T., Winer, I., Hu, G., Van Mater, D., Feng, Y., Smith, I. M., Wu, R., Zhai, Y., Cho, K. R. and Fearon, E. R. ITF-2, a downstream target of the Wnt/TCF pathway, is activated in human

cancers with beta-catenin defects and promotes neoplastic transformation. Cancer Cell (2002) **1** (2): 145-155.
Kraus, S. and Arber, N. Inflammation and colorectal cancer. Curr Opin Pharmacol (2009) **9** (4): 405-410.
Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell (1993) **75** (2): 263-274.
Lee, C. C., Avalos, A. M. and Ploegh, H. L. Accessory molecules for Toll-like receptors and their function. Nat Rev Immunol (2012) **12** (3): 168-179.
Lee, Y. K., Menezes, J. S., Umesaki, Y. and Mazmanian, S. K. Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A (2011) **108 Suppl 1**: 4615-4622.
Lehrer, R. I., Jung, G., Ruchala, P., Andre, S., Gabius, H. J. and Lu, W. Multivalent binding of carbohydrates by the human alpha-defensin, HD5. J Immunol (2009) **183** (1): 480-490.
Lewis, J. D. The utility of biomarkers in the diagnosis and therapy of inflammatory bowel disease. Gastroenterology (2011) **140** (6): 1817-1826 e1812.
Ley, R. E., Turnbaugh, P. J., Klein, S. and Gordon, J. I. Microbial ecology: human gut microbes associated with obesity. Nature (2006) **444** (7122): 1022-1023.
Li, Y., de Haar, C., Chen, M., Deuring, J., Gerrits, M. M., Smits, R., Xia, B., Kuipers, E. J. and van der Woude, C. J. Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis. Gut (2010) **59** (2): 227-235.
Liu, G., Zhang, L. and Zhao, Y. Modulation of immune responses through direct activation of Toll-like receptors to T cells. Clin Exp Immunol (2010) **160** (2): 168-175.
Liu, Z., Yang, L., Cui, Y., Wang, X., Guo, C., Huang, Z., Kan, Q. and Liu, Y. Il-21 enhances NK cell activation and cytolytic activity and induces Th17 cell differentiation in inflammatory bowel disease. Inflamm Bowel Dis (2009) **15** (8): 1133-1144.
Liu, Z., Qin, H., Yang, Z., Xia, Y., Liu, W., Yang, J., Jiang, Y., Zhang, H., Wang, Y. and Zheng, Q. Randomised clinical trial: the effects of perioperative probiotic treatment on barrier function and post-operative infectious complications in colorectal cancer surgery - a double-blind study. Aliment Pharmacol Ther (2011) **33** (1): 50-63.
Louis, P., Young, P., Holtrop, G. and Flint, H. J. Diversity of human colonic butyrate-producing bacteria revealed by analysis of the butyryl-CoA:acetate CoA-transferase gene. Environ Microbiol (2010) **12** (2): 304-314.
Ludviksson, B. R., Gray, B., Strober, W. and Ehrhardt, R. O. Dysregulated intrathymic development in the IL-2-deficient mouse leads to colitis-inducing thymocytes. J Immunol (1997) **158** (1): 104-111.
Macfarlane, G. T. and Macfarlane, S. Bacteria, colonic fermentation, and gastrointestinal health. J AOAC Int (2012) **95** (1): 50-60.
Macpherson, A. J., Geuking, M. B., Slack, E., Hapfelmeier, S. and McCoy, K. D. The habitat, double life, citizenship, and forgetfulness of IgA. Immunol Rev (2012) **245** (1): 132-146.
Maldonado-Contreras, A. L. and McCormick, B. A. Intestinal epithelial cells and their role in innate mucosal immunity. Cell Tissue Res (2011) **343** (1): 5-12.
Maloy, K. J. and Powrie, F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature (2011) **474** (7351): 298-306.
Marchesi, J. R. Human distal gut microbiome. Environ Microbiol (2011) **13** (12): 3088-3102.
Marchesi, J. R., Dutilh, B. E., Hall, N., Peters, W. H., Roelofs, R., Boleij, A. and Tjalsma, H. Towards the human colorectal cancer microbiome. PLoS One (2011) **6** (5): e20447.
Mashimo, H., Wu, D. C., Podolsky, D. K. and Fishman, M. C. Impaired defense of intestinal mucosa in mice lacking intestinal trefoil factor. Science (1996) **274** (5285): 262-265.
Mestecky, J., Moro, I., Kerr, M.A., Woof, J.M. (2005). Mucosal immunoglobulins. Mucosal Immunology. J. Mestecky, Bienenstock, J., Lamm, M.E., McGhee, J., Strober, W., Mayer, L. Amsterdam, Elsevier - Academic Press**:** pp 153-182.
Miyoshi, Y., Nagase, H., Ando, H., Horii, A., Ichii, S., Nakatsuru, S., Aoki, T., Miki, Y., Mori, T. and Nakamura, Y. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Human molecular genetics (1992) **1** (4): 229-233.
Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science (1997) **275** (5307): 1787-1790.
Morris, G. P., Beck, P. L., Herridge, M. S., Depew, W. T., Szewczuk, M. R. and Wallace, J. L. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology (1989) **96** (3): 795-803.
Moser, A. R., Luongo, C., Gould, K. A., McNeley, M. K., Shoemaker, A. R. and Dove, W. F. ApcMin: a mouse model for intestinal and mammary tumorigenesis. Eur J Cancer (1995) **31A** (7-8): 1061-1064.

Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M. and Cheroutre, H. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science (2007) **317** (5835): 256-260.
Nava, G. M. and Stappenbeck, T. S. Diversity of the autochthonous colonic microbiota. Gut Microbes (2011) **2** (2): 99-104.
Neufert, C., Becker, C. and Neurath, M. F. An inducible mouse model of colon carcinogenesis for the analysis of sporadic and inflammation-driven tumor progression. Nat Protoc (2007) **2** (8): 1998-2004.
O'Mahony, L., Feeney, M., O'Halloran, S., Murphy, L., Kiely, B., Fitzgibbon, J., Lee, G., O'Sullivan, G., Shanahan, F. and Collins, J. K. Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice. Aliment Pharmacol Ther (2001) **15** (8): 1219-1225.
Oldenburg, M., Kruger, A., Ferstl, R., Kaufmann, A., Nees, G., Sigmund, A., Bathke, B., Lauterbach, H., Suter, M., Dreher, S., Koedel, U., Akira, S., Kawai, T., Buer, J., Wagner, H., Bauer, S., Hochrein, H. and Kirschning, C. J. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. Science (2012) **337** (6098): 1111-1115.
Osawa, E., Nakajima, A., Fujisawa, T., Kawamura, Y. I., Toyama-Sorimachi, N., Nakagama, H. and Dohi, T. Predominant T helper type 2-inflammatory responses promote murine colon cancers. Int J Cancer (2006) **118** (9): 2232-2236.
Osterlund, P., Ruotsalainen, T., Korpela, R., Saxelin, M., Ollus, A., Valta, P., Kouri, M., Elomaa, I. and Joensuu, H. Lactobacillus supplementation for diarrhoea related to chemotherapy of colorectal cancer: a randomised study. Br J Cancer (2007) **97** (8): 1028-1034.
Pala, V., Sieri, S., Berrino, F., Vineis, P., Sacerdote, C., Palli, D., Masala, G., Panico, S., Mattiello, A., Tumino, R., Giurdanella, M. C., Agnoli, C., Grioni, S. and Krogh, V. Yogurt consumption and risk of colorectal cancer in the Italian European prospective investigation into cancer and nutrition cohort. Int J Cancer (2011) **129** (11): 2712-2719.
Paolillo, R., Romano Carratelli, C., Sorrentino, S., Mazzola, N. and Rizzo, A. Immunomodulatory effects of Lactobacillus plantarum on human colon cancer cells. Int Immunopharmacol (2009) **9** (11): 1265-1271.
Pierik, M., Joossens, S., Van Steen, K., Van Schuerbeek, N., Vlietinck, R., Rutgeerts, P. and Vermeire, S. Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases. Inflamm Bowel Dis (2006) **12** (1): 1-8.
Pollard, J. W. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer (2004) **4** (1): 71-78.
Popivanova, B. K., Kitamura, K., Wu, Y., Kondo, T., Kagaya, T., Kaneko, S., Oshima, M., Fujii, C. and Mukaida, N. Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. J Clin Invest (2008) **118** (2): 560-570.
Rafter, J., Bennett, M., Caderni, G., Clune, Y., Hughes, R., Karlsson, P. C., Klinder, A., O'Riordan, M., O'Sullivan, G. C., Pool-Zobel, B., Rechkemmer, G., Roller, M., Rowland, I., Salvadori, M., Thijs, H., Van Loo, J., Watzl, B. and Collins, J. K. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. Am J Clin Nutr (2007) **85** (2): 488-496.
Rakoff-Nahoum, S. and Medzhitov, R. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. Science (2007) **317** (5834): 124-127.
Ramensky, V., Bork, P. and Sunyaev, S. Human non-synonymous SNPs: server and survey. Nucleic Acids Res (2002) **30** (17): 3894-3900.
Rescigno, M. and Di Sabatino, A. Dendritic cells in intestinal homeostasis and disease. J Clin Invest (2009) **119** (9): 2441-2450.
Rizzo, A., Pallone, F., Monteleone, G. and Fantini, M. C. Intestinal inflammation and colorectal cancer: a double-edged sword? World J Gastroenterol (2011) **17** (26): 3092-3100.
Robinson, C. J., Bohannan, B. J. and Young, V. B. From structure to function: the ecology of host-associated microbial communities. Microbiol Mol Biol Rev (2010) **74** (3): 453-476.
Rodriguez-Concepcion, M. and Boronat, A. Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. Plant Physiol (2002) **130** (3): 1079-1089.
Roediger, W. E., Duncan, A., Kapaniris, O. and Millard, S. Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. Gastroenterology (1993) **104** (3): 802-809.
Round, J. L., Lee, S. M., Li, J., Tran, G., Jabri, B., Chatila, T. A. and Mazmanian, S. K. The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. Science (2011) **332** (6032): 974-977.
Ruemmele, F. M., Targan, S. R., Levy, G., Dubinsky, M., Braun, J. and Seidman, E. G. Diagnostic accuracy of serological assays in pediatric inflammatory bowel disease. Gastroenterology (1998) **115** (4): 822-829.
Saito, H., Kanamori, Y., Takemori, T., Nariuchi, H., Kubota, E., Takahashi-Iwanaga, H., Iwanaga, T. and Ishikawa, H. Generation of intestinal T cells from progenitors residing in gut cryptopatches. Science (1998) **280** (5361): 275-278.

Salcedo, R., Worschech, A., Cardone, M., Jones, Y., Gyulai, Z., Dai, R. M., Wang, E., Ma, W., Haines, D., O'HUigin, C., Marincola, F. M. and Trinchieri, G. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. J Exp Med (2010) **207** (8): 1625-1636.

Sartor, R. B. Microbial influences in inflammatory bowel diseases. Gastroenterology (2008) **134** (2): 577-594.

Savoia, E., Testa, M. A., Biddinger, P. D., Cadigan, R. O., Koh, H., Campbell, P. and Stoto, M. A. Assessing public health capabilities during emergency preparedness tabletop exercises: reliability and validity of a measurement tool. Public Health Rep (2009) **124** (1): 138-148.

Segditsas, S., Rowan, A. J., Howarth, K., Jones, A., Leedham, S., Wright, N. A., Gorman, P., Chambers, W., Domingo, E., Roylance, R. R., Sawyer, E. J., Sieber, O. M. and Tomlinson, I. P. APC and the three-hit hypothesis. Oncogene (2009) **28** (1): 146-155.

Sechi, L. A., Gazouli, M., Sieswerda, L. E., Molicotti, P., Ahmed, N., Ikonomopoulos, J., Scanu, A. M., Paccagnini, D. and Zanetti, S. Relationship between Crohn's disease, infection with Mycobacterium avium subspecies paratuberculosis and SLC11A1 gene polymorphisms in Sardinian patients. World J Gastroenterol (2006) **12** (44): 7161-7164.

Shanahan, F. The host-microbe interface within the gut. Best Pract Res Clin Gastroenterol (2002) **16** (6): 915-931.

Shen, X. J., Rawls, J. F., Randall, T., Burcal, L., Mpande, C. N., Jenkins, N., Jovov, B., Abdo, Z., Sandler, R. S. and Keku, T. O. Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. Gut Microbes (2010) **1** (3): 138-147.

Sheridan, B. S. and Lefrancois, L. Intraepithelial lymphocytes: to serve and protect. Curr Gastroenterol Rep (2010) **12** (6): 513-521.

Shibolet, O. and Podolsky, D. K. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. Am J Physiol Gastrointest Liver Physiol (2007) **292** (6): G1469-1473.

Shida, K., Kiyoshima-Shibata, J., Nagaoka, M., Watanabe, K. and Nanno, M. Induction of interleukin-12 by Lactobacillus strains having a rigid cell wall resistant to intracellular digestion. J Dairy Sci (2006) **89** (9): 3306-3317.

Shirafuji, Y., Tanabe, H., Satchell, D. P., Henschen-Edman, A., Wilson, C. L. and Ouellette, A. J. Structural determinants of procryptdin recognition and cleavage by matrix metalloproteinase-7. J Biol Chem (2003) **278** (10): 7910-7919.

Siegmund, B. Interleukin-18 in intestinal inflammation: friend and foe? Immunity (2010) **32** (3): 300-302.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L. G., Gratadoux, J. J., Blugeon, S., Bridonneau, C., Furet, J. P., Corthier, G., Grangette, C., Vasquez, N., Pochart, P., Trugnan, G., Thomas, G., Blottiere, H. M., Dore, J., Marteau, P., Seksik, P. and Langella, P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A (2008) **105** (43): 16731-16736.

Spor, A., Koren, O. and Ley, R. Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Rev Microbiol (2011) **9** (4): 279-290.

Standiford, T. J., Kuick, R., Bhan, U., Chen, J., Newstead, M. and Keshamouni, V. G. TGF-beta-induced IRAK-M expression in tumor-associated macrophages regulates lung tumor growth. Oncogene (2011) **30** (21): 2475-2484.

Stecher, B. and Hardt, W. D. Mechanisms controlling pathogen colonization of the gut. Curr Opin Microbiol (2011) **14** (1): 82-91.

Stepankova, R., Sinkora, J., Hudcovic, T., Kozakova, H. and Tlaskalova-Hogenova, H. Differences in development of lymphocyte subpopulations from gut-associated lymphatic tissue (GALT) of germfree and conventional rats: effect of aging. Folia Microbiol (Praha) (1998) **43** (5): 531-534.

Stepankova, R., Tonar, Z., Bartova, J., Nedorost, L., Rossman, P., Poledne, R., Schwarzer, M. and Tlaskalova-Hogenova, H. Absence of microbiota (germ-free conditions) accelerates the atherosclerosis in ApoE-deficient mice fed standard low cholesterol diet. J Atheroscler Thromb (2010) **17** (8): 796-804.

Suzuki, R., Kohno, H., Sugie, S., Nakagama, H. and Tanaka, T. Strain differences in the susceptibility to azoxymethane and dextran sodium sulfate-induced colon carcinogenesis in mice. Carcinogenesis (2006) **27** (1): 162-169.

Swidsinski, A., Ladhoff, A., Pernthaler, A., Swidsinski, S., Loening-Baucke, V., Ortner, M., Weber, J., Hoffmann, U., Schreiber, S., Dietel, M. and Lochs, H. Mucosal flora in inflammatory bowel disease. Gastroenterology (2002) **122** (1): 44-54.

Talham, G. L., Jiang, H. Q., Bos, N. A. and Cebra, J. J. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. Infect Immun (1999) **67** (4): 1992-2000.

Tanaka, T., Kohno, H., Suzuki, R., Yamada, Y., Sugie, S. and Mori, H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Sci (2003) **94** (11): 965-973.

Tanaka, T., Kohno, H., Suzuki, R., Hata, K., Sugie, S., Niho, N., Sakano, K., Takahashi, M. and Wakabayashi, K. Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc(Min/+) mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. Int J Cancer (2006) **118** (1): 25-34.

Tezuka, H. and Ohteki, T. Regulation of intestinal homeostasis by dendritic cells. Immunol Rev (2010) **234** (1): 247-258.

Tlaskalova-Hogenova, H., Sterzl, J., Stepankova, R., Dlabac, V., Veticka, V., Rossmann, P., Mandel, L. and Rejnek, J. Development of immunological capacity under germfree and conventional conditions. Ann N Y Acad Sci (1983) **409**: 96-113.

Tlaskalova-Hogenova, H., Stepankova, R., Kozakova, H., Hudcovic, T., Vannucci, L., Tuckova, L., Rossmann, P., Hrncir, T., Kverka, M., Zakostelska, Z., Klimesova, K., Pribylova, J., Bartova, J., Sanchez, D., Fundova, P., Borovska, D., Srutkova, D., Zidek, Z., Schwarzer, M., Drastich, P. and Funda, D. P. 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) **8** (2): 110-120.

Tlaskalova, H., Kamarytova, V., Mandel, L., Prokesova, L., Kruml, J., Lanc, A. and Miler, I. The immune response of germ-free piglets after peroral monocontamination with living Escherichia coli strain 086. I. The fate of antigen, dynamics and site of antibody formation, nature of antibodies and formation of heterohaemagglutinins. Folia Biol (Praha) (1970) **16** (3): 177-187.

TlaskalovaHogenova, H., FarreCastany, M. A., Stepankova, R., Kozakova, H., Tuckova, L., Funda, D. P., Barot, R., Cukrowska, B., Sinkora, J., Mandel, L., Karska, K. and Kolinska, J. The gut as a lymphoepithelial organ: The role of intestinal epithelial cells in mucosal immunity. Folia Microbiol (1995) **40** (4): 385-391.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R. and Gordon, J. I. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature (2006) **444** (7122): 1027-1031.

Turner, J. R. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol (2009) **9** (11): 799-809.

Uronis, J. M., Muhlbauer, M., Herfarth, H. H., Rubinas, T. C., Jones, G. S. and Jobin, C. Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. PLoS One (2009) **4** (6): e6026.

van Wijk, F. and Cheroutre, H. Intestinal T cells: facing the mucosal immune dilemma with synergy and diversity. Semin Immunol (2009) **21** (3): 130-138.

Vannucci, L., Stepankova, R., Kozakova, H., Fiserova, A., Rossmann, P. and Tlaskalova-Hogenova, H. Colorectal carcinogenesis in germ-free and conventionally reared rats: different intestinal environments affect the systemic immunity. Int J Oncol (2008) **32** (3): 609-617.

Velcich, A., Yang, W., Heyer, J., Fragale, A., Nicholas, C., Viani, S., Kucherlapati, R., Lipkin, M., Yang, K. and Augenlicht, L. Colorectal cancer in mice genetically deficient in the mucin Muc2. Science (2002) **295** (5560): 1726-1729.

Vergara-Castaneda, H. A., Guevara-Gonzalez, R. G., Ramos-Gomez, M., Reynoso-Camacho, R., Guzman-Maldonado, H., Feregrino-Perez, A. A., Oomah, B. D. and Loarca-Pina, G. Non-digestible fraction of cooked bean (Phaseolus vulgaris L.) cultivar Bayo Madero suppresses colonic aberrant crypt foci in azoxymethane-induced rats. Food Funct (2010) **1** (3): 294-300.

Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., Brown, D., Stares, M. D., Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A. M., Lobley, G. E., Parkhill, J. and Flint, H. J. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J (2011) **5** (2): 220-230.

Wang, F., Graham, W. V., Wang, Y., Witkowski, E. D., Schwarz, B. T. and Turner, J. R. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol (2005) **166** (2): 409-419.

Wang, K. C. and Ohnuma, S. Isoprenyl diphosphate synthases. Biochim Biophys Acta (2000) **1529** (1-3): 33-48.

Wehkamp, J., Harder, J., Weichenthal, M., Schwab, M., Schaffeler, E., Schlee, M., Herrlinger, K. R., Stallmach, A., Noack, F., Fritz, P., Schroder, J. M., Bevins, C. L., Fellermann, K. and Stange, E. F. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. Gut (2004) **53** (11): 1658-1664.

Wells, J. M., Rossi, O., Meijerink, M. and van Baarlen, P. Epithelial crosstalk at the microbiota-mucosal interface. Proc Natl Acad Sci U S A (2011) **108 Suppl 1**: 4607-4614.

Wirtz, S., Neufert, C., Weigmann, B. and Neurath, M. F. Chemically induced mouse models of intestinal inflammation. Nat Protoc (2007) **2** (3): 541-546.

Worthley, D. L., Le Leu, R. K., Whitehall, V. L., Conlon, M., Christophersen, C., Belobrajdic, D., Mallitt, K. A., Hu, Y., Irahara, N., Ogino, S., Leggett, B. A. and Young, G. P. A human, double-blind, placebo-controlled, crossover trial of prebiotic, probiotic, and synbiotic supplementation: effects on luminal, inflammatory, epigenetic, and epithelial biomarkers of colorectal cancer. Am J Clin Nutr (2009) **90** (3): 578-586.

Wu, H. J., Ivanov, II, Darce, J., Hattori, K., Shima, T., Umesaki, Y., Littman, D. R., Benoist, C. and Mathis, D. Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity (2010) **32** (6): 815-827.

Xie, Q., Gan, L., Wang, J., Wilson, I. and Li, L. Loss of the innate immunity negative regulator IRAK-M leads to enhanced host immune defense against tumor growth. Mol Immunol (2007) **44** (14): 3453-3461.

Yamada, M., Noguchi-Shinohara, M., Hamaguchi, T., Nozaki, I., Kitamoto, T., Sato, T., Nakamura, Y. and Mizusawa, H. Dura mater graft-associated Creutzfeldt-Jakob disease in Japan: clinicopathological and molecular characterization of the two distinct subtypes. Neuropathology (2009) **29** (5): 609-618.

Yan, J., Greer, J. M., Etherington, K., Cadigan, G. P., Cavanagh, H., Henderson, R. D., O'Sullivan, J. D., Pandian, J. D., Read, S. J. and McCombe, P. A. Immune activation in the peripheral blood of patients with acute ischemic stroke. J Neuroimmunol (2009) **206** (1-2): 112-117.

Yang, D., Chertov, O., Bykovskaia, S. N., Chen, Q., Buffo, M. J., Shogan, J., Anderson, M., Schroder, J. M., Wang, J. M., Howard, O. M. and Oppenheim, J. J. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science (1999) **286** (5439): 525-528.

Zeissig, S., Burgel, N., Gunzel, D., Richter, J., Mankertz, J., Wahnschaffe, U., Kroesen, A. J., Zeitz, M., Fromm, M. and Schulzke, J. D. Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease. Gut (2007) **56** (1): 61-72.

Zhu, Y., Michelle Luo, T., Jobin, C. and Young, H. A. Gut microbiota and probiotics in colon tumorigenesis. Cancer Lett (2011) **309** (2): 119-127.

2 Rationale and Aims

Recent research revealed some new aspects of complex microbial ecosystem residing gastrointestinal tract. Microbiota contributes in many physiological as well as pathological processes of the host. It's commonly accepted that gut microbiota plays an important role in the pathogenesis of chronic intestinal inflammation like inflammatory bowel diseases (IBD; ulcerative colitis and Crohn's disease), and, concurrently, in inflammation-related colorectal cancer. Therefore study of the engaged mechanisms means deepening the current knowledge with further possible applications in screening, prevention and treatment of intestinal inflammation and cancer.

- Commensal bacteria have been shown to modulate the host mucosal immune system. We tested if oral treatment of BALB/c mice with components from the commensal *Parabacteroides distasonis* influences the severity of intestinal inflammation in murine models of acute and chronic colitis induced by dextran sulfate sodium (DSS).
- Probiotics can be used for the prevention and treatment of human inflammatory diseases including IBD. However, the nature of active components and exact mechanisms of these beneficial effects have not been fully elucidated. We investigated if lysate of probiotic *Lactobacillus casei* DN-114 001 decreases the severity of intestinal inflammation in a murine model of acute DSS colitis, and related mechanisms.
- We reviewed the role of microbiota in modulating gut environment and mucosal immunity, and its importance in colorectal cancer development with especial accent on experiments in germ-free animals.
- Microbial sensing by Toll-like receptors (TLR) and its negative regulation have important role in the pathogenesis of inflammation-related cancer. We investigated the role of negative regulation of TLR signaling and gut microbiota in the development of colitis-associated cancer in mouse model.
- In the intestine, the Wnt signaling pathway plays two seemingly opposing roles. Under physiological conditions it is required for the intestinal stem cells proliferation and differentialtion. Conversely, aberrant Wnt signaling leads to intestinal cancer development. We studied the regulation of the Wnt pathway in gut epithelium during malignant transformation.

3 Oral administration of *Parabacteroides distasonis* antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition

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3.1 Abstract

Commensal bacteria have been shown to modulate the host mucosal immune system. Here, 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). The membranous fraction of *P. distasonis* (mPd) prevented DSS-induced increases in several proinflammatory cytokines, increased mPd-specific serum antibodies and stabilized the intestinal microbial ecology. The anti-colitic effect of oral mPd was not observed in severe combined immunodeficient mice and probably involved induction of specific antibody responses and stabilization of the intestinal microbiota. Our results 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.

3.2 Introduction

The current hypothesis for the pathogenesis of Crohn's disease and ulcerative colitis, the two main forms of inflammatory bowel disease (IBD), involves an aberrant host immune response to luminal antigens. Although the precise cause of IBD remains unclear, the pathogenic mechanisms are multi-factorial and additional factors such as increased virulence of commensal bacterial species, disruption of the intestinal mucosal barrier and genetic susceptibility have been proposed (1).

Three lines of evidence suggest a crucial role of the intestinal microbiota in IBD pathogenesis. First, lesions in IBD predominate in areas of highest bacterial exposure (2). Secondly, manipulation of luminal content using selective antibiotics, or fecal stream diversion, improves inflammation in IBD patients (3, 4). Thirdly, in some models of IBD intestinal inflammation is attenuated, or fails to develop, if the animals are maintained under germ-free conditions (5-9). It remains to be determined whether IBD can be triggered by the presence of a disbalanced microbiota composition with enhanced proinflammatory capacity. Interestingly, the intestinal microbiota is altered (dysbiosis) in a proportion of patients with IBD, and fecal samples from patients with Crohn's disease exhibit greater temporal instability (10) and decreased number of commensal bacteria with reduction in the Firmicutes phylum (11).

The current treatment of IBD targets the effector phase of the intestinal inflammatory response. In a proportion of patients, however, the disease is refractory to conventional medical treatment, or the effectiveness of the treatment is limited by serious side effects (12). Probiotics are commensal bacteria with proven health beneficial effects. Thus, several probiotic candidates have been evaluated as an alternate and safe treatment option for IBD (13). Some randomized, placebo-controlled studies, using *Escherichia coli* Nissle 1917 and a combination of eight probiotic strains, have demonstrated a beneficial effect in IBD (14-16). However, others have failed to demonstrate significant therapeutic benefit and therefore the overall efficacy of probiotics in active chronic inflammatory conditions of the gut remains a matter of controversy (17). More importantly, mechanistic insight linked to a specific potential probiotic strain has been difficult to establish. Growing evidence indicates that experimental colitis can be mitigated not only with oral administration of live probiotic bacteria, but with bacterial components and by-products of bacteria as well (18-20). Our previous results suggest that orally administered lysates from anaerobic microbiota decrease the severity of experimental colitis (21). The aim of this study was to test the effect of oral

administration of components of a specific anaerobic strain on experimental colitis, and determine the underlying mechanisms.

3.3 Materials and Methods

3.3.1 Mice

Female BALB/c mice (6–8 weeks old) or female severe combined immunodeficient (SCID) mice BALB/cJHanHsd-SCID were obtained from a breeding colony at the Institute of Physiology (Academy of Sciences of the Czech Republic, Prague, Czech Republic) or at the Institute of Microbiology (Academy of Sciences of the Czech Republic, Novy Hradek, Czech Republic), respectively. Flow cytometry was used to exclude SCID mice that had detectable T cells. Mice were reared under conventional conditions at the Institute of Microbiology. The studies were approved by the Animal Care and Use Committee of the Institute of Microbiology.

3.3.2 Identification of candidate bacteria and preparation of bacterial components

Anaerobic bacteria from mouse intestinal microbiota were grown at 37°C in liquid medium (see Supplementary materials and methods), separated into monocultures, lysed in a French press and tested for anti-inflammatory activity in an acute colitis model. To identify single candidate anaerobic strains for subsequent experiments, groups of mice (n = 5–10/group) were orally treated with isolates of anaerobic bacteria lysates (*Parabacteroides distasonis, Bacteroides thetaiotamicron, Veillonella alcalescens*, *B. ovatus, B. vulgatus* and *B. stercoris*; see supplementary data). Their individual effect on the prevention of acute dextran sulphate sodium (DSS) colitis was evaluated (see Supplementary Tables S4 and S5). Because only the crude lysate of *P. distasonis* significantly improved clinical parameters of acute DSS colitis, all subsequent experiments in the study were performed using this isolate and its components. After cell disruption with the French press, the lysate was separated by centrifugation into two fractions, membranous (insoluble) and cytoplasmic (soluble). Lipopolysaccharides (LPS) and DNA from *P. distasonis* were isolated as described previously (22, 23).

3.3.3 Evaluation of anti-inflammatory effects of mPd on macrophages *in vitro*

Because macrophages have been proposed to play a role in acute intestinal inflammation (24, 25), we tested the antiinflammatory effect of bacterial components on the LPS-activated macrophage cell line, RAW 264·7.We cultured the cells in the presence of LPS and different concentrations of *P. distasonis* lysate or its components (see Supplementary materials and methods) and measured tumour necrosis factor (TNF)-α in supernatants by enzyme-linked immunosorbent assay (ELISA).

3.3.4 Induction and evaluation of acute and chronic colitis

Acute colitis was induced by 3% (wt/vol) DSS (mol wt = 36–50 kDa; MP Biomedicals, Irvine, CA, USA) dissolved in drinking water for 7 days *ad libitum*. For chronic colitis, mice received four cycles of DSS as described previously (25). Each cycle consisted of 3% DSS in drinking water for 7 days, followed by a 7-day interval with normal drinking water. Colitis was evaluated on the last day of the experiment using a disease activity index (DAI) described by Cooper et al. (26), a histological scoring system (see Supplementary materials and methods), and by measuring colon length. The level of acute-phase protein haptoglobin was determined in mouse serum using the modified human haptoglobin ELISA quantitation kit (GenWay Biotech, Inc., San Diego, CA, USA) (see Supplementary materials and methods). Water consumption was measured during DSS administration.

3.3.5 Overall study design

To test whether bacterial components of *P. distasonis* prevent acute DSS colitis, we administered 1.5 mg of whole lysate, LPS, membranous or cytoplasmic fraction or 200 μg of DNA in 50 μl of sterile phosphate-buffered saline (PBS) to mice by gavage. To reduce proteolytic activity in the gut, the components were co-administered with 1 mg of soybean trypsin inhibitor (Sigma-Aldrich, St Louis, MO, USA) dissolved in 50 μl of 0.15 m sodium bicarbonate buffer (pH 8.0). Control mice were given sterile PBS with soybean trypsin inhibitor in bicarbonate buffer. We repeated the administration every 7 days for a total of four doses (on days 0, 7, 14 and 21). Seven days after the last dose we induced acute DSS colitis, as explained above.

To determine whether a gut-dependent pathway is necessary for bacterial components to modulate acute colitis, additional mice were treated by four intraperitoneal (i.p.) or subcutaneous (s.c.) injections with mPd before acute colitis induction [5 mg of mPd or PBS, together with incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA)]. The dose of mPd was chosen based on preliminary experiments that determined an optimal antibody response when doses of 2.5 to 1500 μg were used.

To investigate mechanisms underlying the anti-colitic effect of mPd, serum transfer experiments from orally treated mice to untreated mice were performed. Specifically, 200 μl of the serum from either PBS or mPd-treated mice were transferred intravenously to untreated mice before acute DSS colitis induction.

To test the possible effect of mPd administration on established and chronic colitis, we administered 21 doses (as described above) of mPd by daily gavage once chronic DSS colitis had been induced, starting after the third cycle of DSS.

3.3.6 Assessment of *P. distasonis* antibodies by ELISA

We used indirect ELISA assay, optimized in our laboratory, to compare serum antibody [immunoglobulin (Ig) G, IgM and IgA] titres against *P. distasonis* lysate between PBS and mPd-treated groups (see Supplementary materials and methods).

3.3.7 Gut tissue culture and measurement of cytokines

Five sections of the intestine were obtained (Peyer's patches, jejunum, ileum, caecum and colon), and cultivated for 48 h in complete RPMI-1640 media (see Supplementary materials and methods). The supernatants were collected and frozen at -20°C until analysis for cytokine production. To evaluate changes in cytokine levels induced by DSS treatment and mPd therapy in the colon, we used the RayBio^{TM} Mouse Cytokine Array II (Raybiotech, Inc., Norcross, GA, USA) capable of detecting 32 cytokines, chemokines and growth factors (see Supplementary materials and methods; Table S2). We also used commercial ELISA kits to measure the concentrations of selected cytokines [interleukin (IL)-10, TNF-α, transforming growth factor (TGF)-β, IL-6 and interferon (IFN)-γ] (see Supplementary materials and methods).

3.3.8 Flow cytometry

Single-cell suspensions of spleens, mesenteric lymph nodes and Peyer's patches were prepared and stained for regulatory T cells (T_{regs}) using forkhead box P3 (FoxP3) staining buffer set (eBioscience, San Diego, CA, USA) with these fluorochrome-labelled anti-mouse monoclonal antibodies (mAbs): CD4-Qdot® 605 (Invitrogen, Carlsbad, CA, USA; clone RM4-5), CD25-allophycocyanin (eBioscience; clone PC61·5) and FoxP3-phycoerythrin (eBioscience; clone FJK-16 s) according to the manufacturer's recommendations. Flow cytometric analysis was performed on LSRII (BD Biosciences, San Jose, CA, USA), and data were analysed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

3.3.9 Evaluation of intestinal microbiota

We collected stool samples from five mice chosen randomly from PBS and mPd-treated groups on days 0, 28 (just before DSS administration) and 35 (the last day), and analysed the samples by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), as described (see Supplementary materials and methods). We used quantitative PCR to determine the content of all *Eubacteria*, *Bacteroides-Prevotella* group and *P. distasonis* in mouse faeces (see Supplementary materials and methods).

3.3.10 Statistical analysis

The distributions of variables were tested for normality using the D'Agostino-Pearson omnibus normality test. Differences in colon length, DAI, histological score, haptoglobin levels and TNF-α production of multiple groups were compared to the control group (PBS/DSS) by one-way analysis of variance with Dunnett's multiple comparison test. Differences in specific antibody levels, bacteria numbers, DGGE profiles, cytokine production and T_{reg} numbers between the two groups were evaluated using an unpaired two-tailed Student's *t*-test. Serum levels of *P. distasonis*-specific antibodies were compared to the amount of *P. distasonis* in the stool samples using the Pearson correlation coefficient (*r*). The values are expressed as means ± standard deviation (s.d.) 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.

3.4 Results

3.4.1 Anti-inflammatory properties of *Parabacteroides distasonis in vivo* and *in vitro*

From the anaerobic lysates tested, only the crude lysate obtained from *Parabacteroides distasonis*, and especially its membranous fraction (mPd), was able to decrease disease severity significantly after induction of acute DSS colitis (Table 1 and Supplementary Table S4). To test whether the anti-colitic activity observed with *P. distasonis* lysate (Pd) and mPd involved innate immune cells, we treated LPS-activated RAW264·7 macrophage cells with Pd or mPd *in vitro*. In concentrations above 10 ng/l, Pd and mPd decreased the production of TNF-α, suggesting that they can both directly decrease the inflammatory activity of RAW264.7 macrophages (Supplementary Fig. S5). Neither cytoplasmic fraction of *P. distasonis* lysate (cPd) nor DNA decreased TNF-α production *in vitro* (data not shown).

Table 1. Evaluation of acute dextran sulphate sodium (DSS)-induced colitis in BALB/c and SCID mice orally treated with Parabacteroides distasonis components.

Mouse strain	*Experimental group*	*Colon length (cm)*	*Disease activity index*	*Histological grade*	*Serum haptoglobin (g/l)*
BALB/c	PBS (control)	5.97±0.46	3.13±0.74	2.05±0.58	1.49±0.64
	mPd	6.77±0.40**	1.77±0.97**	1.36±0.51*	0.38±0.35*
	cPd	5.96±0.42	3.47±0.53	2.14±0.69	0.84±0.80
	DNA	7.32±0.44**	1.57±0.61**	1.01±0.36**	0.05±0.06**
	LPS	6.70±0.36**	2.07±1.30*	1.68±0.43	0.36±0.08**
	mPd without DSS	8.97±0.40**	0.00±0.00**	0.15±0.13**	0.00±0.01**
	PBS without DSS	9.26±0.41**	0.00±0.00**	0.10±0.20**	0.02±0.01**
SCID	PBS (control)	6.20±0.55	4.00±0.00	2.69±0.49	*Not done*
	mPd	6.13±0.63	3.89±0.17	2.58±0.46	*Not done*

Data are representative of one experiment. Similar results were obtained from three independent experiments. Values are expressed as means ± standard deviation from six to 10 mice per group. One-way analysis of variance with Dunnett's multiple comparison test (in BALB/c mice) or unpaired Student's t-test [severe combined immunodeficient (SCID) mice] were used to evaluate differences between experimental groups and phosphate-buffered saline (PBS)-treated controls. cPd: cytoplasmic fraction of P. distasonis lysate; mPd: membranous fraction of P. distasonis; LPS: lipopolysaccharide (*P < 0.05; **P < 0.01).

3.4.2 Components of *P. distasonis* attenuate DSS colitis in BALB/c mice

Both oral mPd and DNA isolated from *P. distasonis* were effective in preventing acute DSS colitis in BALB/c mice, improving clinical, serological and morphological markers of colitis (Table 1). This effect was seen only with oral administration and was not observed with intraperitoneal or subcutaneous administration of mPd (see Supplementary information; Table S6). In contrast to mPd and DNA treatments, oral administration of cPd did not have a protective effect (Table 1). Orally administered mPd did not prevent colitis in SCID mice (Table 1), suggesting that mechanisms of adaptive immunity are necessary for this effect.

Therapeutic administration of mPd improved colonic length and the severity of clinical scores, but did not affect histological scores (Table 2).

Table 2. Evaluation of chronic dextran sulphate sodium (DSS)-induced colitis in BALB/c mice orally treated with Parabacteroides distasonis components.

Experimental group	*Colon length (cm)*	*Disease activity index*	*Histological grade*	*Serum haptoglobin (g/l)*
PBS	6.25±0.37	3.08±0.39	1.56±0.35	0.91±0.60
mPd	6.79±0.51*	2.25±0.56*	1.59±0.28	0.20±0.15**
cPd	6.46±0.40	3.08±0.39	1.63±0.31	0.09±0.05**
LPS	6.96±0.34*	2.82±0.65	1.51±0.37	0.06±0.02**
PBS without DSS	9.22±0.83**	0.73±0.26**	0.14±0.14**	0.01±0.01**
mPd without DSS	8.89±0.60**	0.45±0.40**	0.32±0.20**	0.01±0.01**

Data are representative of one experiment. Similar results were obtained from two independent experiments. Values are expressed as means ± standard deviation (10 mice per group). One-way analysis of variance with Dunnett's multiple comparison test was used to evaluate differences between experimental groups and phosphate-buffered saline (PBS)-treated controls (*P < 0.05; **P < 0.01). mPd: membranous fraction of *P. distasonis*; cPd: cytoplasmic fraction of P. distasonis lysate; LPS: lipopolysaccharide.

3.4.3 Effect of oral mPd on specific antibodies in serum

Serum titres of anti-*P. distasonis* antibodies were significantly higher ($P < 0.001$) in mice treated orally with mPd compared to PBS-treated mice (IgA: 0.19 ± 0.07 *versus* 0.05 ± 0.02; IgM: 0.56 ± 0.19 *versus* 0.15 ± 0.05 and IgG: 0.46 ± 0.17 *versus* 0.02 ± 0.02; n = 10). Furthermore, serum antibody titres correlated strongly with the amount of *P. distasonis* in faeces on day 28 ($r = 0.99$ for IgA, $r = 0.92$ for IgG and $r = 0.90$ for IgM; $P < 0.01$). The concentration of the specific co-proantibodies was below the detection level in all groups.

To investigate the potential protective role of specific antibodies in serum we performed serum transfer experiments. Indeed, serum transfer from mice orally treated with mPd to naive mice decreased the severity of DSS colitis (Table 3).

Table 3. Evaluation of acute dextran sulphate sodium (DSS)-induced colitis in conventional BALB/c mice, after transfer of serum from mice treated orally with membranous fraction of *Parabacteroides distasonis* (mPd) or phosphate-buffered saline (PBS).

Experimental group	*Colon length (cm)*	*Disease activity index*	*Histological grade*
Serum from PBS treated	6.10±0.58	2.67±1.08	1.45±0.36
Serum from mPd treated	7.82±0.15**	0.40±0.37**	0.35±0.06*

Data are representative of one experiment. Similar results were obtained from three independent experiments. Values are expressed as means ± standard deviations (five mice per group). An unpaired Student's t-test was used to calculate the significance of differences between the mPd-treated group and the PBS-treated group (*P < 0.05; **P < 0.01).

3.4.4 The production of cytokines in gut tissues

To determine the effect of mPd therapy on cytokine production, colonic cytokine profiles from PBS-treated healthy mice, DSS/PBS-treated mice and DSS/mPd-treated mice were compared using cytokine antibody array (Fig. 1). Treatment with mPd prevented DSS-induced increases in several proinflammatory cytokines, including IFN-γ, IL-12, IL-17 and IL-6. Similarly, an overall decrease in both proinflammatory and anti-inflammatory cytokines was detected in Peyer's patches (IL-6, TGF-β and IFN-γ), caecum (IL-10, TNF-α, TGF-β and IFN-γ) and colon (IL-10, TNF-α, IL-6, TGF-β and IFN-γ) of DSS/mPd-treated mice compared to the DSS/PBS group (Fig. 2), as measured by ELISA. No significant differences in cytokine production were found in the ileum, jejunum mucosa (data not shown) or spleen cells (data not shown). mPd treatment did not change the production of cytokines in SCID mice except for an increase in IL-10 production in the colon (see Supplementary information; Fig. S6).

3.4.5 Effect of oral mPd on T_{regs}

We measured the number of T_{regs} ($CD4^+CD25^+FoxP3^+$ cells) in the spleen, mesenteric lymph nodes and Peyer's patches of control and mPd-treated mice. We found that after DSS treatment, mice treated with mPd had significantly more $CD4^+CD25^+FoxP3^+$ cells in their

mesenteric lymph nodes (mean ± s.d.; 3.40 ± 0.50 *versus* 4.81 ± 0.30; $P = 0.014$) than PBS-treated (control) mice (Supplementary information; Fig. S3). There were no differences between mPd-treated and control groups in T_{regs} numbers in spleen or Peyer's patches (data not shown).

Figure 1. Pretreatment with membranous fraction of *Parabacteroides distasonis* (mPd) decreases the dextran sulphate sodium (DSS)-related increase in cytokine production in colon tissue as measured by cytokine antibody array. Values represent the percentage of the intensity of positive control. Granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-1–12p40p70, macrophage inflammatory protein-1α (MIP), stem cell factor (SCF), growth-regulated alpha protein precursor (KC), tissue inhibitor of metalloproteinases-1 (TIMP), tumour necrosis factor-α (TNF), thrombopoietin (TPO) and vascular endothelial growth factor (VEGF). Data are means (bars) and standard deviation (whisker) of three samples [*P < 0·05 *versus* DSS/phosphate-buffered saline (PBS)].

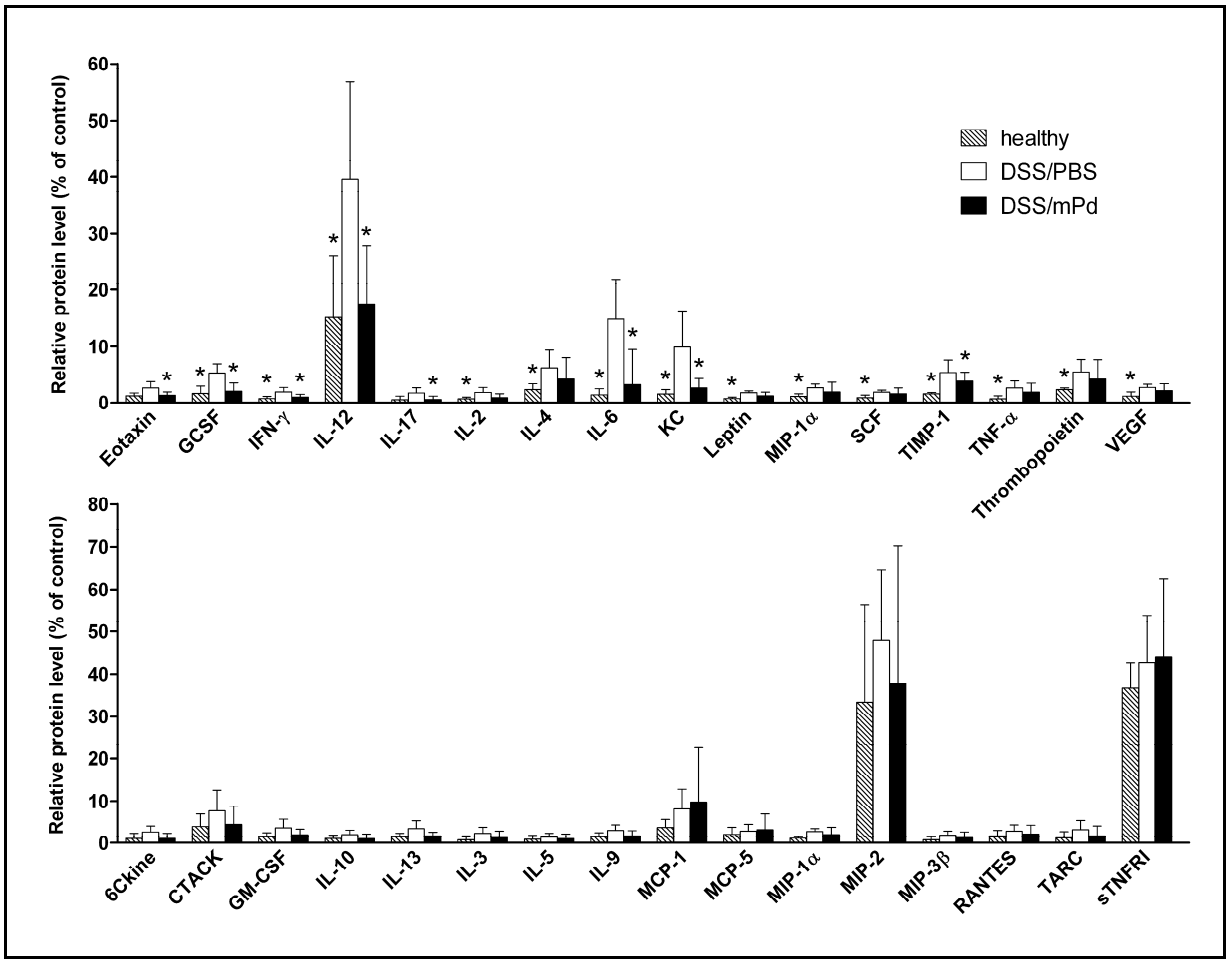
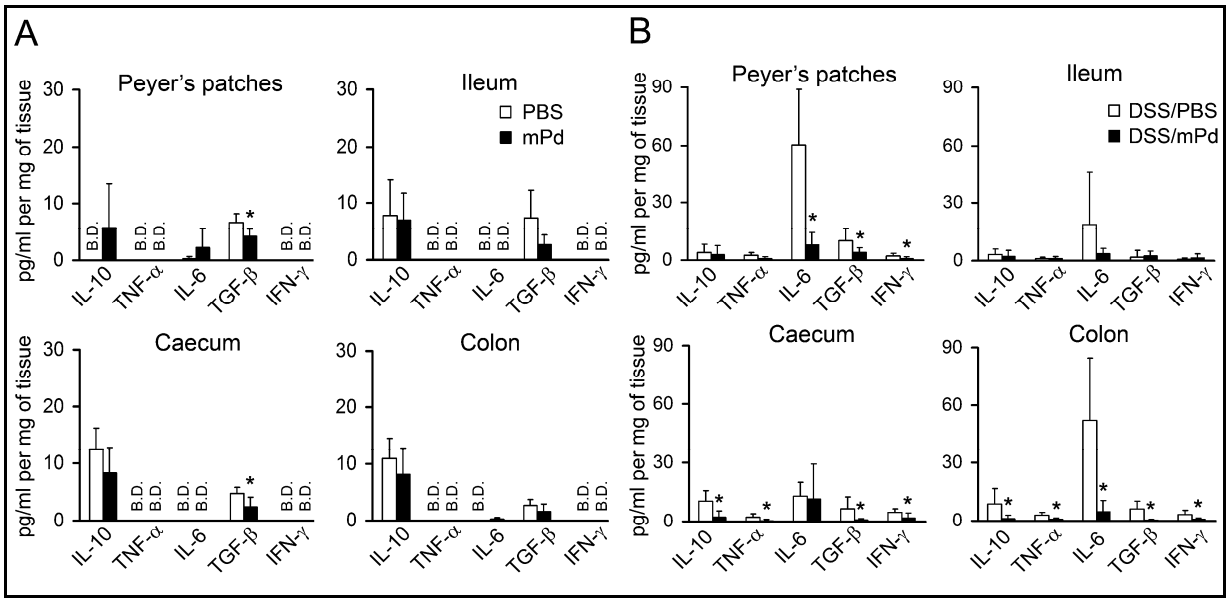


Figure 2. Pretreatment with membranous fraction of *Parabacteroides distasonis* (mPd) decreases cytokine production (pg/mg of tissue) in different parts of the gut in orally treated BALB/c mice as measured by enzyme-linked immunosorbent assay (ELISA). *$P < 0{\cdot}05$ between mPd and phosphate-buffered saline (PBS) (a) or dextran sulphate sodium (DSS)/mPd and DSS/PBS (b)-treated mice. (B.D.) Values below the detection limit; $n = 5$ mice per group.

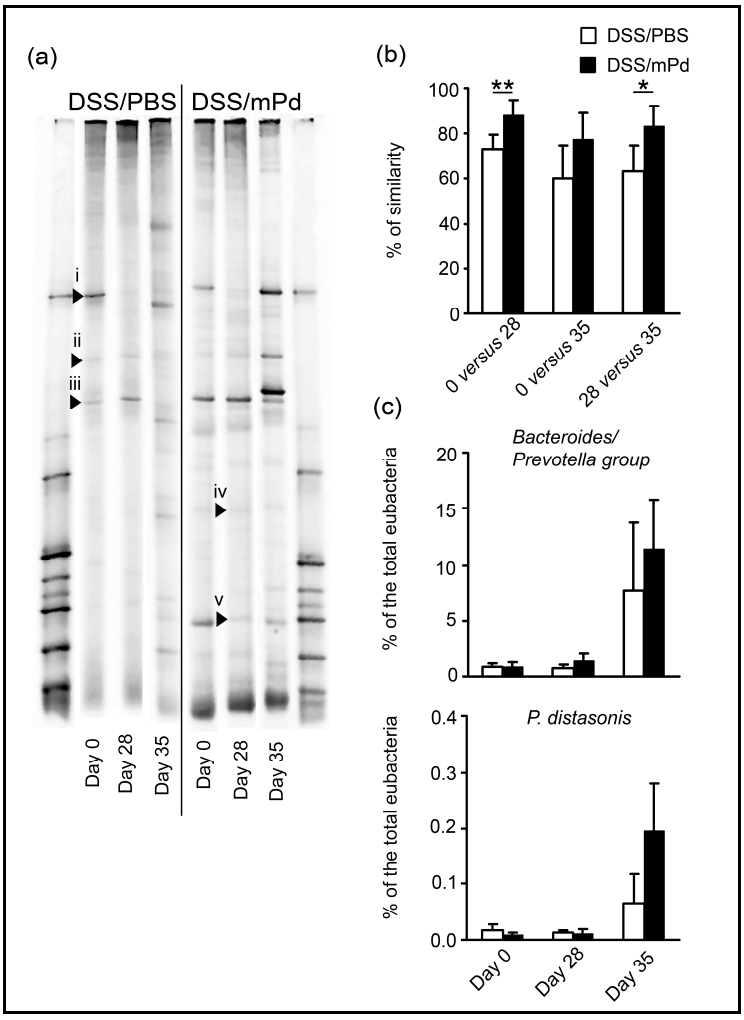


3.4.6 Oral treatment with mPd does not affect the *P. distasonis* or *Bacteroides/Prevotela* group stool content but stabilizes the gut microbial ecology

Before colitis induction, the microbiota composition in PBS and mPd-treated groups was similar. In both groups *Lactobacillus* sp. and *Bacteroides acidofaciens* were present, as assessed by PCR-DGGE (Fig. 3a). In contrast, marked changes in microbiota composition were observed during DSS administration in the PBS-treated control group, as demonstrated in Fig. 3b.

Quantitative PCR on the *Bacteroides/Prevotela* group and *P. distasonis* showed a statistically significant increase of these bacteria during DSS treatment from 0.84 ± 0.39% to 9.58 ± 6.12% ($P < 0.05$) and from 0.01 ± 0.01% to 0.13 ±_0.11% ($P < 0.05$) of total *Eubacteria*, respectively. However, no statistically significant differences in bacteria numbers were found between the mPd-treated and control groups on the same day of the experiment (Fig. 3c).

Figure 3. Oral treatment with membranous fraction of *Parabacteroides distasonis* (mPd) stabilizes the intestinal microbiota without changing the *P. distasonis* or *Bacteroides/Prevotela* group stool content. (a) Changes in fecal bacterial populations of dextran sulphate sodium/phosphate-buffered saline (DSS/PBS) and DSS/mPd-treated mice were measured by 16S rRNA gene polymerase chain reaction- denaturing gradient gel electrophoresis (PCR-DGGE) before the treatment day 0 (before colitis induction), day 28 and at the end of the experiment (day 35). (b) Comparison of changes in DGGE profiles between PBS and mPd-treated mice at different time-points. (c) Representative quantitative changes in the *Bacteroides/Prevotela* group or *P. distasonis* in stool were measured by quantitative PCR. Data are expressed as a percentage of total *Eubacteria* [bar (mean); whisker (standard deviation)]. Samples from the same mouse at different time-points were arranged together. The bands marked with arrows were identified as *B. acidofaciens*, 100% (i), uncultured bacterium related to Clostridium (ii), *Lactobacillus johnsonii*, 98% and *L. gasseri*, 98% (iii), *H. muridarum*, 95% (iv) and *Lactobacillus* sp. 97% (v) by 16S rRNA sequence analysis.



3.5 Discussion

Manipulation of the intestinal microbiota by oral administration of probiotic bacteria or selective antibiotics has emerged as a potentially useful therapeutic strategy for human IBD (4, 14-16). However, the clinical utility of such an approach remains controversial, as the link between specific mechanisms of action and therapeutic effects of specific strains or bacterial components has been difficult to establish. We have shown previously that oral administration of crude lysates from anaerobic bacteria attenuates the severity of experimental colitis (21). In this study we identified a specific anaerobic lysate of *P. distasonis* and its cellular components with anti-inflammatory capacity. We investigated the possible mechanisms of action using in vitro techniques and in vivo acute and chronic DSS colitis.

The intestinal microbiota is a complex ecosystem which consists of high levels of obligate anaerobes (making upmore than 90% of the microbiota). This system is in constant interaction with the host's immune system. Commensal bacteria play an essential role in mucosal immune system development, and dysregulated immune responses to opportunistic commensals have been suggested to play a role in intestinal inflammation (1, 5, 27-29). Moreover, it has been proposed that microbiota composition can be regulated by the host's immune system (29-31).

We found that oral administration of the membranous fraction of *P. distasonis* (mPd) and its DNA were effective in suppressing acute DSS colitis. mPd also attenuated chronic colitis; however, the effect was less marked. The results are consistent with previous observations that prevention of inflammatory bowel disease is achieved more easily than treatment of ongoing inflammation (19). Several mechanisms may underlie the protective effect of oral mPd in colitis. We investigated whether oral mPd (a) changes local gut cytokine production, resulting in a nonspecific anti-inflammatory milieu (b) stimulates adaptive immune mechanisms (vaccination effect) and/or (c) stabilizes intestinal microbiota composition, thereby rendering the mice less susceptible to DSS colitis. Acute DSS colitis is believed to be driven initially by innate immunity mechanisms and, in particular, the role of macrophages has been suggested (5, 24, 25, 32). We tested the ability of Pd and mPd to decrease the TNF-α production by LPS-activated macrophages in vitro. We found that at concentrations above 10 ng/l both Pd and mPd decreased TNF-α production by macrophages, suggesting a possible direct effect of Pd and mPd on innate cell immunity. This mechanism may contribute to the attenuation of acute DSS injury observed in vivo by Pd and mPd. Because probiotics and their isolated DNA have been shown to attenuate the DSS colitis via

Toll-like receptor (TLR)-dependent pathways (20, 33), the involvement of pattern recognition receptors in the initiation of the protective effect by mPd and DNA cannot be ruled out.

Our results show that oral mPd decreases the production of many proinflammatory cytokines, including TNF-α, but also of anti-inflammatory cytokines in the colon of treated mice. The changes were observed in Peyer's patches, caecum and colon of mPd-treated mice compared to control mice, suggesting local mucosal effects of mPd throughout the intestine. This immunomodulatory activity of mPd may interfere with both leucocyte accumulation in intestinal mucosa and with barrier function failure, and contribute to decrease inflammation (34, 35). Our results are consistent with previous work that reported a decrease in proinflammatory, as well as anti-inflammatory, cytokine production in mice treated with DSS and live probiotic *E. coli Nissle* 1917 (33).

It is known that some components of the indigenous microbiota have immunomodulatory properties and affect cytokine production (20, 36-38). Mazmanian et al. have shown that changes in local cytokine production caused by the common commensal bacterium *B. fragilis* and its polysaccharide A protected mice from experimental colitis (39). Unfortunately, changes in microbiota composition or a polysaccharide A-specific immune response were not investigated in that study. Anti-TNF-α-based therapies have been shown to be effective in flare-ups of chronic inflammatory disorders, including IBD and rheumatoid arthritis (40, 41).

The presence of serum antibodies directed against commensals in IBD patients suggests that some patients may exhibit systemic priming against microbiota (42). The pathophysiological significance of this priming remains unclear, but may suggest a role of adaptive immune mechanisms in the luminal containment of microbiota components in patients with mucosal damage. Indeed, a recent study has proposed that systemic immune responses may compensate for innate immune deficiency and constitute a novel homeostatic mechanism in host–microbiota mutualism (43). Antibodies could occur as a consequence of increased penetration of microbiota components through impaired mucus and epithelial layers. These antibodies may play not only a diagnostic (42), but also a protective role against microbes that could perpetuate intestinal inflammation. Moreover, if bacterial epitopes are shared among several bacterial species (molecular mimicry) generation of antibodies against common commensals could constitute potential therapeutic targets in IBD. After oral administration of mPd, we found increased levels of *P. distasonis*-specific antibodies in sera compared to controls, suggesting the possibility that specific immune responses to *P. distasonis* are protective against experimental colitis. The identification of the specific

antibody fraction responsible for this effect is being examined currently in our laboratories. Although both oral and parenteral forms of administration of mPd increased the levels of specific serum antibodies, only oral administration had an effect on colitis prevention. This suggests that the gut microenvironment during antigen priming is essential for colitis prevention, but once established it can be transferred with serum. To investigate further the role of adaptive immunity in the anti-colitic effect of mPd, we studied immunodeficient mice lacking T and B lymphocytes. Although the severity of DSS-induced acute inflammation in SCID mice was similar to that in immunocompetent mice (5, 32), colitis was not prevented in SCID mice with oral mPd. One limitation of the comparison between BALB/c and SCID mice relates to differences in gut microbiota composition and/or innate immune cell activity between strains (44). The innate and adaptive immune systems work in synergy to mount appropriate immune responses to the commensal microbiota and maintain homeostasis (43). As suggested by our in vitro experiments, we cannot rule out the involvement of innate immune mechanisms in the initiation of mPd-induced protection. However, the in vivo experiments show clearly that the adaptive immune response is required for mPd-induced protection.

Our results also suggest a role for Tregs in this mPd-induced protection. We show that there is an increase in both CD4+FoxP3+ and CD4+FoxP3– T cells in MLN of mPd-treated mice. The increase in CD4+FoxP3+ cells is, however, proportionally higher, therefore the increase in absolute numbers of Tregs cannot be explained solely by the increase in CD4+ cells. The significance of this finding requires confirmation in future experiments. Previous work has proposed that the presence of microbiota and bacterial components in the gut can influence Treg activity (36, 45). Thus, future studies will address the relative importance of induction of protective immunity (vaccination) and of tolerance induction in the prevention and treatment of acute and chronic colitis by mPd.

In accordance with previous work (25), we found that DSS colitis alters the intestinal ecosystem with an increase in *P. distasonis*. Interestingly, we found that oral treatment with mPd prevents the microbiota changes caused by DSS. The mechanism by which mPd stabilizes the microbiota composition during DSS is not clear, but could be secondary to improvement in inflammation. Alternatively, mPd may directly affect other bacteria, by a direct antimicrobial effect, or indirectly, by improving epithelial barrier function or regulation of the mucosal immune system. The latter has been demonstrated previously for probiotic candidates *P. freudenreichii* and *F. prausnitzii* (37, 38).

In conclusion, oral administration of *P. distasonis* components (mPd) protects from experimentally induced intestinal inflammation through several innate and adaptive immunomodulatory mechanisms. Oral mPd promotes an increase in the level of mPd-specific antibodies and in the numbers of Tregs. In addition, oral mPd inhibits TNF-α production by macrophages in vitro and stabilizes the intestinal microbiota. These results highlight the importance of individualizing and characterizing the potential capacity of commensal bacteria as immunomodulatory agents. Moreover, oral administration of sterile bacterial components, in contrast to live bacteria, may be safer in severely ill or immunocompromised patients. Our results support the hypothesis that oral supplements consisting of components from specific commensals may lead to the development of new therapeutic approaches for chronic intestinal inflammation.

3.6 Acknowledgement

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Disclosure

The authors declare that they have no conflict of interest related to the publication of this manuscript.

3.7 References

1. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology. 2008 Feb;134(2):577-94.
2. Seksik P, Sokol H, Lepage P, Vasquez N, Manichanh C, Mangin I, et al. Review article: the role of bacteria in onset and perpetuation of inflammatory bowel disease. Aliment Pharmacol Ther. 2006 Oct;24 Suppl 3:11-8.
3. Rutgeerts P, Goboes K, Peeters M, Hiele M, Penninckx F, Aerts R, et al. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. Lancet. 1991 Sep 28;338(8770):771-4.
4. Prantera C, Scribano ML. Antibiotics and probiotics in inflammatory bowel disease: why, when, and how. Curr Opin Gastroenterol. 2009 Jul;25(4):329-33.
5. Hudcovic T, Stepankova R, Cebra J, Tlaskalova-Hogenova H. 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). 2001;46(6):565-72.
6. Rath HC, Herfarth HH, Ikeda JS, Grenther WB, Hamm TE, Jr., Balish E, et al. Normal luminal bacteria, especially Bacteroides species, mediate chronic colitis, gastritis, and arthritis in HLA-B27/human beta2 microglobulin transgenic rats. J Clin Invest. 1996 Aug 15;98(4):945-53.
7. Sadlack B, Merz H, Schorle H, Schimpl A, Feller AC, Horak I. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell. 1993 Oct 22;75(2):253-61.
8. Stepankova R, Powrie F, Kofronova O, Kozakova H, Hudcovic T, Hrncir T, et al. Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. Inflamm Bowel Dis. 2007 Oct;13(10):1202-11.
9. Taurog JD, Richardson JA, Croft JT, Simmons WA, Zhou M, Fernandez-Sueiro JL, et al. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. J Exp Med. 1994 Dec 1;180(6):2359-64.
10. Seksik P, Rigottier-Gois L, Gramet G, Sutren M, Pochart P, Marteau P, et al. Alterations of the dominant faecal bacterial groups in patients with Crohn's disease of the colon. Gut. 2003 Feb;52(2):237-42.
11. Scanlan PD, Shanahan F, O'Mahony C, Marchesi JR. Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease. J Clin Microbiol. 2006 Nov;44(11):3980-8.
12. Van Assche G, Vermeire S, Rutgeerts P. Safety issues with biological therapies for inflammatory bowel disease. Curr Opin Gastroenterol. 2006 Jul;22(4):370-6.
13. Mahida YR, Rolfe VE. Host-bacterial interactions in inflammatory bowel disease. Clin Sci (Lond). 2004 Oct;107(4):331-41.
14. Bibiloni R, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, et al. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am J Gastroenterol. 2005 Jul;100(7):1539-46.
15. Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, et al. Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut. 2004 Nov;53(11):1617-23.
16. Mimura T, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, et al. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. Gut. 2004 Jan;53(1):108-14.
17. Seksik P, Dray X, Sokol H, Marteau P. Is there any place for alimentary probiotics, prebiotics or synbiotics, for patients with inflammatory bowel disease? Mol Nutr Food Res. 2008 Aug;52(8):906-12.
18. Kokesova A, Frolova L, Kverka M, Sokol D, Rossmann P, Bartova J, et al. Oral administration of probiotic bacteria (E. coli Nissle, E. coli O83, Lactobacillus casei) influences the severity of dextran sodium sulfate-induced colitis in BALB/c mice. Folia Microbiol (Praha). 2006;51(5):478-84.
19. Obermeier F, Dunger N, Strauch UG, Grunwald N, Herfarth H, Scholmerich J, et al. Contrasting activity of cytosin-guanosin dinucleotide oligonucleotides in mice with experimental colitis. Clin Exp Immunol. 2003 Nov;134(2):217-24.
20. Rachmilewitz D, Katakura K, Karmeli F, Hayashi T, Reinus C, Rudensky B, et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. Gastroenterology. 2004 Feb;126(2):520-8.
21. Verdu EF, Bercik P, Cukrowska B, Farre-Castany MA, Bouzourene H, Saraga E, et al. Oral administration of antigens from intestinal flora anaerobic bacteria reduces the severity of experimental acute colitis in BALB/c mice. Clin Exp Immunol. 2000 Apr;120(1):46-50.

22. Ali IK, Zaki M, Clark CG. Use of PCR amplification of tRNA gene-linked short tandem repeats for genotyping Entamoeba histolytica. J Clin Microbiol. 2005 Dec;43(12):5842-7.
23. Westphal O, Lüderitz O, Bister F. Extraction of bacteria with phenol/water. Zeitschrift für Naturforschung. 1952;7b:148-55.
24. Murano M, Maemura K, Hirata I, Toshina K, Nishikawa T, Hamamoto N, et al. Therapeutic effect of intracolonically administered nuclear factor kappa B (p65) antisense oligonucleotide on mouse dextran sulphate sodium (DSS)-induced colitis. Clin Exp Immunol. 2000 Apr;120(1):51-8.
25. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology. 1990 Mar;98(3):694-702.
26. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest. 1993 Aug;69(2):238-49.
27. Monteleone G, Peluso I, Fina D, Caruso R, Andrei F, Tosti C, et al. Bacteria and mucosal immunity. Dig Liver Dis. 2006 Dec;38 Suppl 2:S256-60.
28. Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, et al. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunol Lett. 2004 May 15;93(2-3):97-108.
29. Williams AM, Probert CS, Stepankova R, Tlaskalova-Hogenova H, Phillips A, Bland PW. Effects of microflora on the neonatal development of gut mucosal T cells and myeloid cells in the mouse. Immunology. 2006 Dec;119(4):470-8.
30. Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci U S A. 2009 Sep 15;106(37):15813-8.
31. Salzman NH, Hung K, Haribhai D, Chu H, Karlsson-Sjoberg J, Amir E, et al. Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol. 2010 Jan;11(1):76-83.
32. Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology. 1994 Dec;107(6):1643-52.
33. Grabig A, Paclik D, Guzy C, Dankof A, Baumgart DC, Erckenbrecht J, et al. Escherichia coli strain Nissle 1917 ameliorates experimental colitis via toll-like receptor 2- and toll-like receptor 4-dependent pathways. Infect Immun. 2006 Jul;74(7):4075-82.
34. Atreya R, Mudter J, Finotto S, Mullberg J, Jostock T, Wirtz S, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. Nat Med. 2000 May;6(5):583-8.
35. Wang F, Graham WV, Wang Y, Witkowski ED, Schwarz BT, Turner JR. Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. Am J Pathol. 2005 Feb;166(2):409-19.
36. Hrncir T, Stepankova R, Kozakova H, Hudcovic T, Tlaskalova-Hogenova H. Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice. BMC Immunol. 2008;9:65.
37. Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008 Oct 28;105(43):16731-6.
38. Okada Y, Tsuzuki Y, Miyazaki J, Matsuzaki K, Hokari R, Komoto S, et al. 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. 2006 May;55(5):681-8.
39. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008 May 29;453(7195):620-5.
40. Papadakis KA, Targan SR. Tumor necrosis factor: biology and therapeutic inhibitors. Gastroenterology. 2000 Oct;119(4):1148-57.
41. Siddiqui MA, Scott LJ. Infliximab: a review of its use in Crohn's disease and rheumatoid arthritis. Drugs. 2005;65(15):2179-208.
42. Adams RJ, Heazlewood SP, Gilshenan KS, O'Brien M, McGuckin MA, Florin TH. IgG antibodies against common gut bacteria are more diagnostic for Crohn's disease than IgG against mannan or flagellin. Am J Gastroenterol. 2008 Feb;103(2):386-96.
43. Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson MA, et al. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science. 2009 Jul 31;325(5940):617-20.
44. Keilbaugh SA, Shin ME, Banchereau RF, McVay LD, Boyko N, Artis D, et al. 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. 2005 May;54(5):623-9.

45. Singh B, Read S, Asseman C, Malmstrom V, Mottet C, Stephens LA, et al. Control of intestinal inflammation by regulatory T cells. Immunol Rev. 2001 Aug;182:190-200.
46. Westphal O, Lüderitz O, Bister F. Über die Extraktion von Bakterien mit Phenol/Wasser Zeitschrift für Naturforschung. 1952;7b:148-55.
47. deVos T, Dick TA. A rapid method to determine the isotype and specificity of coproantibodies in mice infected with Trichinella or fed cholera toxin. J Immunol Methods. 1991 Aug 9;141(2):285-8.
48. Kverka M, Burianova J, Lodinova-Zadnikova R, Kocourkova I, Cinova J, Tuckova L, et al. Cytokine profiling in human colostrum and milk by protein array. Clin Chem. 2007 May;53(5):955-62.
49. Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol. 1993 Mar;59(3):695-700.
50. Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci U S A. 1979 Oct;76(10):5269-73.
51. Pavlicek A, Hrda S, Flegr J. Free-Tree--freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of genus Frenkelia. Folia Biol (Praha). 1999;45(3):97-9.
52. Bartosch S, Fite A, Macfarlane GT, McMurdo ME. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol. 2004 Jun;70(6):3575-81.
53. Kreader CA. Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution. Appl Environ Microbiol. 1995 Apr;61(4):1171-9.

3.8 Supplementary data

3.8.1 Suplemental materials and methods

Preparation of bacterial lysates and bacterial components

Cultures were grown on Wilkins-Chalgren Anaerobe Agar (Oxoid, Basingstoke, UK) and enriched with defibrinated blood. After 24 hours (h) of incubation in an anaerobic atmosphere (Oxoid), a small portion of agar was cut out and placed into Brain Heart Infusion Broth (Oxoid) for 48 h at 37°C in an anaerobic atmosphere. The cells were harvested by centrifugation (4000 x g, 30 min) and washed twice with sterile PBS to minimise contamination of the sample with the culture media.

After disruption of the bacteria with the French press, part of the lysate was separated by centrifugation (8500 x g, 30 min) into two fractions, membranous (insoluble; mPd) and cytoplasmic (soluble; cBd). The lysate and its fractions were lyophilised and diluted to a working concentration of 15 g/l. Lipopolysaccharide of the P. distasonis was isolated by phenol-water extraction according to the procedure described by Westphal et al (46). DNA from P. distasonis was isolated using a shortened version of the cetyltrimethylammonium bromide (CTAB) DNA isolation method described previously (22). Sterility of all components was verified by both aerobic and anaerobic cultivation before administration.

Evaluation of colitis

Colitis was evaluated on the last day of the experiment using a clinical activity score, histological score and colon length measurement. The clinical activity score represents the sum of separate scores ranging from 0 to 4 and was calculated using the following parameters: body weight decrease (none 0 points, weight loss of 1 to 5% as 1 point, 5 to 10% as 2 points, 10 to 20% as 3 points, and 20% as 4 points), stool consistency (solid 0 points, loose stool that did not stick to the anus 2 points, and 4 points for liquid stools that did stick to the anus), and bleeding (none 0, positive guaiacum reaction 2 points, and 4 points for gross bleeding). These scores were added and divided by 3, forming a total clinical score that ranged from 0.0 (healthy) to 4.0 (maximal activity of colitis), as described previously by Cooper et al (26).

Postmortem, the entire colon was removed (from caecum to anus) and placed without tension on a ruler and colon length measured. Colon descendens were fixed in 4% buffered formalin and embedded in paraffin for histological evaluation. Sections were stained with

hematoxylin/eosin. Four transversal sections, separated with 100 µm gaps, were evaluated from each sample. Histological scoring was performed for each section in a blinded fashion by 2 expert pathologists (K. K. and P. R.) and a score combining the degree of leukocyte infiltration in lamina propria and submucosa and the extent of mucosal defect (Table S1 and Fig. S1) was obtained. The final score represents the mean of four sections ranging from 0 (no signs of colitis) to 3 (severe colitis). Evaluation of acute DSS-induced colitis in parenterally (s.c. subcutaneous, i.p. intraperitoneal) treated BALB/c mice is sumarised in table S6.

Figure S1 Histological examples of different grades of mucosal damage in DSS treated mice (H&E stained colon descendens; magnification, ×40). See table S1 for detailed description.

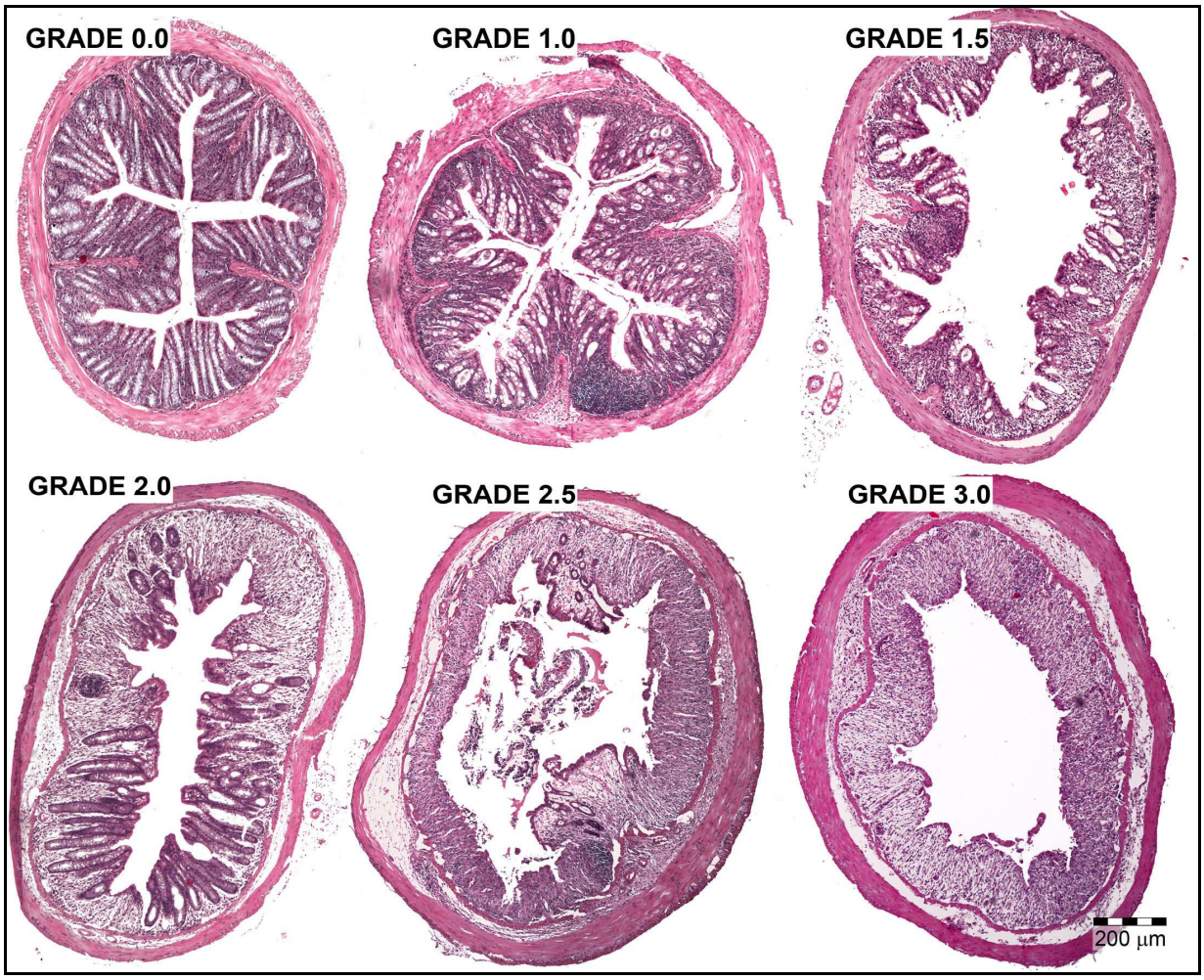


Table S1. Detailed description of individual histological grades.

Grade		*Description*
0	normal mucosa	Thin colon wall without oedema or infiltration, crypt without defects with well-preserved mucus production.
0.5	borderline	Discrete focal infiltration by the crypt basis without any defect in mucosa. Also encountered in some control animals.
1.0	mild	Extension of cellular infiltrate to the superficial layer of lamina propria and to submucosa. Mild oedema of lamina propria and flattening of crypts without defects of epithelium.
1.5	medium	Confluence of inflammatory cells and oedema in lamina propria and patchy infiltrate in submucosa. The mucosa is markedly flat with discrete errosion(s) or ulcers covering less than 10% of colon diameter.
2.0	medium to severe	Same as above, but the ulcers extend to 10%-50% of diameter, mostly with the purulent exudate in the lumen. Crypts are regressed and the mucus production is suppressed.
2.5	very severe	Same as above, but the ulcers cover over 50% of diameter. Massive inflammatory infiltration and oedema of both lamina propria and submucosa with pseudoabscesses and intravascular leukostasis.
3.0	extreme	Same as above, but with subtotal/total denudation of the mucosa.

Determination of serum and faecal antibodies

Faecal pellets were collected and processed as previously described (47). We then used indirect ELISA, optimised in our laboratory, to compare serum or fecal antibody (IgG, IgM and IgA) titres against *P. distasonis* lysate between PBS and mPd-treated groups.

Briefly, 96-well ELISA plates (Nunc, Roskilde, Denmark) coated overnight with mPd (100 µl/well at 10 mg/l in PBS) and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in PBS were incubated for 2 h with serum samples diluted 1:50 (1% BSA was used as a blank, normal mouse serum as a negative control and a pool of the hyper-immune mouse sera as a positive control). After washing (three times with PBS containing 0.05% Tween 20 (Sigma-Aldrich)), secondary antibodies (50 µl/well) were added and incubated for 1 h at room temperature. We used horseradish peroxidase (HRP)-labelled anti-mouse IgG (The Binding Site Ltd, Birmingham, UK) diluted 1:2000 in 1% BSA, HRP-labelled anti-mouse IgM (The Binding Site Ltd) diluted 1:500 in 1% BSA, or biotinylated anti-mouse IgA (Sigma-Aldrich) diluted to a concentration of 1:2000 in 1% BSA and 5% fetal bovine serum (BioClot GmbH, Aidenbach, Germany). After a washing step, we added 50 µl/well of streptavidin-HRP (R&D Systems Inc., Minneapolis, MN) diluted 1:200 in 1% BSA into the IgA plate. The plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich) and the optical density (OD) was measured at 450 nm. The OD of the background (1% BSA) was subtracted and resulting adjusted ODs of the treated groups were

compared with those of PBS-treated groups. We used 10 mice per group for the analysis of serum and 5 mice per group for analysis of coproantibodies.

Determination of serum haptoglobin levels

The level of the acute-phase protein haptoglobin was determined in mouse serum using a modified Human Haptoglobin ELISA Quantitation Kit (GenWay Biotech, Inc., San Diego, CA). Antibodies used in this kit have high cross-reactivity with mouse haptoglobin; the recovery for the mouse reference serum was 94%. The kit was used according to the manufacturer's recommendation, with minor modifications. Briefly, a 96-well ELISA plate (Nunc) was coated with Chicken anti-Human Haptoglobin antibody (100 μl/well at 5 mg/l) diluted in 0.05 M Carbonate-Bicarbonate buffer (pH 9.6) and incubated for 1 h at room temperature. After washing (three times with PBS containing 0.05% Tween 20), the plate was blocked with 1% nonfat dry milk in PBS. The samples were diluted 1:1000 in 1% nonfat dry milk and incubated for 1 h at room temperature. Serial dilutions of mouse reference haptoglobin serum (ICL, Inc., Newberg, OR, USA) were used as calibrator instead of the pure human haptoglobin provided in the kit. Then the plates were washed five times and incubated with HRP conjugated detection antibody (100 μl/well at 61.3 μg/l) for 1 h. The plates were developed with TMB (Sigma-Aldrich) and the OD was measured at 450 nm. The quantitative determination was performed between 39-2500 μg/l.

Gut tissue fragment culture

Five sections of mouse intestine were obtained (Peyer’s patches, jejunum, ileum, caecum and colon), cut open longitudinally, washed in PBS containing penicillin and streptomycin and weighed.

The tissue fragments were then cultivated for 48 h in a humidified incubator at 37°C and 5% CO_2 in RPMI-1640 (Sigma-Aldrich) containing 10% fetal bovine serum (BioClot GmbH, Aidenbach, Germany) and 1% Antibiotic-Antimycotic solution (Sigma-Aldrich). The supernatants were collected and stored at -20°C until analysis. During our preliminary experiments, we found that there is still significant production of IL-10 and TGF-β after 48h cultivation (Fig. S2).

Macrophage cell line culture

Mouse macrophage RAW 264.7 cells, originally obtained from the American Type Culture Colection (ATCC TIB-71), were cultured in Dulbecco´s modified Eagle´s medium (Institute of Molecular Genetics AS CR, Prague, Czech Republic) supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany), penicillin (100 U/ml), streptomycin (100 mg/l, Sigma-Aldrich), 4.5 g/l glucose, 1.5 g/l sodium bicarbonate and 4 mM glutamine (Institute of Molecular Genetics AS CR). The cells were cultured in a humidified incubator at 37°C and 5% CO_2. Cell viability was evaluated by flow cytometry. The cell density was then adjusted to 10^6 cells/ml and the cells were seeded in the wells of flat-bottom, 96-well plates (200 µl/well). The plates were incubated with LPS (*Salmonella typhimurium*, 1 mg/l, Sigma-Aldrich), LPS together with either bacterial lysate from *P. distasonis*, or mPd or cPd or DNA for 24 h (37°C, 5% CO_2). To address the question of dose dependence, serial decreasing dilutions of lysate and mPd were used, ranging from 1 µg/l to 100 fg/l. Supernatants were collected and stored at - 20°C until analysis. The supernatants were screened semiquantitatively with the RayBio™ Mouse Cytokine Array 3 (Raybiotech, Inc., Norcross, GA), capable of detecting 62 cytokines, or quantitatively for TNF-α with ELISA (Invitrogen Corp., Carlsbad, CA), similarly as described below.

Determination of cytokine production

To determine the changes in cytokine spectra induced by DSS treatment and mPd therapy in the colon of mice, we used the RayBio™ Mouse Cytokine Array II (Raybiotech, Inc.) (see Table S2 for array layout). For this purpose, we used three samples of media after 48 h of colon cultivation (see above) from healthy, DSS/PBS (sham) and DSS/mPd-treated groups of mice. Chemiluminescence was detected by a luminescence detector LAS-1000 (Fujifilm, Tokyo, Japan), and quantitation of spots was performed by AIDA (3.28, Raytest, Straubenhardt, Germany) software as described previously (48). Values from different arrays were first normalized using the intensity of positive controls, which are made of biotinylated antibody directly spotted on the array. Furthermore, the levels of selected cytokines were determined using commercially available ELISA sets purchased from Invitrogen (TNF-α, IFN-γ, TGF-β, IL-10; Invitrogen Corp.) or R&D Systems (IL-6; R&D Systems Inc., Minneapolis, MN). All tests were performed according to the manufacturers' recommendations.

Table S2. Layout of the RayBio™ Mouse Cytokine Array II.

	A	B	C	D	E	F	G	H	I	J	K	L
1 2	Positive control	Positive control	Negative control	Negative control	6-Ckine	CTACK	Eotaxin	G-CSF	GM-CSF	IL-2	IL-3	IL-4
3 4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13	IL-17	IFN-γ	KC	Leptin	MCP-1
5 6	MCP-5	MIP-1α	MIP-2	MIP-3β	RANTES	SCF	sTNFRI	TARC	TIMP-1	TNF-α	TPO	VEGF
7 8	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Positive control

Abbreviations used in this table stand for the following cytokines: 6-Ckine, 6-Cysteine chemokine; CTACK, Cuteaneous T-cell attracting chemokine; G-CSF, Granulocyte-colony stimulating factor; GM-CSF, Granulocyte-macrophage colony stimulating factor; IFN (Interferon) -γ, IL (Interleukin) -2, -3, -4, -5, -6, -9, -10 , -12p40p70 (detects both p70 and p40), -12p70 (detects only whole cytokine IL-12), -13, -17, KC, Growth-regulated alpha protein precursor; MCP (Monocyte chemoattractant protein)-1, -5; MIP (Macrophage inflammatory protein)-1α, -2, -3β, RANTES, Regulated upon activation, normal T cell expressed, and presumably secreted; SCF, Stem call factor; sTNFRI, Soluble tumor necrosis factor-α receptor 1; TARC, Thymus and activation-Regulated chemokine; TIMP (Tissue inhibitor of metalloproteinases)-1; TNF (Tumor necrosis factor)-α; TPO, Thrombopoietin; VEGF, Vascular endothelial growth factor.

Figure S2. Cytokine production by colon tissue of healthy mice during the first, second and third days of ex vivo cultivation. Three colon samples were cultivated for 72 h in complete RPMI medium. Every 24 h, the tissue was gently washed in fresh media and transferred to the new cultivation well for next 24 h. The supernatant after the first, second and third 24 h of cultivation was stored for cytokine analysis.

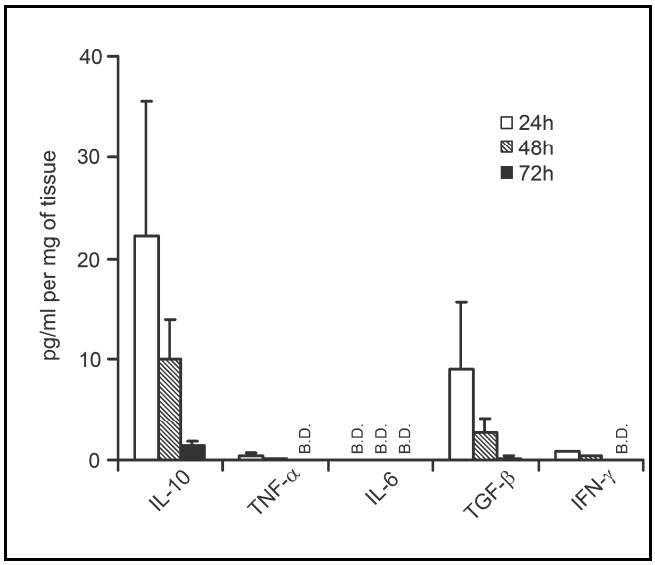
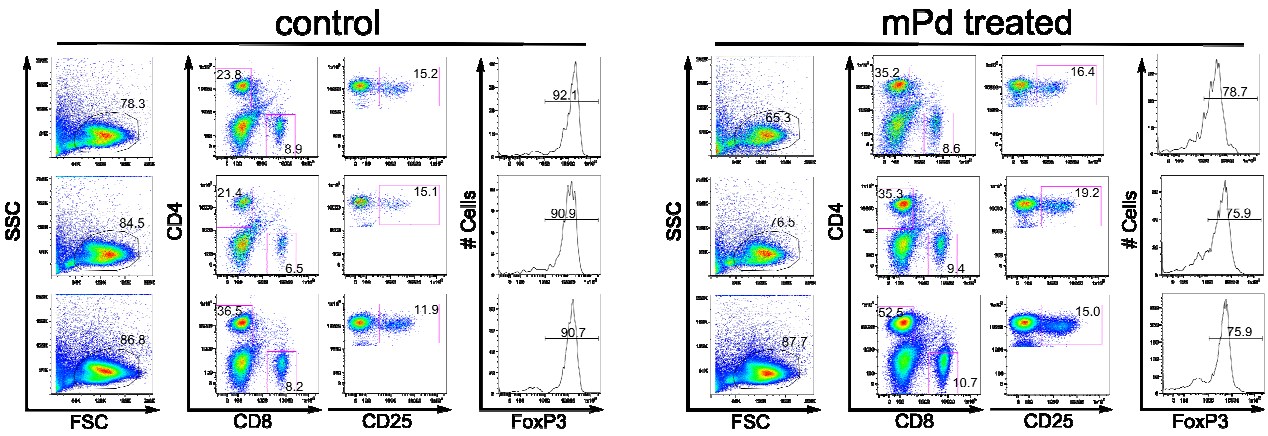


Figure S3. Showing gating strategy in 3 mice (rows) from DSS/PBS and 3 mice from DSS/mPd treated group. Total number of Tregs is increased in mesenteric lymph nodes (MLN) of mPd treated mice is increased in cells (mean±SD; 3.40±0.50 vs. 4.81±0.30; P=0.014 for $CD4^+CD25^+FoxP3^+$ or 4.29±0.26 vs. 5.36±0.10; P=0.019 for $CD4^+FoxP3^+$). First column shows gating on cells, second gating on CD4 and CD8 expression on these cells, third gating on $CD4^+CD25^+$ cells and fourth is a histogram of FoxP3 expression on these $CD4^+CD25^+$ cells.



Evaluation of microbiota changes with PCR-DGGE

Total bacterial DNA was isolated from mice faecal samples by using the ZR Fecal DNA Kit™ (Zymo Research Corp., Orange, CA) according to the manufacturer's description. Fragments of 16S rRNA genes were amplified from total bacterial DNA with primers 338GC and RP534 (49).

PCR products were separated and analysed on the DCodeTM Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA). The denaturating gradient was 35 – 60% and the electrophoresis was carried out for 18 h at 55 V. The gel was stained in SYBR® green I dye for 30 minutes and observed in a Vilber Lourmat system under UV light.

Banding patterns were converted to a binary matrix, taking into account the presence or absence of the individual bands. This binary matrix was used to calculate the distance matrix between individual samples (50) and a dendrogram comparing all 30 samples was obtained with UPGMA (unweighted pair-group method with arithmetic averages) using FreeTree software (51).

The similarity between the DGGE profiles obtained from a single mouse at different time points was determined by calculating Dice's similarity coefficient (D_{SC} =[2j/(a+b)] x 100), where j is the number of DGGE bands found in both profiles, a is the number of bands at first time point, and b is the number of bands at the second time point. A D_{SC} value of 100% indicates that the samples are identical.

To identify the bacteria, bands of interest were cut from the gel, eluted with dH_2O and amplified with PCR. The PCR product was purified with the QIAquick PCR purification kit and analysed on the 3100 Avant Genetic Analyser (Applied Biosystems Inc., Foster City, CA).

Quantitative PCR

Faecal samples were weighed and total bacterial DNA was extracted by using a ZR Fecal kit (Zymo Research, USA) according to the manufacturer's protocol. Real-time PCR analyses were performed on the Mx3005P system (Stratagene, USA) with the qPCR 2x SYBR Master Mix (Top-Bio, Czech Republic). The qPCR reactions were performed in a 20 μL volume, and the primer concentrations were 0.5 μM each. The following bacterial groups were monitored: all *Eubacteria* (with primers Uni331F+ Uni797R) (52), Bacteroides-Prevotella group (primers Bac303F+Bac708R) (52) and *P. distasonis* (Bd180F+Uni797R) (53). We used these amplification conditions: initial denaturation at 95°C (3 min), 35 cycles of denaturation at 95°C (30 seconds) and annealing/elongation (30 seconds) (Table S3), and one final cycle at 95°C (30 seconds) followed by a dissociation curve from 55°C to 95°C (1°C per cycle of 10 s). DNA isolated from a known number of cells from pure cultures of *Bacteroides vulgates* and *P. distasonis* were used as qPCR standards. Because the weight and the consistency of the stool differed among the samples, the results were normalised to the total number of *Eubacteria* and expressed as a percentage of total *Eubacteria*.

Table S3. PCR primer sets used in the study.

Target organism	***Primer set***	***Sequence (5'-3')***	***Product size (bp)***	***Annealing temp (°C)***	***Reference***
All eubacteria (PCR-DGGE)	338GC	CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCGCCGCCGC ACTCCTACGG GAGGCAGCAG	196	58	(49)
	RP534	ATTACCGCGG CTGCTGG			
All eubacteria (qPCR)	Uni331F	TCCTACGGGAGGCAGCAGT	466	58	(52)
	Uni797R	GGACTACCAGGGTATCTATCCTGTT			
Bacteroides-Prevotella group	Bac303F	GAAGGTCCCCCACATTG	418	56	
	Bac708R	CAATCGGAGTTCTTCGTG			
Parabacteroides distasonis	Bd180F	AAT ACC GCA TGA AGC AGG	617	62	(53)
	Uni797R	GGACTACCAGGGTATCTATCCTGTT			(52)

3.8.2 Supplemental tables

Table S4. Evaluation of acute DSS colitis in orally treated BALB/c mice.

Experimental group	*Colon length (cm)*	*Disease activity index*	*Histological grade*
PBS	9.31±0.88	3.33±0.49	1.44±0.71
***P. distasonis* lysate**	10.85±1.26**	0.90±0.77**	0.61±0.50**
***B. ovatus* lysate**	9.48±0.80	2.63±1.15	1.26±0.35
***V. alcalescens* lysate**	9.21±0.66	1.58±0.66	1.58±0.66

Values are expressed as means ± standard deviation (10 mice per group). One-way ANOVA with Dunnett's multiple comparison test was used to evaluate differences between experimental groups and PBS-treated controls (*P<0.05, **P<0.01).

Table S5. Evaluation of acute DSS colitis in orally treated BALB/c mice.

Experimental group	*Colon length (cm)*	*Disease activity index*	*Histological grade*
PBS	7.83±0.55	3.78±0.27	1.81±0.28
***B. vulgatus* lysate**	8.13±1.06	2.87±1.04	1.44±0.89
***B. stercoris* lysate**	8.60±0.60	3.40±0.80	1.33±0.41
***B. stercoris* confidence level *Capnocytophaga spp.* lysate**	9.00±0.70	3.13±0.65	0.63±0.25**
***B. thetaiotamicron* lysate**	8.90±0.81	2.13±0.96	1.25±0.47

Values are expressed as means ± standard deviation (5 mice per group). One-way ANOVA with Dunnett's multiple comparison test was used to evaluate differences between experimental groups and PBS-treated controls (*P<0.05, **P<0.01).

Table S6. Evaluation of acute DSS colitis in parenterally treated BALB/c mice.

Experimental group	*Colon length (cm)*	*Disease activity index*	*Histological grade*
PBS/IFA s.c.	6.02±0.46	3.40±0.37	1.61±0.84
mPd/IFA s.c.	6.20±0.51	2.87±0.80	1.43±0.53
PBS/IFA i.p.	6.72±0.54	3.13±0.69	1.69±0.81
mPd/IFA i.p.	6.72±0.83	2.73±0.89	1.29±0.62

Values are expressed as means ± standard deviation (5 mice per group). One-way ANOVA with Dunnett's multiple comparison test was used to evaluate differences between experimental groups and PBS-treated controls (*P<0.05, **P<0.01).

3.8.3 Supplemental figures

Figure S4. Cytokine profiling of the supernatants after the cultivation of untreated RAW264.7 cells or cells after treatment with LPS, Pd+LPS or mPd+LPS, as measured by RayBio Mouse Cytokine Antibody Array 3. Only cytokines positive at least in one sample are shown. As compared with LPS-activated cells, the Pd and mPd decrease TNF-α, IL-6, MCP-1 and MCP-5, and increase in CXCL16.

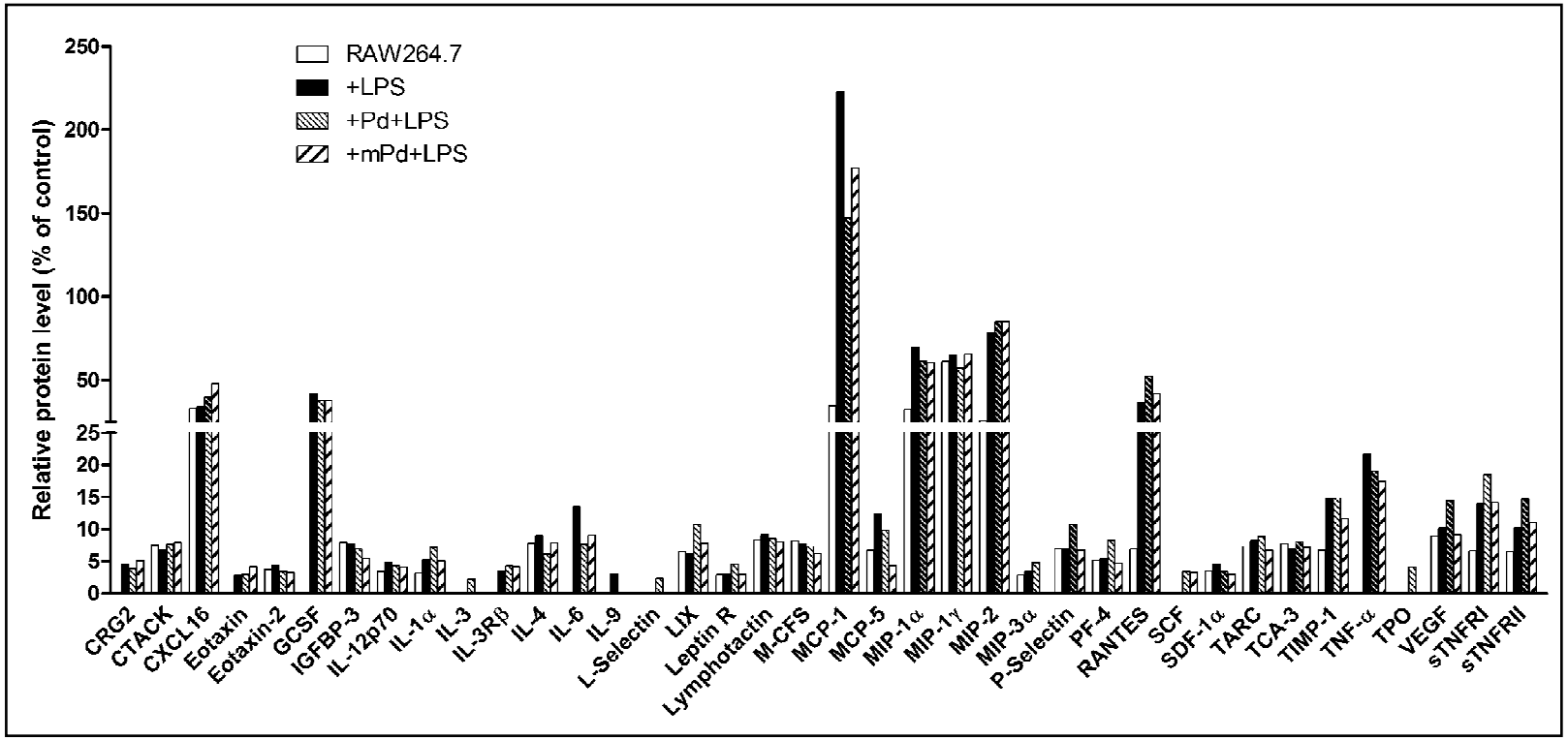


Figure S5. The effect of Pd and mPd on TNF-α production by LPS-activated macrophage cell line RAW 264.7 was measured by ELISA. TNF-α production with sterile PBS with 1 mg/l of LPS is set at 100%. Data are means of five independent experiments. Error bars are SEM. P < 0.05: [a] (for LPS+Pd versus controls); [b] (for LPS+mPd versus controls) using ANOVA with a Dunnett's post-hoc test.

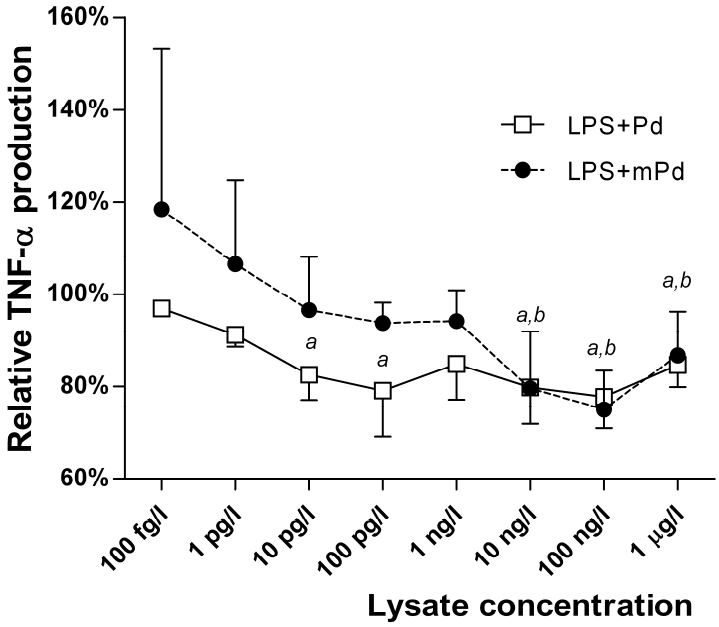
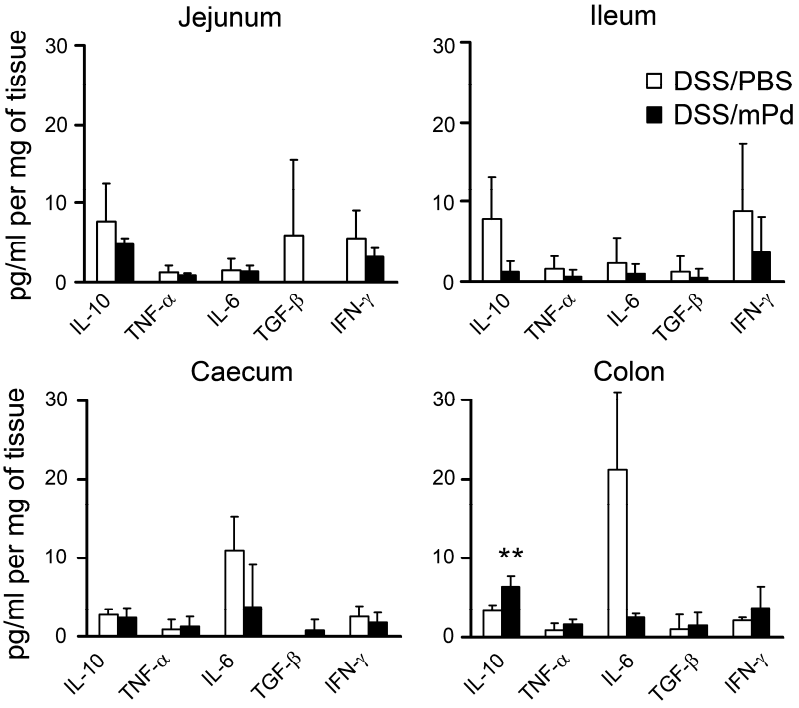


Figure S6. Pretreatment with mPd decreases cytokine production (pg/mg of tissue) in different parts of the gut in orally treated SCID mice as measured by ELISA. **P<0.01: DSS/mPd versus DSS/PBS-treated mice; n = 5 mice per group.



4 Lysate of probiotic *Lactobacillus casei* DN-114 001 ameliorates colitis by strengthening the gut barrier function and changing the gut microenvironment

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4.1 Abstract

BACKGROUND: Probiotic bacteria can be used for the prevention and treatment of human inflammatory diseases including inflammatory bowel diseases (IBD). However, the nature of active components and exact mechanisms of these beneficial effects have not been fully elucidated. Our aim was to 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. METHODOLOGY/PRINCIPAL FINDINGS: 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. In order to analyze how this beneficial effect interferes with well-known phases of intestinal inflammation pathogenesis *in vivo* and *in vitro*, we evaluated intestinal permeability using the FITC-labeled dextran method and analysed tight junction proteins expression by immunofluorescence and PCR. We also measured $CD4^+FoxP3^+$ regulatory T cells proportion by FACS analysis, microbiota composition by pyrosequencing, and local cytokine production by ELISA. Lc leads to a significant protection against increased intestinal permeability and barrier dysfunction shown by preserved ZO-1 expression. We found that the Lc treatment increases the numbers of $CD4^+FoxP3^+$ regulatory T cells in mesenteric lymph nodes (MLN), decreases production of pro-inflammatory cytokines TNF-α and IFN-γ, and anti-inflammatory IL-10 in Peyer's patches and large intestine, and changes the gut microbiota composition. Moreover, Lc treatment prevents lipopolysaccharide-induced TNF-α expression in RAW 264.7 cell line by down-regulating the NF-κB signaling pathway. CONCLUSION/SIGNIFICANCE: 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.

4.2 Introduction

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 (1). Moreover, dysbiosis, an imbalance in the intestinal bacterial ecosystem, has been found in IBD and linked to its pathogenesis (2). 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 (3).

When administered in adequate amounts, probiotics, defined as live microorganisms, confer a health benefit to the host (4), and have been successfully used in treatment of IBD (5). 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 (6). First, probiotics may exclude or inhibit the growth of certain pathogens (7); second, they may improve the gut barrier function (8); and third, they can modulate mucosal and/or systemic immune response or metabolic functions (9). 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 live microorganisms may lead to severe infections, and therefore represents considerable risk especially in severely ill patients (10). There is increasing evidence, that similar beneficial effects could be achieved with sterile lysates or components isolated from probiotic or even commensal microbes (11).

Colitis induced by dextran sulfate sodium (DSS) is a well established and reliable model of IBD because its clinical features resemble the ulcerative colitis (12). Acute DSS colitis starts with epithelial cell barrier dysfunction which causes the antigens from the gut lumen to enter the lamina propria and stimulate the immune response. The dysfunction of the epithelial barrier starts as early as the first day after DSS treatment by gradual decrease in the tight junction protein ZO-1 production, which in turn leads to increased gut permeability (13, 14). In the acute phase, DSS-induced colitis is driven mainly by cells of innate immunity, because it also occurs in the absence of functional T, B and NK cells (15). The functional adaptive immune system, however, plays an important role in the chronic phase of the

inflammation and might be necessary for its preventive treatment with microbial antigens (11, 16).

The most intensively studied and used probiotic bacteria are lactobacilli (17, 18). Oral treatment with probiotic bacterium *L. casei* DN-114 001 has been found to reduce the duration and severity of diarrhea and common infectious diseases in children (19). Moreover, supernatant of this probiotic strain was described to exert immunological activities and strong inhibitory effect on epithelial cell adhesion of virulent *E. coli* strain (20). These studies clearly show the beneficial potential of this bacterium, however, the clinical utility of such approach remains controversial, as neither the specific mechanisms of action nor the active component responsible for its beneficial properties has been established.

In our previous study, we have shown that the preventive treatment with live probiotic bacterium *L. casei* DN-114001 protects mice from subsequent acute DSS-induced colitis in BALB/c mice (21). Here, we show that oral treatment with lysate of this bacterium (Lc) has a similar effect, and that this effect is associated with change in the intestinal microbiota composition, modulation of mucosal immune system, and induction of regulatory T cells in mesenteric lymph nodes (MLN). Our results show that even killed probiotic bacteria can decrease the severity of the intestinal inflammation, which represents safer and more practical therapeutic intervention than the use of live bacteria in the treatment of intestinal inflammation.

4.3 Materials and Methods

4.3.1 Preparation of bacteria

Lactobacillus casei DN-114 001 (Danone Institute, Palaiseau Cedex, France), *Lactobacillus plantarum* CCDM 185 (Culture Collection of Dairy Microorganisms, Milcom a.s., Prague), were grown in an anaerobic chamber in De Man, Rogosa, and Sharpe broth (Oxoid, Basingstoke, UK) at 37°C until the cultures were in the late log phase of growth. Both lactobacilli 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, lactobacilli 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 and the sterility of all components was verified by both aerobic and anaerobic 48 hours cultivation before administration.

4.3.2 Animals

Ethics statement: All animal experiments were approved by the Animal Care and Use Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic, approval ID: 10/2005, 94/2008 and 211/2009. Female BALB/c mice (8-12 weeks old) or severe combined immunodeficient mice BALB/cJHanHsd-SCID (SCID) were obtained from a breeding colony at the Institute of Physiology (Academy of Sciences of the Czech Republic, Prague, Czech Republic) or at the Institute of Microbiology (Academy of Sciences of the Czech Republic, Novy Hradek, Czech Republic), respectively, and reared under conventional conditions.

4.3.3 Study design and DSS induced 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. To reduce proteolytic activity in the gut, the Lc components were co-administered with 1 mg of soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 50 µl of 0.15 M sodium bicarbonate buffer (pH 8.0). Control mice were given only sterile PBS with soybean trypsin inhibitor in bicarbonate buffer. 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, Irvine, CA, USA) dissolved in tap water for 7 days, and on the last day of the experiment the colitis was evaluated by using a clinic al activity score, colon length, and the histological scoring system as described previously (11). Furthermore, to analyze if the protective effect of Lc could be achieved also by parenteral administration, four subcutaneus doses of Lc or PBS (25 µg per dose) were injected in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) before colitis induction. For chronic colitis, mice received four cycles of DSS as described previously (12).

4.3.4 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 (22). Briefly, each mouse received 440 mg/kg of body weight of FITC-dextran (molecular weight 4.4 kDa; Sigma-Aldrich) by gavage. A blood sample, obtained 5h later, was first centrifuged (3,000 rpm at 4

°C) for 30 min, and serum was collected and added to a 96-well microplate. The concentration of FITC-dextran was determined by spectrophotofluorometry (Safire^2, Tecan Group Ltd., Männedorf, Switzerland) with an excitation wavelength of 483 nm and an emission wavelength of 525 nm using serially diluted samples of the marker as standard.

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 mounted on the poly-L-lysine-coated slides. Then the slides were dried and fixed in 4% buffered paraformaldehyde (pH 7.4) for 10 min at room temperature. Fixed sections were washed in PBS and blocked with 2% donkey serum (Sigma-Aldrich) in PBS for 20 min at room temperature. The slides were incubated with the rabbit polyclonal anti-mouse ZO-1 or occludin antibodies (both from Invitrogen, Camarillo, CA, USA) overnight at 4 °C. The negative controls were performed similarly using 1% bovine serum albumin (BSA) in PBS instead of primary antibody. After washing, the sections were incubated with donkey anti-rabbit antibody conjugated either with Texas Red or with DyLight 488 fluorochrome (both from Jackson ImmunoResearch Laboratories, West Grove, USA). Nuclei were counterstained using DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich) stain. Finally, the sections were mounted in Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA, USA) and viewed with a fluorescence microscope Olympus AX-70 (Olympus, Tokyo, Japan).

Determination of ZO-1 mRNA expression in intestinal tissue

Intestinal mucosa from terminal ileum and colon was placed in RNAlater stabilization reagent (QIAGEN GmbH, Hilden, Germany). Total messenger RNA (mRNA) was extracted by using the RNeasy Mini isolation kit (QIAGEN GmbH) following the manufacturer's instructions. RNA integrity was determined by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The purity of the RNA was assessed by the ratio of absorbance at 260 and 280 nm. RNA purity was within a range of 2.0–2.1. 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 (23). Briefly, RNA was converted to cDNA using Taq-Man reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). Beta-actin was used as an endogenous control and its expression was similar in all tested samples. A reaction mix for real-time PCR was made with Taq-Man Universal PCR master mix, water, and assays on demand gene

expression products for ZO-1 and β-actin (all Applied Biosystems, Foster City, CA, USA). The master mix (20 µl) was aliquoted to the wells on a real-time PCR plate; and each sample was analyzed in duplicate. A volume of 5 µl of cDNA was added to each well, and the PCR reaction was run on a 7300 real-time PCR System (Applied Biosystems, Foster City, CA, USA) using standard conditions. The data was analyzed with Genex software (version 4.3.8).

4.3.5 Production of cytokines

Intestinal tissue culture and measurement of cytokines

Sections of Peyer's patches (PP), ileum, cecum, and colon were taken from every mouse. The intestines were then opened longitudinally, washed in ice cold PBS containing antibiotics and cultivated for 48 hours at 37°C and 5% CO_2 in complete RPMI medium with 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and 100,000 U/l penicillin, 100 mg/l streptomycin (Sigma-Aldrich), as described previously (11). Commercially available ELISA sets were used to measure the levels of TNF-α, IFN-γ, TGF-β, IL-10 (Invitrogen Corp.) and IL-6 (R&D Systems Inc., Minneapolis, MN, USA) in these supernatants. All tests were performed according to the manufacturers' recommendations.

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, Foster City, CA, USA.

4.3.6 Determination of specific antibodies

Sera and small intestine washings were collected for specific antibody evaluation. Gut washings were obtained by flushing the content of isolated small intestine with 2 ml of sterile PBS containing a mixture of proteinase inhibitors (Sigma-Aldrich). The samples were then vortexed and centrifuged at 4°C, and the supernatant was collected and stored at -80°C until analysis. Indirect ELISA, optimized in our laboratory as previously described (11), was used to assess the specific antibody response against Lc in serum (IgG, IgM, and IgA) and gut washings (secretory IgA; SIgA). Briefly, Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific Inc., Rochester, NY, USA) were coated overnight with Lc (100 µl/well at 10 mg/l in PBS) and blocked with 1% BSA (Sigma-Aldrich) in PBS. Serum and gut washing samples

diluted 1:50 and 1:10 in 1% BSA, respectively, were added and incubated for 2 hours. As control sera normal reference serum purchased from Bethyl Laboratories (TX, USA) and hyperimmune serum prepared by four subcutaneous injections of Lc in incomplete Freund's adjuvant within 14 days intervals (50μg of Lc in the each dose) were used. After washings (three times with PBS containing 0.05% Tween 20 (Sigma-Aldrich)), secondary antibodies (50 μl/well) were added and incubated for 1 hour at room temperature. Antibody combinations were used as follows: 1) rabbit anti-mouse SIgA (Uscn Life Science Inc., China) and horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Cell Signaling Technology Inc., Danvers, MA, USA); 2) biotinylated anti-mouse IgA (Sigma-Aldrich) and streptavidin-HRP (R&D Systems Inc.); 3) HRP-labeled anti-mouse IgG; 4) HRP-labeled anti-mouse IgM (both The Binding Site Ltd, Birmingham, UK). All reagents were diluted in 1% BSA in PBS except anti-IgA antibody that was diluted in 1% BSA with 5% fetal bovine serum (BioClot GmbH, Aidenbach, Germany). The plates were developed with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) and the optical density (OD) was measured at 450 nm. The OD of the background (1% BSA) was subtracted and resulting adjusted ODs of the treated groups were compared with those of PBS-treated groups.

4.3.7 Flow cytometry

Single-cell suspensions of spleens, MLNs and PPs were prepared and stained for T_{regs} using FoxP3 Staining Set (eBioscience, San Diego, CA, USA) with fluorochrome-labeled anti-mouse mAbs: CD4-Qdot® 605 (Invitrogen, Carlsbad, CA, USA), CD8-BD Horizon^{TM} V500 (BD Biosciences, San Jose, CA, USA), CD3-FITC and FoxP3-Phycoerythrin (both from eBioscience) according to the manufacturer's recommendation.

RAW 264.7 cells were cultivated and stained for IL-7R-Alexa647 (a gift from Pavel Otahal, IMG AS CR, Prague, Czech Republic), CD206-PE (AbD Serotec, Oxford, UK), CD-11c-NC625 and F4/80-APC780 (both from eBioscience). 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., Ashland, OR, USA).

4.3.8 Evaluation of the anti-inflammatory properties of Lc *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, as previously described (11). Briefly, the cells were cultured for 24 hour at 37°C and 5% CO_2 in Dulbecco´s modified Eagle´s medium (Institute of Molecular Genetics AS CR, Prague, Czech Republic) containing 10% heat-inactivated fetal bovine serum (Biochrom AG), 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 4 mM glutamine (Institute of Molecular Genetics AS CR), 100,000 U/l penicillin and 100 mg/l streptomycin (both Sigma-Aldrich). The cells were cultured together with Lc, lysate of *L. plantarum* (Lp) or sterile PBS in the presence or absence of LPS (*Salmonella typhimurium*, 1 mg/l, Sigma-Aldrich). After cultivation, the concentration of TNF-α in the supernatant was measured with ELISA (Invitrogen). The nuclear proteins were extracted from stimulated RAW264.7 cells by a nuclear extract kit (Active Motif, Rixensart, Belgium) and used to quantify the DNA binding activity of p65 subunit using the TransAM NF-κB family transcription factor assay kit (Active Motif). In NF-κB assay, only the concentration with the strongest immunomodulatory properties of Lc was used, i.e. 10 pg/l. All assays were performed according to the manufacturer's recommendation.

4.3.9 Evaluation of microbiota changes by pyrosequencing

Stool samples from PBS or Lc-treated mice, on day 0, 28 (just before DSS administration) and 35 (the last day of experiment) were collected. Total DNA from these samples was then isolated with ZR Fecal DNA Kit™ (Zymo Research Corp., Orange, CA) according to the manufacturer's recommendation.

DNA was subsequently gel-purified and PCR was performed in triplicate for each primer pair, and pooled to minimize random PCR bias. The reaction mixture contained 1 μl of DNA (10 ng/μl), 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of dNTPs, 1x PCR buffer and 1U platinum TAQ DNA polymerase (Invitrogen) and 0.40 μmol/l of forward modified primer consisting of 454 adaptor A (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3'; Genome Sequencer FLX system), unique 10-base tag sequence (ATATCGCGAG, CGTGTCTCTA, CTCGCGTGTC, TAGTATCAGC, TCTCTATGCG) and universal broad-range bacterial primer 5'-AYTGGGYDTAAAGNG and 0.40 μmol/l of reverse primer consisting of adaptor B (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3') and universal primer

TACNVGGGTATCTAATCC. PCR conditions were as follows: 1 ×: 95 °C, 3 min; 35 ×: 94 °C, 50 sec; 40 °C, 30 sec; 72 °C, 60 sec; 1 ×: 72 °C, 5 min and final hold at 4 °C. The length of PCR product was checked on the agarose gel electrophoresis. PCR product was subsequently purified using magnetic beads (AMPure beads, Beckman Coulter Genomics, Danvers, USA). Concentration was measured on Qubit fluorometer (Invitrogen, Carlsbad, CA, USA). Equimolar amounts of PCR product from each sample were used for unidirectional 454 FLX amplicon pyrosequencing using LIB-L emPCR kits following the manufacturer's protocols (Roche Diagnostics, Basel, Switzerland).

4.3.10 Metagenomic data processing

Flowgrams were processed using amplicon analysis option in data processing software from Roche. The sequencing resulted in 161551 overall number of reads. Quality trimmed sequences obtained from the FLX sequencing run were processed using RDP pyrosequencing pipeline. Beforehand, data files were depleted of chimeras by Black Box chimera Checker (24) using default settings. Processing involved aligning of sequences with fast, secondary-structure aware Infernal aligner, subsequent clustering with max distance of 3 % based on complete-linkage clustering method and classifying of incurred clusters by naïve Bayesian rDNA classifier (25). Bootstrap cutoff was set to 50 %, which was sufficient for accurate classification at the genus level. Generated designations of clustered sequences together with their relative abundances within the given samples were used for comparing bacterial diversity.

4.3.11 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.

4.4 Results

4.4.1 Oral administration of lysate *L. casei* attenuate the acute colitis in BALB/c mice but not in SCID mice

In our previous study we showed that oral treatment with *L. casei* DN-114 001 attenuates the severity of acute experimental colitis (21). To test if its lysate have similar activity, we pretreated mice with four weekly oral doses of Lc and induced colitis by DSS in BALB/c and SCID mice. Oral (Table 1) but not parenteral (data not shown) administration of Lc is effective in preventing the acute DSS colitis in BALB/c mice, improving clinical and morphological markers of colitis. In contrast, when colitis was induced in SCID mice (Table 1) pretreatment with Lc failed to improve acute colitis in all tested parameters. Also no significant effects of Lc were found when the model of chronic colitis was used (data not shown).

Table 1. Lc improves the severity of DSS-induced colitis in BALB/c, but not in SCID mice.

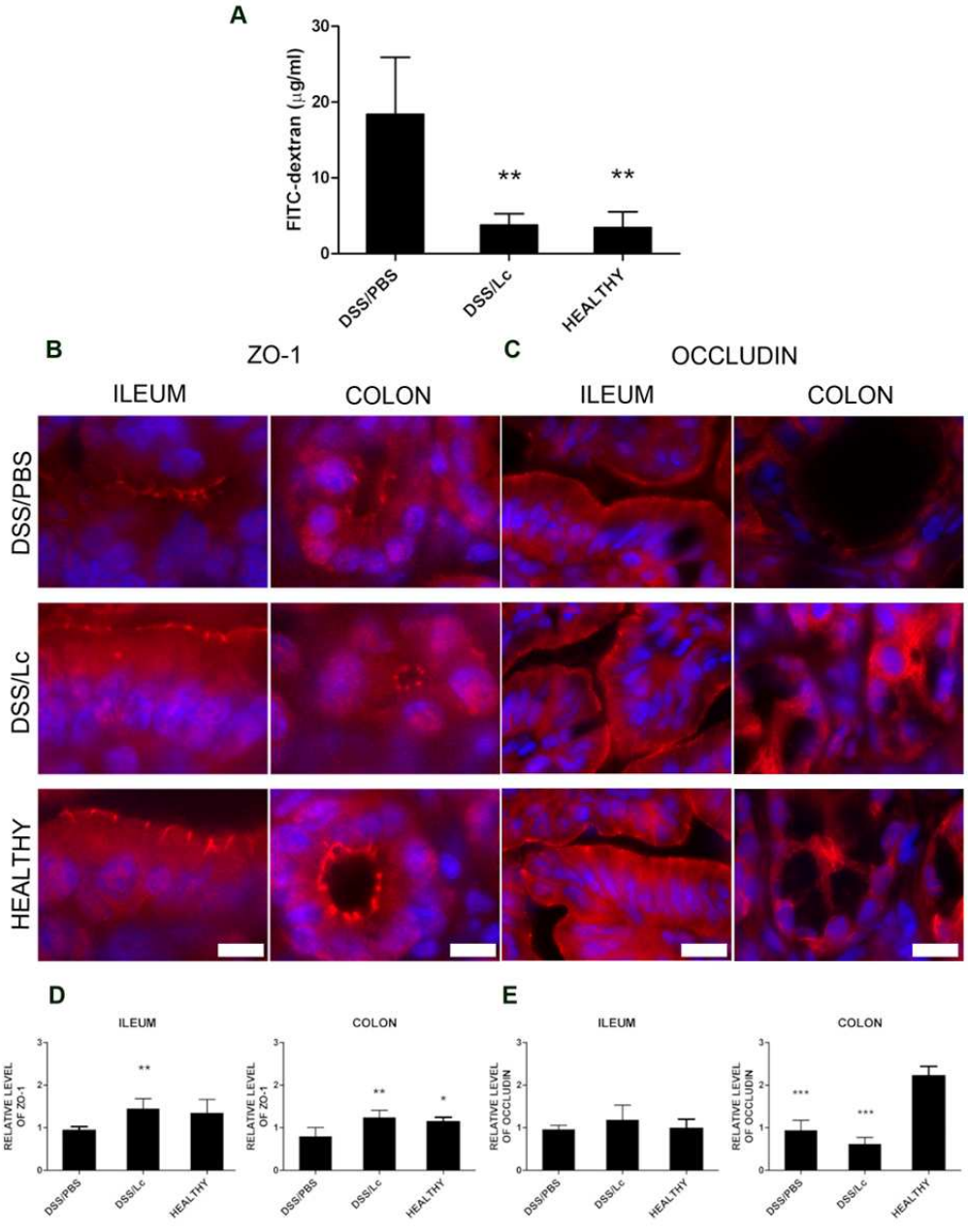
Mouse strain	*Experimental group*	*Disease activity index*	*Colon length (cm)*	*Histological grade*
BALB/c	DSS/PBS	2.80 ± 0.68	6.35 ± 0.62	1.59 ± 0.54
	DSS/Lc	1.67 ± 1.09***	7.14 ± 0.34***	1.20 ± 0.51*
SCID	DSS/PBS	1.95 ± 1.66	6.63 ± 0.55	1.2 ± 0,84
	DSS/Lc	1.99± 1.54	7.17 ± 1.21	1.26 ±0.85

Values are expressed as means ± SD (5 BALB/c mice per group) of one representative experiment out of three independent experiments. Unpaired Student's t-test in BALB/c mice was used to evaluate the significance of differences between experimental groups and the PBS-treated control group (*P<0.05, ***P<0.001).

4.4.2 Lysate of *L. casei* prevents the increase in intestinal permeability and preserves ZO-1 expression in acute colitis

Increased intestinal permeability caused by impairment of the gut barrier function drives the pathogenesis of intestinal inflammation in both DSS-induced colitis and human IBD (26, 27). To investigate the effect of Lc on the gut barrier function in acute DSS-induced colitis, we

Figure 1. Oral treatment with Lc strengthens the gut barrier function as compared to PBS control mice. (A) Measurement of intestinal permeability by FITC-dextran. Serum levels of 4.4-kDa FITC-dextran 5 hour after administration by gavage in DSS/PBS, DSS/Lc-treated group and healthy controls. Immunohistological detection of tight junction proteins ZO-1 (B) and occludin (C) in representative sections of colon and terminal ileum from DSS/PBS-, DSS/Lc-treated group and healthy controls. Fluorescent signal of ZO-1 or occludin (red) is merged with DAPI counterstained nuclei (blue). mRNA expression of ZO-1(D) and occludin (E) evaluated in DSS/PBS, DSS/Lc treated group and healthy controls in colon and terminal ileum. RT-PCR was performed using TaqMan® gene expression assay for ZO-1. β-actin was used as the internal control. One-way ANOVA with Dunnett's multiple comparison test was used to evaluate the significance of differences between experimental groups and the DSS/PBS-treated control group (*P<0.05, **P<0.01, ***P<0.001). Data represent means (bar) ± SD (whisker) of five mice of one representative experiment out of three independent experiments. Scale bars are 10 μm in ZO-1 and 20 μm in occludin figures.

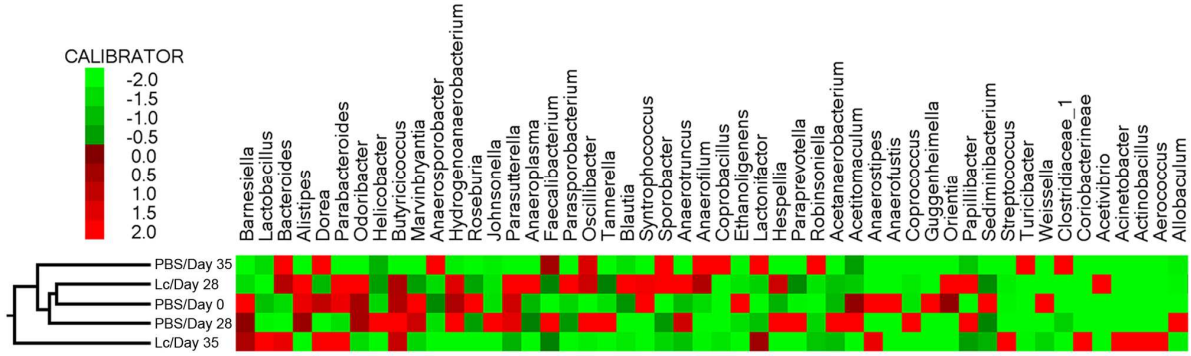


administered a single dose of FITC–dextran by gavage and measured the intensity of fluorescence in mouse serum 5h later. Oral pretreatment with Lc significantly decreased the intestinal permeability to macromolecules on the last day of DSS (day 35) to the same extent as found in healthy mice (Figure 1A). One possible mechanism by which this effect could be mediated is the reinforcement of tight junctions. Previous studies have demonstrated that DSS causes the extensive decrease in ZO-1 expression and occludin redistribution and that this effect could be prevented by live bacteria or their components in the murine colonic epithelium (28, 29). Therefore, we investigated whether treatment with Lc interferes with changes in the tight junction proteins production and distribution. As shown by immunohistochemistry and RT-PCR, treatment with Lc could completely prevent the loss of expression and changes in distribution of ZO-1 in both colon and terminal ileum (Figure 1B and D). Interestingly, in PBS-treated mice with subsequent induction of colitis (DSS/PBS), or in Lc-treated mice with subsequent induction of colitis (DSS/Lc) was a substantial loss of occludin in colon but not in terminal ileum (Figure 1C and E). Nevertheless, its distribution in colon seems to be slightly less affected in DSS/Lc- as compared with DSS/PBS-treated mice. Thus, we are able to demonstrate that the expression of ZO-1 in the colon and terminal ileum was significantly preserved following Lc treatment and probably contributes to reduced permeability of FITC-dextran. These findings suggest that treatment with Lc enhances the intestinal barrier function.

4.4.3 Oral treatment with lysate of *Lactobacillus casei* results in important changes in the gut microbial ecology

Changes in the intestinal gut microbial ecology are expected to be associated with the state of disease and could be influenced by probiotic treatment (30). To determine the impact of oral treatment with Lc on the intestinal microbiota, we used pyrosequencing of segments of genes for bacterial 16S rRNA. We collected feces before the treatment (day 0), before the colitis induction (day 28), and at the end of the experiment (day 35). We found that oral treatment with Lc resulted in significant changes in the intestinal microbial ecology (Figure 2). The frequently present genus in our fecal samples was a little-studied genus *Barnesiella*, from the Bacteroidetes phylum, one of the most abundant phylum in intestinal microbiota. The next most abundant genus with very well described capability to ameliorate intestinal inflammation *Lactobacillus* increased in abundance after exposure to DSS and the Lc

Figure 2. Oral treatment with Lc changes the intestinal microbiota composition. Normalized and z scored heat map and clustering dendrogram comparing relative abundance of the top 50 most abundant bacterial species in fecal microbiota of PBS (pool of 5 mice) and Lc-treated mice (pool of 5 mice) before the treatment (Day 0), before colitis induction (Day 28) and at the end of the experiment (Day 35). Horizontal columns represent the day of the experiment and or the treatment; vertical rows depict genus sorted from the most abundant species from left to right. The color scale for the heat maps is shown in upper left corner. The samples were clustered on the basis of their similarity by unsupervised clustering in the package CLUTO 2.1.1 (http://glaros.dtc.umn.edu/gkhome/cluto/cluto/download), as described previously (56).



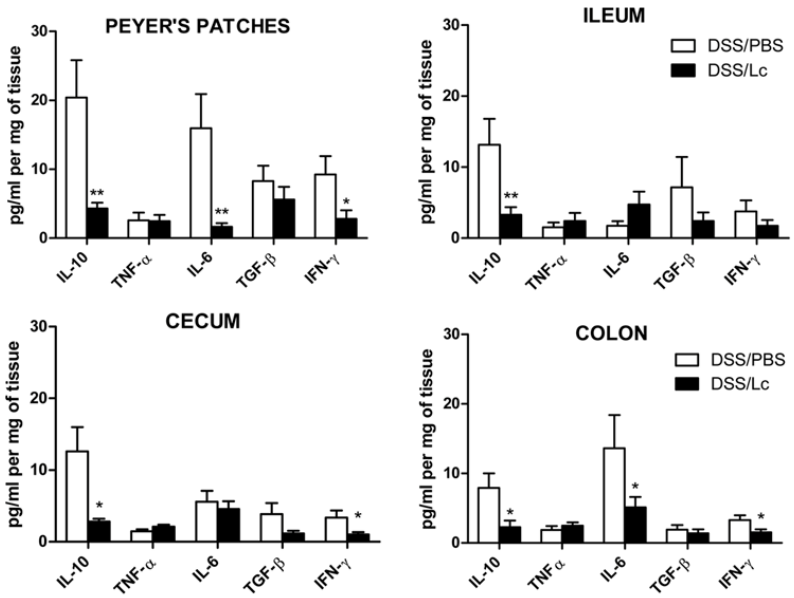
treatment. This increase in abundance was not observed in the control PBS group. The *Bacteroides,* known to be increased during DSS-induced colitis, proliferated after intestinal inflammation, was induced in Lc and PBS treated groups. Moreover, there is an increase in the biggest group of genera from Clostridium cluster: butyrate producing *Butyricicoccus, Coprococcus* and *Anaerostipes*. Butyrate is crucial for energy homeostasis of mammalian colonocytes, capable to prevent their autophagy (31). Dynamic changes in microbiota composition were observed before and during DSS administration in both Lc-treated and PBS-treated control group. Therefore, we can suggest that these microbial changes lead to improvement in gut barrier function and decrease susceptibility to intestinal inflammation by producing active substances such as lactate and butyrate.

4.4.4 Oral administration of Lc changes the immune response of gut mucosa

Changes in cytokine microenvironment in the gut mucosa can influence the mucosal immune response to luminal antigens leading to the decrease of intestinal inflammation. Therefore, we investigated if the protective effect of Lc is associated with modifications in inflammatory response in the key compartments of the gut. We cultivated tissues from four distinct parts of

the gut of either DSS/PBS or DSS/Lc-treated mice for 48h and then measured the cytokines in supernatants by ELISA. We found that pretreatment with DSS/Lc decreased the production of pro-inflammatory cytokines (IL-6, IFN-γ) and anti-inflammatory cytokine IL-10 in PPs, cecum and colon as compared to DSS/PBS-treated mice (Figure 3). These results were confirmed at mRNA level by RT-PCR (data not shown).

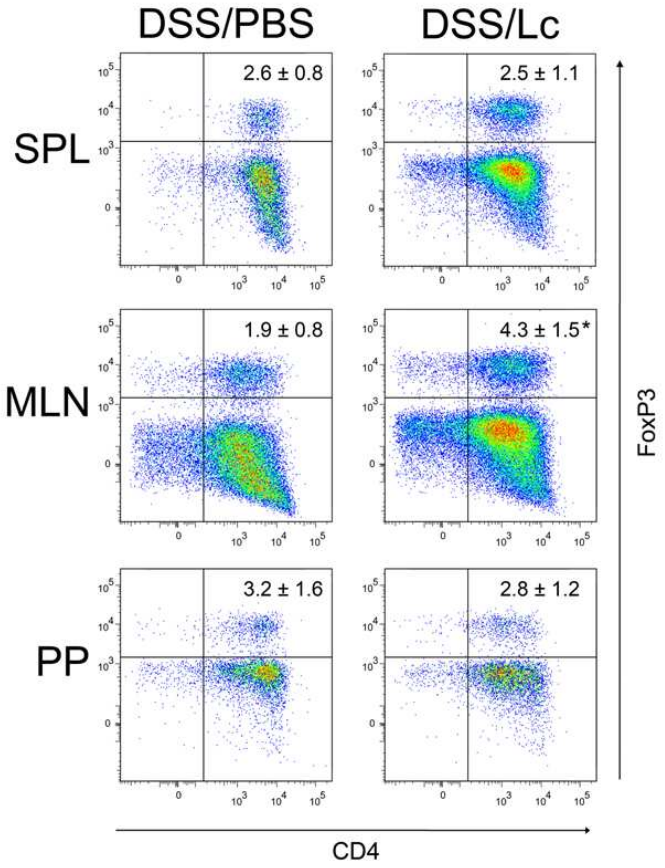
Figure 3. Pretreatment with Lc changes cytokine production in different parts of the gut. After DSS treatment and 24 hours cultivation, the production of cytokines TNF-α, TGF-β, IL-6, IL-10, IFN-γ differs in various parts of the gut as measured by ELISA. *P < 0.05, **P < 0.01 between DSS/PBS and DSS/Lc-treated mice in the same part of the gut was compared by unpaired Student's t-test (n = 10 per group).



4.4.5 Lc treatment increased the number of regulatory T cells

Since the intestinal inflammation in acute DSS-induced colitis is triggered by microbial antigens (32), the induction of oral tolerance to microbiota could be the one of the potential mechanisms of Lc protective effects. As the oral tolerance is maintained mainly at the periphery by T_{regs} , we analyzed the changes in $CD4^{+}FoxP3^{+}$ T_{regs} in the spleen, MLNs and PPs of DSS/PBS-, DSS/Lc-treated mice. We found a statistically significant increase in T_{regs} in MLN of DSS/Lc-treated mice as compared to DSS/PBS-treated mice. There were no statistically significant differences in the numbers of T_{regs} in spleen and PPs between these groups (Figure 4).

Figure 4. Oral treatment with Lc increases the number of $CD4^+FoxP3^+$ T_{regs} in MLNs. No significant changes were found in spleen or Peyer's patches. The plots shows the expression of CD4 *versus* FoxP3 on gated Th cells ($CD3^+CD8^-$), and the values within the plots represent the mean ± standard deviation of the total numbers of $CD4^+FoxP3^+$ T cells from one representative experiment out of three independent experiments (3-5 mice per group). One-way ANOVA with Dunnett's multiple comparison test was used to evaluate the significance of differences in numbers of $CD3^+CD8^-$ $CD4^+FoxP3^+$ cells between DSS/Lc-treated groups and the DSS/PBS-treated (control) group (*P<0.05).



4.4.6 Lysate of *L. casei*, but not *L. plantarum*, decreases the production of TNF-α and down-regulates NF-κB activity in LPS-activated macrophages

Because probiotics have an immunomodulatory effect on cells involved in innate immunity (33) and because the macrophages play a role in the pathogenesis of DSS-induced colitis (12), we analyzed the anti-inflammatory effect of Lc in LPS-activated macrophages in vitro. We found that doses below 100 pg/l significantly decrease the production of TNF-α by LPS-

stimulated RAW 264.7 cells in vitro, while similarly prepared Lp did not (Figure 5A). Using the FACS analysis of cultured cells, we found that neither Lc nor Lp changes the viability of RAW 264.7 cells (data not shown). The treatment with either lysate of bacteria in the absence of LPS did not change the TNF-α production (data not shown), this data is in agreement with a study using *L. casei* 3260 (34). As published by others (34, 35), this result suggests that Lc could interfere with the intracellular proinflammatory signaling cascade leading to activation of NF-κB transcription factor. To test this hypothesis, we isolated the nuclear extract from the untreated RAW 264.7 cells or from cells treated with either LPS (1 mg/L), or LPS with Lc and measured the activity of the NF-κB signaling pathway. Lc significantly decreased the NF-κB/DNA binding activity of p65 subunit as compared to the LPS-only or Lp+LPS treated cells (Figure 5B).

Since Lc treatment decreased production of TNF-α by LPS-activated macrophages, we decided to characterize macrophages further by investigating their stage of polarization by FACS. We found that M2 phenotype marker, the mannose receptor CD206 was significantly upregulated and M1 phenotype marker IL-7R downregulated in LPS+Lc treated macrophages as compared to either LPS or LPS+Lp treated macrophages. Therefore, Lc seems to counteract the LPS mediated M1 polarization. Neither Lc nor Lp without the addition of LPS changes the macrophage polarization.

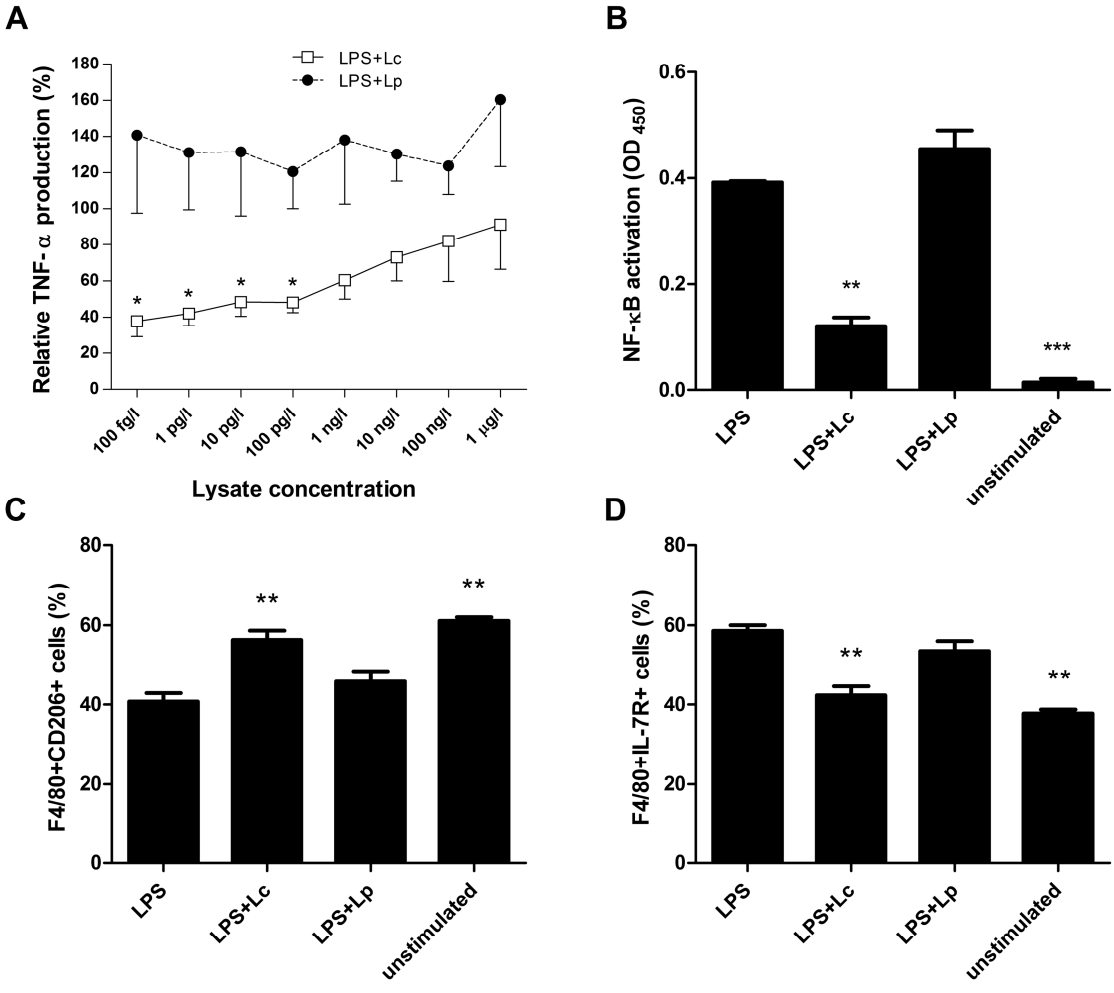
4.5 Discussion

Oral treatment with probiotic bacteria has emerged recently as a potentially useful therapeutic strategy for human IBD (5, 36). However, the clinical utility of such approach remains controversial, as the link between specific mechanisms of action and therapeutic effects of specific bacterium has been difficult to establish. We have shown previously that repeated oral administration of probiotic bacteria *L. casei* DN-114 001 protects BALB/c mice from severe forms of acute intestinal inflammation (21). In this study we demonstrated that not only live probiotic bacteria, but also its lysate protects BALB/c, but not SCID, mice from severe forms of DSS-induced inflammation.

The lack of protective effect in SCID mice suggests that mechanisms of adaptive immunity are essential for the beneficial effect of Lc. We did not find any changes neither in Lc-specific serum IgA, IgG and IgM, nor in gut SIgA during our experiments (data not shown), so we analyzed another mechanism executed by adaptive immune response, oral tolerance. Major role in this mechanism is played by Tregs, whose protective role in

inflammation control has been clearly established (37). In this study, we found that Lc treatment leads to significant increase in Tregs in MLNs, but not PPs. This might be because MLNs are crossroads between mucosal and systemic immunity, because even naïve T cells (L-selectin expressing cells) can enter and after the interaction with gut-committed cells

Figure 5. Lc exerts anti-inflammatory effect on LPS-activated macrophage cell line RAW 264.7. (A) Lc decreases the production of TNF-α in LPS-activated macrophages while Lp does not. TNF-α production by cells stimulated with 1 mg/l of LPS is set as 100% and data are expressed as means ± standard error of the mean of three independent experiments. *P<0.05: the means were compared against a hypothetical mean of 100% by one sample t-test (B) The effect of Lc on NF-κB binding activity in LPS-stimulated RAW 264.7 cells. Lc and Lp was co-cultured with LPS-activated cells for 24 h, and then the binding activity of NF-κB subunit p65 was analyzed by colorimetric assay. Data are expressed as mean ± standard deviation of three independent experiments. One-way ANOVA with Dunnett's multiple comparison test was used to evaluate the significance of differences between experimental groups and the LPS-treated cells group (**P<0.01, ***P<0.001). (C, D) Lc counteracts the LPS mediated M1 polarization. Expression of F4/80, CD206, IL-7R was determined by flow cytometry. One-way ANOVA with Dunnett's multiple comparison test was used to evaluate the significance of differences between experimental groups and the LPS-treated cells group (**P<0.01).



(α4β7-integrin expressing cells) from intestine became Tregs (38).

The intestinal barrier prevents viable enteric bacteria and the microbiota derived components from excessive interaction with the immune system. Here, we demonstrated that increase in intestinal permeability and the decrease in local ZO-1 expression, typical for DSS-treated mice, are both significantly improved by oral application of Lc. These results are in agreement with several studies showing that *L. casei* and other probiotics can strengthen the gut barrier function (28, 39). Probiotic *E. coli Nissle* 1917 provided protection against DSS-mediated leakiness and was capable to produce specific up-regulation of ZO-1 expression in the intestinal epithelial barrier (29). In addition, treatment with probiotic mixture VSL#3, where one of included bacterial strain is *L. casei*, prevents changes in expression and distribution of tight junction proteins ZO-1 and occludin (40). It is well known that inadequate function of intestinal barrier could lead to inflammatory and neoplastic diseases (41, 42). The disruption of the gut barrier has been identified as one of the crucial steps in IBD pathogenesis, causing excessive host-microbiota interaction during the initial phases of the IBD (26). Protection of the gut barrier from disruption by induction of changes in expression and distribution of tight junction proteins and mucus was proposed as a key mechanism of probiotic function (29, 43).

Several studies showed that there is a marked difference in the gut microbiota composition in IBD patients ("dysbiosis") as compared to healthy individuals. These changes in microbiota composition, or presence of certain microbial species with increased virulence, cause or perpetuate the intestinal inflammation in IBD (44). Here, we report that oral treatment with Lc significantly changes the composition of gut microbiota. Similar effects have been already described as mechanisms involved in the probiotics-mediated protection from intestinal inflammation (29, 45). Some of them are attributed to the fact, that probiotics can grow and colonize the gut, which could not be achieved with the non-living bacteria. The clear protective effect of bacterial lysate administration in intestinal inflammation is, therefore, rather indirect by shaping the gut microbial community or influencing the immune response. Nevertheless, similar mechanisms as in live bacteria could be involved to explain this effectiveness. Probiotics (or certain bacteria in general) can produce substances with antibiotic properties, such as bacteriocins, and molecules capable to signal to other members of the ecosystem to adjust their growth (quorum sensing modifiers), as recently reviewed (46). These molecules could be present in the lysates of bacteria and, selectively modify the bacterial populations (47). Moreover, certain probiotics can induce long-term production of anti-microbial peptides in vivo, which can shape the gut microbiota composition long time

after the probiotic therapy has ended (48). These mechanisms cause more favorable microbiota composition thus renders the Lc-treated mice less susceptible to intestinal inflammation.

By using the pyrosequencing technique we observed an increase in Bacteroides genus after induction of intestinal inflammation as shown previously (11). DSS/Lc compared to DSS/PBS-treated group has shown a substantial increase in Lactobacillus genus which suggests that treatment with Lc promotes this genus among others. This effect could be caused by formation of niche ideal to lactobacilli. These and other differences in microbiota could be also explained by decreased inflammation in Lc-treated mice mediated by differing immunological mechanisms.

The cytokines produced in the gut mucosa greatly influence the resulting immunological outcome. The production of anti-inflammatory cytokines induces the mucosal unresponsiveness and tolerance and high levels of pro-inflammatory cytokines induce protective immune response and inflammation (49). Here, we report that Lc treatment decrease the production of pro-inflammatory cytokines IL-6 and IFN-γ as well as anti-inflammatory cytokine IL-10 in both PP and the large intestine. This suggests that Lc can influence both the induction and effectors' functions of the mucosal immune system. We did not find significant decrease in local production of TNF-α, despite the clear differences in the colitis severity between Lc/DSS and PBS/DSS treated mice. This is consistent with our previous experiments (11), and suggests that, despite being crucial pro-inflammatory cytokine produced by macrophages, TNF-α could be either exhausted or downregulated by IL-10, cytokine inhibiting TNF-α production, at this stage of colitis. Interestingly, since IFN-γ increases the gut permeability (50), a decrease in its local production can be responsible for strengthening of the gut barrier function as we found in Lc-treated mice. This is in agreement with findings that live *L. casei* can downregulate the pro-inflammatory mediators in the lamina propria of inflamed mucosa from Crohn's disease patients during ex vivo cultivation (51). However, various strains of lactobacilli could differ in their immunological activities (52), and some lactobacilli are capable to induce T cells toward Th1 (53) or Th2 (54) immune responses.

Acute DSS colitis is believed to be driven initially by innate immunity mechanisms and, in particular, the role of macrophages has been proposed (12, 15, 32). Therefore we tested the ability of bacterial lysates to decrease the inflammatory response of LPS-activated macrophages in vitro. We found that Lc, but not Lp, decreases the production of TNF-α, and the activation of NF-κB cascade and polarizes macrophages to M2 phenotype, suggesting a

possible direct effect of Lc on the cells of the innate immunity. It is not excluded that negative regulators are involved in beneficial anti-inflammatory effects of probiotics (55).

In conclusion, our data provide evidence that even lysate of *L. casei* DN-114 001 can protect from induction of intestinal inflammation, thus confer a health benefit for the host. This is achieved by mechanisms that comprise: a) improvement in the gut barrier function, b) correction of the dysbiosis, and c) modulation of the mucosal immune response. These complex immunomodulatory properties of bacterial lysates may lead to the development of new therapeutic approaches for treatment of chronic intestinal inflammation. Moreover, oral administration of sterile bacteria, in contrast to live bacteria, may be safer in severely ill or immunocompromised patients.

4.6 Acknowledgements

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4.7 References

1. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol. 2006 Jul;3(7):390-407.
2. Packey CD, Sartor RB. Commensal bacteria, traditional and opportunistic pathogens, dysbiosis and bacterial killing in inflammatory bowel diseases. Curr Opin Infect Dis. 2009 Jun;22(3):292-301.
3. Sheil B, Shanahan F, O'Mahony L. Probiotic effects on inflammatory bowel disease. J Nutr. 2007 Mar;137(3 Suppl 2):819S-24S.
4. FAO/WHO. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Cordoba (Argentina): Food and Agriculture Organization of the United Nations and World Health Organization2001 1-4 Oct 2001.
5. Bibiloni R, Fedorak RN, Tannock GW, Madsen KL, Gionchetti P, Campieri M, et al. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am J Gastroenterol. 2005 Jul;100(7):1539-46.
6. Lebeer S, Vanderleyden J, De Keersmaecker SC. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microbiol. 2010 Mar;8(3):171-84.
7. Servin AL. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev. 2004 Oct;28(4):405-40.
8. Gupta P, Andrew H, Kirschner BS, Guandalini S. Is lactobacillus GG helpful in children with Crohn's disease? Results of a preliminary, open-label study. J Pediatr Gastroenterol Nutr. 2000 Oct;31(4):453-7.
9. Reiff C, Delday M, Rucklidge G, Reid M, Duncan G, Wohlgemuth S, et al. Balancing inflammatory, lipid, and xenobiotic signaling pathways by VSL#3, a biotherapeutic agent, in the treatment of inflammatory bowel disease. Inflamm Bowel Dis. 2009 Nov;15(11):1721-36.
10. Besselink MG, van Santvoort HC, Buskens E, Boermeester MA, van Goor H, Timmerman HM, et al. Probiotic prophylaxis in predicted severe acute pancreatitis: a randomised, double-blind, placebo-controlled trial. Lancet. 2008 Feb 23;371(9613):651-9.
11. Kverka M, Zakostelska Z, Klimesova K, Sokol D, Hudcovic T, Hrncir T, et al. Oral administration of Parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. Clin Exp Immunol. 2011;163(2):250-9.
12. Okayasu I, Hatakeyama S, Yamada M, Ohkusa T, Inagaki Y, Nakaya R. A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. Gastroenterology. 1990 Mar;98(3):694-702.
13. Poritz LS, Garver KI, Green C, Fitzpatrick L, Ruggiero F, Koltun WA. Loss of the tight junction protein ZO-1 in dextran sulfate sodium induced colitis. J Surg Res. 2007 Jun 1;140(1):12-9.
14. Yan Y, Kolachala V, Dalmasso G, Nguyen H, Laroui H, Sitaraman SV, et al. Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. PLoS One. 2009;4(6):e6073.
15. Dieleman LA, Ridwan BU, Tennyson GS, Beagley KW, Bucy RP, Elson CO. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology. 1994 Dec;107(6):1643-52.
16. Dieleman LA, Palmen MJ, Akol H, Bloemena E, Pena AS, Meuwissen SG, et al. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. Clin Exp Immunol. 1998 Dec;114(3):385-91.
17. Kleerebezem M, Vaughan EE. Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. Annu Rev Microbiol. 2009;63:269-90.
18. Hanniffy S, Wiedermann U, Repa A, Mercenier A, Daniel C, Fioramonti J, et al. Potential and opportunities for use of recombinant lactic acid bacteria in human health. Adv Appl Microbiol. 2004;56:1-64.
19. Merenstein D, Murphy M, Fokar A, Hernandez RK, Park H, Nsouli H, et al. Use of a fermented dairy probiotic drink containing Lactobacillus casei (DN-114 001) to decrease the rate of illness in kids: the DRINK study. A patient-oriented, double-blind, cluster-randomized, placebo-controlled, clinical trial. Eur J Clin Nutr. 2010 Jul;64(7):669-77.
20. Ingrassia I, Leplingard A, Darfeuille-Michaud A. Lactobacillus casei DN-114 001 inhibits the ability of adherent-invasive Escherichia coli isolated from Crohn's disease patients to adhere to and to invade intestinal epithelial cells. Appl Environ Microbiol. 2005 Jun;71(6):2880-7.
21. Kokesova A, Frolova L, Kverka M, Sokol D, Rossmann P, Bartova J, et al. Oral administration of probiotic bacteria (E. coli Nissle, E. coli O83, Lactobacillus casei) influences the severity of dextran sodium sulfate-induced colitis in BALB/c mice. Folia Microbiol (Praha). 2006;51(5):478-84.
22. Wang Q, Fang CH, Hasselgren PO. Intestinal permeability is reduced and IL-10 levels are increased in septic IL-6 knockout mice. Am J Physiol Regul Integr Comp Physiol. 2001 Sep;281(3):R1013-23.

23. Zanvit P, Tichopad A, Havlickova M, Novotna O, Jirkovska M, Kolostova K, et al. 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. 2010 2010 Nov 30;134(1):26-34.
24. Gontcharova V, Youn E, Wolcott RD, Hollister EB, Gentry TJ, Dowd SE. Black Box Chimera Check (B2C2): a Windows-Based Software for Batch Depletion of Chimeras from Bacterial 16S rRNA Gene Datasets. Open Microbiol J. 2010;4:47-52.
25. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007 Aug;73(16):5261-7.
26. Munkholm P, Langholz E, Hollander D, Thornberg K, Orholm M, Katz KD, et al. Intestinal permeability in patients with Crohn's disease and ulcerative colitis and their first degree relatives. Gut. 1994 Jan;35(1):68-72.
27. Kitajima S, Takuma S, Morimoto M. Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. Exp Anim. 1999 Jul;48(3):137-43.
28. Ewaschuk JB, Diaz H, Meddings L, Diederichs B, Dmytrash A, Backer J, et al. Secreted bioactive factors from Bifidobacterium infantis enhance epithelial cell barrier function. Am J Physiol Gastrointest Liver Physiol. 2008 Nov;295(5):G1025-34.
29. Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W, et al. Probiotic Escherichia coli Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One. 2007;2(12):e1308.
30. Fava F, Danese S. Intestinal microbiota in inflammatory bowel disease: friend of foe? World J Gastroenterol. 2011 Feb 7;17(5):557-66.
31. Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab. 2011 May 4;13(5):517-26.
32. Hudcovic T, Stepankova R, Cebra J, Tlaskalova-Hogenova H. 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). 2001;46(6):565-72.
33. Mileti E, Matteoli G, Iliev ID, Rescigno M. Comparison of the immunomodulatory properties of three probiotic strains of Lactobacilli using complex culture systems: prediction for in vivo efficacy. PLoS One. 2009;4(9):e7056.
34. Lee JM, Hwang KT, Jun WJ, Park CS, Lee MY. Antiinflammatory effect of lactic acid bacteria: inhibition of cyclooxygenase-2 by suppressing nuclear factor-kappaB in Raw264.7 macrophage cells. J Microbiol Biotechnol. 2008 Oct;18(10):1683-8.
35. Matsumoto S, Hara T, Hori T, Mitsuyama K, Nagaoka M, Tomiyasu N, et al. Probiotic Lactobacillus-induced improvement in murine chronic inflammatory bowel disease is associated with the down-regulation of pro-inflammatory cytokines in lamina propria mononuclear cells. Clin Exp Immunol. 2005 Jun;140(3):417-26.
36. Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, et al. Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut. 2004 Nov;53(11):1617-23.
37. Singh B, Read S, Asseman C, Malmstrom V, Mottet C, Stephens LA, et al. Control of intestinal inflammation by regulatory T cells. Immunol Rev. 2001 Aug;182:190-200.
38. Mowat AM, Donachie AM, Parker LA, Robson NC, Beacock-Sharp H, McIntyre LJ, et al. The role of dendritic cells in regulating mucosal immunity and tolerance. Novartis Found Symp. 2003;252:291-302; discussion -5.
39. Liu Z, Zhang P, Ma Y, Chen H, Zhou Y, Zhang M, et al. Lactobacillus plantarum prevents the development of colitis in IL-10-deficient mouse by reducing the intestinal permeability. Mol Biol Rep. 2011 Feb;38(2):1353-61.
40. Mennigen R, Nolte K, Rijcken E, Utech M, Loeffler B, Senninger N, et al. Probiotic mixture VSL#3 protects the epithelial barrier by maintaining tight junction protein expression and preventing apoptosis in a murine model of colitis. Am J Physiol Gastrointest Liver Physiol. 2009 May;296(5):G1140-9.
41. Fasano A. Zonulin and its regulation of intestinal barrier function: the biological door to inflammation, autoimmunity, and cancer. Physiol Rev. 2011 Jan;91(1):151-75.
42. Tlaskalova-Hogenova H, Stepankova R, Kozakova H, Hudcovic T, Vannucci L, Tuckova L, et al. 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.
43. Chen HQ, Yang J, Zhang M, Zhou YK, Shen TY, Chu ZX, et al. Lactobacillus plantarum ameliorates colonic epithelial barrier dysfunction by modulating the apical junctional complex and PepT1 in IL-10 knockout mice. Am J Physiol Gastrointest Liver Physiol. 2010 Sep 30.

44. Sokol H, Seksik P, Rigottier-Gois L, Lay C, Lepage P, Podglajen I, et al. Specificities of the fecal microbiota in inflammatory bowel disease. Inflamm Bowel Dis. 2006 Feb;12(2):106-11.
45. Tannock GW, Munro K, Harmsen HJ, Welling GW, Smart J, Gopal PK. Analysis of the fecal microflora of human subjects consuming a probiotic product containing Lactobacillus rhamnosus DR20. Appl Environ Microbiol. 2000 Jun;66(6):2578-88.
46. Boyer M, Wisniewski-Dye F. Cell-cell signalling in bacteria: not simply a matter of quorum. FEMS Microbiol Ecol. 2009 Oct;70(1):1-19.
47. Jamuna M, Jeevaratnam K. Isolation and characterization of lactobacilli from some traditional fermented foods and evaluation of the bacteriocins. J Gen Appl Microbiol. 2004 Apr;50(2):79-90.
48. Mondel M, Schroeder BO, Zimmermann K, Huber H, Nuding S, Beisner J, et al. Probiotic E. coli treatment mediates antimicrobial human beta-defensin synthesis and fecal excretion in humans. Mucosal Immunol. 2009 Mar;2(2):166-72.
49. MacDonald TT, Monteleone G, Pender SL. Recent developments in the immunology of inflammatory bowel disease. Scand J Immunol. 2000 Jan;51(1):2-9.
50. Madara JL, Stafford J. Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. J Clin Invest. 1989 Feb;83(2):724-7.
51. Llopis M, Antolin M, Carol M, Borruel N, Casellas F, Martinez C, et al. Lactobacillus casei downregulates commensals' inflammatory signals in Crohn's disease mucosa. Inflamm Bowel Dis. 2009 Feb;15(2):275-83.
52. Thomas CM, Versalovic J. Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbes. 2010 May;1(3):148-63.
53. Chuang L, Wu KG, Pai C, Hsieh PS, Tsai JJ, Yen JH, et al. Heat-killed cells of lactobacilli skew the immune response toward T helper 1 polarization in mouse splenocytes and dendritic cell-treated T cells. J Agric Food Chem. 2007 Dec 26;55(26):11080-6.
54. Delcenserie V, Martel D, Lamoureux M, Amiot J, Boutin Y, Roy D. Immunomodulatory effects of probiotics in the intestinal tract. Curr Issues Mol Biol. 2008;10(1-2):37-54.
55. Biswas A, Wilmanski J, Forsman H, Hrncir T, Hao L, Tlaskalova-Hogenova H, et al. Negative regulation of Toll-like receptor signaling plays an essential role in homeostasis of the intestine. Eur J Immunol. 2011 Jan;41(1):182-94.
56. Zhao Y, Karypis G. Clustering in life sciences. Methods Mol Biol. 2003;224:183-218.

5 Colorectal carcinoma: Importance of colonic environment for anti-cancer response and systemic immunity

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5.1 Abstract

The intestinal environment is considered to play an important role both in colorectal tumor development and in the evolution and modulation of mucosal immunity. Studies in animals reared in germ-free (GF, without any intestinal microflora) versus conventional (CV, with regular microflora in bowel) conditions can aid in clarifying the influence of bacteria on carcinogenesis and anti-cancer immune responses *in situ*. The lower incidence of colon cancers and better immunological parameters in GF animals versus CV ones after chemically-induced carcinogenesis raises questions about specific characteristics of the immunological networks in each respective condition. Different levels of tolerance/regulatory mechanisms in the GF versus CV animals may influence the development of immune responses not only at the level of mucosal, but also at the systemic, immunity. We hypothesize that GF animals can better recognize and respond to evolving neoplasias in the bowel as a consequence of their less-tolerogenic immunity (i.e., due to their more limited exposure to antigens to become tolerated against at the intestinal level). In this paper, we review the role of bacteria in modulating gut environment and mucosal immunity, their importance in cancer development, and aspects of immune regulation (both at local and systemic level) that can be modified by bacterial microflora. Lastly, the use of GF animals in comparison with conventionally-raised animals is proposed as a suitable and potent model for understanding the inflammatory network and its effect on cancer immunity especially during colorectal cancer development.

5.2 Introduction

Colorectal carcinoma is a cancer still characterized by high incidence and mortality worldwide, especially in the Western world (extensively reviewed in (1)). In Europe alone, 412,900 cases were reported (12.9% of all cancers), along with 207,400 deaths in 2006 (2). In the United Kingdom, it is the second most commonly diagnosed cancer in females (34.8/100,000) and third in males (54.8/100,000). Similarly, it is the third most commonly diagnosed cancer in both males (62.7/100,000) and females (45.8/100,000) in the United States and the second most commonly diagnosed cancer for males (60.7/100,000) and females (52.1/100,000) in Australia.

An increase of colorectal cancer incidence in Asiatic countries has also been reported (i.e., it is now the third most common cancer). For example, in Japan and China (Hong Kong, specifically), incidence levels have been attained that were very similar to those in the United States (3). Investigators have indicated that most of this increase in incidence was very likely associated with the populations' progressive adoption of a Western lifestyle and diet. Interestingly, in Africa, while the colorectal cancer incidence has been found to range from 3.7% to 10% of all diagnosed cancer, the cancers were seen to occur among a larger number of young patients (relative to the incidence in this population in Western countries) as well as among the increasingly urbanized populations (4). As a result, it has been suggested that general environmental factors (i.e., lifestyle, alimentation [diet]) were likely critical for enhanced carcinogenesis in the gut and colorectal carcinoma development, and these outcomes were being influenced through possible changes in the local intestinal environment (e.g., commensal microflora) that variously impacted upon the mucosal components, including immune system cells (5, 6).

The potential role of bacteria and inflammation in carcinogenesis and cancer progression has raised increasing interest among researchers over the last 15 years (7-9). A number of studies have shown inflammation to be a central event in various phases of cancer evolution due to the double-faceted activity of inflammatory cells (macrophages) and molecules (interleukin [IL]-1β, tumor necrosis factor [TNF]-α, transforming growth factor [TGF]-β, IL-4, metalloproteinases, nitric oxide [NO], etc.) in the cancer microenvironment (10-13).

Intestinal bacterial flora has been described as displaying a double-edged activity, either assisting or preventing carcinogenesis, depending on the metabolic activities and degree of saprophytism of the bacterial species (e.g., Clostridia, Lactobacillus) (5, 14). The

composition of the gut commensal microbiota and the prevalence of bacterial species are influenced by various factors during the lifetime of an individual (type of diet, traveling, use of antibiotics), with effects on the mucosal immune system of the gut and also the possible modulation of systemic immunity (15, 16).

To study how microbiota, mucosal components, and immunity might crosstalk, comparative research has been developed using gnotobiologic animals versus individuals of the same strain reared under conventional conditions (i.e., with regular commensal microbiota in the gut). In fact, gnotobiologic animals can be either reared in GF conditions (with completely sterile gut) or be selectively contaminated with known bacteria. As it has been documented for more than 25 years, these animal models represent an extraordinary investigative tool for immunological and oncological studies on colorectal pathologies that occur in humans. (Readers are directed to papers from (17, 18) as good examples.)

5.3 Intestinal microbiota and local immunity

The intestinal environment is largely determined by the commensal microbiota normally present in the individual gut. The billions of bacteria forming the composite intestinal microflora start to colonize the gut after birth and undergo variations during their lifespan (19-21). Their establishment and different proportion of species can influence the development of local immunity (mucosal immunity), with concomitant effects that can extend to systemic immunity (see review of (22)). An example of this phenomenon is the induction of genes involved in innate immunity after co-colonization of the gut of gnotobiologic animals with *Bifidobacterium longum* and/or *Bacterioides thetaiotaomicron* (23). Since only a small part of the intestinal bacterial species (≈500 species) can be cultured in vitro, there is still a lack of complete information about effects exerted by many species on the biology of the intestinal mucosa and, consequently, on mucosal and systemic immunity. Nevertheless, very recent "-omic" approaches (i.e., metagenomics, metabonomics) are trying to elucidate these aspects (24-27).

In the bowel, especially the colon, the major micro-biota (more than 90%) are obligate anaerobes, including Bacteroides, Eubacterium, Bifidobacterium. Both *Escherichia coli* and Lactobacillus are also present, but in more limited proportions. Many studies have shown that the balance between the bacterial populations can influence many carcinogenic processes, either by inducing tumor promotion (Clostridia) or inhibition (Lactobacillus) (5, 28). The "eutrophism" of certain bacterial populations follows the dietary habits of the host, and the

resulting metabolic products of the microbiota can possibly act as tumor promoting (e.g., nitrosamine, polyamines, etc.) or inhibiting (e.g., butyrate, etc.) factors (5, 29).

The antigens derived from the various species of bacteria (e.g., lipopolysaccharides [LPS]), from the bacterial metabolism, and from the very large amount of ingested substances (from food, drugs, etc.), make essential the activation of immunological mechanisms permitting (oral) tolerance in order to avoid detrimental effects on the bowel in particular and the organism in general (i.e., chronic inflammatory diseases, alimentary intolerances, autoimmunity) (6, 30). Simultaneously while maintaining the symbiotic tolerance toward commensal microflora, the host's immune system must still be able to counter any pathogenic bacteria assaulting the gut. The mechanisms underlying this "double play" approach are not yet completely elucidated. However, the possibility that efficient immune responses can be elicited at regional level in the bowel (involving mucosal cells, gut-associated lymphatic tissue [GALT], intraepithelial lymphocytes [IEL], dendritic cells [DC], and cells of the mesenteric lymph nodes [MLN]) (31, 32) has to be considered.

Within the gut, if antigens pass through the mucosal barrier and are directly presented to GALT (i.e., via a damaged lamina propria), they may escape the regulatory network and are then able to elicit a rapid and vigorous immune response. This response at the systemic level would give rise to the appearance of antigen-specific antibodies in the serum and, locally, an antigen-specific IgA response in the bile. Breaking the mucosal barrier and its tolerant environment, the GALT appears capable of active reactions to foreign antigens penetrating the gut wall (33). Because certain bacteria types (e.g., *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus viridians*, *Lactobacillus brevis*) induce modification of the mucosal permeability, the balance among commensal microbiota species is of considerable importance (20, 28, 34).

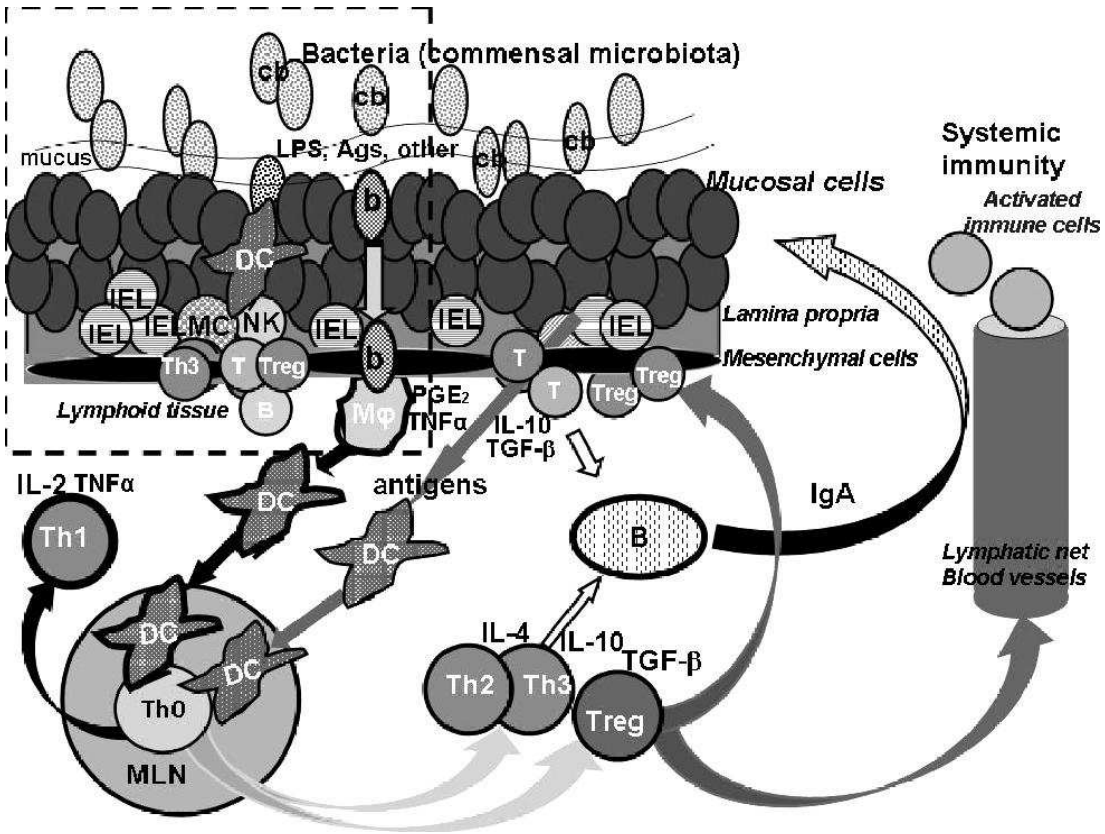
The GALT-articulated immunological barrier and, in particular, the IEL, DC, and macrophages, constitute a very reactive network. Because of the continuous attempts of bacteria to penetrate the mucosa, it is understandable that there must be a constant activation of the mucosal immunity to preserve mucosal integrity and immunological homeostasis. In general, this activated network has the characteristics of a "controlled" inflammatory environment, also referred to as a "physiological inflammation" (35-37). The regulation of this inflammation within "physiological" limits involves secretion of at least three important cytokines: TGF-β, IL-4, and IL-10. In particular, these TH2 cytokines are (physiologically) involved in the termination of inflammatory responses and in the termination of local wound reparation processes (38, 39).

Immune cells associated to the lamina propria along with intestinal epithelial cells (IEC) can collaborate in the production of TGF-β. There are several examples of this collaborative effect in the literature. For example, it is well known that IEC are able to secrete a variety of cytokines, permitting their active participation to the modulation of local immune responses. The IEC, because of their close proximity to B-lymphocytes in the lamina propria, may, and do, affect local antibody production (e.g., IgA, IgG, and IgM isoforms) via some of their cytokines, i.e., IL-6 and TGF-β (40). Smythies et al. (2006) showed that IL-8 and TGF-β, that was being produced by gut epithelial cells and lamina propria mast cells, were critical factors in the regulation of mononuclear cell recruitment to both non-inflamed and inflamed intestinal mucosa (41). Lastly, even the mucosal cells can be harnessed by pathogens to help in thwarting the normal immune response required to encounter/remove the bacteria, in part, influencing of the cells' abilities to release TGF-β. Specifically, Wu and colleagues recently showed that there were soluble proteins released by Helicobacter pylori that were able to preferentially induce TGF-β production by gastric epithelial cells (42).

Antigen tolerance, critical to maintain the symbiotic bal-ance between mucosal cells and commensal microbiota, is enabled by the function of TGF-β-producing T-regulatory lymphocytes ($CD4^+CD25^+Foxp3^+$) of the lamina propria and a special population of IL-10-producing DC. These DC are able to polarize naïve T-helper (TH0) lymphocytes to become regulatory cells producing IL-4, IL-10, and TGF-β and also stimulate B-lymphocytes to produce IgA (43, 44). Furthermore, B-lymphocytes are also stimulated by BAFF/APRIL ligands released by IEC. The IL-10-producing DCs exert their function by migrating in the Peyer's patches and in the MLN where the tolerance to the antigens originating from the alimentary tract is developed. In this way, these DC contribute to the control of the homeostasis in the bowel and permit the maintenance of a tolerant environment leading also to systemic tolerance and local IgA production (45-47); Figure 1).

It is also clear that the MLN represent a critical center both for the elimination of translocated bacteria and for processing of their products and other various antigens without direct challenge to the systemic immunity (31). In fact, the commensal bacteria are largely pre-vented from reaching the systemic immune compartment by efficient macrophage killing activity and by sequestering in DC at the MLN level. Intestinal inflammatory responses induced by pathogenic bacteria are driven by recognition of pathogen-associated molecular patterns (PAMPs) with involvement of Toll-like receptor (TLR) expressed on mucosal cells, mesenchymal cells, and mucosal immune cells. The pro-inflammatory cytokines (i.e., IL-1, IL-6, IL-8, and CCL5) collaborate with the activation of macrophages by lipopolysaccharides

Figure 1. Interplay between microbiota and mucosal immunity in the gut. The figure shows a schematic view of the multiple relationships between commensal bacteria (cb), mucosal cells, and immune cells involved in the gut immune responses and homeostasis. Bacteria (b) on the mucosal surface are inhibited from invading the mucosa by both mechanical-anatomical barrier (mucus, tight intercellular junctions) and mucosal immunity activation. Immune cells involved in this network are mainly located in the lamina propria and within mucosal epithelial structures (intraepithelial lymphocytes, IEL). Innate immunity cells (e.g., macrophages, Mφ; mast cells, MC; natural killer cells, NK) are involved (along with local dendritic cells [DC]) in rec-ognition, killing, processing, and presentation of bacterial-/food-derived antigens to adaptive immunity T-cells (T) that are then primed and activated. These innate immunity cells, once having been activated following contact with the bacteria, generate pro-inflammatory molecules like TNF-α, IL-1β, NO, and/or PGE_2. If the antigens are processed passing through the lamina propria defense network, DC activation of T-helper cells in the mesenteric lymph nodes (MLN, cell maturation centers) will lead naïve (T_H0) cells to develop into regulatory/tolerant (i.e., T_H2, T_H3, T_{reg}) cells. The cytokines, that these specific cell types produce (e.g., IL-4, IL-10, TGF-β) are essential in modulating the continuously activated mucosal immunity ("physiologic inflammation") and for inducing IgA production by lamina propria-associated B-cells. These IgA are secreted in the mucosa (to protect it from the target microflora) and circulate systemically to induce/partake in general immunologic effects. Part of the gut local immune response is an acute inflammatory response. This is elicited by direct contact of either bacteria or intestinal lumen anti-gens with immune cells "skipping" along the lamina propria barrier or via stimulation of these cells by bacterial products like lipopolysaccharides. In this case, the response is the maturation of T_H1 cells, the release of T_H1 cytokines (IL-2, IFN-γ, TNF-α), and the elicitation of cytotoxic activity. If these responses are not rigorously controlled and regularly terminated, damage to the mucosa can develop and the "physiological inflammation" can exceed the homeostatic limit and so become pathological. Activated (including tolerant) cells can pass into the lymphatics and blood vessels, entering the general circulation to impact upon systemic immune responses.



to stimulate DC (34, 45). After uptake of the pathogenic anti-gens and migration in the MLN, the DC mature and produce IL-12. The primed naïve CD4+ T-helper (TH0) lymphocytes develop as TH1 and sustain the inflammatory process. The TH1 lymphocytes can express their function both locally and at the systemic level. Therefore, the tolerogenic response, produced by the DC and T-helper lymphocyte crosstalk with the epithelial cells and bacteria, induces modulation of the cytokine balance (TH1, TH2, and TH3 cytokines) as well as the inflammatory reaction elicited by TLR involvement, and, in the end, may produce effects that can influence systemic immunity (30, 48).

Under these conditions, the highly efficient regulatory network generated through the chronic challenge of the mucosal immune cells by the commensal microbiota may affect the activation of mechanisms leading to the recognition of transformed cells (e.g., danger signals) (49). We can also hypothesize that the higher the number of antigens to be tolerated, the higher should be the possibility of cross-recognition of molecules that when expressed during the cancer cell transformation may mimic tolerated antigens.

5.4 Effects of intestinal microbial environment on carcinogenesis and natural immunity

Bacteria of different strains, including commensal micro-biota, can support environmental conditions that can lead to mucosal cell transformation and/or the sustaining of tumor progression. Epidemiologic studies have estimated that ≈15% of the worldwide cancer incidence is attributable in a secondary manner to infectious agents (50). In general, bacteria have been linked to cancer by two mechanisms: induction of chronic inflammation following bacterial infection and production of toxic bacterial metabolites. Some studies in human patients have revealed an interesting correlation between specific bacteria and gastrointestinal cancers (51, 52). In 2004, Heavey and Rowland detailed some of the relationships between specific bacteria and gastrointestinal cancers, as well as pathologies (non-cancerous) that could give rise to enhanced bacterial contributions to the onset/development of neoplasias in the gut (7). For example, in patients with reduced gastric acid secretion (hypochlorhydria), the subjects often ultimately become achlorhydric. Hypochlorhydria is a condition that is common after gastric surgery and occurs with aging, diseases such as pernicious anaemia and hypogammaglobulinaemia, and, in patients with atrophic gastritis associated with chronic *H. pylori* infection. The evolution to achlorhydria allows for diverse flora to establish in the

stomach (i.e., usually species of salivary Neisseria, Streptococcus, and Staphylococcus, as well as Escherichia, Bacteroides, and Lactobacillus). This condition increases the probability of xenobiotic metabolism by bacteria, particularly since gastric emptying rates are often prolonged in these patients. It has been suggested (see Hill, 1988) that the increased gastric cancer risk seen in achlorhydric patients is linked, in part, to a now-increased formation of N-nitroso compounds by these gastric bacteria.

With respect to this one pathogen, both epidemiological and clinical evidence has indicated that *H. pylori* is associated with an increased risk of gastric carcinoma (53, 54). In fact, the International Agency for Research into Cancer (IARC) declared in 1997 "There is sufficient evidence in humans for the carcinogenicity of infection with *H. pylori*" (55), though similar conclusions in animal models were lacking. Furthermore, in developed countries, strains of *H. pylori* that carry the cag pathogenicity island are associated with an increased risk of adenocarcinoma compared with strains negative for cag. This effect appears to be dependent upon alterations in IL-4 balance and activity induced in the mucosa infected by cag island + *H. pylori*, a status that could then promote a chronic inflammation and, ultimately, lead to metaplasia of the gastric mucosa (56, 57). Even if it is generally accepted that *H. pylori* infection plays a significant role in the etiology of gastric cancer, however, the precise mechanisms have not yet been defined. Several proposed for *H. pylori*-associated carcinogenesis in humans have included: gastric epithelial cell cycle dysregulation, increased DNA adduct and free radical generation, altered growth factor secretion/cytokines, and effects that evolved secondarily to induced decreases in gastric secretion(s).

In their 2004 review, Heavey and Roland also postulated about the bacterial involvement in colorectal cancer. As they noted, most of the information on this phenomena was derived from animal work and some human studies and that evidence from a wide range of sources supported the view that the colonic microflora was involved in the etiology of local cancers (7). Among the major categorizations of evidence, it was indicated that: intestinal bacteria can produce, from dietary components, substances with genotoxic, carcinogenic, and tumor-promoting activities (58); gut bacteria can activate pro-carcinogens to DNA-reactive forms; germ-free (GF) rats fed 1,2-dimethylhydrazine or fed human diets had a lower incidence of, respectively, colon tumors and of DNA adducts than similarly-treated rats with a normal microflora (59, 60); and, that human fecal matter has been documented to contain mutagenic and genotoxic substances of bacterial origin (61). Moreover, a direct effect of *Enterococcus faecalis* as an inducer of aneuploidy and tetraploidy was demonstrated in intestinal cell cultures (62). The DNA damage and instability was associated to the oxidative

stress due to the production of extracellular superoxide, also by induction of macrophage COX-2, generating chromosomal instability. Antioxidants were able to attenuate this effect while it was worsened by the administration of inhibitors of glutathione synthase. Moreover, epithelial cells were induced to G2 cell cycle arrest by the direct contact with the bacteria (63).

The importance of specific bacterial strains in exerting pro-carcinogenic activities is demonstrated also by studies that utilized gnotobiological animals. For example, colonization with a single strain or a selected mix of Bacteroides and Clostridium species increased the incidence of chemically-induced intestinal cancers (64, 65). These Investigators noted that the cancer-promoting activity of bacteria like *Mitsuokella multiacida, Clostridium butyricum, Bifidobacterium longum, C. paraputrificum, C. butyricum,* and *E. coli* was related to the de-conjugation of biliary acids. The expansion of these particular species of bacteria in the colon can be related to dietary factors. In particular, the prevalence of fat and red meat in the diet appear to create a favorable substrate for the survival and proliferation of such bacteria. Their metabolic products can also be mutagenic, and the risk of colorectal cancer was enhanced by their formation of polyamines and the increased formation of secondary bile acids such as deoxy- and lithocholic acids (5, 66-68). Moreover, it is possible that these (and other) products may modify the mucosal barrier, favoring antigen penetration, deregulation of the physiological inflammation, and oxidative stress, ultimately resulting in damage to and genetic instability within the mucosal cells. In turn, the subsequent elicitation of reparative responses can additionally lead to the increased production of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF), factors useful for sustaining tumor development (69).

On the other hand, probiotics like *Lactobacillus rhamnosus - GG, L. reuteri*, bifidobacteria and certain strains of *L. casei* or the *L. acidophilus* group have been shown to have protective effect in both animal cancer models and in some clinical trials. However, their effectiveness in anti-cancer prevention is still under evaluation. Interestingly, not only the use of living probiotic bacteria but also of some of their components should be helpful in promoting possible anti-carcinogenic effects. Choi and colleagues (2006) proved this concept by showing that both *L. acidophilus 606* and the soluble polysaccharide components of this strain were able to exert anti-oxidative effects and to induce apoptosis in colon cancer cell lines (70).

Lactic acid bacteria were also found to significantly enhance natural killing activity of spleen cells in vivo. NK1.1 positive natural killer (NK) cells and natural killer-T (NKT) cells

produced interferon (IFN)-γ after stimulation with this bacterium in vitro. The IFN-γ-producing cells stimulation was secondary to IL-12 production by CD11c+ DC in a TLR2- and/or TLR4-dependent manner. A direct interaction between DC and NK1.1+ cells was necessary, confirming also in this context the importance of the DC-NK cell cross-talk (71). NK1.1+ T-lymphocytes in the liver are mostly CD1d-dependent, whereas in the large intestine they are mostly CD1d-independent. While Lactobacilli (L. casei, L. rhamnosus) administered to mice stimulated selective expansion and IFN-γ expression of this subpopulation of NK1.1+ T-lymphocytes in the colon, bacteria like Pseudomonas aeruginosa, E. coli, Staphylococcus aureus, or Lactobacillus gasseri elicited activation of CD1d-dependent NKT (72, 73).

Some studies have also shown that the association of probiotics with prebiotics (e.g., inulin) can better stimulate NK cell activities. In the mouse, saccharides like inulin, oligofructose, and dextran administered together with Lactobacilli or Bifidobacterium lactis more effectively enhanced NK cytotoxicity and cytokine production of spleen derived mononuclear cells, indicating systemic effectiveness of environmental manipulations at the gut level. Applied in colon cancer models in vivo, they reduced tumor incidence (74, 75).

Some evidence was also found about the capability of NK cells to inhibit effectors CD4+CD45RBhigh T-lymphocytes (producing IL-2) by a perforin-dependent mechanism in a mouse model of colitis. Consequently, the bacterial modulation of NK cell activity should exert further effects, including regulation of effector T-lymphocytes in their responses to gut–bacteria interactions (76). Finally, the increase of NK cell activities related to administration of pro- and pre- biotics was also documented in humans (healthy volunteers) and related to an increase in IL-12, as was evidenced in murine models (77).

When the local conditions permit the predominance of bacterial strains able to modify the mucosal barrier, we can suppose a shift from the physiologically controlled inflammatory network toward a more aggressive inflammation. Both LPS and living bacteria can induce phosphorylation of STAT3 in the mucosa activating JAK/STAT pathway and leading to IL-1β and IL-6 production (78). The binding of LPS to TLR4 receptor also induces Neu1 sialidase with following activation of NF-κB. The consequent macrophage production of nitric oxide and pro-inflammatory IL-6 and TNFα cytokines can further collaborate to sustain inflammation. Additional proliferative effects and resistance to apoptosis can be induced in autocrine manner by the IL-6 through STAT3, enabling the establishment of a cancer-promoting circle (79-81).

5.5 Germ-free animals and cancer

The field of gnotobiology utilizes GF raised animals (with-out any microflora) that are maintained either in amicrobial conditions or conditions associated with one/multiple known or specified bacteria. These animal models are especially useful to generically study host–bacteria inter-actions but, critically, are also very useful in performing detailed mechanistic studies of the immune responses to specific pathogens (akin to how knockout mice are important for helping to define/predict very specific aspects of human immune responses to pathogens or environmental toxicants, pharmaceuticals, etc.). The advantages of this particular approach (i.e., use of gnotobiotic hosts) are clear, including: the possibility to evaluate and compare immune responses in normally contaminated hosts versus GF counterparts; to elucidate how immunity works as a result of either physiological or pathological stimulation(s); the possibility to colonize a sterile host with one/more select bacterial species and, thus, uniquely clarify specific inter-actions with the specific pathogen(s); and, the possibility of performing investigations using animals that possess a completely efficient immune system that arises from a "natural" exposure to a very limited antigenic challenge rather than via genetic engineering (knockout/knockdown/ silenced models) (36, 82-84).

As underlined in two recent reviews on gut bacteria and their role in early human development (85, 86), these animal models are consistent to enlightening the importance of the cross-talk between bacteria, mucosal immunity and gut structure also in relation to human conditions. As observed by the Authors, bacterial colonization of the gut appears to be able to shape human immunity and organ development. Similarities in immunological, biological, and pathological factors and mechanisms were found between gnotobiotic animals and humans. In particular, the bowel environment of human neonates appears be mimicked by gnotobiotic animals. In fact, newborns are initially germ-free, then show mono-association with bifidobacteria. Later on, a progressively more complex panel of bacteria become associated to the gut reaching the maximal level of colonization ≈ 4 years after birth. Thus, during these first years of life, bacteria (together with food components) contribute to the progressive maturation of the immature immune system and tolerance to the gut environment-related antigenic challenge(s). This is a not yet fully clarified process; thus, the gnotobiologic animals can provide an important model especially for understanding the possible long-lasting biological and immunological effects from these early-life processes and, more generally, after contact with specific bacteria (34, 87-90).

GF animals present some anatomical differences of the gut in comparison to conventionally (CV)-reared animals. For example, they present a much larger cecum (3−4 times than in CV) and their intestinal walls appear subtler (Figure 2). Furthermore, the mesenteric lymph nodes in these animals are less developed than in their CV counterparts. These features suggest that the presence of com-mensal microbiota (and the local stimulation by them) may assist in the modeling of gut structures and the stimulation of lymphatic organ(s) development.

It is known that chronic inflammation, through the release of mediators, can induce hypertrophy/hyperplasia of smooth muscle cells in the gut as in other organs (e.g., airways). A similar mechanism, as a consequence of the "physiological" inflammation activated by the commensal microbiota, may also be suggested for the (re)modeling of intestinal wall structures (6, 82, 84, 91-93).

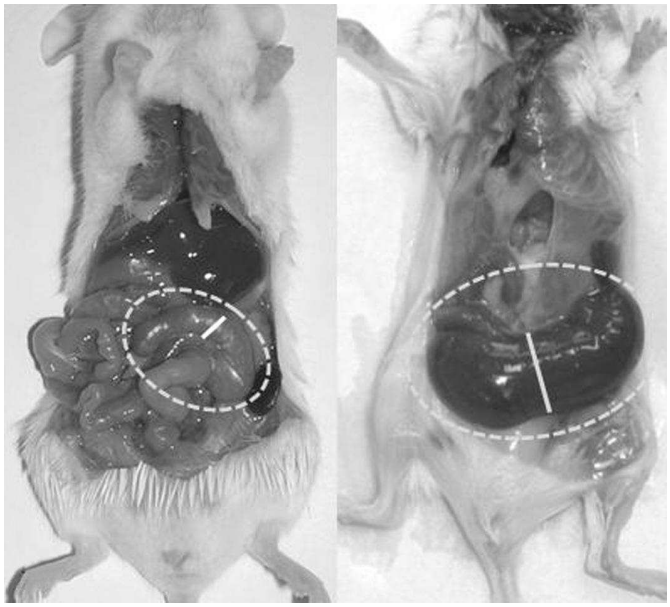
In GF animals, the number of lamina propria cells (namely T-lymphocytes) is reduced. These cells appear also to be less sensitive to mitogen-induced proliferation. Same hypo-responsiveness was found in the splenocytes (94, 95). We have also found higher numbers of T-lymphocytes in the spleen and blood of CV rats than in GF rats. An opposite situation was found with NK and NKT cells; in this case, these cell types were more numerous in GF than in CV rats (96).

After conventionalization of GF rats by gut colonization with regular commensal flora, the composition of intestinal intraepithelial lymphocyte subsets (IEL) showed an increase in, respectively, CD4+, CD8a+, CD8b+, TCRa/b+ cells. A "conventional" lymphocytic pattern was induced also in blood and spleen (94, 95). These differences suggest that the relative prevalence of innate immunity cells versus adaptive immunity cells in the GF animals should be a consequence of the more limited antigenic challenges they have to sustain. This might be also a factor that is then able to influence the local immune responses toward newly expressed antigens, like cancer antigens (96).

More than 30 years ago, studies about the incidence of tumors in GF versus CV animals evidenced a reduced capability of GF animals to develop solid tumors. In 1976, Sacksteder wrote in his report on the spontaneous tumors occurring in GF rats that "for unknown reasons, significantly fewer solid tumors were observed in germfree than in conventional male rats" (97). In the same and following years, Reddy et al. published various papers about the induction of colorectal cancers either with dimethylhydrazine (DMH) or azoxymethane (AOM; an active derivative of DMH) in CV and GF rats under various experimental conditions (i.e., using variations in route of administration, association of

carcinogens, hyperlipidic alimentation, etc.) (98-100). These investigators confirmed the reduced occurrence of colon cancers in GF rats (20% incidence in GF versus 93% in CV groups).

Figure 2. Conventional and germ-free (GF) reared mice. (A) Conventionally reared mouse (CV); (B) GF reared mouse. The cecum of both mice is framed by the interrupted line; the continuous line indicates the diameter of the cecum in its middle part. Differences in dimension are clearly evident. The GF animal has a very large and smooth cecum; its dark color is due to visibility of fecal content through a very transparent wall, and also due to a biliary acid metabolism that differs from that in conventional mice. In addition, the small bowel appears larger than in the CV animal control.



In 2008, our own studies confirmed the lower incidence of colorectal tumors in cancer-induced GF rats but also showed evidence of a different degree of immune reactivity between GF and CV hosts (96). This was documented at the systemic level (i.e., in the blood and spleen) by evaluating both the cytotoxic cell activity and pro-portions of lymphocyte subpopulations. Healthy GF and CV rats were, respectively, compared with rats that developed cancer or those that were resistant to developing cancer, after carcinogen administration. Interestingly, the cytotoxic activity in the GF rats was higher than that in CV rats, even under the “cancer conditions”. Levels of cytotoxic cells (NK and CD8+ T-lymphocytes [CTL]) were also significantly increased in the peripheral blood of cancer-resistant GF rats, both in comparison to healthy controls and CV rats with the same condition. It was noted that the colorectal cancers that developed in the GF rats were smaller than those in the CV rats, and tended to be singular entities rather than multiple tumors synchronously developed in various segment of the large bowel as was found in the CV animals (96). The

different susceptibility to cancer development and the smaller tumor dimensions in the absence of bacteria can be explained in terms of various factors, both non-immunological and immunological. Specifically, the absence of bacteria would lead to a different pattern of metabolism of food components and biliary acids, thereby resulting in a more limited production of cholic acid, fatty acids, and polyamine molecules that could potentially be active pro-/ co-carcinogens (29, 67, 69, 101, 102).

Still, from an immunological viewpoint, the absence of chronic pro-inflammatory environment, the reduced regulatory network in the lamina propria, the much lower amount of molecules to be tolerated, deriving from the bacterial metabolisms of alimentary products and from the same bacteria, can lead to a more naïve and plastic immunity (96, 103). This should be indicated by the relatively lower proportion of T-lymphocytes versus the innate immunity cells in healthy conditions, the hypoplastic mesenteric lymph nodes, but also by a more valid immune reactivity against transformed cells, as described above. The absence of the pro-inflammatory background should permit a more prompt reaction to danger signals and the constitutive lower needs of regulatory molecules in the gut environment could permit more efficient anti-cancer responses. The earlier and more efficient immune response assumed to be elicited in these conditions should also lead to a more effective antigen presentation to DC, adaptive immunity priming and extension of the response from local also to systemic level.

5.6 Conclusions and perspectives

In conclusion, the balance between bacterial species of commensal microbiota is important for intestinal homeostasis. The mucosal homeostasis is maintained by a complex immunological network of activation-regulatory mechanisms with local and systemic effects. To dissect the role that environmental factors and bacterial balance can have on gut and organism, the GF animals represent a unique and suit-able model. They aid in highlighting important mechanisms addressing local and systemic immune responses against pathological conditions, e.g., cancer. Comparative studies in CV and GF animals can efficaciously address the development of new possibilities of therapeutic interventions (e.g., manipulation of the intestinal microbiota by pre- and pro-biotics in immunomodulation or anti-cancer prevention), as well as the identification of immunological therapeutic targets and their degree of dependence on local environ-mental factors involved in colorectal cancer development. The comparison between GF with conventionally raised animals can also introduce new models for studying inflammatory network and its regulation.

5.7 Acknowledgements

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***Declaration of interest*:** The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

5.8 Erratum

Colorectal carcinoma: Importance of colonic environment for anti-cancer response and systemic immunity, *Journal of Immunotoxicology*, 2009; 6(4): 217–226.

An error in the print and online versions of this article has been brought to our attention by the authors. The following sentence was written incorrectly in the printed and online version as:

These Investigators noted that the cancer-promoting activity of bacteria like *Mitsuokella multiacida, Clostridium butyricum, Bifidobacterium longum, C. paraputrificum, C. butyricum,* and *E. coli* was related to the de-conjugation of biliary acids.

The corrected sentence should read as:

These Investigators noted that the biological activity of bacteria like *Mitsuokella multiacida, Clostridium butyricum, Bifidobacterium longum, C. paraputrificum, C. butyricum,* and *E. coli* was related to the de-conjugation of biliary acids.

The publishers would like to apologize for any inconvenience caused.

5.9 References

1. Hewitson P, Glasziou P, Irwig L, Towler B, Watson E. Screening for colorectal cancer using the faecal occult blood test, Hemoccult. Cochrane Database Syst Rev. 2007(1):CD001216.
2. Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol. 2007 Mar;18(3):581-92.
3. Sung JJ, Lau JY, Goh KL, Leung WK. Increasing incidence of colorectal cancer in Asia: implications for screening. Lancet Oncol. 2005 Nov;6(11):871-6.
4. Abdulkareem FB, Abudu EK, Awolola NA, Elesha SO, Rotimi O, Akinde OR, et al. Colorectal carcinoma in Lagos and Sagamu, Southwest Nigeria: a histopathological review. World J Gastroenterol. 2008 Nov 14;14(42):6531-5.
5. McGarr SE, Ridlon JM, Hylemon PB. Diet, anaerobic bacterial metabolism, and colon cancer: a review of the literature. J Clin Gastroenterol. 2005 Feb;39(2):98-109.
6. Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, et al. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunol Lett. 2004 May 15;93(2-3):97-108.
7. Heavey PM, Rowland IR. Microbial-gut interactions in health and disease. Gastrointestinal cancer. Best Pract Res Clin Gastroenterol. 2004 Apr;18(2):323-36.
8. Potter JD. Risk factors for colon neoplasia--epidemiology and biology. Eur J Cancer. 1995 Jul-Aug;31A(7-8):1033-8.
9. Yu L, Wang L, Chen S. Toll-like receptors, inflammation and tumor in the human female reproductive tract. Am J Reprod Immunol. 2009 Jul;62(1):1-8.
10. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001 Feb 17;357(9255):539-45.
11. Borrello MG, Degl'Innocenti D, Pierotti MA. Inflammation and cancer: the oncogene-driven connection. Cancer Lett. 2008 Aug 28;267(2):262-70.
12. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest. 2007 May;117(5):1175-83.
13. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008 Jul 24;454(7203):436-44.
14. Hope ME, Hold GL, Kain R, El-Omar EM. Sporadic colorectal cancer--role of the commensal microbiota. FEMS Microbiol Lett. 2005 Mar 1;244(1):1-7.
15. Cebra JJ, Jiang, H. Q., Boiko, N., and Tlaskalova-Hogenova, H. The role of mucosal microbiota in the development, maintenance, and pathologies of the mucosal immune system. Mucosal Immunology. 3rd ed. Amsterdam: Elsevier/Academic Press; 2005.
16. Guarner F, Malagelada JR. Gut flora in health and disease. Lancet. 2003 Feb 8;361(9356):512-9.
17. Stepankova R. Rearing germ-free rats, mice, and rabbits. Immunology Methods Manual: New York: Academy Press; 1997. p. 1537-42.
18. Tlaskalova-Hogenova H, Sterzl J, Stepankova R, Dlabac V, Veticka V, Rossmann P, et al. Development of immunological capacity under germfree and conventional conditions. Ann N Y Acad Sci. 1983 Jun 30;409:96-113.
19. Fanaro S, Chierici R, Guerrini P, Vigi V. Intestinal microflora in early infancy: composition and development. Acta Paediatr Suppl. 2003 Sep;91(441):48-55.
20. Umesaki Y, Setoyama H. Structure of the intestinal flora responsible for development of the gut immune system in a rodent model. Microbes Infect. 2000 Sep;2(11):1343-51.
21. Williams AM, Probert CS, Stepankova R, Tlaskalova-Hogenova H, Phillips A, Bland PW. Effects of microflora on the neonatal development of gut mucosal T cells and myeloid cells in the mouse. Immunology. 2006 Dec;119(4):470-8.
22. Smith K, McCoy KD, Macpherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin Immunol. 2007 Apr;19(2):59-69.
23. Turroni F, Ribbera A, Foroni E, van Sinderen D, Ventura M. Human gut microbiota and bifidobacteria: from composition to functionality. Antonie Van Leeuwenhoek. 2008 Jun;94(1):35-50.
24. Handelsman J. Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev. 2004 Dec;68(4):669-85.
25. Hord NG. Eukaryotic-microbiota crosstalk: potential mechanisms for health benefits of prebiotics and probiotics. Annu Rev Nutr. 2008;28:215-31.
26. Kinross JM, von Roon AC, Holmes E, Darzi A, Nicholson JK. The human gut microbiome: implications for future health care. Curr Gastroenterol Rep. 2008 Aug;10(4):396-403.

27. Tuohy KM, Gougoulias C, Shen Q, Walton G, Fava F, Ramnani P. Studying the human gut microbiota in the trans-omics era--focus on metagenomics and metabonomics. Curr Pharm Des. 2009;15(13):1415-27.
28. Christensen HR, Frokiaer H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol. 2002 Jan 1;168(1):171-8.
29. Olaya J, Neopikhanov V, Uribe A. Lipopolysaccharide of Escherichia coli, polyamines, and acetic acid stimulate cell proliferation in intestinal epithelial cells. In Vitro Cell Dev Biol Anim. 1999 Jan;35(1):43-8.
30. Dubois B, Goubier A, Joubert G, Kaiserlian D. Oral tolerance and regulation of mucosal immunity. Cell Mol Life Sci. 2005 Jun;62(12):1322-32.
31. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nature Reviews Immunology. 2004 Jun;4(6):478-85.
32. Macpherson AJ, Uhr T. Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. Ann N Y Acad Sci. 2004 Dec;1029:36-43.
33. Nicklin S, Miller K. Local and systemic immune responses to intestinally presented antigen. Int Arch Allergy Appl Immunol. 1983;72(1):87-90.
34. Macpherson AJ, Geuking MB, McCoy KD. Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. Immunology. 2005 Jun;115(2):153-62.
35. Forchielli ML, Walker WA. The role of gut-associated lymphoid tissues and mucosal defence. Br J Nutr. 2005 Apr;93 Suppl 1:S41-8.
36. Gordon HA, Pesti L. The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriol Rev. 1971 Dec;35(4):390-429.
37. Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol. 2003 Apr;3(4):331-41.
38. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev. 2002 Aug-Oct;13(4-5):413-21.
39. Liu X, Conner H, Kobayashi T, Abe S, Fang Q, Wen FQ, et al. Synergetic effect of interleukin-4 and transforming growth factor-beta1 on type I collagen gel contraction and degradation by HFL-1 cells: implication in tissue remodeling. Chest. 2003 Mar;123(3 Suppl):427S-8S.
40. Goodrich ME, McGee DW. Regulation of mucosal B cell immunoglobulin secretion by intestinal epithelial cell-derived cytokines. Cytokine. 1998 Dec;10(12):948-55.
41. Smythies LE, Maheshwari A, Clements R, Eckhoff D, Novak L, Vu HL, et al. Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells. J Leukoc Biol. 2006 Sep;80(3):492-9.
42. Wu MS, Lin JT, Hsu PN, Lin CY, Hsieh YT, Chiu YH, et al. Preferential induction of transforming growth factor-beta production in gastric epithelial cells and monocytes by Helicobacter pylori soluble proteins. J Infect Dis. 2007 Nov 1;196(9):1386-93.
43. Makita S, Kanai T, Nemoto Y, Totsuka T, Okamoto R, Tsuchiya K, et al. Intestinal lamina propria retaining CD4+CD25+ regulatory T cells is a suppressive site of intestinal inflammation. J Immunol. 2007 Apr 15;178(8):4937-46.
44. Tezuka H, Abe Y, Iwata M, Takeuchi H, Ishikawa H, Matsushita M, et al. Regulation of IgA production by naturally occurring TNF/iNOS-producing dendritic cells. Nature. 2007 Aug 23;448(7156):929-33.
45. Baba N, Samson S, Bourdet-Sicard R, Rubio M, Sarfati M. Commensal bacteria trigger a full dendritic cell maturation program that promotes the expansion of non-Tr1 suppressor T cells. J Leukoc Biol. 2008 Aug;84(2):468-76.
46. Bilsborough J, Viney JL. Gastrointestinal dendritic cells play a role in immunity, tolerance, and disease. Gastroenterology. 2004 Jul;127(1):300-9.
47. Woof JM, Mestecky J. Mucosal immunoglobulins. Immunol Rev. 2005 Aug;206:64-82.
48. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol. 2003;21:685-711.
49. Gallucci S, Matzinger P. Danger signals: SOS to the immune system. Curr Opin Immunol. 2001 Feb;13(1):114-9.
50. Kuper H, Adami HO, Trichopoulos D. Infections as a major preventable cause of human cancer. J Intern Med. 2000 Sep;248(3):171-83.
51. Rowland IR. The role of the gastrointestinal microbiota in colorectal cancer. Curr Pharm Des. 2009;15(13):1524-7.
52. Swidsinski A, Khilkin M, Kerjaschki D, Schreiber S, Ortner M, Weber J, et al. Association between intraepithelial Escherichia coli and colorectal cancer. Gastroenterology. 1998 Aug;115(2):281-6.
53. Atherton JC. H. pylori virulence factors. Br Med Bull. 1998;54(1):105-20.
54. Kikuchi S, Wada O, Nakajima T, Nishi T, Kobayashi O, Konishi T, et al. Serum anti-Helicobacter pylori antibody and gastric carcinoma among young adults. Research Group on Prevention of Gastric Carcinoma among Young Adults. Cancer. 1995 Jun 15;75(12):2789-93.

55. IARC. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Geneva: World Health Organization; 1997.
56. Asonuma S, Imatani A, Asano N, Oikawa T, Konishi H, Iijima K, et al. Helicobacter pylori induces gastric mucosal intestinal metaplasia through the inhibition of interleukin-4-mediated HMG box protein Sox2 expression. Am J Physiol Gastrointest Liver Physiol. 2009 Aug;297(2):G312-22.
57. Moss SF. The carcinogenic effect of H. pylori on the gastric epithelial cell. J Physiol Pharmacol. 1999 Dec;50(5):847-56.
58. Hambly RJ, Rumney CJ, Cunninghame M, Fletcher JM, Rijken PJ, Rowland IR. Influence of diets containing high and low risk factors for colon cancer on early stages of carcinogenesis in human flora-associated (HFA) rats. Carcinogenesis. 1997 Aug;18(8):1535-9.
59. Reddy BS, Narisawa T, Maronpot R, Weisburger JH, Wynder EL. Animal models for the study of dietary factors and cancer of the large bowel. Cancer Res. 1975 Nov;35(11 Pt. 2):3421-6.
60. Rumney CJ, Rowland IR, Coutts TM, Randerath K, Reddy R, Shah AB, et al. Effects of risk-associated human dietary macrocomponents on processes related to carcinogenesis in human-flora-associated (HFA) rats. Carcinogenesis. 1993 Jan;14(1):79-84.
61. Venturi M, Hambly RJ, Glinghammar B, Rafter JJ, Rowland IR. Genotoxic activity in human faecal water and the role of bile acids: a study using the alkaline comet assay. Carcinogenesis. 1997 Dec;18(12):2353-9.
62. Wang X, Allen TD, May RJ, Lightfoot S, Houchen CW, Huycke MM. Enterococcus faecalis induces aneuploidy and tetraploidy in colonic epithelial cells through a bystander effect. Cancer Res. 2008 Dec 1;68(23):9909-17.
63. Wang X, Huycke MM. Extracellular superoxide production by Enterococcus faecalis promotes chromosomal instability in mammalian cells. Gastroenterology. 2007 Feb;132(2):551-61.
64. Horie H, Kanazawa K, Okada M, Narushima S, Itoh K, Terada A. Effects of intestinal bacteria on the development of colonic neoplasm: an experimental study. Eur J Cancer Prev. 1999 Jul;8(3):237-45.
65. Onoue M, Kado S, Sakaitani Y, Uchida K, Morotomi M. Specific species of intestinal bacteria influence the induction of aberrant crypt foci by 1,2-dimethylhydrazine in rats. Cancer Lett. 1997 Feb 26;113(1-2):179-86.
66. Mastromarino A, Reddy BS, Wynder EL. Metabolic epidemiology of colon cancer: enzymic activity of fecal flora. Am J Clin Nutr. 1976 Dec;29(12):1455-60.
67. Reddy BS, Sharma C, Darby L, Laakso K, Wynder EL. Metabolic epidemiology of large bowel cancer. Fecal mutagens in high- and low-risk population for colon cancer. A preliminary report. Mutat Res. 1980 Aug;72(3):511-22.
68. Rohrmann S, Hermann S, Linseisen J. Heterocyclic aromatic amine intake increases colorectal adenoma risk: findings from a prospective European cohort study. Am J Clin Nutr. 2009 May;89(5):1418-24.
69. Cianchi F, Cortesini C, Fantappie O, Messerini L, Sardi I, Lasagna N, et al. Cyclooxygenase-2 activation mediates the proangiogenic effect of nitric oxide in colorectal cancer. Clin Cancer Res. 2004 Apr 15;10(8):2694-704.
70. Choi SS, Kim Y, Han KS, You S, Oh S, Kim SH. Effects of Lactobacillus strains on cancer cell proliferation and oxidative stress in vitro. Lett Appl Microbiol. 2006 May;42(5):452-8.
71. Koizumi S, Wakita D, Sato T, Mitamura R, Izumo T, Shibata H, et al. Essential role of Toll-like receptors for dendritic cell and NK1.1(+) cell-dependent activation of type 1 immunity by Lactobacillus pentosus strain S-PT84. Immunol Lett. 2008 Oct 30;120(1-2):14-9.
72. Nieuwenhuis EE, Matsumoto T, Lindenbergh D, Willemsen R, Kaser A, Simons-Oosterhuis Y, et al. Cd1d-dependent regulation of bacterial colonization in the intestine of mice. J Clin Invest. 2009 May;119(5):1241-50.
73. Takahashi S, Kawamura T, Kanda Y, Taniguchi T, Nishizawa T, Iiai T, et al. Activation of CD1d-independent NK1.1+ T cells in the large intestine by Lactobacilli. Immunol Lett. 2006 Jan 15;102(1):74-8.
74. Roller M, Pietro Femia A, Caderni G, Rechkemmer G, Watzl B. Intestinal immunity of rats with colon cancer is modulated by oligofructose-enriched inulin combined with Lactobacillus rhamnosus and Bifidobacterium lactis. Br J Nutr. 2004 Dec;92(6):931-8.
75. Watzl B, Girrbach S, Roller M. Inulin, oligofructose and immunomodulation. Br J Nutr. 2005 Apr;93 Suppl 1:S49-55.
76. Fort MM, Leach MW, Rennick DM. A role for NK cells as regulators of CD4+ T cells in a transfer model of colitis. J Immunol. 1998 Oct 1;161(7):3256-61.
77. Ogawa T, Asai Y, Tamai R, Makimura Y, Sakamoto H, Hashikawa S, et al. Natural killer cell activities of synbiotic Lactobacillus casei ssp. casei in conjunction with dextran. Clin Exp Immunol. 2006 Jan;143(1):103-9.

78. Samavati L, Rastogi R, Du W, Huttemann M, Fite A, Franchi L. STAT3 tyrosine phosphorylation is critical for interleukin 1 beta and interleukin-6 production in response to lipopolysaccharide and live bacteria. Mol Immunol. 2009 May;46(8-9):1867-77.
79. Amith SR, Jayanth P, Franchuk S, Siddiqui S, Seyrantepe V, Gee K, et al. Dependence of pathogen molecule-induced toll-like receptor activation and cell function on Neu1 sialidase. Glycoconj J. 2009 Dec;26(9):1197-212.
80. Bromberg J, Wang TC. Inflammation and cancer: IL-6 and STAT3 complete the link. Cancer Cell. 2009 Feb 3;15(2):79-80.
81. Slattery ML, Wolff RK, Herrick JS, Caan BJ, Potter JD. IL6 genotypes and colon and rectal cancer. Cancer Causes Control. 2007 Dec;18(10):1095-105.
83. Trexler PC. A rationale for the development of gnotobiotics. Lab Anim. 1978 Oct;12(4):257-62.
84. Tlaskalova-Hogenova H. Gnotobiology as a tool - An introduction. Immunology Methods Manual: New York: Academic Press; 1997. p. 1524-29.
85. Tannock GW. What immunologists should know about bacterial communities of the human bowel. Semin Immunol. 2007 Apr;19(2):94-105.
86. Wilks M. Bacteria and early human development. Early Hum Dev. 2007 Mar;83(3):165-70.
87. Boudry G, Peron V, Le Huerou-Luron I, Lalles JP, Seve B. Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. J Nutr. 2004 Sep;134(9):2256-62.
88. Cario E, Podolsky DK. Intestinal epithelial TOLLerance versus inTOLLerance of commensals. Mol Immunol. 2005 May;42(8):887-93.
89. Kelly D, Conway S, Aminov R. Commensal gut bacteria: mechanisms of immune modulation. Trends Immunol. 2005 Jun;26(6):326-33.
90. Prescott SL, Holt PG. Abnormalities in cord blood mononuclear cytokine production as a predictor of later atopic disease in childhood. Clin Exp Allergy. 1998 Nov;28(11):1313-6.
91. Blennerhassett MG, Bovell FM, Lourenssen S, McHugh KM. Characteristics of inflammation-induced hypertrophy of rat intestinal smooth muscle cell. Dig Dis Sci. 1999 Jul;44(7):1265-72.
92. Blennerhassett MG, Vignjevic P, Vermillion DL, Collins SM. Inflammation causes hyperplasia and hypertrophy in smooth muscle of rat small intestine. Am J Physiol. 1992 Jun;262(6 Pt 1):G1041-6.
93. Chiappara G, Gagliardo R, Siena A, Bonsignore MR, Bousquet J, Bonsignore G, et al. Airway remodelling in the pathogenesis of asthma. Curr Opin Allergy Clin Immunol. 2001 Feb;1(1):85-93.
94. Stepankova R, Sinkora J, Hudcovic T, Kozakova H, Tlaskalova-Hogenova H. Differences in development of lymphocyte subpopulations from gut-associated lymphatic tissue (GALT) of germfree and conventional rats: effect of aging. Folia Microbiol (Praha). 1998;43(5):531-4.
95. Woolverton CJ, Holt LC, Mitchell D, Sartor RB. Identification and characterization of rat intestinal lamina propria cells: consequences of microbial colonization. Vet Immunol Immunopathol. 1992 Oct;34(1-2):127-38.
96. Vannucci L, Stepankova R, Kozakova H, Fiserova A, Rossmann P, Tlaskalova-Hogenova H. Colorectal carcinogenesis in germ-free and conventionally reared rats: different intestinal environments affect the systemic immunity. Int J Oncol. 2008 Mar;32(3):609-17.
97. Sacksteder MR. Occurrence of spontaneous tumors in the germfree F344 rat. J Natl Cancer Inst. 1976 Dec;57(6):1371-3.
98. Reddy BS, Narisawa T, Weisburger JH. Colon carcinogenesis in germ-free rats with intrarectal 1,2-dimethylhydrazine and subcutaneous azoxymethane. Cancer Res. 1976 Aug;36(8):2874-6.
99. Reddy BS, Narisawa T, Wright P, Vukusich D, Weisburger JH, Wynder EL. Colon carcinogenesis with azoxymethane and dimethylhydrazine in germ-free rats. Cancer Res. 1975 Feb;35(2):287-90.
100. Reddy BS, Ohmori T. Effect of intestinal microflora and dietary fat on 3,2'-dimethyl-4-aminobiphenyl-induced colon carcinogenesis in F344 rats. Cancer Res. 1981 Apr;41(4):1363-7.
101. Rao CV, Hirose Y, Indranie C, Reddy BS. Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. Cancer Res. 2001 Mar 1;61(5):1927-33.
102. Weidema WF, Deschner EE, Cohen BI, DeCosse JJ. Acute effects of dietary cholic acid and methylazoxymethanol acetate on colon epithelial cell proliferation; metabolism of bile salts and neutral sterols in conventional and germfree SD rats. J Natl Cancer Inst. 1985 Mar;74(3):665-70.
103. Hrncir T, Stepankova R, Kozakova H, Hudcovic T, Tlaskalova-Hogenova H. Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice. BMC Immunol. 2008;9:65.

6 Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M deficient mice

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6.1 Abstract

Background: Microbial sensing by Toll-like receptors (TLR) and its negative regulation have important role in the pathogenesis of inflammation-related cancer. In this study, we investigated the role of negative regulation of TLR signaling and gut microbiota in the development of colitis-associated cancer in mouse model.

Methods: Colitis-associated cancer was induced by azoxymethane and dextran sodium sulfate in wild-type and in Interleukin-1 receptor associated kinase-M (IRAK-M) deficient mice with or without antibiotic (ATB) treatment. Local cytokine production was analyzed by multiplex cytokine assay or ELISA, and regulatory T cells were analyzed by flow cytometry. Changes in microbiota composition during tumorigenesis were analyzed by pyrosequencing, and β-glucuronidase activity was measured in intestinal content by fluorescence assay.

Results: 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. However, the β-glucuronidase activity was not as low as in germ-free mice. 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.

Conclusions: We conclude 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 conditions of altered microbiota. Therefore, gut microbiota and its metabolic activity could be potential targets for colitis-associated cancer therapy.

6.2 Introduction

Chronic gut inflammation, as seen in patients with inflammatory bowel diseases (IBD), is a strong risk factor for colon cancer. Therefore, the increase in number of IBD patients observed in last decades, will ultimately lead to increase in number of patients with colitis-associated cancer (CAC) (1, 2). Although the pathogenesis of IBD and the development of CAC are still not completely understood, it is generally accepted that an aberrant immune reaction to intestinal commensal microbiota and subsequent chronic inflammatory responses in the gut play major roles (3).

Microbial stimulation in the gut is important for maintaining physiological functions, including intestinal epithelium growth, mucosal permeability and production of antimicrobial agents, as well as regulation and development of the immune system (3, 4). Various resident bacteria have protective role in the process of inflammation and cancer development but on the other hand there is a great amount of potentially harmful species that can be derived from normal microbiota under specific conditions of imbalance in gut milieu (4-6). As an example of a potentially harmful microbe, can serve *Helicobacter pylori,* which is confirmed triggering agent of the chronic gastric inflammation and cancer and where antibiotic treatment leads to cancer prevention (7). Use of broad-spectrum antibiotics, like ciprofloxacin and metronidazole, also brought positive results in the therapy of certain forms of Crohn's disease and pouchitis (8), decreasing the risk of colon cancer development.

Metabolic activity of microbiota is an important detail of the gut ecosystem. Microbes are equipped with a broad-spectrum of enzymes and therefore can metabolize various substrates (5). Among final products belong potential pro-carcinogens as well as beneficial substances such as short-chain fatty acids (SCFA). SCFA are a crucial source of energy for colonic epithelium and their lack has been implicated in pathogenesis of colorectal carcinoma (9). Protective role is also ascribed to probiotics defined as live bacteria beneficial to health, that have stabilizing effect on gut microbiota during administration with potential to reduce pro-inflammatory response (10, 11). Manipulation of the microbiota therefore brings wide possibilities, although not fully elucidated, of influencing intestinal homeostasis and immune system reactivity.

Detection of conserved microbe-associated molecular patterns is provided by different cellular pattern-recognition receptors, such as family of the Toll-like receptors (TLR) (12). TLR signalization is important in maintaining gut epithelium homeostasis but the stimulation of TLR may also induce cancer development or promote tumor growth (13). Myeloid

differentiation factor 88 (MyD88), which is responsible for signal transduction from all TLRs except for TLR3, has been shown to interfere with the pathogenesis of colon inflammation and cancer by triggering pro-inflammatory response via transcription factor NF-κB (14, 15). Interleukin-1 receptor associated kinase-M (IRAK-M) is a molecule crucial in regulation of gut immune response through negative feedback. IRAK-M binds and blocks MyD88/IRAK-4 protein complex thus negatively regulating pro-inflammatory signal transduction by IRAK-1/TRAF6 in various immune cells and gut epithelium (16, 17). The expression of *Irak-m* gene is closely associated with the presence of intestinal microbiota and TLR signaling (18). Its deficiency enhances the production of pro-inflammatory cytokines in macrophages and intensifies experimentally-induced dextran sodium sulfate (DSS) colitis (19). Recent studies showed that single immunoglobulin IL-1 receptor-related molecule (SIGIRR), another negative regulator of TLR signaling, is involved in inflammation and cancer development (20), which suggests the important role of these molecules in tumorigenesis.

In our previous studies, we found that germ-free condition protected rats from colonic inflammation as well as from cancer (21). Since recognition of microbiota by TLRs plays important role in tumorigenesis, we hypothesized that both dysbiosis and negative regulation of TLR signaling via IRAK-M interferes with colon cancer development. By using inflammation-related mouse model of colon cancer induced by azoxymethane and DSS, we were able to follow the "inflammation-dysplasia-carcinoma" sequence, typical for CAC, under different microbial conditions. Here, we show the impact of gut microbiota composition on colon cancer development and immune system reactivity, and analyze the role of negative regulator IRAK-M in this process.

6.3 Materials and Methods

6.3.1 Animals and experimental schedule

We used two-month-old C57BL/6 male mice reared either in conventional (Institute of Physiology AS CR, Prague, Czech Republic) or in germ-free (GF) conditions (Institute of Microbiology AS CR, Novy Hradek, Czech Republic). The GF mice were reared in controlled sterile conditions, as described previously (22). IRAK-M deficient mice (obtained from the laboratory of Koichi S. Kobayashi) were backcrossed to C57BL/6 background for eleven generations and were held in specific pathogen-free facility in Novy Hradek. All mice received the same diet (ST-1, Velaz, Czech Republic) and tap water ad libitum, and were used

according to the procedures approved by the Institute of Microbiology animal care and use committee (No. 094/2008 and 053/2010).

We initiated tumorigenesis using modified protocol published previously by Clapper et al. (23). 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 3% dextran sodium sulfate (DSS, MW 36–50 kDa; MP Biomedicals, Illkirch, France) in their drinking water continuously for up to 4 days. DSS was subsequently replaced by tap water for the rest of the experiment. 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). Four independent experiments with five mice per group and three independent experiments with at least five mice per group were done in wild-type and IRAK-M deficient mice, respectively.

We recorded changes in body weight, stool consistency and gross bleeding every week, every day during DSS administration, and sacrificed the mice 5 weeks after AOM injection.

6.3.2 Histopathology evaluation

At the end of the experiments, the colon length was measured and fecal samples were tested for occult blood using Okult-Viditest Rapid (Vidia s.r.o., Jesenice u Prahy, Czech Republic). The colon was cut open longitudinally and macroscopically inspected for the presence of pathological lesions. Proximal and distal colon and rectum were fixed in 4% buffered formalin, dehydrated and embedded in paraffin. Histopathological examinations were performed in 4μm sections after hematoxylin/eosin staining. The degree of intestinal alteration was examined by two experienced pathologists (P.R., K.K.) using conventional criteria to determine normal mucosa; low or high-grade dysplasia; non-invasive or invasive carcinoma.

6.3.3 Cultivation and cytokine measurement

We took a part of the colon from every mouse, i.e. from tumor in tumor-bearing mice and from similar locality in the other mice, washed it in cold phosphate-buffered saline (PBS) and

weighed. These tissues were then cultivated for 48h at 37°C in RPMI-1640 media (Sigma-Aldrich) containing 10% fetal bovine serum (BioClot GmbH, Aidenbach, Germany) and 1% Antibiotic-Antimycotic solution (Sigma-Aldrich). The supernatants were collected and frozen at -20°C until analysis.

Cytokine profiles were determined using a multiplex cytokine analyzer – Luminex. The Fluorokine MAP Mouse Base Kit was used in accordance with manufacturer's instructions in combination with recommended bead sets for selected cytokines: IL-1β/IL-1F2, IL-6, IL-10, IL-12 p70, IL-17, TNF-α and IFN-γ (all R&D Systems, Minneapolis, MN) and analyzed using Luminex 200 instrument (Luminex Corporation, Austin, TX). The concentrations of analytes were determined by monitoring the spectral properties of the beads and the amount of phycoerythrin fluorescence.

Levels of TGF-β were measured by commercially available ELISA kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

6.3.4 Haptoglobin determination

The level of haptoglobin in mouse sera was assessed by Human Haptoglobin ELISA Quantitation Kit (GenWay Biotech., Inc., San Diego, CA). Antibodies used in this kit have high cross-reactivity with mouse haptoglobin that allowed us to use the kit according to the manufacturer's instructions with minor modifications as described previously (24).

6.3.5 Real-time polymerase chain reaction (PCR)

Samples of colon were placed in RNAlater stabilization reagent (QIAGEN GmbH, Hilden, Germany) and stored in -80°C. Total RNA was extracted by using the RNeasy Mini isolation kit (QIAGEN GmbH) following the manufacturer's instructions. RNA integrity was determined by gel electrophoresis in 1% agarose gel stained with SYBR-green (Life Technologies) and the concentration of the RNA was assessed by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). First strand cDNA was synthesized from 0.5 µg of RNA using SuperScript II reverse transcriptase (Life Technologies). Real-time PCR was performed using iQ SYBR-green Supermix (Bio-Rad Laboratories, Hercules, CA) on iQ5 cycler (Bio-Rad). The samples were analyzed in doublets and the expression was normalized to ribosomal protein S12 using iQ5 software (Bio-Rad). All primers were purchased from Generi Biotech (Hradec Kralove, Czech Republic);

sequences of the primers were as follows: ribosomal protein S12 forward: 5‘-CCTCGATGACATCCTTGGCCTGAG-3‘, ribosomal protein S12 reverse: 5‘-GGAAGGCATAGCTGCTGGAGGTGT-3‘; cyclooxygenase (COX)-2 forward: 5‘-AGTGGGGTGATGAGCAACTA-3‘, COX-2 reverse: 5‘-GGCAATGCGGTTCTGATACT-3‘; IL-18 forward: 5‘-ACGTGTTCCAGGACACAACA-3‘, IL-18 reverse: 5‘-ACAAACCCTCCCCACCTAAC-3‘.

6.3.6 Flow cytometric analysis

Spleens, mesenteric lymph nodes (MLN), Peyer’s patches (PP) and colonic tissue were collected and processed into single cell suspension. Briefly, cells from MLN, PP and colon were centrifuged and resuspended in 300 µL of cold FACS buffer (PBS containing 0.1% NaN3, 0.5% fetal bovine serum and 0.5M EDTA; pH 7.2 – 7.4). Splenocytes were treated by 5 mL of ACK lysis buffer (0.15M NH4Cl, 10mM KHCO3, 0.5M EDTA in distilled water; pH 7.2 – 7.4), resuspended in 5 mL of cold FACS buffer and kept on ice until staining. Cells were blocked with 10% normal mouse sera and then stained for surface molecules with fluorescent-labeled monoclonal antibodies cocktail containing anti-mouse CD3 – fluorescein isothiocyanate (BD Bioscience, Heidelberg, Germany; clone 145-2C11), CD4 – Qdot 605 (Life Technologies; clone RM4-5), CD8 – PerCP-Cy5.5 (BD Bioscience; clone 53-6.7) and CD25 – allophycocyanin (eBioscience; clone PC61.5). Subsequent intracellular staining for mouse Foxp3 was performed with phycoerythrin-labeled anti-mouse/rat Foxp3 staining set according to the manufacturer’s instructions (eBioscience; clone FJK-16s). Cells were measured using LSRII (BD Bioscience) and data were evaluated by FlowJo software (Tree Star Inc., Ashland, OR).

6.3.7 Microbiota analysis

Stool samples were collected from all mice on days 1, 23 (just before DSS administration), 30 (after DSS treatment) and 50 (the last day of experiment), and total DNA was isolated using ZR Fecal DNA Kit (Zymo Research Corp., Orange, CA) according to the manufacturer’s instruction. The isolated DNA was stored at -20°C for further analyses.

For the detailed determination of microbiota composition, we used high-throughput pyrosequencing described in detail elsewhere (25). Briefly, previously isolated DNA was gel-purified and PCR with broad-range bacterial primers for 16S rDNA including tags for

pyrosequencing was performed. PCR product was purified using magnetic beads (AMPure beads, Beckman Coulter Genomics, Danvers, MA) and concentration was measured on Qubit fluorometer (Life Technologies). Equimolar amounts of PCR product from each sample were then used for unidirectional 454 FLX amplicon pyrosequencing using LIB-L emPCR kits following the manufacturer's protocols (Roche Diagnostics, Basel, Switzerland). Sequence reads were processed using RDP's pyrosequencing pipeline (http://rdp.cme.msu.edu/) and Greengenes workbench compatible with ARB (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) following all standard procedures as sequence quality trimming, chimera check, phylotype identification, phylogenetic analysis and diversity analysis.

We performed quantitative PCR (qPCR) with specific primers to determine the numbers of the total bacteria (Eubacteria), *Parabacteroides distasonis* (*P. distasonis*) and *Faecalibacterium prausnitzii* (*F. prausnitzii*) in the stool samples. The conditions for PCR reactions are listed in Table 1. The qPCR 2x SYBR Master mix (Top-Bio, Prague, Czech Republic) was used along with Stratagene mx3005P (Agilent Technologies, Santa Clara, CA) equipment. Three-log diluted DNA isolated from known number of cells was used as standard for absolute quantification.

Table 1. Quantitative PCR conditions.

Target taxon	**Primers**	**Sequence (5' to 3')**	**Annealing temperature (°C)**	**Standard strain**	**Reference**
All Eubacteria	Uni331F Uni797R	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTATCCTGTT	58	*Clostridium leptum* ATCC 29065	(43)
F. prausnitzii	Fprau223F Fprau420R	GATGGCCTCGCGTCCGATTAG CCGAAGACCTTCTTCCTCC	58	*F. prausnitzii* A2165	(43)
P. distasonis	Bd180F Bd463R	AATACCGCATGAAGCAGG GACACGTCCCGCACTTTA	62	*P. distasonis*	(44)

F. prausnitzii, *Faecabacterium prausnitzii*; *P. distasonis*, *Parabacteroides distasonis*.

6.3.8 β-glucuronidase determination

To analyze the activity of β-glucuronidase enzyme in the intestine, the stool samples were collected on the day of AOM injection from wild-type and IRAK-M deficient mice with and without ATB treatment, and from GF mice. Enzyme was extracted from lyophilized and weighed stool pellets into one mL of acetate buffer (50 mM; pH 7) and incubated for two hours at 4°C. 50 μL of extract was added into 100 μL of acetate buffer (50 mM; pH 5) with 50 μL of 2.5 mM MUG substrate (4-methylumbelliferyl-β-D-glucuronide; Glycosynth, Warrington, England) and incubated at 37°C. Product fluorescence was measured at the beginning and after two hours on microplate reader (Tecan GmbH, Grödig, Austria) using 388 nm as excitation and 480 nm as emission wavelength.

6.3.9 Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare multiple experimental groups. Two-way ANOVA with Bonferoni post-test was used in determination of significant weight changes. Differences between two groups were evaluated using an unpaired two-tailed Student's t test, and the incidence of invasive carcinoma between AOM/DSS and ATB/AOM/DSS treated IRAK-M deficient mice were compared by Fisher's exact test. The data are presented either as the mean ± standard deviation, or as percentage of mice with invasive tumors, and differences were considered statistically significant at $P \leq 0.05$. GraphPad Prism statistical software (version 5.0, GraphPad Software, Inc., La Jolla, CA) was used for analyses.

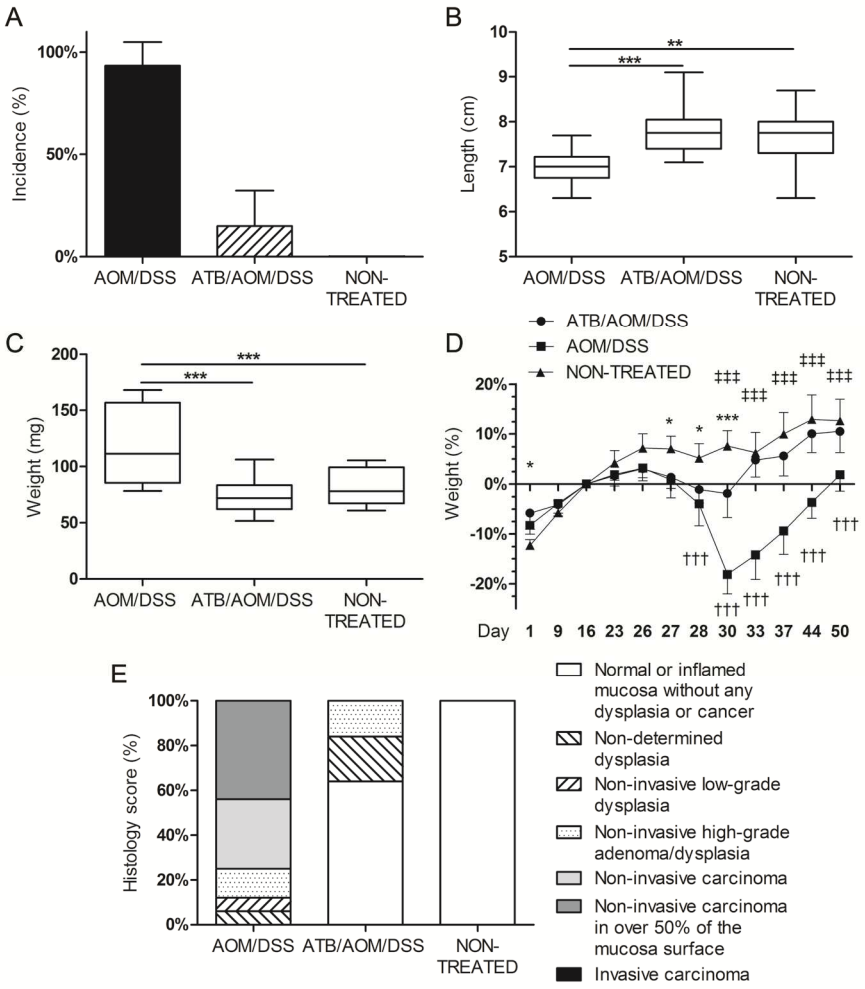
6.4 Results

6.4.1 Colon cancer incidence is significantly decreased in GF and ATB-treated mice

To follow the effect of microbiota on tumorigenesis, we induced the CAC by AOM/DSS in C57BL/6 mice reared either under conventional, or germ-free conditions, or in conventionally-reared ATB-treated mice. After 5 weeks from AOM injection, the histological examination revealed that either graded dysplasia or carcinoma were present in over 93% of conventionally-reared mice and were not accompanied by any sign of tumor dissemination

(Fig. 1A). In all cases, the tumors were situated in the descendent portion of colon and/or in rectum, while no lesions were found in proximal colon. This relatively early tumor development can be ascribed to experimental conditions including sensitive mouse strain, its gut microbiota, diet, or high DSS concentration we used. Therefore, we used shorter

Figure 1. Commensal microbiota is required for the progression of colitis-associated cancer in AOM/DSS-induced colorectal carcinoma model. (A) Total percentage of cases of high-grade dysplasia and tumor incidence was higher in antibiotic (ATB)-non-treated group. (B, C) The colon length and spleen weight were measured at the end of the experiment. Significant shortening of colon length and increase in spleen weight was found in ATB-non treated mice. ** P<0.01, *** P<0.001. (D) ATB-treated mice showed significantly smaller decrease in body weight after DSS treatment and during tumorigenesis when compared to ATB-non-treated mice. The values are relative to the weight before DSS treatment. * P<0.05, ** P<0.01, *** P<0.001 for ATB/AOM/DSS compared with non-treated control, † P<0.05, †† P<0.01, ††† P<0.001 for AOM/DSS compared with non-treated control, ‡ P<0.05, ‡‡ P<0.01, ‡‡‡ P<0.001 for ATB/AOM/DSS compared with AOM/DSS. (E) Histology screening showed decrease of lesions severity in ATB-treated mice as compared with ATB-non-treated mice. Data shown are combined results from three separate experiments with at least five mice per group.



experimental period to see shifts in the incidence in early tumor development and to prevent unnecessary loss of mice due to the mortality. We found significantly shorter colon and heavier spleen in AOM/DSS-treated mice as compared with healthy mice (Fig. 1B and 1C). Interestingly, antibiotic treatment of these mice mitigated the DSS-induced weight loss and significantly decreased both the incidence of tumors (16%) and severity of tumor lesions (Fig. 1D, 1A and 1E). Moreover, we did not observe the significant increase in weight of spleen and shortening of colon in these ATB-treated mice (ATB/AOM/DSS-treated) (Fig. 1B and 1C).

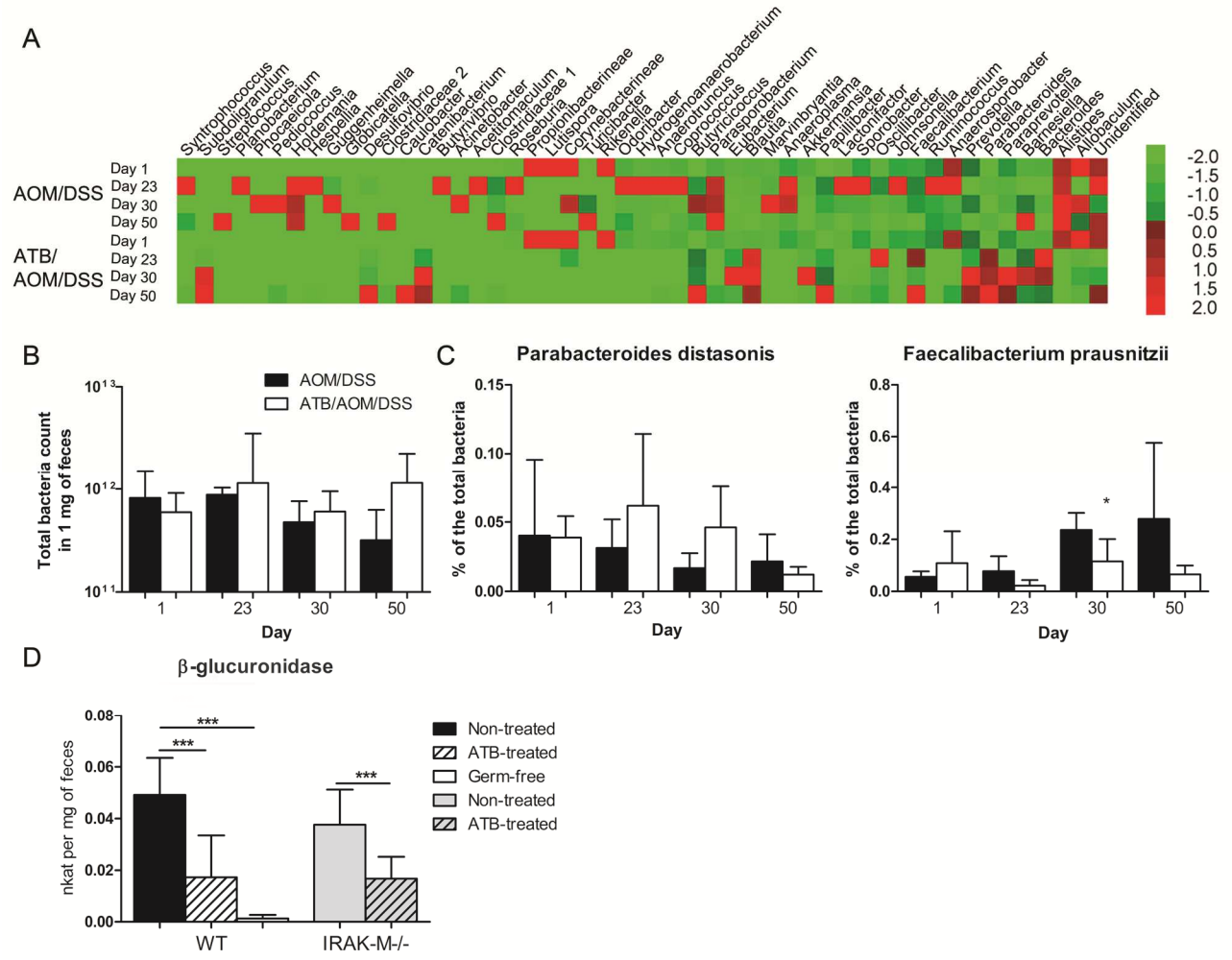
To investigate the role of microbiota in carcinogenesis further, we used the same protocol to induce the colon cancer in GF mice. After 5 weeks, the incidence of tumors in GF mice (10%) was even lower than in ATB-treated conventional animals, confirming the significance of microbiota in the pathogenesis of inflammation associated colorectal carcinoma. Moreover, if present, the tumors had polypoid exophytic character, which corresponded to low-grade dysplasia (data not shown), they were less numerous and smaller in size as compared with those in conventionally-reared mice.

6.4.2 Effect of ATB on microbiota during tumorigenesis

Since dysbiosis is characteristic for IBD and colon cancer, uncovering changes in microbiota composition seems to be crucial for understanding of immune mechanisms involved in pathogenesis of these diseases (4). To address this issue, we compared the fecal microbiota composition between ATB-treated (ATB/AOM/DSS-treated) and control (AOM/DSS-treated) wild-type mice by high-throughput pyrosequencing at different time-points during tumorigenesis (Fig. 2A). ATB treatment caused an increase in occurrence of Bacteriodes, Parabacteroides, Prevotella, Blautia, Desulfovibrio and Subdoligranulum genera and decrease in occurrence of Allobaculum, Alistipes, Ruminoccocus and Johnsonella genera as compared with non-treated mice. In accordance with the previous report, we observed decreasing levels of Bacteroidetes and increasing levels of Firmicutes at the end of the experiment during tumor progression (26). In order to quantify these changes, we performed quantitative analysis with qPCR. Using all Eubacteria and species specific primers, we found that ATB treatment did not decrease the total count of bacteria (Fig. 2B), but resulted in changes of particular bacteria (Fig. 2C). We analyzed *P. distasonis* and *F. prausnitzii* as prevalent representatives of Bacteroidetes and Firmicutes phyla, respectively, because of their evident biological activity associated with intestinal inflammation (24, 27). *P. distasonis* showed increase in abundance

during the first 30 days of ATB treatment whereas *F. prausnitzii* was markedly reduced. On the other hand, ATB-non-treated mice showed opposite trend as *P. distasonis* moderately decreased whereas *F. prausnitzii* abundance continuously grew during tumorigenesis.

Figure 2. Microbiota changes play an important role in the induction of colitis-associated cancer. We collected and analyzed feces at the beginning, before and after DSS treatment and at the end of the experiment to describe the bacterial profile changes during tumor development. (A) Pyrosequencing demonstrates changes among the main fecal phyla of Bacteroidetes, Firmicutes and Proteobacteria during tumorigenesis determined on the level of genera. The heatmap is colored according to the relative abundance of the bacterial genera (green for low, red for high). (B, C) Continuous changes of fecal microbiota were assessed by quantitative PCR using specific primers. The data are normalized by the day 1. The total count of bacteria and the relative numbers of *Parabacteroides distasonis* and *Faecalibacterium prausnitzii* showed variability during tumorigenesis. (D) Activity of β-glucuronidase was measured in the colon content collected in the day of AOM injection from germ-free (GF), antibiotic (ATB)-treated and ATB-non-treated mice. The enzyme activity is significantly decreased by ATB treatment as well as in GF mice. The data are presented as mean ± standard deviation with * P<0.05, *** P<0.001.



6.4.3 Microbiota metabolism increases tumor incidence

The genotoxic effect of AOM can be increased by microbial β-glucuronidase, which releases active compound – methylazoxy-methanol from AOM metabolite (28). Thus, we analyzed the specific activity of this enzyme in germ-free mice and in ATB-treated and non-treated wild-type and IRAK-M deficient mice at the time of carcinogen introduction. For this purpose, we collected samples of feces just before AOM injection and compared the metabolic activity by measuring specific fluorescent product. We found decrease of β-glucuronidase activity in GF as well as in ATB-changed conditions (Fig. 2D). Almost 3-times lower activity, when compared with non-treated mice, was linked with lower tumor incidence in the group of ATB-treated wild-type mice and confirmed that ATB treatment decreased the amount of β-glucuronidase-equipped bacteria in the gut. Although the β-glucuronidase activity was more than 2-times lower in ATB-treated IRAK-M deficient mice, this change was insufficient to reduce the tumor development in these mice. Therefore, the IRAK-M seems to be the effector molecule promoting tumor resistance after ATB treatment in this model, i.e. under the conditions of reduced carcinogen load.

6.4.4 IRAK-M deficient mice developed invasive tumors and ATB treatment was insufficient to protect them from cancer

Since negative regulator of TLR-signaling, IRAK-M, and gut microbiota regulate each other and since IRAK-M molecule mitigates intestinal inflammation in chronic colitis model, as we recently published (18, 19), we further analyzed the importance of IRAK-M in ATB-associated protection against colon cancer development. We used AOM/DSS to induce colon cancer and performed the ATB treatment in conventionally-reared IRAK-M deficient mice. An increased infiltration of mononuclear cells was observed in lamina propria of control non-treated IRAK-M deficient mice as compared with wild-type controls. IRAK-M deficient mice were also more sensitive to AOM/DSS treatment and the incidence of tumors reached 100% after 5 weeks (Fig. 3A). Moreover, the favorable effect of ATB treatment on tumor development was not presented in IRAK-M deficient mice where there was no difference in the tumor incidence, colon length, spleen weight and body weight between ATB-treated and ATB-non-treated group (Fig. 3).

Detailed histological analysis showed widespread flat tumor lesions with high infiltration of inflammatory cells and invasion of crypt bases into submucosa, which had never been seen in wild-type mice (Fig. 4). The invasive carcinoma are significantly more common in the AOM/DSS as compared to ATB/AOM/DSS-treated IRAK-M deficient mice (53% vs. 10%, P=0.005, Fisher's exact test) (Fig. 4D). We also followed the mice for additional 6 weeks observing that both the 100 % incidence of tumors and histological finding are similar as 6 weeks earlier (data not shown).

Figure 3. IRAK-M regulation was important for ATB-induced colon cancer resistance. We used the same protocol as in wild-type mice to induce colitis-associated cancer in IRAK-M deficient mice. (A) IRAK-M deficient mice were more sensitive to the AOM/DSS treatment, which is documented by the tumor incidence. (B, C) Significant shortening of the colon and increase in the weight of spleen were found in both AOM/DSS and antibiotic (ATB)/AOM/DSS-treated mice when compared with non-treated mice. * P<0.05, *** P<0.001. (D) Weight changes during tumorigenesis. The values are relative to the weight before DSS treatment. *** P<0.001 ATB/AOM/DSS compared with non-treated control, ††† P<0.001 AOM/DSS compared with non-treated control. Data shown are compiled from two independent experiments with at least six mice per group.

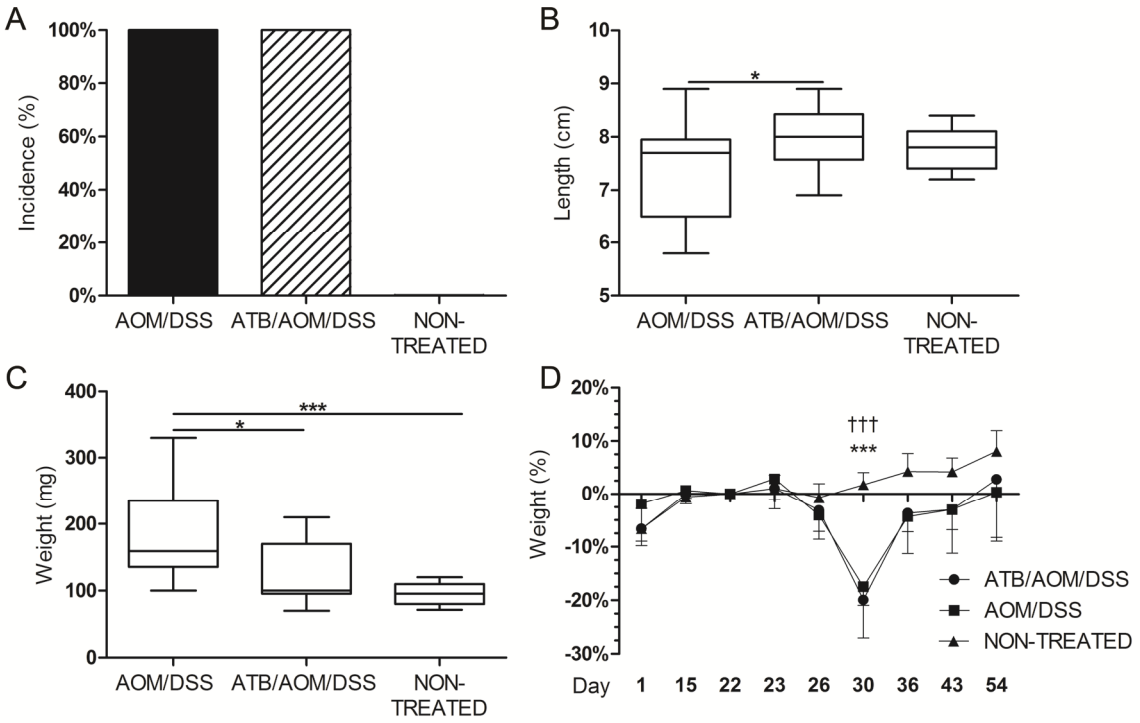
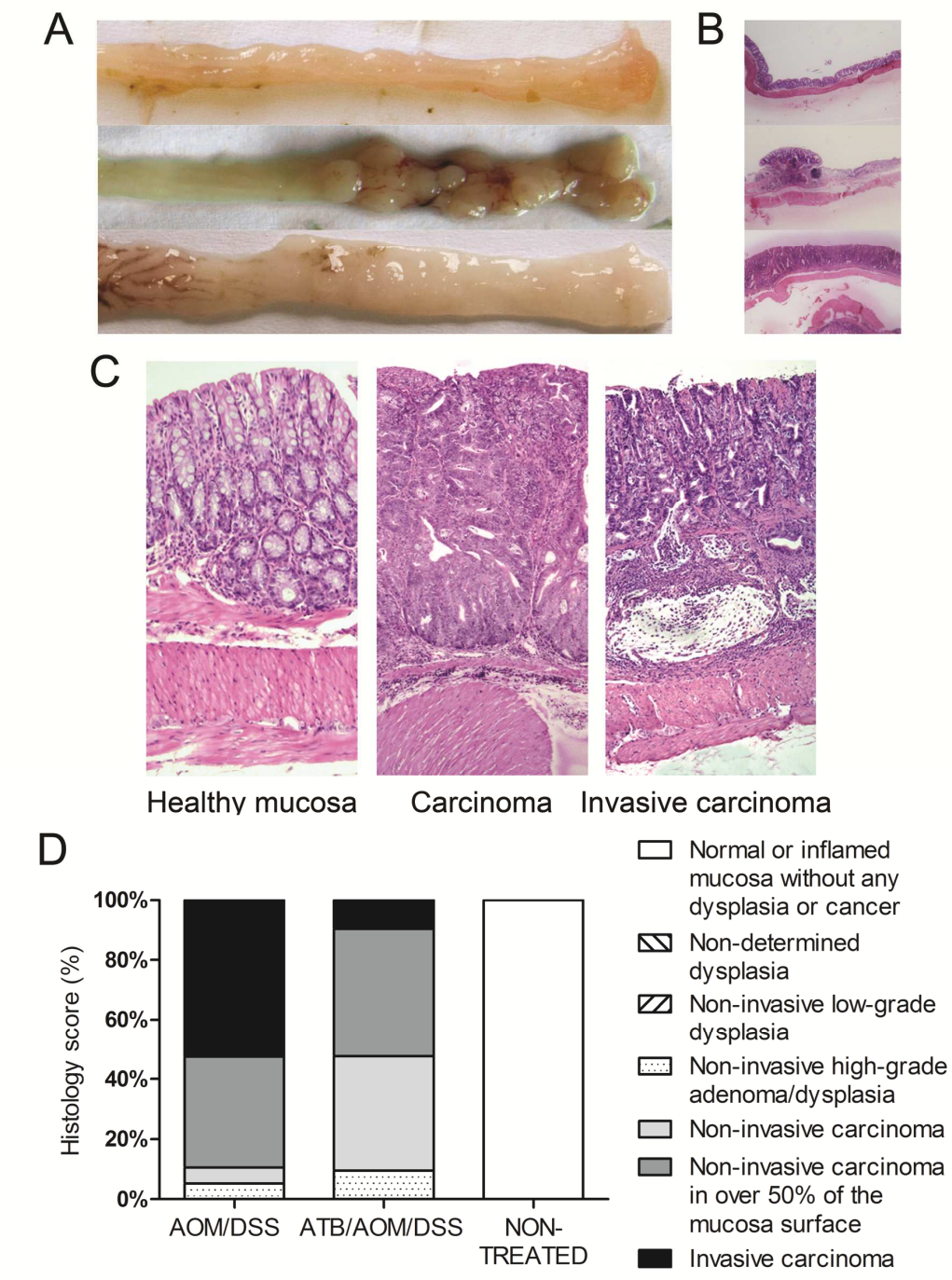


Figure 4. IRAK-M deficient mice developed invasive type of tumor lesions in AOM/DSS model. (A) Macroscopic views of distal colon and rectum of IRAK-M deficient mice show healthy intestine, polypoid lesion and flat lesion. (B) Hematoxylin/eosin-stained representative images of intestines in low magnification show the differences among normal mucosa, polypoid and flat tumor lesions. (C) Detail of the sections shows normal mucosa of non-treated mice, and high-grade carcinoma and invasive character of tumor lesions in AOM/DSS-treated IRAK-M deficient mice. Magnification 10x. (D) Histological examination of colon tissue showed that only 10% of ATB-treated IRAK-M deficient mice have massive widespread tumor lesions with invasion to submucosa, as compared to 53% of ATB-non-treated mice (P=0.005, Fisher's exact test).

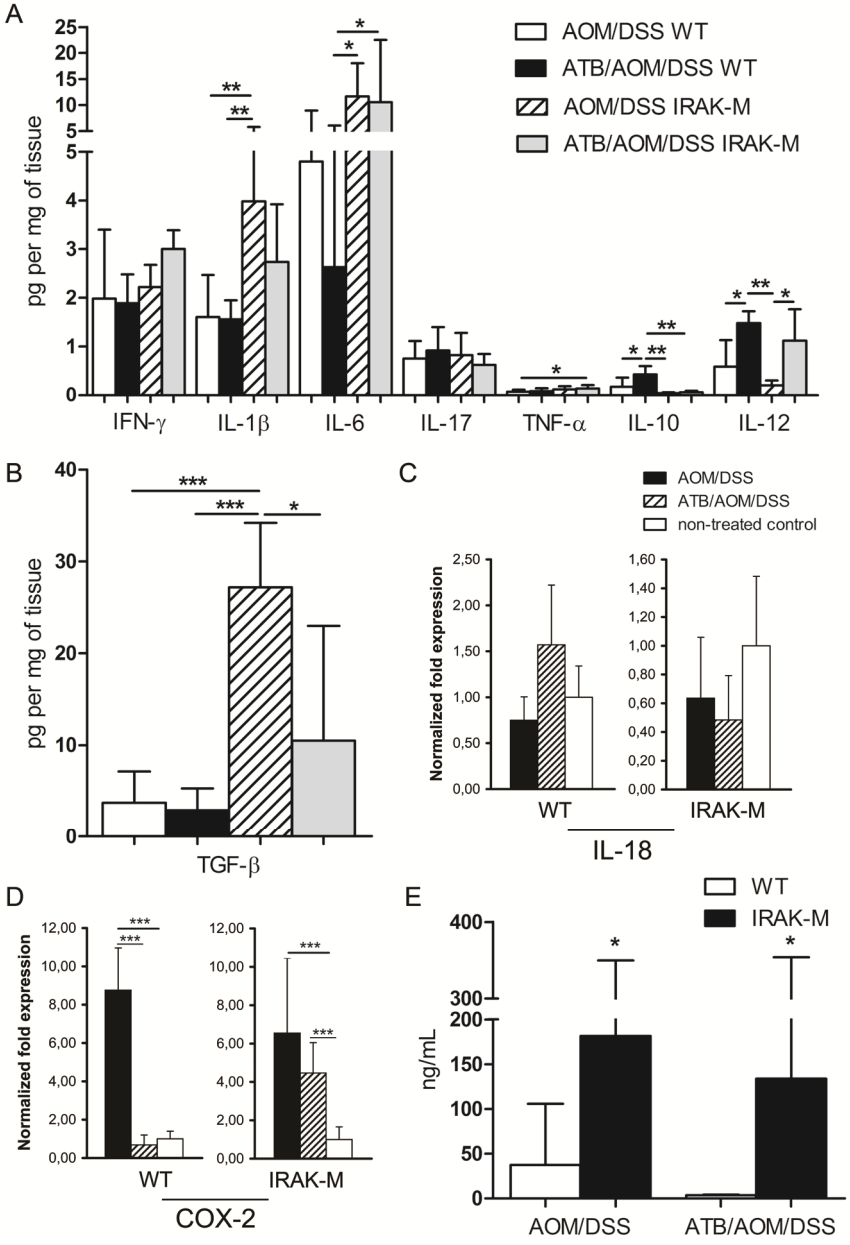


6.4.5 IRAK-M deficient mice showed enhanced pro-inflammatory response

As the chronic inflammation plays a crucial role in tumorigenesis (1), we analyzed the inflammatory response on both local (production of cytokines in the gut) and systemic level (serum acute phase protein – haptoglobin). We found a significant increase in the pro-inflammatory cytokines – IL-1β, IL-6, TGF-β and TNF-α in supernatants from tumor tissues of AOM/DSS-treated IRAK-M deficient mice as compared with wild-type mice (Fig. 5A). The increased inflammatory reaction in IRAK-M deficient mice became more visible in ATB-treated groups where the inflammation and tumorigenesis were reduced in wild-type mice. On the other hand, ATB-treated wild-type mice had significantly higher production of IL-10 and IL-12 than IRAK-M deficient mice. TGF-β is an important factor for regulatory T cells response and function under chronic inflammatory conditions, and for tumor invasion (29). In our experiments we observed low levels of TGF-β at the sites of cancer and inflammation in wild-type mice, while we found much higher production in tumor-bearing IRAK-M deficient mice (Fig. 5B). Moreover, we found unchanged expression of IL-18 in colon tissue of both wild-type and IRAK-M deficient mice, which correlates with the lower levels of IFN-γ found in supernatants (Fig. 5C). These findings suggest increased suppression of anti-tumor immunity in IRAK-M deficient mice. The expression of COX-2 in colon tissue was significantly increased in tumor-bearing wild-type as well as IRAK-M deficient mice confirming local pro-inflammatory activation (Fig. 5D).

To determine the systemic response on tumorigenesis we measured serum haptoglobin levels and we confirmed increased inflammatory activity in tumor-bearing mice in both wild-type and IRAK-M deficient mice (Fig. 5E). Moreover, IRAK-M deficient mice showed significantly higher levels of haptoglobin when compared with wild-type mice. Thus, IRAK-M deficiency seems to maintain an increased pro-inflammatory reactivity affecting the whole organism, and a decreased anti-tumor immunity, regardless of ATB treatment.

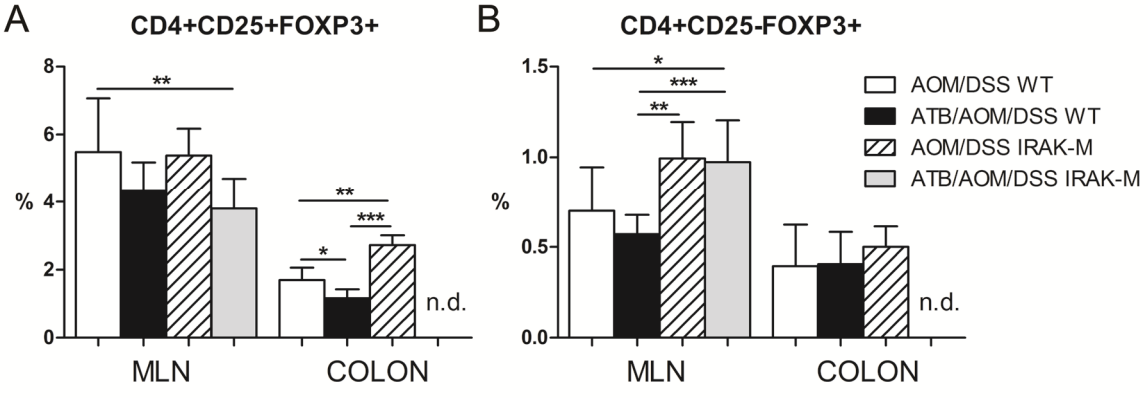
Figure 5. IRAK-M deficient mice showed increased pro-inflammatory response to AOM/DSS treatment at the end of the experiment. (A, B) Cytokine levels in colonic tissue culture supernatants from wild-type (WT) and IRAK-M deficient mice with or without antibiotic (ATB) treatment were measured by Luminex and ELISA. (C, D) The changes in the expression of IL-18 and COX-2 in the colon tissue of WT and IRAK-M deficient mice were analyzed using real-time PCR. (E) Haptoglobin levels were determined by ELISA method in the sera of wild-type and IRAK-M deficient mice ± ATB treatment. Data are presented as mean ± standard deviation with *P<0.05, **P<0.01, ***P<0.001. Data shown are compiled from two independent experiments with at least six mice per group.



6.4.6 Regulatory T cells accumulated in tumor tissue and local lymph nodes

Tumor-bearing mice are known to have different organ distribution and numbers of regulatory T (Treg) cells as compared with healthy controls (30). Therefore, we measured the numbers of Treg cells ($CD4^+Foxp3^+$) in spleen, MLN, PP, and directly in the tumor tissue. We did not observe any significant differences in Treg cell populations in spleens and PPs among the experimental groups (data not shown). In contrast to significant increase of Treg cells populations that we found in MLN and colon tissue of tumor-bearing wild-type as well as IRAK-M deficient mice (Fig. 6A). Generation of $Foxp3^+$ Tregs in the periphery is associated with TGF-β-induced expression of Foxp3 transcription factor in $CD4^+$ cells (31). Interestingly, we found significant increase in this initial population of $CD4^+CD25^-Foxp3^+$ cells in MLN of IRAK-M deficient mice (Fig. 6B). These findings are consistent with other measured factors inducing immune suppression in tumor microenvironment (e.g. high TGF-β, low IL-12).

Figure 6. Tumor-bearing mice showed higher levels of Treg cells. Percentage of $CD4^+Foxp3^+$ regulatory T (Treg) cells was measured by flow cytometry in mesenteric lymph nodes (MLN), and colon tissue of treated wild-type (WT) mice and IRAK-M deficient mice. (A) Significantly increased population of $CD4^+CD25^+Foxp3^+$ Treg cells was found in colon tissue of treated IRAK-M deficient mice. (B) Local induction of immunosuppressive milieu is documented by increase in $CD4^+CD25^-Foxp3^+$ cells in the MLN of treated IRAK-M deficient mice. Differences in Treg cells are presented as bars ± standard deviation and *P<0.05, **P<0.01, ***P<0.001. n.d.: not done. Data shown are compiled from two independent experiments with at least six mice per group.



6.5 Discussion

Microbiota-derived signals are crucial for the development and setting of mucosal immune system and the gut is an important primary site of host – microbe interaction. Disruption of balance between intestinal mucosa and commensal bacteria can result in inflammatory disorders (4). Chronic inflammation, as IBD has long been recognized as a risk factor for cancer development at various sites. For example, individuals suffering from ulcerative colitis have a 2 – 8 fold increased risk of developing colorectal cancer through inflammation-related mechanisms, which increases with the extent and duration of the disease (2). Both these processes, intestinal homeostasis and colitis-associated carcinogenesis, are closely associated with the presence of stimulatory signals derived from intestinal bacteria. In our study, we showed that defect in regulation of signaling cascades involved in bacteria recognition resulted in increased pro-inflammatory response and extensive colon tumorigenesis. This is in agreement with the fact that epithelial cell damage triggers the TLR/MyD88 dependent pathway to enhance regeneration of the epithelium and in the situation of IRAK-M deficiency, which means lack of negative regulation, can cause uncontrolled proliferation resulting in dysplasia and cancer (32). The suggestion that defect in IRAK-M regulation could play a role in inflammatory disease pathogenesis was later supported by Balaci et al., who found polymorphisms in genes encoding IRAK-M in early-onset persistent asthma patients (33). Nevertheless, the function of IRAK-M may not be the same in lung as it is in colon. Xie and coworkers in their study didn't find any tumor growth in IRAK-M deficient mice after simple inoculation of tumor cells (34) and another recently published study about IRAK-M function in lung macrophages confirmed these results (35). Therefore, further research is needed to reveal the exact role of IRAK-M in different types of tissues. We have previously shown that the expression of IRAK-M in colon is low in GF condition and increases after colonization (18). One can hypothesize that changes in the type and level of antigenic stimulation after antibiotic treatment will increase the tumor development either because of lower negative regulation or by inhibition of pro-inflammatory signals. Surprisingly, we found that ATB treatment had protective effect on tumor development in wild-type mice. Subsequent investigation confirmed the important role of IRAK-M in tumorigenesis, because we showed that ATB treatment was not sufficient to decrease the tumor incidence in IRAK-M deficient mice. We also observed increased production of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), and COX-2 expression in the colons, and haptoglobin level in the sera of IRAK-M deficient mice, which suggests their increased sensitivity to inflammatory stimuli.

These results suggest that not only microbiota composition but also fine tuning of MyD88-dependent signaling plays important role in the development of colon cancer in this model.

It is known that administration of ATB influences the diversity of intestinal microbiota (36). Using high-throughput pyrosequencing, we showed that the microbiota composition changes in both groups of ATB-treated and ATB-non-treated mice during tumor development. We showed that the lower tumor incidence is associated with these changes in the microbiota like an increase in proportions of Bacteroidetes and decrease in Firmicutes and Proteobacteria. The capability of certain microbes to metabolize carcinogens in the intestine can increase the exposure of the epithelium by cancer causing substances (5, 37). Bile acids can serve as an example of endogenous substances influencing tumor development. Mainly secondary bile acids, products of bacterial metabolism, are suspect to promote epithelial cells mutations and its transition to cancer (38, 39). High fat diet leads in increased secretion of bile and enhances its prolonged contact with the intestinal mucosa, which is, thus, exposed to oxidative stress, DNA damage, and activation of NF-κB pathway. Similar mechanisms are known for pro-carcinogen AOM, but may not be limited to this particular compound. The AOM is metabolized in liver to methylazoxy-methanol, which is conjugated with glucuronic acid and eliminated from organism in urine or bile. Epithelial and bacterial enzyme β-glucuronidase can dissociate the conjugate and release free methylazoxy-methanol, which is the main DNA methylating compound (28). We found that there is significant decrease in β-glucuronidase enzymatic activity after ATB treatment, which correlates with the decrease in tumor incidence, size and severity. Analysis of microbiota composition confirmed that our combination of ATB decreased the bacteria having β-glucuronidase activity – e.g. Clostridiaceae. It is important to mention, that β-glucuronidase could be produced also by gut epithelium (40). But since the β-glucuronidase activity is extremely low in GF mice, the majority of this activity seems to be generated by gut microbiota and not by epithelium. Changing the microbiota composition with ATB could therefore decrease the local production of β-glucuronidase, thus decreasing the exposure of gut epithelium to carcinogens.

The impaired regulation of TLR downstream pathway promotes the activation of mucosal and systemic immune response, which results in chronic pro-inflammatory stimulation. This effect, together with potent immune suppression, then leads to massive tumor progression (41). Here, we showed that IRAK-M deficient mice have lower levels of IFN-γ and decreased expression of IL-18 in their colons, which suggests inhibition of anti-tumor immunity. Host's immune response is also tuned by regulatory T cells, which maintain the immune tolerance to harmless antigens in gut lumen; this is substantially influenced by

microbiota presence and composition. As germ-free mice, which are known to have decreased counts of lymphocytes including regulatory T cells (42), have also reduced tumor incidence. Thus, immune suppression becomes a disadvantage in such disorders like cancer, when active suppression of immune response enables cancer cells to grow and spread (30). In our study, we found higher levels of $Foxp3^+$ regulatory T cells in tumors and local lymph nodes of tumor-bearing mice as compared with healthy controls. We also observed higher counts of $CD4^+CD25^-Foxp3^+$ cells in tumor-bearing mice suggesting induction of local suppression immune response. Interestingly, as compared with wild-type controls, IRAK-M deficient mice showed increased immune suppression. Our data are consistent with the observation made by Berglund et al. who found upregulated expression of Foxp3 transcription factor, a marker of regulatory T cells, in IRAK-M deficient mice during acute DSS-induced colitis (19). This suggests that impaired regulation of TLR downstream pathway renders IRAK-M deficient mice more susceptible to tumorigenesis due to the active immune suppression, which is enhanced by inflammation.

We conclude that metabolic activity of certain commensal microbes substantially influences the process of CAC. We showed that antibiotic treatment changes the microbiota composition, and that this change is responsible for the beneficial effect on tumorigenesis. IRAK-M deficient mice developed increased local and systemic pro-inflammatory response to microbial stimulation via TLR. Although the antibiotic treatment reduced the population of bacteria with beta-glucuronidase in the intestine, IRAK-M deficiency, enhancing inflammatory response of the host, led to the aggressive tumor development. We showed that IRAK-M is one of the important effector molecules promoting tumor resistance under the conditions of reduced carcinogen load. Further investigation is needed to reveal how host's genetic background shapes the intestinal microbiota and its role in maintaining the balance in local immune response, and in the induction of inflammation or CAC. Therefore, understanding of this host-microbiota crosstalk could bring new strategies in IBD-related cancer therapy and prevention.

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6.7 References

1. Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. Am J Physiol Gastrointest Liver Physiol. 2004;287:G7-17.
2. Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut. 2001;48:526-535.
3. Sartor RB. Microbial influences in inflammatory bowel diseases. Gastroenterology. 2008;134:577-594.
4. Tlaskalova-Hogenova H, Stepankova R, Kozakova H, et al. 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;8:110-120.
5. Arthur JC, Jobin C. The struggle within: microbial influences on colorectal cancer. Inflamm Bowel Dis. 2011;17:396-409.
6. Tannock GW. The bowel microbiota and inflammatory bowel diseases. Int J Inflam. 2010;2010:954051.
7. Parsonnet J. Helicobacter pylori and gastric cancer. Gastroenterol Clin North Am. 1993;22:89-104.
8. Gionchetti P, Rizzello F, Lammers KM, et al. Antibiotics and probiotics in treatment of inflammatory bowel disease. World J Gastroenterol. 2006;12:3306-3313.
9. Thangaraju M, Cresci GA, Liu K, et al. GPR109A is a G-protein-coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon. Cancer Res. 2009;69:2826-2832.
10. Barc MC, Charrin-Sarnel C, Rochet V, et al. Molecular analysis of the digestive microbiota in a gnotobiotic mouse model during antibiotic treatment: Influence of Saccharomyces boulardii. Anaerobe. 2008;14:229-233.
11. Azcarate-Peril MA, Sikes M, Bruno-Barcena JM. The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer? Am J Physiol Gastrointest Liver Physiol. 2011;301:G401-424.
12. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34:637-650.
13. Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol. 2010;10:131-144.
14. Fukata M, Abreu MT. TLR4 signalling in the intestine in health and disease. Biochem Soc Trans. 2007;35:1473-1478.
15. Salcedo R, Worschech A, Cardone M, et al. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. J Exp Med. 2010;207:1625-1636.
16. Hubbard LL, Moore BB. IRAK-M regulation and function in host defense and immune homeostasis. Infect Dis Rep. 2010;2.
17. Kobayashi K, Hernandez LD, Galan JE, et al. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell. 2002;110:191-202.
18. Biswas A, Wilmanski J, Forsman H, et al. Negative regulation of Toll-like receptor signaling plays an essential role in homeostasis of the intestine. Eur J Immunol. 2011;41:182-194.
19. Berglund M, Melgar S, Kobayashi KS, et al. IL-1 receptor-associated kinase M downregulates DSS-induced colitis. Inflamm Bowel Dis. 2010;16:1778-1786.
20. Xiao H, Gulen MF, Qin J, et al. The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. Immunity. 2007;26:461-475.
21. Vannucci L, Stepankova R, Kozakova H, et al. Colorectal carcinogenesis in germ-free and conventionally reared rats: Different intestinal environments affect the systemic immunity. Int J Oncol. 2008;32:609-617.
22. Stepankova R, Powrie F, Kofronova O, et al. Segmented filamentous bacteria in a defined bacterial cocktail induce intestinal inflammation in SCID mice reconstituted with CD45RBhigh CD4+ T cells. Inflamm Bowel Dis. 2007;13:1202-1211.
23. Clapper ML, Cooper HS, Chang WC. Dextran sulfate sodium-induced colitis-associated neoplasia: a promising model for the development of chemopreventive interventions. Acta Pharmacol Sin. 2007;28:1450-1459.
24. Kverka M, Zakostelska Z, Klimesova K, et al. Oral administration of Parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. Clin Exp Immunol. 2011;163:250-259.
25. Zakostelska Z, Kverka M, Klimesova K, et al. 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:e27961.

26. Shen XJ, Rawls JF, Randall T, et al. Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. Gut Microbes. 2010;1:138-147.
27. Sokol H, Pigneur B, Watterlot L, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A. 2008;105:16731-16736.
28. Fiala E. Investigations into the metabolism and mode of action of the colon carcinogen 1, 2-dimethylhydrazine. Cancer. 1975;36:2407-2412.
29. Chen W, Perruche S, Li J. CD4+CD25+ T regulatory cells and TGF-beta in mucosal immune system: the good and the bad. Curr Med Chem. 2007;14:2245-2249.
30. Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. Int J Cancer. 2010;127:759-767.
31. Murai M, Krause P, Cheroutre H, et al. Regulatory T-cell stability and plasticity in mucosal and systemic immune systems. Mucosal Immunol. 2010;3:443-449.
32. Asquith M, Powrie F. An innately dangerous balancing act: intestinal homeostasis, inflammation, and colitis-associated cancer. J Exp Med. 2010;207:1573-1577.
33. Balaci L, Spada MC, Olla N, et al. IRAK-M is involved in the pathogenesis of early-onset persistent asthma. Am J Hum Genet. 2007;80:1103-1114.
34. Xie Q, Gan L, Wang J, et al. Loss of the innate immunity negative regulator IRAK-M leads to enhanced host immune defense against tumor growth. Mol Immunol. 2007;44:3453-3461.
35. Standiford TJ, Kuick R, Bhan U, et al. TGF-beta-induced IRAK-M expression in tumor-associated macrophages regulates lung tumor growth. Oncogene. 2011;30:2475-2484.
36. Dethlefsen L, Huse S, Sogin ML, et al. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 2008;6:e280.
37. Hamer HM, De Preter V, Windey K, et al. Review article: Functional analysis of colonic bacterial metabolism: Relevant to health? Am J Physiol Gastrointest Liver Physiol. 2011.
38. Venturi M, Hambly RJ, Glinghammar B, et al. Genotoxic activity in human faecal water and the role of bile acids: a study using the alkaline comet assay. Carcinogenesis. 1997;18:2353-2359.
39. Bernstein C, Holubec H, Bhattacharyya AK, et al. Carcinogenicity of deoxycholate, a secondary bile acid. Arch Toxicol. 2011;85:863-871.
40. Rod TO, Midtvedt T. Origin of intestinal beta-glucuronidase in germfree, monocontaminated and conventional rats. Acta Pathol Microbiol Scand B. 1977;85:271-276.
41. Vannucci L, Stepankova R, Grobarova V, et al. Colorectal carcinoma: Importance of colonic environment for anti-cancer response and systemic immunity. J Immunotoxicol. 2009;6:217-226.
42. Hrncir T, Stepankova R, Kozakova H, et al. Gut microbiota and lipopolysaccharide content of the diet influence development of regulatory T cells: studies in germ-free mice. Bmc Immunol. 2008;9:-.
43. Bartosch S, Fite A, Macfarlane GT, et al. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol. 2004;70:3575-3581.
44. Kreader CA. Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution. Appl Environ Microbiol. 1995;61:1171-1179.

7 Troy, a Tumor Necrosis Receptor Family Member 19, Interacts with Lgr5 to Inhibit Wnt Signaling in Intestinal Stem Cells

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7.1 Abstract

Background & Aims

In the intestine, the Wnt signaling pathway plays two seemingly opposing roles. Under physiological conditions the pathway is required for maintenance of the intestinal epithelia and its blocking diminishes the proliferative capacity of the intestinal stem cells. Conversely, aberrant Wnt signaling leads to intestinal cancer.

Methods

To investigate the role of the Wnt pathway in gut epithelium homeostasis and its malignant transformation, we employed the chromatin immunoprecipitation method in combination with DNA microarrays (so-called ChIP-on-chip) to identify genes regulated by Wnt signaling in colorectal cancer (CRC) cells.

Results

Promoter regions of 960 genes interacting with the Wnt pathway nuclear effector T-cell factor (TCF) 4 were identified in four different human CRC-derived cell lines; 18 of these promoters were present in all chromatin precipitates. One of the most prominent targets of canonical Wnt signaling was *TROY*, a member of the tumor necrosis factor receptor superfamily (TNFRSF). *TROY*messenger RNA (mRNA) was increased in human cells deficient in the *adenomatous polyposis coli* (*APC*) gene andin cells stimulated with the Wnt3a ligand. Moreover, *Troy* expression was significantly upregulated in neoplastic tissues in two mouse models of intestinal tumorigenesis. Lineage tracing experiments revealed that *Troy* is produced specifically in the fast-cycling intestinal stem cells. TROY associates with a unique marker of these cells, leucine-rich-repeat containing G-protein coupled receptor (LGR) 5, and suppresses Wnt signaling.

Conclusions

TROY functions as a negative modulator of the Wnt pathway to reduce the levels of Wnt signaling in LGR5-positive stem cells.

7.2 Introduction

Development of neoplasia is a multi-step process requiring sequential acquisition of mutational events for its completion. In human colorectal carcinomas, the inheritance of a single mutation inactivating the *APC* gene can result in a significant predisposition to tumor formation and cancer. Moreover, mutations of the *APC* tumor suppressor are frequently found in sporadic colorectal carcinoma (1). It has been well established that *APC*-deficient cells lose control over β-catenin turnover and display constitutive activation of the Wnt pathway (2).

The central feature of the canonical Wnt pathway – also known as Wnt/β-catenin signaling - is the post-transcriptional control of the β-catenin protein stability (reviewed in (3)). In the absence of the Wnt ligand, the cytoplasmic level of free β-catenin is low due to the activity of a β-catenin degradation complex that includes Apc, axis inhibition protein (Axin), glycogen synthase kinase (GSK) 3β and casein kinase (CK) 1α. β-catenin is phosphorylated and subsequently destroyed in the ubiquitin-proteasome pathway. The binding of Wnt to the receptor Frizzled (Fz) and the Wnt co-receptor low-density lipoprotein receptor-related protein (Lrp) leads to disruption of the β-catenin degradation complex. Consequently, β-catenin accumulates in the cytoplasm and nucleus, where it associates with members of the lymphoid enhancer-binding factor (Lef)/Tcf family of transcriptional factors (further referred to as Tcfs). Tcf/β-catenin heterocomplexes function as transcriptional activators of Wnt-responsive genes such as *Axin2*, *c-myc*, *Cyclin D1*, *CD44* and *naked cuticle homolog* (*Nkd*) *1*.

The gastrointestinal epithelia represent the most rapidly self-renewing tissue in the adult mammalian body and completely renew approximately every five days (reviewed in (4)). The single-layer epithelium of the small intestine is ordered into invaginations called crypts and microscopic projections called villi. Each crypt contains several long-lived stem cells. The progenitors [transit-amplifying (TA) cells] generated from these stem cells frequently divide and move upwards from the crypt. TA cells that have reached the edge of the crypts start to differentiate. When the differentiated cells arrive at the top of the villus (in the small intestine) or to the luminal surface (in the colon), they undergo apoptosis and are shed to the intestinal lumen. Paneth cells of the small intestine are the only exception to this scheme.These antibacterial agent-producing cells stay at the crypt base, where they persist for approximately three to six weeks. Two types of intestinal stem cells have been described based on their markers, cycling rate and location in the crypt. *Lgr5*-positive fast-cycling stem cells [also known as crypt base columnar (CBC) cells] are interspersed among the Paneth

cells (5). The second pool consists of *Bmi1*-expressing cells. These relatively slowly dividing cells represent the reserve stem population and reside several cell diameters from the bottom of the crypt (6). The physiological role of Wnt signaling in the gut was revealed by gene targeting experiments in mice. Disruption of the Wnt signaling pathway components *Tcf4* or *β-catenin* resulted in the terminal differentiation of stem cells followed by complete loss of the intestinal proliferative compartments of the crypts (7, 8).

To investigate the role of the Wnt pathway in the intestine, we employed ChIP-on-chip on chromatin isolated from human CRC cells. We identified TROY [alternative names *the tumor necrosis factor receptor superfamily, member 19* (TNRFSF19) or TAJ] (9), as a target of canonical Wnt signaling. In addition, we showed that Troy is produced in fast-cycling stem cells of the small intestine and interacts with LGR5, a recently described receptor for the Wnt agonists R-spondins (Rspos) (10, 11). Interestingly, Troy-deficient organoids derived from the intestinal crypts retained proliferation even in the absence of exogenous Rspo. This observation was in concordance with results showing that TROY inhibits signaling induced by Wnt and Rspo ligands. In conclusion, Troy represents a membrane modulator of the Wnt pathway that fine-tunes the levels of the signaling in the signal-receiving cell.

7.3 Materials and Methods

7.3.1 Cell Lines and Transfections

Human HEK293, Colo320, DLD1, HCT116, HeLa, LS74T, SW480, U2OS cells and mouse 3T3, C57MG and Wnt3a-producing L cells were purchased from ATCC. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics. Transfections were performed using Lipofectamine RNAiMAX or Lipofectamine 2000 reagent (Invitrogen).

7.3.2 ChIP-on-chip

Formaldehyde-crosslinked chromatin isolated from approximately $2x10^9$ Colo320, DLD1, SW480 and LS17T cells was processed as described previously (12) and immunoprecipitated using an anti-human TCF4 rabbit polyclonal antibody or anti-EGFP polyclonal antibody (negative control). Immunoprecipitated DNA was processed onto Human RefSeq Promoter Arrays (4226 HG18; Nimblegen) covering 2000 bps upstream and 500 bps downstream of the

putative transcription start sites of all known human genes. Processing of the results is described in Supplemental Methods. The primers used for the PCR amplification are listed in Supplemental Table S1.

7.3.3 RNA purification and Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNAs were isolated from cells and tissues using the Trizol reagent (Invitrogen) and reversely transcribed as previously described (12). The primers are listed in Supplemental Table S1.

7.3.4 Plasmids and Constructs

Complementary DNAs (cDNAs) encoding human and mouse Troy, human LGR5 and Ectodysplasin A receptor (EDAR) were purchased from Open Biosystems. Additional information is given in Supplemental Methods.

7.3.5 Experimental Animals

$Apc^{+/Min}$, Lgr5-EGFP-IRES-CreERT2 and Rosa26 “reporter” (Rosa 26R) mice were purchased from the Jackson Laboratory; $Apc^{CKO/CKO}$ mice were obtained from the Mouse Repository (NCI, Frederick, US); $Troy^{-/-}$ mice (13) were obtained from MMRRC (University of California, Davis, US). Tumors of the colon and rectum were collected from adult C57BL/6J mice five weeks after a single subcutaneous injection of azoxymethane [(AOM); 10 mg/kg; Sigma] followed by a 5-day dextran sulphate (DS) treatment in drinking water [3% (w/v) DS; MW 36-50 kDa; MP Biomedicals]. The TROY-CreERT2 bacterial artificial chromosome (BAC) transgenic construct was generated using homologous recombination in bacteria according to the previously published protocol (14). Details are given in Supplemental Methods. Tamoxifen (Sigma) was administrated by a single intraperitoneal injection (1 mg dissolved in 0.1 ml of corn oil). Animals were housed and handled according to guidelines approved by the Institutional committee.

7.3.6 β-Galactosidase (LacZ) Staining

Detection of the LacZ activity was performed as described elsewhere (5).

7.3.7 In Situ Hybridization (ISH) and Immunohistochemistry (IHC)

Both techniques were performed as described in detail previously (15). The antisense RNA probes were derived from the following regions of the analyzed genes: *Axin2* (NM_015732.4), nucleotides 2069-2938; *Lgr5* (NM_010195.2), nucleotides 373-887, *Troy* (NM_013869.5), nucleotides 763-1924. Antibodies for IHC: a mouse monoclonal anti-β-catenin antibody (E5; Santa Cruz); anti-ChromograninA (Abcam) and anti-Lysozyme (DAKO) rabbit polyclonal antibodies.

7.3.8 Patient Samples

Ethical approval was obtained from the Institute for Clinical and Experimental Medicine (ICEM) and the Thomayer University Hospital Research Ethics Committee and the Ethics Committee of the Third Faculty of Medicine, Charles University. A cohort of 35 patients with precancerous colorectal lesions and 20 non-consecutive patients with primary colorectal cancer diagnosed at ICEM were enrolled into the study. Additional information is given in Supplemental Methods.

7.3.9 Coimmunoprecipitations and Western Blotting

The experiments were carried out as described previously (12). The antibodies are listed in Supplemental Methods.

7.3.10 Confocal and Fluorescence Resonance Energy Transfer (FRET) Microscopy

Protein colocalizations and FRET were measured by the acceptor photobleaching method (16) using a Leica SP5 confocal microscope. A brief protocol is given in Supplemental Methods.

7.3.11 Recombinant Wnt3a purification; Wnt3a and Rspo1 Treatment

Mouse Wnt3a ligand was isolated from the culture medium of Wnt3a-producing L cells according to the detailed protocol of Willert (17). Wnt3a and/or mouse RSpo1 (R&D; final concentration 250 ng/ml) stimulations lasted 18 hours. Control stimulations were performed by "empty" ligand buffers.

7.3.12 Small Inhibitory RNA (siRNA) and Reporter Gene Assays

Two siRNAs targeting *β-CATENIN* (s437 and s438; Ambion) and two *TROY*-specific siRNAs (DharmaconsiGENOME SMART pool; Ambion) were used and gave similar results. *LGR5*-specific (siGENOME SMART pool) and control non-silencing siRNAs were purchased from Dharmacon. A detailed protocol of the assay was described previously (12). A brief description is given in Supplemental Methods.

7.3.13 Organoid Culture and Proliferation Assay

Isolated crypts were cultured according to reference (18) using various concentrations (0 to 500 ng/ml) of Rspo1. Proliferation rate of crypt-derived multicellular structures called organoids was assessed on day 6 after plating. Details are given in Supplemental Methods.

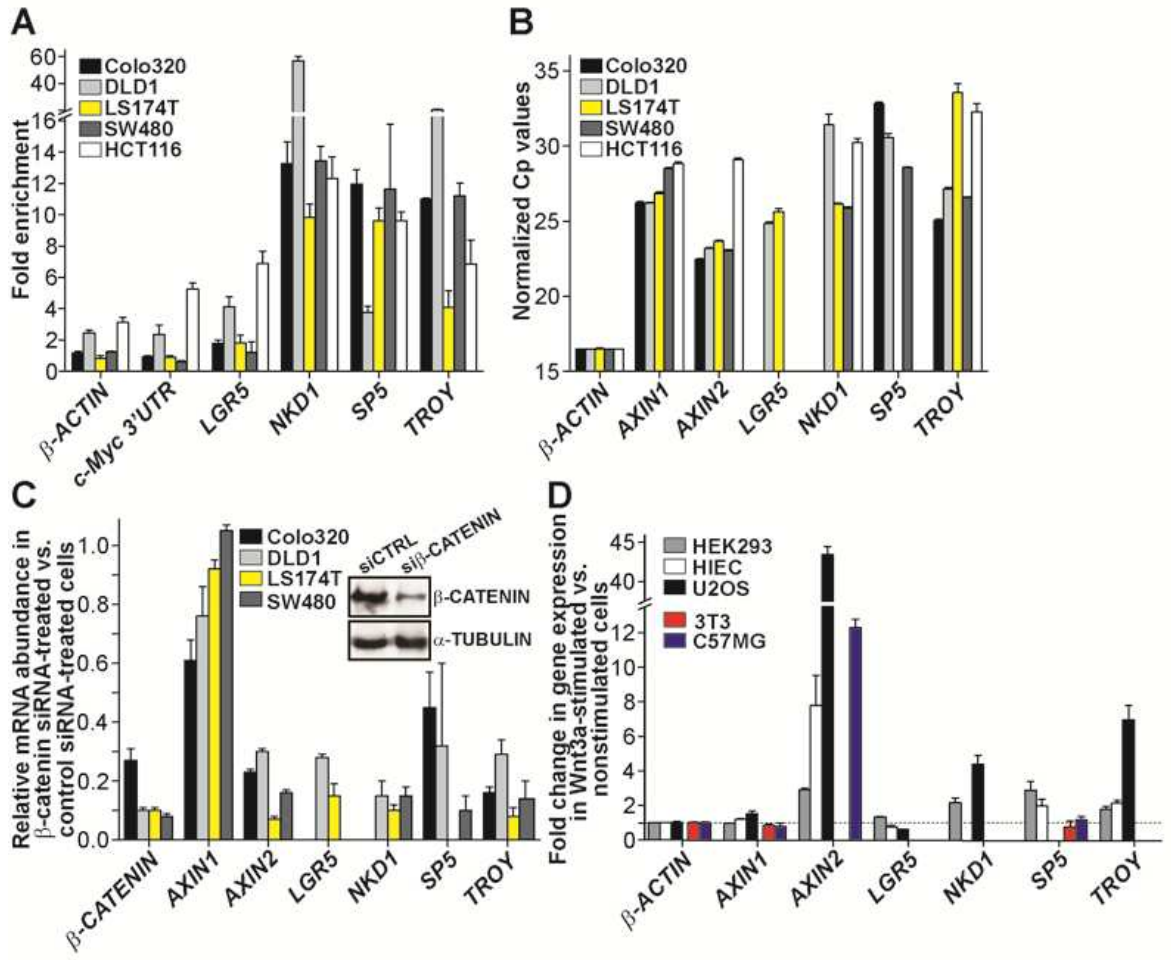
7.4 Results

7.4.1 TROY is a Wnt/β-catenin target gene in APC-deficient cancer cells

ChIP-on-chip was performed using chromatin isolated from human DLD1, SW480, Colo320 and LS174T cells. These CRC cells produce distinct truncated versions of the APC protein; LS174T cells are the only exception as they harbor wild-type *APC*, but carry instead an oncogenic mutation in *β-catenin* (19). Subsequent analysis revealed that promoter regions of 960 genes were retained on the anti-TCF4 antibody-coupled beads; promoters of only 18 genes were positive in all four cell lines tested (Figure 1, Supplemental Figure S1 and

Supplemental Table S8). Among these putative TCF target genes we identified *TROY*. The gene encodes a type I transmembrane protein from the TNFRSF family that was described previously as a Wnt target in dermomyotome of mouse embryos (20). In contrast to some other putative Wnt/β-catenin-activated genes, the *TROY* promoter (containing multiple consensus Tcf binding sites) was precipitated reproducibly with the TCF4-specific antibody

Figure 1. *TROY* is a Wnt/β-catenin target gene. (*A*) ChIP analysis in human CRC cells. Genomic DNA including promoter regions of the presumptive Wnt signaling target genes *LGR5*, *NKD1*, *SP5*, *TROY* were immunoprecipitated from chromatin isolated from the indicated cells. The promoter sequences of the *β-ACTIN* gene and 3' untranslated region (UTR) of *c-MYC* were used as negative controls. Bars represent enrichment of the tested DNA elements in precipitates obtained with the TCF4-specific versus control antibody. (*B*) *Troy* expression is abundant in *APC*-deficient cells. Results of the RT-qPCR analysis of mRNA isolated from indicated CRC cells. Crossing point (Cp) values were normalized to the *β-ACTIN* expression levels (Cp=16.5). The Wnt/β-catenin non-responsive gene *AXIN1* was also included in the test. (*C*) *β-CATENIN* knockdown reduces *TROY* expression. (*D*) In human cells, *TROY* acts as a Wnt responsive gene. Cells of human (HEK293, HIEC, U2OS) or mouse (C57MG, 3T3) origin were stimulated using Wnt3a or Wnt storage buffer (vehicle) and assayed in RT-qPCR. All experiments were performed in triplicates and repeated three times. Histograms represent mean values of a representative experiment with the corresponding standard deviations (SDs). Missing bars stand for unexpressed gene (normalized Cp≥35).



(Figure 1*A*). A subsequent RT-qPCR analysis showed that *TROY* mRNA is abundant mainly in *APC*-deficient cells (Colo320, DLD1, SW480), although its expression in cells with intact *APC* (but harboring *β-CATENIN* activating mutation: LS174T, HCT116) was still detected (Figure 1*B*). In addition, we observed robust downregulation of *TROY* mRNA upon *β-CATENIN* knockdown (Figure 1*C*). Interestingly, when different mammalian cells were stimulated with recombinant Wnt3a, the *TROY* gene was – contrary to the "general" Wnt signaling target gene *Axin2* – upregulated only in cells of human origin [HEK293, U2OS, primary human intestinal epithelial cells (HIEC)] and not in mouse cells [3T3, C57MG, (Figure 1*D*)]. Taken together, these results indicated that *TROY* is a tissue-specific target of canonical Wnt signaling.

7.4.2 Troy is a tumor marker in mouse intestinal neoplasia

Next we analyzed *Troy* expression in tumors in multiple intestinal neoplasia (Min) mice (further referred to as the $Apc^{+/Min}$ strain) and in neoplastic tissue generated in wild-type animals upon treatment with AOM and DS. $Apc^{+/Min}$ animals carry a truncation mutation in one allele of the *Apc* gene and all adult individuals eventually develop large amounts of intestinal polyps and die of cancer (21). Although AOM/DS treatment induces tumors displaying aberrant Wnt signaling (22), these tumors (in contrast to $Apc^{+/Min}$ animals that suffer mainly from the neoplasia of the small intestine) are localized in the distal colon and rectum. We observed a substantial increase in *Troy* mRNA and protein in tumor tissue in both cancer models (Figure 2*AB*). The same expression pattern was noted for another Wnt signaling target gene, *Nkd1*. The analysis of some additional genes regulated by the Wnt/β-catenin pathway was less conclusive. Whereas mRNAs encoding *Axin2* and *Lgr5* were more abundant in the small intestinal lesions of $Apc^{+/Min}$ animals, another Wnt target gene, *Sp5*, was expressed predominantly in the large intestine tumors induced by AOM/DS (Figure 2*A*). Subsequently, we performed ISH using the *Troy*-specific antisense RNA probe. Similarly as *Axin2*, *Troy* expression was detected through the tumor mass, although the signal was less intense. Interestingly, the positive staining was detected not only in tumors but also in the crypts of healthy mucosa of the small intestine (Figure 2*C* and Supplemental Figure S4*A*).

Recently, Lgr5 was established as a specific marker of the intestinal epithelium stem cells. Moreover, knock-in mice designated Lgr5-EGFP-IRES-CreERT2 expressing a tamoxifen-regulated variant of Cre recombinase from the *Lgr5* locus were produced (5). To evaluate the Troy expression at early stages of tumor development we intercrossed Lgr5-

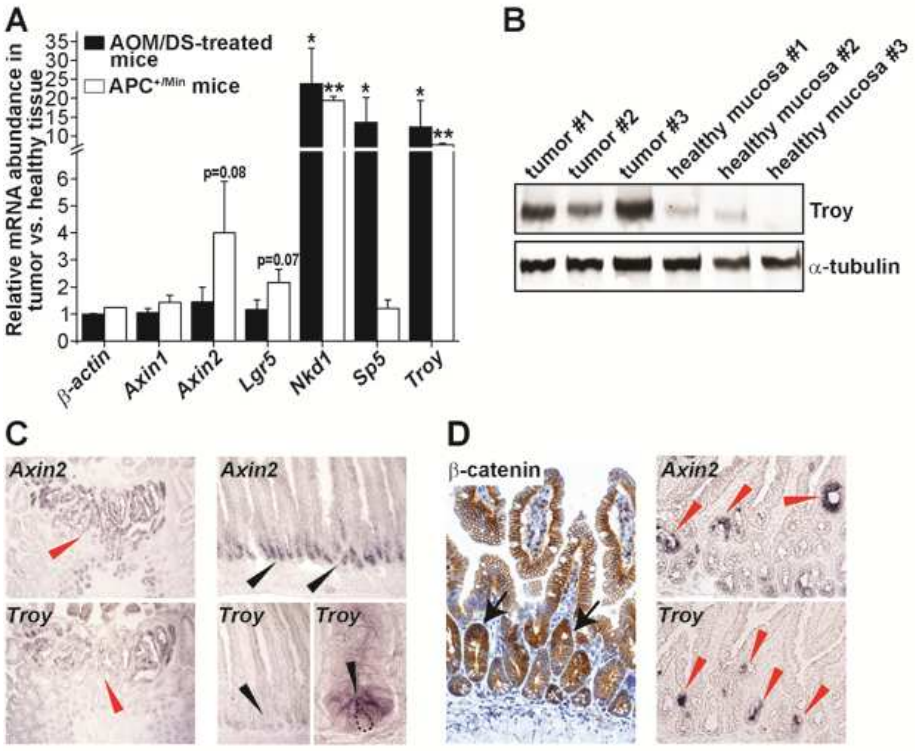
Table 1. List of the genes bound by TCF4 in chromatin isolated from all four CRC cell lines tested.

Cell line:	**Colo320**		**DLD1**		**LS174T**		**SW480**		Peak position	Gene identified by Hatzis et al. (33)
Gene	Fold change	p-value	Fold change	p-value	Fold change	p-value	Fold change	p-value		
LEF1	4.45	1.08E-09	3.73	1.57E-09	2.11	6.78E-10	3.62	9.87E-10	chr4: 09310227-109310282	+
TROY	4.03	1.08E-09	3.56	1.57E-09	2.69	6.78E-10	3.16	9.87E-10	chr13: 23042725-23042786	+
RPS27	3.91	7.58E-08	3.4	1.01E-07	2.75	5.25E-08	2.43	7.05E-08	chr1: 152229752-152229801	-
MOBKL3	3.37	1.08E-09	2.88	1.57E-09	2.24	6.78E-10	2.78	9.87E-10	chr2: 198088264-198088315	-
GGH	2.45	3.16E-07	3.36	1.57E-09	2.6	5.95E-09	2.84	9.87E-10	chr8: 64114840-64114890	-
C12orf35	3.45	7.58E-08	2.26	2.51E-06	2.33	2.24E-07	2.78	4.14E-07	chr12: 32002831-32002889	-
NT5C2	2.21	1.10E-06	3.98	1.57E-09	1.72	7.98E-07	2.21	3.51E-08	chr10: 104943406-104943455	-
SUPT4H1	2.9	1.08E-09	3.09	1.57E-09	1.59	2.56E-08	2.05	9.87E-10	chr17: 53785562-53785611	+
C9orf103	2.88	2.66E-06	2.26	3.34E-06	1.52	1.98E-06	2.87	7.05E-08	chr9: 85426185-85426234	-
SYMPK	2.65	7.58E-08	3.22	1.57E-09	1.7	2.24E-07	1.77	2.51E-06	chr19: 51057357-51057406	-
PCTK2	2.33	3.16E-07	2.58	4.14E-07	2.08	2.24E-07	2.32	8.48E-06	chr12: 95319254-95319303	-
CBWD3	2.46	6.42E-07	2	6.79E-06	2.67	4.23E-06	2.02	5.23E-06	chr9: 70047011-70047060	-
TOM1L1	1.69	1.08E-09	2.79	5.16E-08	2.22	7.98E-07	2.44	9.87E-10	chr17: 50332402-50332451	-
KLF5	2.94	1.08E-09	2.49	1.25E-08	1.77	2.24E-07	1.83	3.51E-08	chr13: 72530342-72530396	+
CBWD5	2.42	2.66E-06	1.83	6.79E-06	2.4	4.68E-07	2.05	6.03E-07	chr9: 69729627-69729676	-
DACH1	2.72	7.58E-08	2.53	3.34E-06	1.57	1.98E-06	1.87	2.51E-06	chr13: 71340031-71340087	+
CBWD2	2.59	5.51E-06	1.92	6.79E-06	2.37	4.23E-06	1.76	5.23E-06	chr2: 113912252-113912301	-
IFRD1	1.5	3.16E-07	2.73	3.16E-07	2.04	8.31E-09	1.65	4.14E-07	chr7: 111876650-111876702	-
AXIN2	3.52	6.42E-07	3.04	8.23E-07	1.49	4.68-07	2.17	8.31E-09	chr17: 60988928-60988982	+
LGR5	NA	NA	1.69	4.13E-07	1.59	5.94E-09	0.79	2.61E-02	chr12: 70119080-70119131	+
NKD1	2.22	7.57E-08	1.60	1.56E-09	0.83	4.16E-04	2.42	2.50E-06	chr16: 49137941-49137998	+
SP5	1.3	3.81E-03	2.72	1.75E-02	1.45	1.56E-08	1.08	2.18E-03	chr2: 171279606-171280055	+

The left columns indicate the fold change of the signal obtained using the anti-TCF4 antibody vs. negative control antibody. The statistical significance of the results (p-value) was evaluated by the chi-square test (see Supplemental Methods for details). The genes are listed according to the signal intensity recorded in the ChIP-on-chip analysis of Colo320 cells. A region was scored as "TCF4-bound" if the ratio of the signal obtained by ChIP with the anti-TCF4 antibody vs. control antibody was ≥ 1.5. The table includes previously identified TCF/β-catenin target genes *AXIN2*, *LGR5*, *NKD1* and *SP5* (listed below the dashed line), although none of these genes fulfilled completely the selection criterion. The base numbering of chromosomal DNA corresponds to the human genome annotation HG18. For comparison, genes identified by the screen of Hatzis and colleagues (33) are shown in the right column.

EGFP-IRES-CreERT2 mice with animals carrying the conditional alleles (*CKO*) of the *Apc* gene. The Cre-mediated excision of the floxed exon 14 in the *CKO* allele results in production of the truncated non-functional Apc polypeptide (23). Multiple β-catenin-positive dysplastic lesions were observed in $Apc^{CKO/CKO}$/Lgr5-EGFP-IRES-CreERT2 mice as early as 5 days after tamoxifen injection. These microadenomas were also positive for *Axin2* and *Troy* mRNA (Figure 2*D*). To confirm our assumption that Troy possibly represents a reliable marker of the neoplastic growth related to non-physiological Wnt signaling, we performed *TROY* expression analysis in sporadic human colorectal specimens. Both in early and

Figure 2. *Troy* mRNA is enriched in mouse intestinal tumors. (*A*) RT-qPCR analysis of 19 tumors dissected from colons of 12 AOM/DS-treated mice and 23 small intestinal tumors from 9 $Apc^{+/Min}$ animals. Control RNA samples were isolated from matched healthy tissue. (*B*) Western blot analysis of 3 tumors isolated from $APC^{+/Min}$ mice. Intact mucosa adjacent to the corresponding neoplasia was used as a control; α-tubulin, loading control. (*C*) Left, ISH of *Troy* and *Axin2* mRNAs in tumors (red arrowheads) developing in $APC^{+/Min}$ mice. Right, in the healthy intestinal tissue both genes are expressed in the lower parts of the crypts (black arrowheads; the image was taken from the duodenum). The enlarged crypt base is shown in the bottom right of the image (one *Troy*-positive CBC cell was outlined by a dashed line). (*D*) Immunohistochemical detection of β-catenin (left panel; counterstained with hematoxylin) and ISH of *Axin2* and *Troy* (right panels) in the microadenomas developed 5 days after Cre-mediated ablation of the *Apc* gene in Lgr5-EGFP-IRES-CreERT2/$Apc^{CKO/CKO}$ animals. Notice that the neoplastic lesions (black arrows in the right image) produce increased amounts of β-catenin and are also positive for *Axin2* and *Troy* (red arrowheads). Original magnification for panels: (*C*) 100× (the bottom right image 600×); (*D*) 200×.



advanced stages of tumor progression, the presence of the inactivating *APC* mutations was positively correlated with increased expression levels of *AXIN2* (Supplemental Figure S2 and Supplemental Table S6). In comparison, the increased expression of the intestinal stem cell marker *LGR5* was related to the *APC* mutation status only at the early, but not the late, stages of tumorigenesis. Contrary to mouse tumors, *TROY* transcription in human carcinomas did not follow the expected pattern, and no correlation between the levels of *TROY* mRNA and the mutation status of *APC* was observed. In addition, the analysis of *TROY* expression in thirty-five polyps of the colon showed a decrease in *TROY* mRNA in the majority of these premalignant lesions (compared to healthy mucosa; Supplemental Figure S3 and Supplemental Table S7).

Taken together, these data indicate that in contrast to mouse neoplasia in human sporadic lesions the TROY expression is governed possibly by a more complex regulatory mechanism.

7.4.3 Troy is produced in Lgr5-positivive intestinal stem cells

Using ISH, we noted a *Troy* localization in the lower part of the crypt compartments (Figure 2*C*, bottom right panels, Supplemental Figure S4*A*). Since the colocalization of *Troy* and EGFP (a surrogate marker of Lgr5$_+$ CBC cells), using a combination of ISH and IHC, was not conclusive (Supplemental Figure S4*A*), we prepared crypts from Lgr5-EGFP-IRES-CreERT2 knock-in mice and sorted the isolated cells according to the EGFP production (Supplemental Figure S4*B*). *Troy* mRNA was detected mainly in cells with high GFP signal (GFP^{high}) and not in cell populations with low or no GPF expression (GFP^{low} and GFP^{negative}, respectively). The same expression pattern was recorded for *Lgr5* but not for *Bmi1* (Figure 3*A*). In a complementary approach, neither *Troy* nor *Lgr5* were enriched in sorted Paneth cells (Supplemental Figure S4*C*). To study the identity of the Troy-producing cells in detail we used BAC carrying the mouse *Troy* locus to generate transgenic mice (designated Troy-CreERT2) expressing tamoxifen-inducible Cre enzyme inserted in frame at the translation initiation codon of the *Troy* gene (Supplemental Figure S5*ABC*). Then we performed the cell labeling experiments in the intestinal tissue. We intercrossed Troy-CreERT2 with Rosa26R mice. Rosa26R animals produce bacterial *LacZ* mRNA from the ubiquitously active *Rosa26* allele (24). The mRNA is not translated (and the enzyme produced) unless a transcriptional stop signal flanked by a pair of loxP sites is removed from the genome by Cre-mediated excision. Adult Troy-CreERT2$^+$/Rosa26R mice were injected with a single dose of tamoxifen

and sacrificed at several time points later. Interestingly, Cre-mediated recombination was the most efficient in the proximal part of small intestine (duodenum, jejunum) and much less effective in the ileum or colon (Figure 3*B* and Supplemental Figure S5*D*). This observation was rather unexpected as endogenous *Troy* expression (similar to the *CreERT2* production from the transgene) is abundant in the colon (Supplemental Figure 5*E*). A similar

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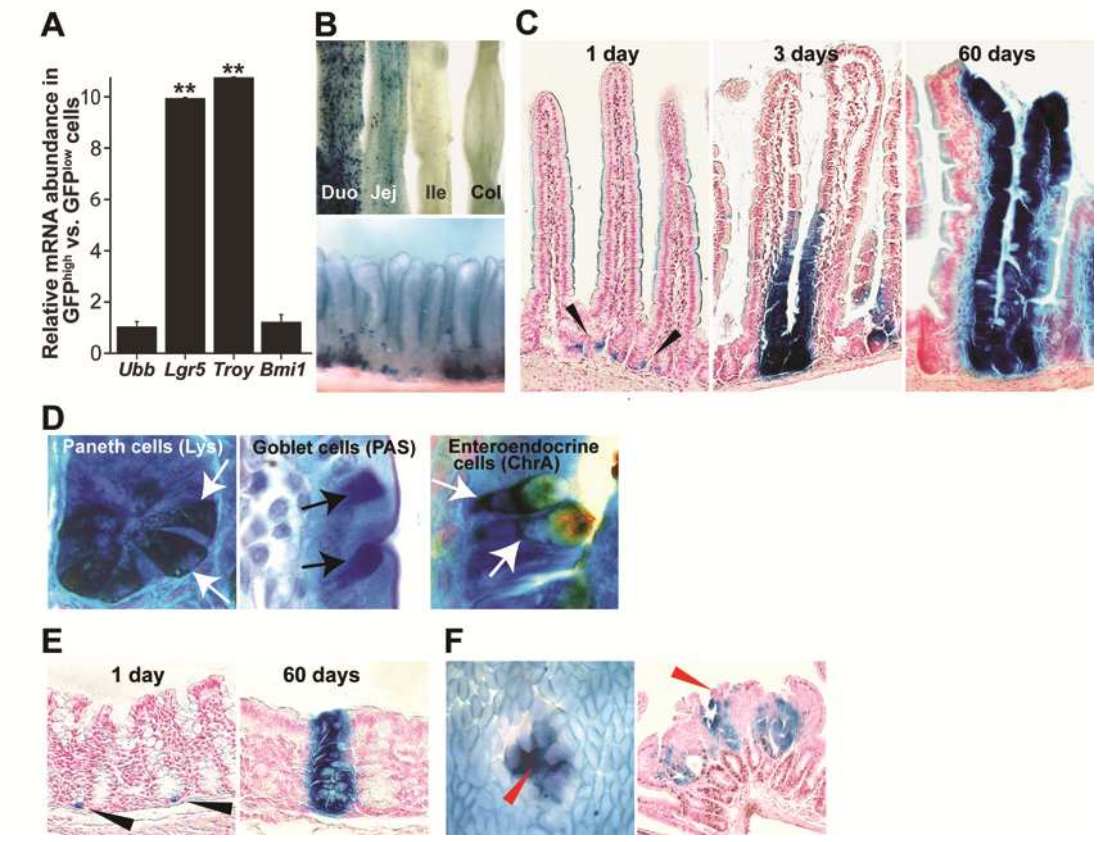
Figure 3. *Troy* expression is confined to the Lgr5-positive cells in the intestinal crypts. (*A*) Expression profiling of GFP^{high} and GFP^{low} cells isolated from the intestinal crypts of 10 Lgr5-EGFP-IRES-CreERT2 knock-in mice (details are given in Supplemental Figure S4*B*). (*B-E*) Lineage tracing in the intestinal epithelium of Troy-CreERT2^{+}/Rosa26R mice. (*B*) Upper image, whole-mount staining of LacZ in different parts of the intestine 1 day after tamoxifen administration. Lower image, whole-mount staining of the jejunum 3 days after tamoxifen administration. Duo, duodenum; Ile, ileum; Jej, jejunum; Col, colon. (*C*) Histochemical detection of the LacZ activity in the duodenum 1 day, 3 days and 60 days after tamoxifen administration. (*D*) Double-labeling demonstrates that LacZ-expressing ("blue") clones produce markers for differentiated cell lineages (arrows) including Paneth, goblet and enteroendocrine cells. Lys, lysozyme; ChrA, chromogranin A; PAS, periodic acid Schiff. (*E*) LacZ staining in the colon 1 day and 60 days after tamoxifen administration. Notice that in both small intestine and colon one-day induction generates LacZ-positive cells located at the crypt base (black arrowheads). (*F*) The tumor-associated LacZ activity (red arrowhead) developed in the small intestine of Troy-CreERT2^{+}/Rosa26R/Apc$^{+/Min}$ mice. The tissue was analyzed 1 day after tamoxifen administration using whole-mount (left panel) or histochemical staining (right). Counterstain: nuclear red. Original magnification: (*A-C*, *I*) 100×, (*D-F*) 600× and (*G*, *H*) 200×.



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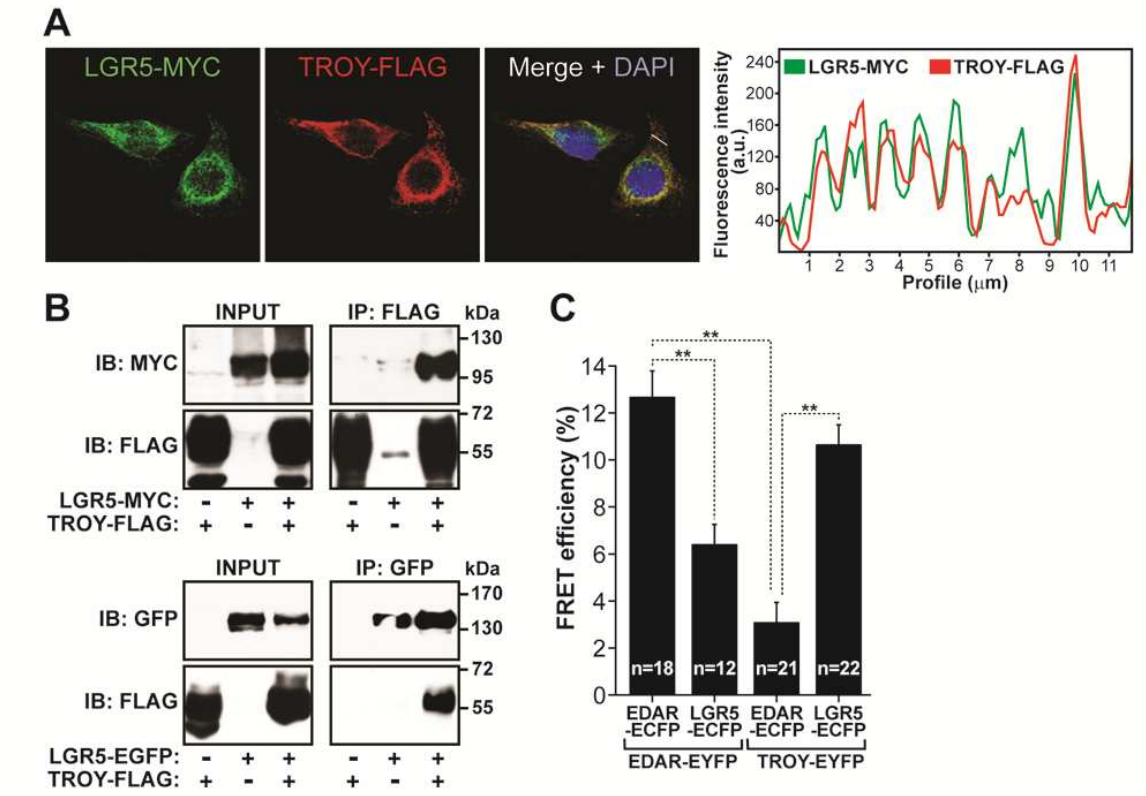
phenomenon was observed in Lgr5-EGFP-IRES-CreERT2 mice (not shown), indicating inefficient recombination of the transcription “roadblock” cassette in the Lgr5- or Troy-positive cells residing in the caudal parts of the intestine. As shown in Figure 3*BD* and Supplemental Figure S5*F*, one day after the induction of Cre, LacZ-expressing cells were located at the crypt bottom of the small intestine and colon (no staining was observed in non-induced animals). At later time points, “blue” cell clones were moving upwards from the crypt and reached the top of the villus or the colonic luminal surface. The labeled cells persisted in the gut for more than two months (Figure 3*BD*) and also descendants of *Troy*-expressing cells differentiated into all major cell lineages of the intestinal epithelium (Figure 3*C*). These data indicated that Troy-positive cells are capable of long-term renewal of the epithelium and are identical to CBC cells. Our conclusion that Troy is a novel marker of CBC stem cells was in concurrence with a recently published analysis of expression profiling of these cells (25, 26).

7.4.4 TROY interacts with LGR5

Next, we visualized the cellular localization of TROY and LGR5 in HeLa cells transfected with TROY and LGR5 expression constructs using confocal microscopy. Interestingly, a clear colocalization of TROY with LGR5 was noted (Figure 4*A*). The possible interaction of TROY with LGR5 was further analyzed using co-immunoprecipitation. Experiments involving tagged variants of TROY and LGR5 demonstrated that MYC-tagged LGR5 could be co-isolated with FLAG-tagged TROY by an anti-FLAG antibody. Conversely, TROY was present in the anti-GFP precipitates when TROY-FLAG and LGR5-EGFP were co-produced in the same cell (Figure 4*B*). To confirm TROY association with LGR5, we employed the FRET acceptor photobleaching approach in HeLa cells expressing various combinations of TROY and LGR5 fused to fluorescent protein at their C-termini. EDAR, a TNFRSF member related to TROY known for its homodimerization, was used as a control (27). Indeed, a high FRET signal was measured in cells co-expressing EDAR-EYFP (acceptor) and EDAR-ECFP (donor). Moreover, the signal was also detected in cells producing TROY-EYFP and LGR5-ECFP, confirming the results obtained by co-immunoprecipitation (Fig 4*C*). Virtually no energy transfer was recorded between EDAR and TROY; however, we noted FRET between EDAR and LGR5. This implied a possible association of these two molecules, which was verified subsequently by co-immunoprecipitation of EDAR with LGR5 in HEK293 cells (Supplemental Figure S6).

Figure 4. TROY interacts with the marker of intestinal CBC cells LGR5. (*A*) Colocalization of ectopically expressed LGR5-EGFP and TROY-FLAG. Confocal microscopy images of HeLa cells transfected with the corresponding constructs and stained with rabbit anti-MYC and mouse anti-FLAG monoclonal antibodies. The panel at the right shows the overlap of fluorescence intensity peaks along the profile as indicated in the merged micrograph. (*B*) Co-immunoprecipitation of MYC-tagged LGR5 and FLAG-tagged TROY (upper panels) or EGFP-tagged LGR5 and FLAG-tagged TROY (bottom panels). In lanes denoted "INPUT", 20% of the total lysate used for one immunoprecipitation was loaded; IP, immunoprecipitation; IB, immunoblotting. (*C*) Detection of TROY binding to LGR5 using FRET microscopy. Fluorescently tagged LGR5, TROY and EDAR proteins were co-expressed in HeLa cells and visualized as indicated under the histogram. EYFP was photo bleached in a selected area of the cytoplasmic membrane and FRET efficiency measured as an increase of ECFP fluorescence upon EYFP photo destruction. The bars indicate the relative FRET efficiency calculated from the given cell numbers (n); $^{**}p<0.01$ (Student's t-test).

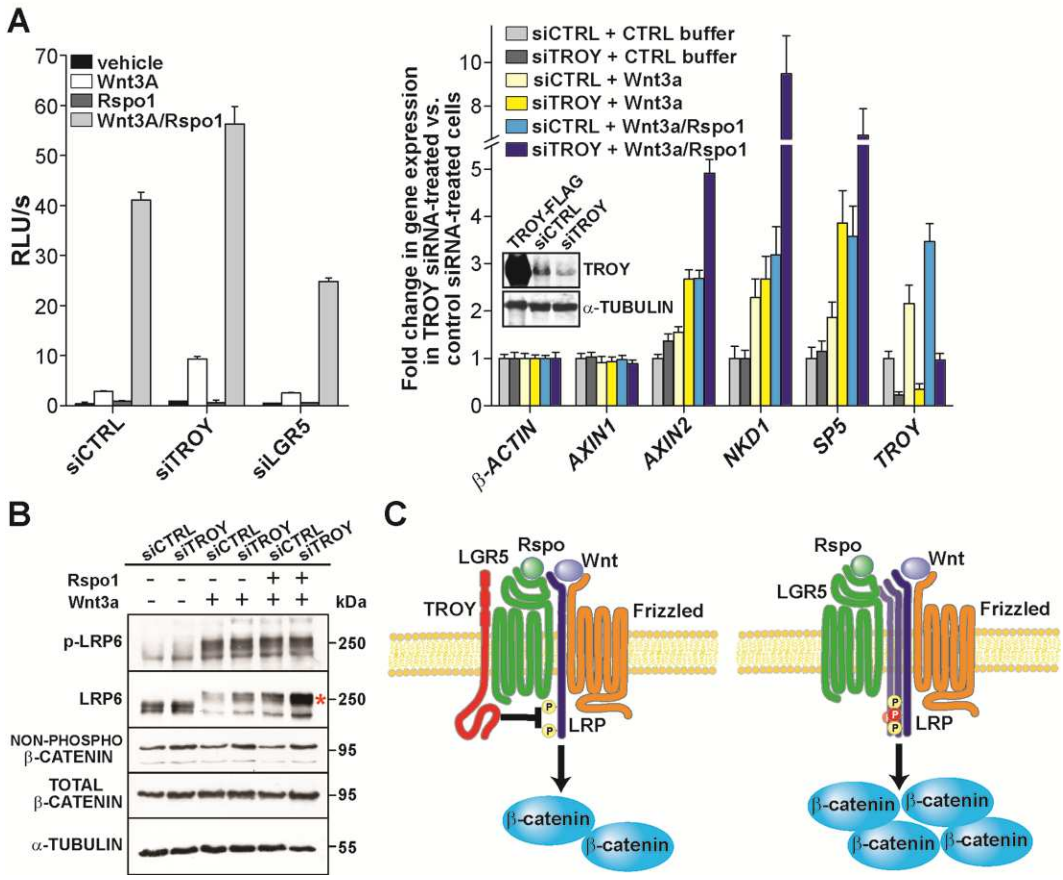


7.4.5 TROY functions as a negative regulator of Wnt signaling

Recently, several studies have shown that Rspos, the secreted agonists of Wnt signaling, bind to Lgr5 and this interaction augments the action of Wnt ligands (10, 11, 28). To examine whether TROY affects the Rspo function, we performed a reporter gene assay and a RT-qPCR analysis in HEK293 cells treated with siRNA specific for TROY. We found that the

TROY mRNA level was reduced to 15-20% upon transfection with two different TROY siRNAs (Figure 5*A* and data not shown). Interestingly, TROY knockdown elevated the Wnt-dependent transcription not only in the cells treated with a combination of Wnt3 and Rspo1, but also in cells stimulated only with Wnt3a ligand. In a parallel control experiment, cells were transfected with LGR5-specific siRNA, and as expected, the potentiating effect of

Figure 5. TROY inhibits Wnt signaling. (*A*) Downregulation of TROY potentiates the responsiveness of HEK293 cells to Wnt3a and Rspo1 stimulation. Left, results of the reporter gene assay using the Wnt/β-catenin reporter pTOPFLASH. Right, RT-qPCR analysis of HEK293 cells treated as indicated. The blot in the inset demonstrates the efficiency of TROY knockdown. The reporter gene activity (left diagram) or expression level of a given gene (right diagram) in cells transfected with control non-silencing siRNA and treated with vehicle (Wnt storage buffer) was arbitrarily set to 1. (*B*) TROY influences cellular levels and phosphorylation of LRP6. The western blot of whole-cell extracts prepared from HEK293 cells treated with non-silencing or TROY siRNA and further stimulated as indicated. The immunoblotting was performed with antibodies recognizing the total cellular pool of LRP6 (bottom panel) or its form phosphorylated at Ser1490 (p-LRP; upper panel). Notice that TROY siRNA increased the amount and phosphorylation of LRP6 (the putative phosphoprotein with changed mobility is marked by red asterisk) but, other residue than Ser1490 is modified. Moreover, TROY siRNA induced stabilization of β-CATENIN including the N-terminally non-phosphorylated form of the protein. (*C*) A possible model of TROY action. The absence of TROY increases levels and phosphorylation of LRP at specific residue (indicated by "P" in red circle). This potentiates the outcome of Wnt/Rspo signaling and leads to β-catenin accumulation.

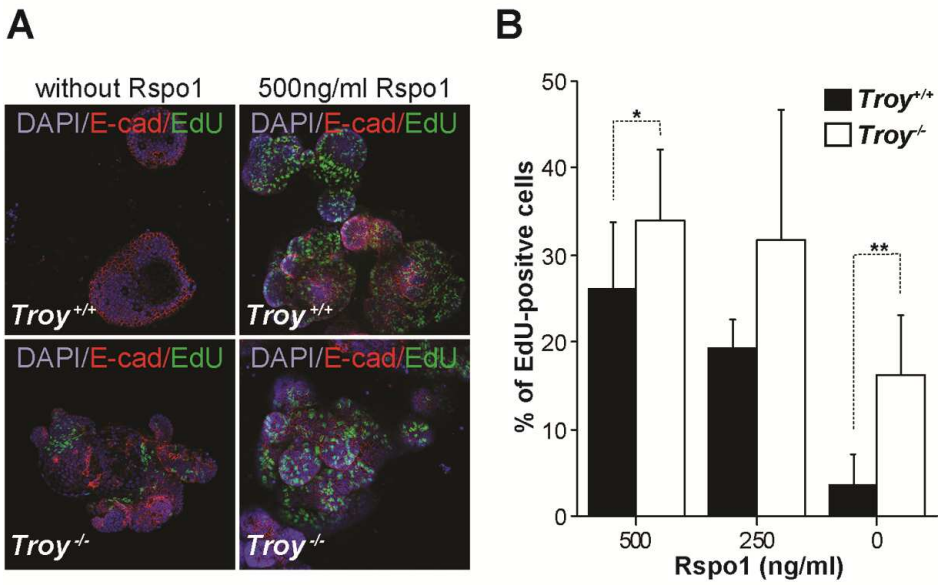


Rspo1 on Wnt signaling was abolished (Figure 5*A*). Conversely, a gradual increase of TROY reduced both Wnt3a- and Wnt3a/Rspo1-mediated signaling (Figure 6*B*). Importantly, TROY knockdown or overexpression had no effect on the transcription from the negative-control reporter pFOPFLASH (not shown) and, furthermore, the TROY downregulation did not potentiate TCF/β-catenin-mediated transcription in human CRC cells with aberrant Wnt signaling (Supplemental Figure S7*A*). The latter finding indicated that TROY functions upstream of the β-catenin degradation complex. Experiments with ectopic TROY were less conclusive. In cells with inducible expression of TROY, only minor effects on transcription of Wnt signaling target genes were recorded (Supplementary Figure S7*B*). Nevertheless, we observed that binding of Rspo1 to LGR5 was not eradicated by the TROY presence (Supplemental Figure S8). We further focused on the signaling downstream of Wnt and Rspo ligands. Interestingly, although Dvl phoshorylation was not changed by TROY knockdown, the treatment of HEK293 cells with TROY siRNA increased phosphorylation and cellular levels of the Wnt co-receptor LRP6 (Supplemental Figure S8*B* and Figure 5*B*). We concluded that TROY might block specific modifications of LRP6 which in turn can change LRP6 stability and/or function. However, western blots with antibodies recognizing phospho-Ser1490 andphospo-Thr1572 of LRP6 protein did not show differential modification of these residues in control and TROY siRNA-treated cells (Figure 5*B* and data not shown). Thus, the putative phoshorylation site(s) remains to be determined. Finally, we functionally tested the role of Troy in defined primary cultures of the intestinal crypts. Interestingly, upon withdrawal of exogenous Rspo1 organoids derived from the wild-type mice stopped to proliferate. In contrast, $Troy^{-/-}$ organoids continued to grow even in the absence of Rspo1 (Figure 6*AB*). These results convincingly confirmed the possible inhibitory role of Troy on Wnt/Rspo1 signaling.

7.5 Discussion

In the present study, we identified TROY as the Wnt/β-catenin pathway target gene in human CRC cells and in mouse tumors. Using expression profiling, ISH and lineage tracing experiments in the mouse we demonstrated that in the healthy small intestine, Troy marks the fast-cycling CBC stem cells. In addition, we showed that TROY interacts with LGR5 and inhibits canonical Wnt signaling.

Figure 6. Troy-/- organoids proliferate in absence of Rspo. (*A*) Confocal microscopy images of organoids derived from Troy$^{+/+}$ and Troy$^{-/-}$ small intestinal crypts. The organoids were grown with or without Rspo1 and stained using an anti-E-cadherin (E-cad) antibody (red) and DAPI nuclear stain (blue); proliferating cells were visualized by EdU incorporation (green). Original magnification: 400×. (*B*) Proliferation assay of organoids. *p<0.05; **p<0.01.



In contrast to mouse neoplasia, *TROY* expression in human sporadic colorectal cancer did not correlate with the mutation status of *APC* (Supplemental Figure S2). Interestingly, we never observed an "hundred percent" correlation between the expression of the given gene and (hyper)active Wnt signaling, although the analysis included several well-defined Wnt/β-catenin signaling target genes. Even *Axin2*, considered to be a "universal" target of the canonical Wnt pathway, was not (significantly) upregulated in AOM/DS-induced neoplasia (Figures 1*BC* and 2*A*). These data indicate that the transcription of the tested genes is regulated by complex mechanisms and cannot be solely related to the status of the Wnt pathway.

The ISH indicated that *Troy* mRNA is uniformly expressed throughout tumor tissues (Figure 2*B*). This result was somehow contradictory to the lineage-tracing experiments in Troy-CreERT2^{+}/Rosa26R/Apc$^{+/Min}$ that showed "patches" of the LacZ-positive cells in tumor tissues (Figure 3*F*). As Cre-mediated recombination displayed different efficiency in distinct parts of the intestine (Figure 3*B*), we suppose that the observed variability in LacZ staining was caused by the inefficient recombination of the "floxed" transcriptional stop signal rather than by the heterogeneous expression of *Troy* (or its surrogate marker Cre) in tumor tissue.

Troy expression was described in the adult central nervous system, the developing hair follicle and embryonic skin (27). In postnatal neurons, Troy interacts with the Nogo-66 receptor 1 (NgR1) and activates RhoA signaling (29). Moreover, TROY in glial tumor or HEK293 cells participates in the Rac1 and JNK pathways, respectively (30, 31). Recently, a study of Hashimoto and colleagues revealed that the cytokine lymphotoxin-α (LTα) binds to Troy and triggers NFκB signaling (32). All these results suggest a complex and pleiotropic role of Troy in various cellular contexts. According to our data, TROY interacts with LGR5 and limits the level of canonical Wnt signaling. Importantly, this conclusion was based on results obtained not only in cell lines but also in primary cultures of epithelial organoids. The Troy-mediated inhibition on the Wnt pathway possibly involves the stability and/or the phoshorylation status of the LRP co-receptor. Nevertheless, the relationship between phosphorylation of specific residue(s) in LRP6 and the stability and function of the protein remains to be determined.

Troy-deficient mice are viable and fertile with no apparent defects in the gut tissue (13, 32). This would implicate a Troy redundancy with another protein. Partial redundancy between TROY and EDAR has already been suggested (13). However, EDAR expression was not observed in CBC cells (L.T. and V.K., unpublished data). These observations rule out the possible Wnt-related role of EDAR in the gut. Alternatively, the Troy absence results in a relatively subtle with survival-compatible phenotype that was not noted during the primary analysis of $Troy^{-/-}$ animals. Finally, $Troy^{-/-}$ mice must be “challenged” in some way to reveal any functional outcomes of the Troy absence.

7.6 Acknowledgements

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7.7 References

1. Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159-70.
2. Korinek V, Barker N, Morin PJ, et al. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science 1997;275:1784-7.
3. Cadigan KM, Peifer M. Wnt signaling from development to disease: insights from model systems. Cold Spring Harbor perspectives in biology 2009;1:a002881.
4. Marshman E, Booth C, Potten CS. The intestinal epithelial stem cell. BioEssays 2002;24:91-8.
5. Barker N, van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007;449:1003-7.
6. Tian H, Biehs B, Warming S, et al. A reserve stem cell population in small intestine tendere Lgr5-positive cells dispensable. Nature 2011;478:255-9.
7. Fevr T, Robine S, Louvard D, et al. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Molecular and cellular biology 2007;27:7551-9.
8. Korinek V, Barker N, Moerer P, et al. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet 1998;19:379-83.
9. Hisaoka T, Morikawa Y, Kitamura T, et al. Expression of a member of tumor necrosis factor receptor superfamily, TROY, in the developing mouse brain. Brain Res Dev Brain Res 2003;143:105-9.
10. de Lau W, Barker N, Low TY, et al. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature 2011;476:293-7.
11. Glinka A, Dolde C, Kirsch N, et al. LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling. EMBO Rep 2011;12:1055-61.
12. Lukas J, Mazna P, Valenta T, et al. Dazap2 modulates transcription driven by the Wnt effector TCF-4. Nucleic Acids Res 2009;37:3007-20.
13. Pispa J, Pummila M, Barker PA, et al. Edar and Troy signalling pathways act redundantly to regulate initiation of hair follicle development. Hum Mol Genet 2008;17:3380-91.
14. Yang XW, Model P, Heintz N. Homologous recombination based modification in Escherichia coli and germline transmission in transgenic mice of a bacterial artificial chromosome. Nature biotechnology 1997;15:859-65.
15. Pospichalova V, Tureckova J, Fafilek B, et al. Generation of two modified mouse alleles of the Hic1 tumor suppressor gene. Genesis 2011;49:142-51.
16. Huranova M, Jablonski JA, Benda A, et al. In vivo detection of RNA-binding protein interactions with cognate RNA sequences by fluorescence resonance energy transfer. RNA 2009;15:2063-71.
17. Willert K, Brown JD, Danenberg E, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 2003;423:448-52.
18. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 2009;459:262-5.
19. Rosin-Arbesfeld R, Cliffe A, Brabletz T, et al. Nuclear export of the APC tumour suppressor controls beta-catenin function in transcription. Embo J 2003;22:1101-13.
20. Buttitta L, Tanaka TS, Chen AE, et al. Microarray analysis of somitogenesis reveals novel targets of different WNT signaling pathways in the somitic mesoderm. Dev Biol 2003;258:91-104.
21. Su LK, Kinzler KW, Vogelstein B, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 1992;256:668-70.
22. Tanaka T, Kohno H, Suzuki R, et al. Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc(Min/+) mice: inflammatory stimuli by dextran sodium sulfate results in development of multiple colonic neoplasms. International journal of cancer 2006;118:25-34.
23. Kuraguchi M, Wang XP, Bronson RT, et al. Adenomatous polyposis coli (APC) is required for normal development of skin and thymus. PLoS Genet 2006;2:e146.
24. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nature genetics 1999;21:70-1.
25. van der Flier LG, van Gijn ME, Hatzis P, et al. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell 2009;136:903-12.
26. Sato T, van Es JH, Snippert HJ, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 2011;469:415-8.
27. Kojima T, Morikawa Y, Copeland NG, et al. TROY, a newly identified member of the tumor necrosis factor receptor superfamily, exhibits a homology with Edar and is expressed in embryonic skin and hair follicles. The Journal of biological chemistry 2000;275:20742-7.
28. Carmon KS, Gong X, Lin Q, et al. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc Natl Acad Sci U S A 2011;108:11452-7.

29. Shao Z, Browning JL, Lee X, et al. TAJ/TROY, an orphan TNF receptor family member, binds Nogo-66 receptor 1 and regulates axonal regeneration. Neuron 2005;45:353-9.
30. Eby MT, Jasmin A, Kumar A, et al. TAJ, a novel member of the tumor necrosis factor receptor family, activates the c-Jun N-terminal kinase pathway and mediates caspase-independent cell death. The Journal of biological chemistry 2000;275:15336-42.
31. Paulino VM, Yang Z, Kloss J, et al. TROY (TNFRSF19) is overexpressed in advanced glial tumors and promotes glioblastoma cell invasion via Pyk2-Rac1 signaling. Mol Cancer Res 2010;8:1558-67.
32. Hashimoto T, Schlessinger D, Cui CY. Troy binding to lymphotoxin-alpha activates NF kappa B mediated transcription. Cell cycle (Georgetown, Tex 2008;7:106-11.
33. Hatzis P, van der Flier LG, van Driel MA, et al. Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. Mol Cell Biol 2008;28:2732-44.

7.8 Supplementary data

7.8.1 Supplemental Materials and Methods

ChIP-on-chip Data Analysis

The results of hybridizations were obtained in a gff file format (Nimblegen). The fluorescent intensity peaks in corresponding areas of the microarray were identified by "triangle shape model" described by Kim and colleagues (Savoia et al. 2009). The peaks were used to define the threshold for "positive" probes. Enrichment of positive probes was then calculated using chi-square of the actual number compared to the expected number of positive probes in a given window (Cubeddu et al. 2009). The p-value listed in Table 1 and Supplementary Table S8 is the best (=smallest) p-value assigned to a probe located in the peak region.

Generation of Troy-CreERT2 Transgenic Mice

To generate transgenic mice, a BAC clone RP23-166C22 was purchased from imaGenes (Berlin, Germany); a construct containing CreERT2 cDNA was kindly provided by M. Cepko via Addgene. The adapters "a" (5'-CCTCTCGTCTTTGACTTGCATCCTTCAGGAATAAA CACGTTTGGTGAGAGCCatgtccaatttactgaccgtac-3'; the *Troy*-specific sequence is highlighted in capital letters, the part complementary to the CreERT2 expression cassette is given in small caps) and "b" (5'-TGATAGGAGTTCTTTCCCCATATTCATATGAAAGAG AAGAGGCAACTTACgccgctctagaactagtggatc-3') were derived from the sequence of the exon containing the translation start site or from the flanking intron, respectively (Supplemental Fig. S5*A*). The adapters were employed for the PCR amplification, the product was purified from agarose gel and electroporated into the *E. coli* strain EL250 harboring the BAC clone RP23-166C22. Bacterial clones containing correctly recombined BACs were verified by PCR. Isolated recombinant BAC DNA was linearized and used for pronuclear injection of fertilized eggs (C57Bl/6J background). Three TROY-CreERT2 transgenic founder lines were produced using the service of the Transgenic Unit of the Institute of Molecular Genetics. All three lines exhibited a virtually identical pattern of the Cre recombinase activity.

Processing of Specimens of Human Sporadic Colorectal Carcinomas and Precancerous Colorectal Lesions

Samples of the following macroscopically distinguishable tissue segments were taken from fresh colectomy specimens: 1) the center (stroma) of the tumor without, if any present, ulcerated luminal portion and the least necrotic parts of the lesion were preferred, and, 2) healthy surrounding mucosa were collected in the distance of a maximum of 5 cm from the primary tumor site. Pathological and morphological criteria included tumor location, pTNM classification, histological subtype, grade of differentiation and presence of vessel invasion (a summary is given in Supplemental Table S2). RNA was isolated from biological replicates of distinct tumor segments and healthy mucosa. Sporadic precancerous colorectal lesions were obtained during colonoscopies carried out at the Second Department of Internal Medicine, Third Faculty of Medicine, Charles University in Prague. Collection of each polyp was accompanied by two biopsies of normal mucosa from the rectosigmoideum region. Histopathology of the lesions was evaluated on hematoxylin and eosin stained sections by two pathologists (a summary is given in Supplemental Table S3). Tissue samples were homogenized in RNA Blue reagent (TopBio, Czech Republic) using a T8 Ultra Turrax disperser (IKA). Total RNAs were extracted according to the manufacturer's protocol and subjected to RNA integrity number (RIN) analysis (Agilent 2100 Bioanalyzer; samples with RIN > 8 were used). Reverse transcription was performed using the RevertAid H Minus reverse transcriptase (Fermentas) with1.5μg of total RNA using random hexanucleotide primers (Invitrogen). Negative controls were represented by input RNA processed in the absence of reverse transcriptase. PCR reactions were run in parallel triplicates for each primer set in Light Cycler 480 (Roche Applied Science). Two housekeeping genes, *UBIQUITIN B* (*UBB*) and *β-ACTIN* were used as internal controls. Primer sequences are listed in Supplemental Table S1.

The APC Mutation Analysis

Genomic DNA was extracted from deep-frozen samples of tumor tissue and healthy surrounding mucosa using the DNeasy Blood & Tissue Kit (Qiagen). Seven PCR fragments partially covering the sequence of human *APC* exon 18 and two fragments corresponding to *β-CATENIN* (*CTNNB1)* exons 3 and 4, including intron-exon boundaries, were amplified using gene-specific primers (Supplemental Table S4) containing overhangs recognized by T7 (5'-TAATACGACTCACTATAG-3') and RP (5'-TGAAACAGCTATGACCATG-3') universal sequencing primers. PCR was performed in 38 cycles with 30s primer annealing at

60°C and 45s extension at 71°C. Each fragment was directly sequenced from both sides using the BigDye® Terminator v3.1 cycle sequencing kit and ABI 3130 Genetic Analyzer (Applied Biosystems).

Statistical analysis of RT-qPCR Data

RNA isolated from each specimen (biological replicate) of tumor or healthy mucosa was subjected to the RT-qPCR analysis; the PCR reactions were performed in triplicates. In each technical replicate, Cp's were normalized using *β-ACTIN* as a reference gene to obtain ΔCp's and averaged. Principal component analysis (PCA) on ΔCp's of biological replicates identified one outlying patient. All samples of this patient were removed and the remaining 76 samples of 19 patients were used in downstream statistical analyses. To detect differences in expression, we applied Wilcoxon's rank-sum test. To account for different genetic background of the patients, we further fitted a linear model (REML) described by the formula ΔCp ~ patient + tissue/(stage * mutation), with biological replicates treated by a random effect and all healthy mucosa samples treated as a single group and validated the results. No correction of multiple testing was applied. Statistical significance was estimated at the level of 0.05 (or 0.01 and 0.001) and marked by asterisk (or ** and ***) in Supplemental Figure S2. All analyses were performed in R language/environment.

Statistical Analysis of RT-qPCR Data of Precancerous Lesions

mRNA obtained from 35 samples of precancerous lesions of the colon and matching healthy mucosa was subjected to RT-qPCR analysis. Cp´s of individual technical triplicates were normalized by geometric average of internal control genes β-*ACTIN*, *AXIN1* and *UBB* and averaged (ΔCp). PCA revealed that specimens of one patient were deemed outlying and were consequently omitted from further statistical analyses. Differential expression was evaluated as described for colorectal cancor samples.

Bisulfite Analysis of Cytosine Methylation

Genomic DNA (0.5 μg) isolated from tumor center and matched healthy surrounding mucosa was subjected to bisulfite conversion using the EZ DNA Methylation™ Kit (Zymo Research). Bisulfite-treated DNA was amplified by nested PCR using the following primers: for primary amplification oligonucleotides 5´-ATTTTATTGGTGGAAAAGTATTTTAT-3´ [forward; priming on nucleotides (nt) -672 to -647 upstream from the *TROY* genes transcription start (according to sequence NM_001204458.1)], and 5´-AACTTACTCTACAACTAAATTCTT

TAA-3´ (reverse; nt -163 to -137). The PCR product was re-amplified using primers 5´-TTTTTTTAGTAAAATTGTTTAGTGAGTTTT-3´ (forward; nt -629 to -600) and 5´-AATAAATATAAAACTAACTAAAAACTTAAAAATA-3´ (reverse; nt -225 to -193). Cytosines (not included in CpG dinucleotides) were changed to T or A in forward and reverse primers, respectively. Amplification products were cloned into the pGEM-T-Easy vector (Promega) and sequenced. Nine to twelve independent PCR clones with at least 95% conversion of cytosine outside of CpG dinucleotides were analyzed.

Plasmids and Constructs

Proteins with a C-terminal FLAG or MYC tag were produced from pCMV-FLAG/MYC vector (Sigma). Fluorescently tagged proteins were generated in pECFP-N, pEGFP-N, pEYFP-N (Clontech) and pTaqBFP (Evrogen) vectors. The construct encoding the N-terminally truncated variant of human LRP6 protein (LRP6ΔN) (3) in the pCS2 vector was kindly provided by V. Bryja.

Coimmunoprecipitations and Western Blotting

The following antibodies were used: mouse anti-β-catenin, (clone E5; Santa Cruz); rabbit anti non-phospho-β-catenin/S33/S37/T41 (polyclonal; Santa Cruz); rabbit anti-Dvl2 (30D2; Cell Signaling); mouse anti-FLAG (M2; Sigma); mouse anti-GFP (JL-8; Clontech); goat anti-GFP (polyclonal; a gift of D. Stanek); mouse anti-MYC (9E10; Roche); rabbit anti-MYC (71D10; Cell Signaling); rabbit anti-LRP6 (C5C7; Cell Signaling); rabbit anti-phospho-LRP6/Ser1490 (polyclonal; Cell Signaling); rabbit anti-phospho-LRP6/T1572 (polyclonal; Millipore); mouse anti-TROY (ab55043, Abcam); mouse anti-α-tubulin (TU-01; Exbio CZ).

Rspo Binding Assay

Human U2OS or HeLa cells were transiently co-transfected with a combination of LGR5-EYFP and TROY-TagBFP or with LGR5-EYFP and “empty” pTagBFP vector. The next day, cells were incubated (1 hour, 37°C) with conditioned medium containing recombinant mouse Rspo1 containing the constant region of the mouse immunoglobulin G2a heavy chain (IgG2a) (4). In control staining, the cells were incubated with recombinant IgG2a alone. Conditioned media were harvested from HEK293 cells transiently transfected with constructs encoding mouse Rspo1-IgG2a or IgG2a proteins (the constructs were kindly provided by V. Bryja; media were harvested 3 days upon transfection). The presence of recombinant proteins in culture media was verified by Western blotting using the goat anti mouse secondary

antibodies (BIO-RAD). Cells were washed twice with PBS and fixed for 10 min in 4% (w/v) paraformaldehyde in PBS. Rspo1-IgG2a or IgG2a retained on the cell surface was visualized by a goat anti-mouse IgG2a Alexa Fluor 594 secondary antibody (Life Sciences; 1:900 dilution, 1 hour at room temperature). Nuclei were counterstained with Draq5 stain (Biostatus Limited).

FRET

HeLa cells were transfected with the corresponding constructs. Twenty-four hours after transfection the cells were fixed in 4% (w/v) paraformaldehyde/PIPES buffer (10 min, at room temperature). After rinsing with PBS and distilled water, cells were embedded in glycerol containing DABCO (Sigma). Intensities of ECFP (excited by 405 nm laser set to 5-10% of maximum power) and EYFP (excited by 514 nm laser line set to 2-10% of maximum power) fluorescence were recorded. Subsequently, EYFP was bleached in the region of interest by four intensive (80% maximum power) pulses of 514 nm laser line and ECFP- and EYFP-emitted fluorescence was measured again. The apparent FRET efficiency was calculated according to the equation: FRET efficiency [%] = (ECFP after -ECFP before) × 100/ECFP after. Unbleached regions of the same cell were used as negative control.

Generation of Stable Cell Lines

To obtain cells with inducible expression of human TROY, the Lenti-X Tet-On Advanced Inducible Expression System (Clontech) was used. HEK293 cells were transduced with recombinant lentiviruses producing the tetracycline-controlled transcription activator (rtTA) and selected without subcloning using G418 (Enzo Life Sciences; 0.5 mg/ml). Resistant cells were further transduced with lentivirus pLVX-Tight-Puro encoding full-length human *TROY* with the C-terminal FLAG tag. HEK293/TROY cells were selected and maintained in puromycin (Alexis; 2 μg/ml) and G418 (0.2 mg/ml).

Reporter Gene Assays

To test the effect of TROY on the Wnt-dependent transcription, TROY expression was induced in HEK293/TROY by doxycycline (Sigma; 1μg/ml). The cells were stimulated simultaneously with doxycycline, Wnt3a and/or human Rspo1 and analyzed 18 hours later. Reporter gene assays in siRNA-treated HEK 293 cells were performed in an analogous manner. To ensure effective downregulation of TROY, two days after the first transfection with TROY or control non-silencing siRNA the cells were lipofected with the same siRNA

together with pTOPFLASH/pFOPFLASH and *Renilla* luciferase expression plasmid. Twenty-four hours upon the second transfection, Wnt3a and Rspo1 ligands were added to culture medium (separately or in combination). The cells were stimulated for an additional 18 hours, then harvested and analyzed.

Paneth Cells Sorting

Single cells from crypts were obtained as in reference (5). Cells were stained with phycoerythrin (PE)-conjugated anti-CD24 antibody (eBioscience), allophycocyanin (APC)-conjugated anti-Epcam antibody (eBioscience) and FITC-conjugated anti-CD45 antibody (ExBio) for 30 min at 4°C, and sorted by Influx cell sorter (BD Biosciences).

Organoid Proliferation Assay

Cell proliferation was visualized by a Click-iT™ EdU Imaging Kit (10 μM EdU incorporation for 1 hour at 37°C) according to manufacturer's instructions (Invitrogen). Organoids were further stained with an anti-E-cadherin antibody (Zymed) and DAPI and imaged using a Leica Sp5 confocal microscope. The proliferation rate was determined by counting of EdU-positive vs. DAPI-positive cells; at least 10 organoids were evaluated per each genotype and Rspo1 concentration. The organoids prepared from 3 mice of corresponding genotype were grown in quadruplicates for each concentration of Rspo1. Statistical significance was estimated by the Student's t-test.

7.8.2 Supplemental Results

TROY expression profiling in sporadic colorectal carcinoma and premalignant lesions

Two biological replicates of both tumor and healthy tissue were obtained from each individual and used for sequence analysis of the *APC* gene. The analyses encompassed the somatic mutation cluster region of *APC* as well as flanking regulatory sequences encoding elements required for the β-CATENIN interaction and downregulation [nucleotides 1959 to 4945 (numbering was taken from the sequence NM_000038.5)] (Miyoshi et al. 1992). Putatively inactivating mutations of *APC* were found in 11 out of 20 patients (55%), which is in concordance with published data (Kolligs et al. 2002). Of note, since the mutational analysis was covering only a "hot spot" of the *APC* gene, we cannot exclude that not all mutations in the *APC* sequence have been detected. No oncogenic mutations were detected in

the somatic hotspot regions of exons 3 and 4 of *β-CATENIN* (summary of the *APC* mutations can be found in Supplemental Table S5).

To analyze differences in the expression of *AXIN2*, *LGR5* and *TROY* genes in tumor tissues versus healthy mucosa, we applied non-parametric Wilcoxon's test. In addition, to account for histopathological variations in specimens obtained from different patients we used a linear model (REML). Both statistical analyses clearly showed significant correlation between the presence of the inactivating *APC* mutations and expression levels of the *AXIN2* gene (Supplemental Figure S2*A*; p- and other values are indicated in Supplemental Table S6). This result was in agreement with previously published data (Yan et al. 2009). On the other hand, *LGR5* mRNA was increased in tumor tissues irrespective of the *APC* status. The *TROY* expression levels did not follow any conclusive expression pattern, moreover we detected a decrease in *TROY* expression in the majority of tumors. To study a molecular mechanism of possible *TROY* silencing in tumor tissue, genomic DNA isolated from specimens with elevated and decreased *TROY* expression was subjected to the cytosine methylation analysis. The analysis of the sequence containing 35 CpG doublets located in the proximity of the *TROY* transcription start (see Supplemental Methods for details) in two carcinomas with reduced *TROY* expression revealed that only 2 and 14% of analyzed CpG dinucleotides were methylated (Supplemental Fig. S2*B*). This implied that the observed reduction in *TROY* expression is not related to changes in the DNA methylation status.

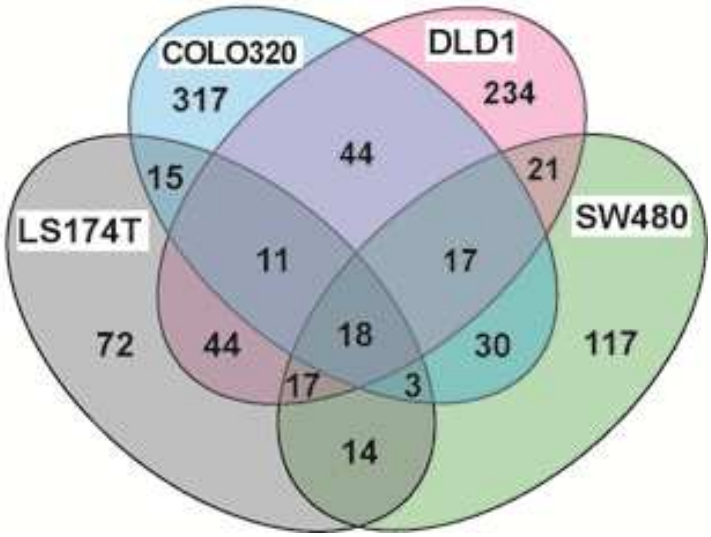
In addition, the analysis of *TROY* expression in thirty-five premalignant lesions of the colon was performed. In polyps, *AXIN2* and *LGR5* displayed a significant increase in mRNA levels (compared to healthy mucosa tissue). Expression of both genes strongly correlated with the grade of the neoplastic tissues showing elevated expression in more progressed lesions. These results were in good concordance with published data (9). In contrast, *TROY* mRNA was decreased in the majority of premalignant lesions irrespective of their histological category (Supplemental Figure S3 and Supplemental Table S7).

7.8.3 Supplemental Acknowledgments

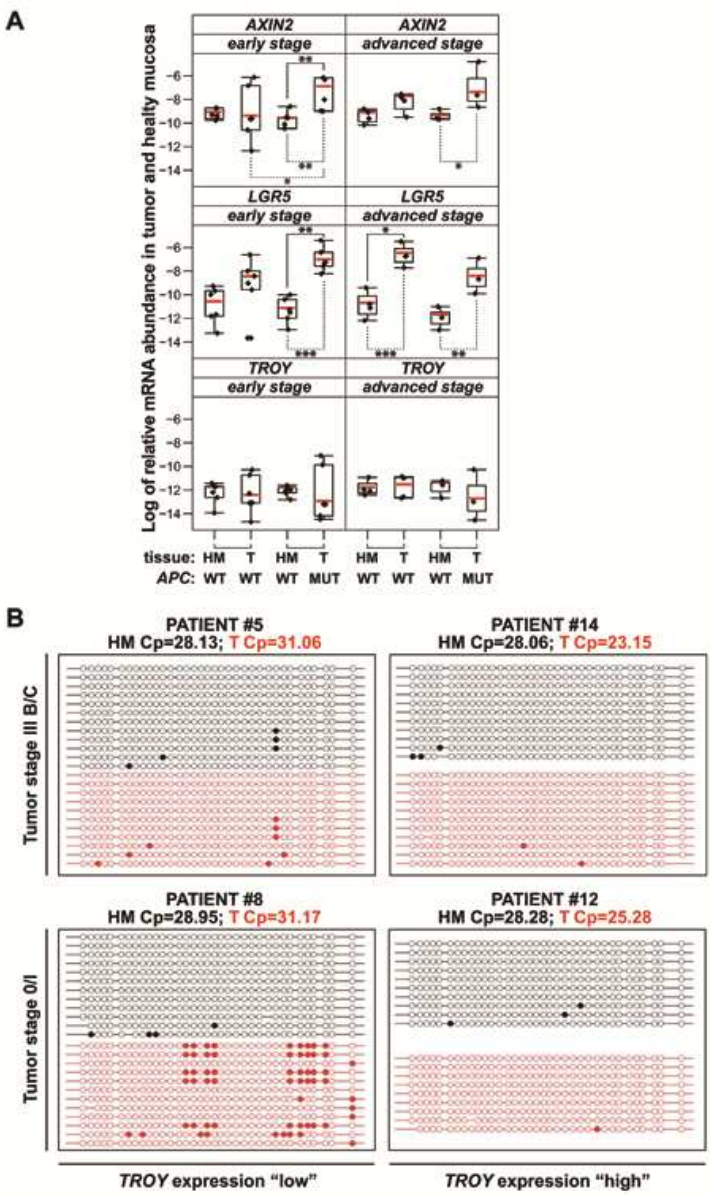
We thank J. Dobes and J. Tureckova for help with sorting and RT-qPCR analysis of Paneth cells. We also thank O. Sebesta for help with microscopy.

7.8.4 Supplemental Figures and Tables

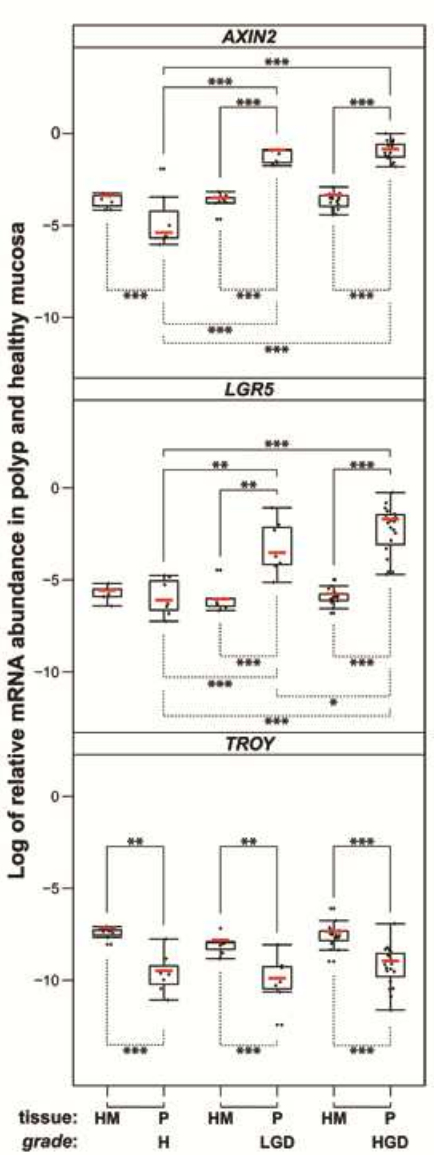
Supplemental Figure S1. Summary of the ChIP-on-chip analysis. Venn diagram depicts the numbers of promoter regions bound by TCF4 in each cell line (the selection criterion is defined in the Table 1 legend). Of 960 genes identified, 18 genes scored positive in all four cell lines.



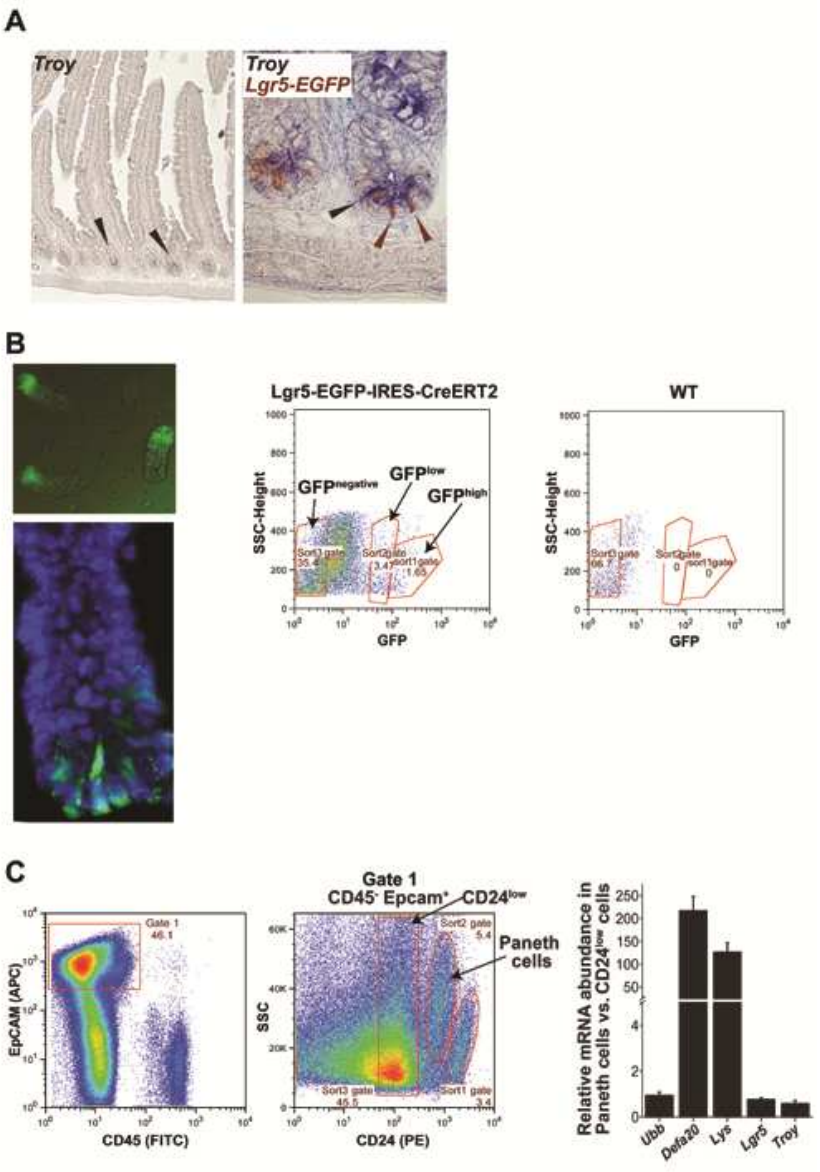
Supplemental Figure S2. *TROY* expression does not correlate with the *APC* status in human colorectal cancer. (*A*) Comparison of relative expression levels of *AXIN2*, *LGR5* and *TROY* genes in sporadic colorectal tumors (T) compared to surrounding healthy mucosa (HM) samples. Individual Cp values were normalized to the level of the housekeeping gene *β-ACTIN* to obtain a log of relative expression levels (-ΔCp) and averaged. Tumors were assigned to four distinct subgroups with respect to the *APC* mutation status (WT, wild-type *APC*; MUT, *APC* mutated) and disease stage. Stage grouping: the "early stage" encompasses tumors classified 0 to IIC according to the 7th Edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual. Stages of advanced colorectal disease (IIIA-IV) constitute the subgroup designated as the "advanced stage". Median of ΔCp values for each respective subgroup is indicated by the red line. The differences in the expression levels of a selected gene in the given groups and their statistical significance were tested by Wilcoxon's rank-sum test (solid line) and a linear model (REML, dashed line), which accounted for tissue type, *APC* status, stage and biological background of the patients. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Data accompanying this figure can be found in Supplemental Table S6. (*B*) Cytosine methylation analysis of the CpG island located upstream of the *TROY* promoter. The analysis was performed on genomic DNA isolated from tumors with "high" (normalized Cp<25.5) or "low" (Cp> 31) *TROY* expression. Both early and advanced stage tumors were assayed as indicated. Open circles mark unmethylated CpG dinucleotides, while closed circles indicate methylated CpGs. Tumor samples (T) are depicted in red, matched healthy surrounding mucosa (HM) in black.



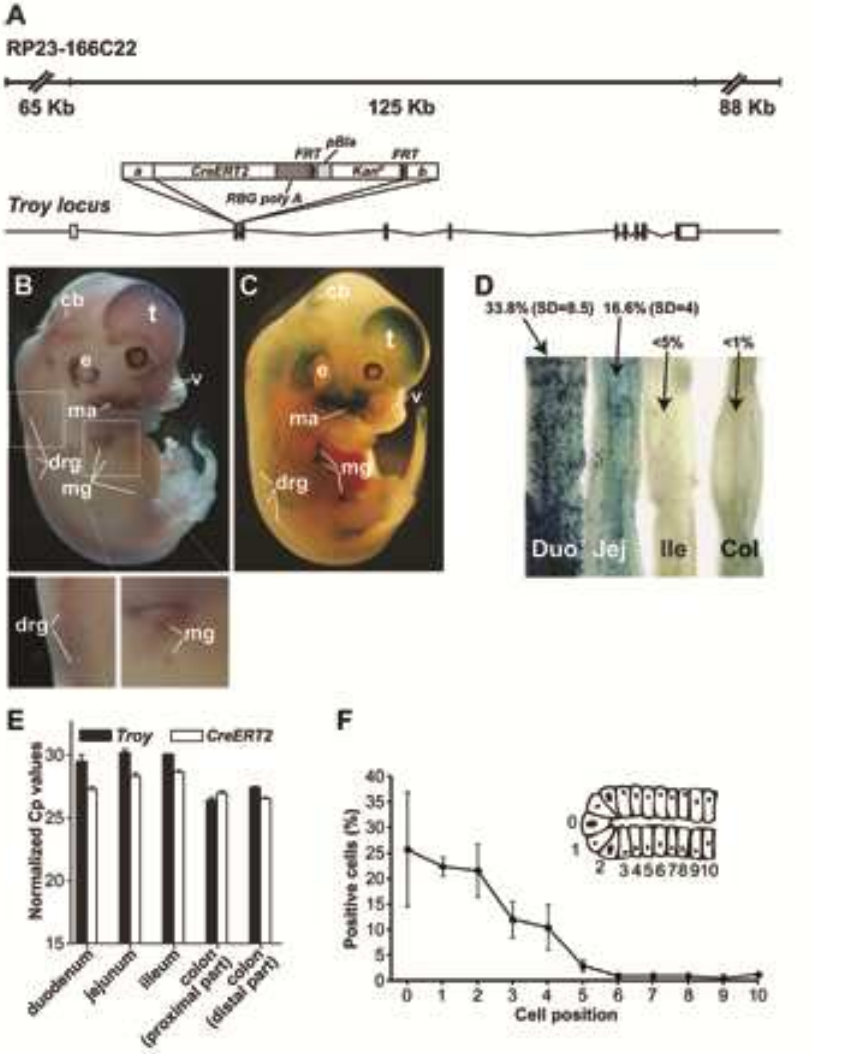
Supplemental Figure S3. *TROY* expression is decreased in precancerous lesions of the human colon. Comparison of relative expression levels of *AXIN2*, *LGR5* and *TROY* genes in sporadic premalignant lesions (P) of human colon and matching healthy mucosa (HM) samples. Based on prevailing microscopic appearance, lesions were subdivided to three histological categories: hyperplasia (H), low grade dysplasia (LGD) and high grade dysplasia (HGD). Median of ΔCp values in individual subgroups is indicated by the red line. Statistical significance of differences in expression levels was examined by nonparametric Wilcoxon´s rank-sum test (solid line). Moreover, a linear model (REML; dashed line) encompassing tissue type, histology and individual biological background of the patients was applied. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Data accompanying this figure can be found in Supplemental Table S7.



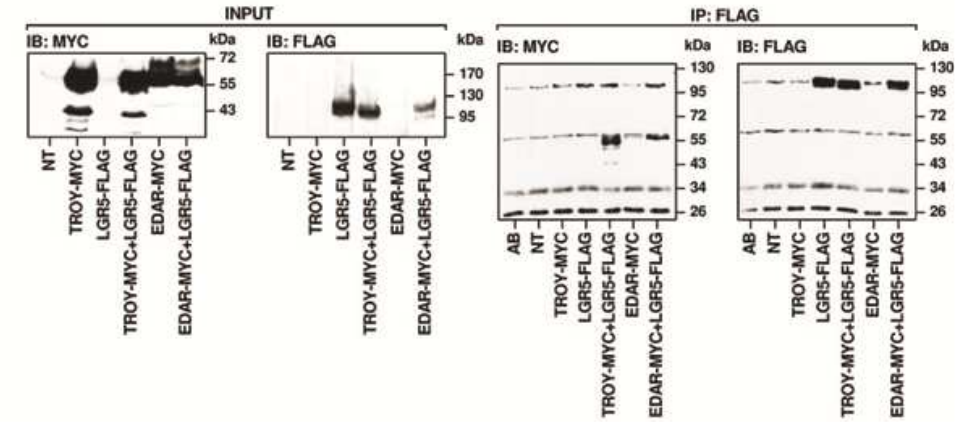
Supplemental Figure S4. Analysis of Troy expression in the small intestine using ISH and fluorescence-activated cell sorting (FACS). (*A*) Left, ISH of *Troy* mRNAs in jejunum showing expression in the lower parts of the crypts (black arrowheads). Right, colocalization of Troy-expressing and CBC cells in the intestine. Frozen sections prepared from Lgr5-EGFP-IRES-CreERT2 mice were stained using ISH for *Troy* mRNA (black arrowhead). The slides were mounted in glycerol and the results recorded. Next, the cover slips were removed and the CBC cells were visualized in the same specimen using IHC detection of GFP protein (brown arrowheads). (*B*) FACS of Lgr^{+} cells. Left, fresh isolates of the crypts from the small intestine of Lgr5-EGFP-IRES-CreERT2 mice. A detailed confocal image of one crypt stained with DAPI nuclear stain is shown in bottom panel. The putative CBC stem cells located at the base of each crypt are marked by EGFP expression. Right, diagrams of the cell-sorting procedure. Three cell populations (GFP^{high}, GFP^{low}, $GFP^{negative}$) were obtained from the crypts of Lgr5-EGFP-IRES-CreERT2 mice. GFP^{high} and GFP^{low} cells, which are not present in the intestine of control wild-type (WT) mice, were used for the subsequent RT-qPCR analysis. The crypt isolations were performed as described in the reference (Yamada et al. 2009). (*C*) Cell sorting of Paneth cells. Left, FACS plots of dissociated single cells from small intestinal crypts of wild-type mice. Viable single cells were gated by forward scatter, side scatter, pulse-width parameter and negative staining for Hoechst 33258. Epithelial cells (CD45-Epcam+) were sorted in three gates. Two CD24 bright populations differed by side-scatter (SSC) pattern. Sorted CD24highSSClow population (Gate 1) corresponds to enteroendocrine cells and CD24highSSChigh cells are Paneth cells (Sort 2 gate). Right, RT-qPCR from sorted cells, Sort 2 vs. Sort 3 gate (CD24low) illustrates that *Troy* (and *Lgr5*), unlike the Paneth cell markers *Lysozyme (Lys)* and *Defensin α 20 (Defa20)*, is not enriched in Paneth cells.



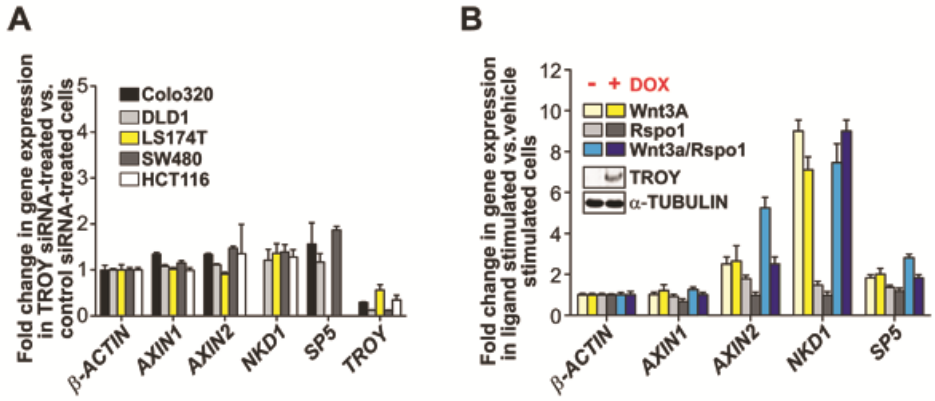
Supplemental Figure S5. Tracking the Troy expression using the *Troy-CreERT2* BAC transgenic allele. (*A*) CreERT2 was inserted in frame into the first coding exon of the mouse *Troy* gene. The non-coding or translated regions are depicted by empty and black boxes, respectively. *a,b*, Troy sequences employed for the homologous recombination into the BAC clone RP23-166C22. *RGB poly A*, a poly A signal derived from the rabbit *β-globulin* gene; *Kan*^{r}, the *kanamycin* resistance gene; *pBla*, *β-lactamase* promoter. Prior to pronuclear injection, the bacterial resistance cassette was excised from recombinant BAC using Flp recombinase and FRT sites (black semicircles) flanking the cassette. (*B*) The whole-mount hybridization of wild-type mouse embryo at embryonic day (E) 14.5 using an antisense probe against *Troy*. (*C*) The activity of CreERT2 recombinase produced from the Troy-CreERT2 BAC transgene was visualized at E 14.5 in embryos derived from crossing of Troy-CreERT2 and Rosa26R mice. Tamoxifen was injected intraperitoneally into pregnant females. The animals were sacrificed one day later, embryos were removed and used to stain the LacZ-expressing tissues. The CreERT2 expression pattern visualized by X-gal substrate phenocopies the sites of expression of endogenous *Troy* mRNA and its localization is also in concordance with the *Troy* expression pattern described previously (11, 12). cb, cerebral plate; drg, dorsal root ganglion; e, ear; ma, maxilla; mg, mammary gland primordium; v, vibrissae. (*D*) Frequency of Cre-mediated recombination in different anatomical parts of the intestine of Troy-CreERT2/Rosa26R mice. Three mice were injected with tamoxifen and sacrificed 60 days later. Percentage of positive (i.e. blue) crypts in duodenum (Duo), jejunum (Jej), ileum (Ile) and colon (Col) is indicated with given SD in parentheses. Notice that the image was taken from Figure3*B*. (*E*) RT-qPCR profiling of the *CreERT2* and *Troy* genes in intestinal mucosa dissected from different parts of the gastrointestinal system. The tissues were obtained from four Troy-CreERT2 transgenic mice or from their wild-type littermates (n=4). (*F*) Localization of LacZ-positive cells in the crypts of Troy-CreERT2/Rosa26R mice 1 day after tamoxifen administration. The diagram depicts frequency of occurrence of blue cells at specific positions in the crypt; the positions are indicated in the scheme in the inset. Four hundred crypts from the proximal part of the small intestine of two mice were counted. Results are depicted as means with SDs. Most of the LacZ-expressing cells occurred in the bottom part of the crypt, whereas less than 6% of these cells were observed at the +5 position or higher.



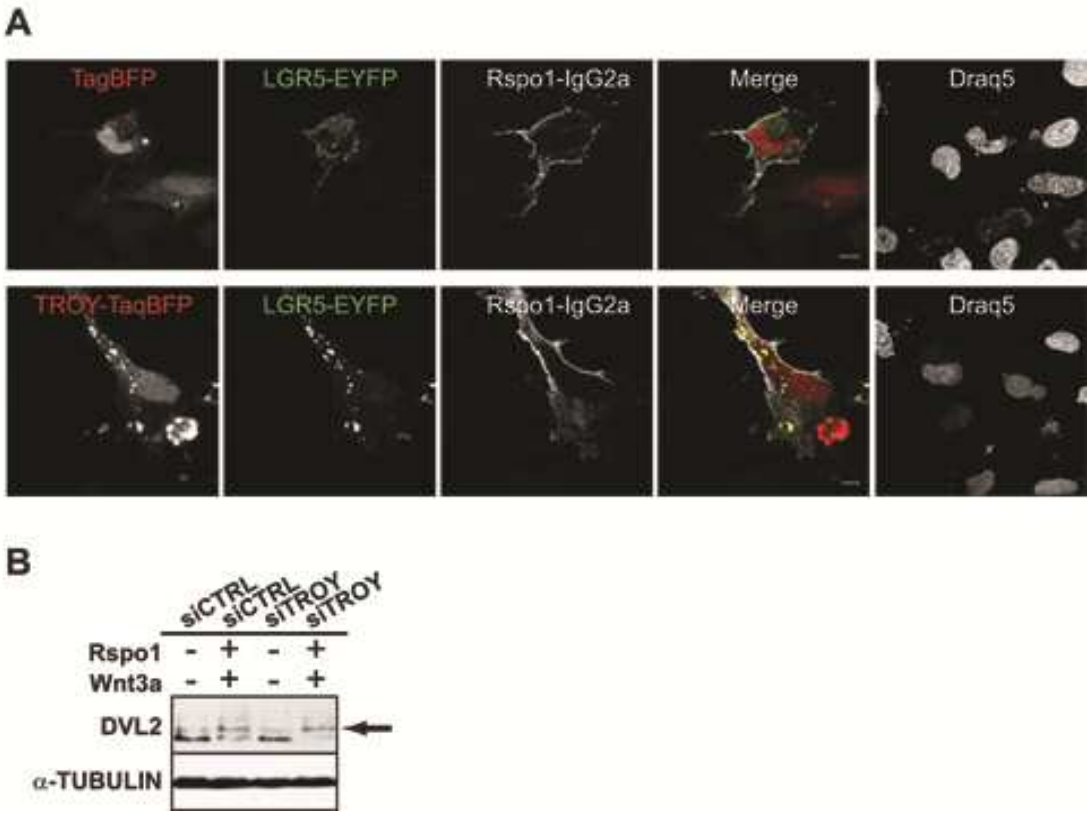
Supplemental Figure S6. LGR5 interacts with the TROY-related receptor EDAR. Co-immunoprecipitation of MYC-tagged EDAR or MYC-tagged TROY with LGR5-FLAG. In panels indicated as "INPUT", 20% of the total lysate used for one immunoprecipitation was loaded. AB, the precipitation was performed without cell lysates; NT, lysate from non-transfected cells was precipitated; IP, immunoprecipitation; IB, immunoblotting.



Supplemental Figure S7. (*A*) Downregulation of TROY does not potentiate TCF/β-catenin-mediated transcription in human CRC cells. Results of the RT-qPCR analysis of indicated genes in TROY siRNA-treated vs. control siRNA-treated cells. The Wnt/β-catenin non-responsive gene *AXIN1* was also included in the test. Cp values were normalized to the β-*ACTIN* expression levels (Cp=16.5). The histogram represents mean Cp values of a representative experiment performed in triplicates (repeated twice). The expression level of a given gene in cells transfected with control non-silencing siRNA and treated with vehicle was arbitrarily set to 1. Missing bars stand for unexpressed gene (normalized Cp>35). (*B*) The effect of ectopic TROY was tested in HEK293/TROY cells with doxycyclineinducible expression of TROY transgene. RT-qPCR analysis of cells grown with or without doxycycline (DOX) and stimulated with Wnt3a and/or Rspo1 ligands.



Supplemental Figure S8. The most proximal events triggered by Wnt or Rspo ligand are not influenced by TROY. (*A*) TROY does not interfere with binding of Rspo1 to LGR5. Laser scanning confocal microscopy images of U2OS cells transfected with equimolar amounts of constructs expressing LGR5-EYFP (green) and TagBFP (pseudocolored in red; upper panels) or LGR5-EYFP and TROY-TagBFP (lower panels). Cells grown on cover slips were incubated with conditioned medium containing Rspo1-IgG2a fusion protein. The cell surface binding of Rspo1-IgG2a to its receptor LGR5 was detected using Alexa Fluor 594 anti-IgG2a antibody (gray). Recombinant IgG2a alone did not display any nonspecific binding to the cells (not shown). Nuclear counterstain Draq5 (gray) is omitted from the overlay images for clarity. Original magnification: 1000×. (*B*) Dvl phosphorylation is not affected by *TROY* knockdown. The western blot of whole-cell extracts prepared from HEK293 cells treated with non-silencing or TROY siRNA and further stimulated as indicated. The putative phosphorylated form of Dvl with changed mobility in the gel is marked by arrow.



Supplemental Table S1. Sequences of primers used for ChIP and RT-qPCR analyses.

Gene	Organism	Sequence	Application
β-ACTIN	Human	GGCATCCTCACCCTGAAGTA AGGTGTGGTGCCAGATTTTC	qRT PCR/ChIP
β-Actin	Mouse	GATCTGGCACCACACCTTCT GGGGTGTTGAAGGTCTCAAA	qRT PCR
β-catenin	Human	TTCCAGACACGCTATCATGC AATCCACTGGTGAACCAAGC	qRT PCR
AXIN1	Human	GAAGGTGAGGACGGCGATCCAT AGGCACCTGGCACCTCGGTGC	qRT PCR
Axin1	Mouse	ACCCAGTACCACAGAGGACG CTGCTTCCTCAACCCAGAAG	qRT PCR
AXIN2	Human	CTGGCTTTGGTGAACTGTTG AGTTGCTCACAGCCAAGACA	qRT PCR
Axin2	Mouse	GGGGGAAAACACAGCTTACA TCTTCATTCAAGGTGGGGAG	qRT PCR
Bmi1	Mouse	TGTGTCCTGTGTGGAGGGTA TTGAAAAGCCCTGGGACTAA	qRT PCR
Cre	P1 phage	GCACTGATTTCGACCAGGTT GCTAACCAGCGTTTTCGTTC	qRT PCR/genotyping
c-MYC	Human	CTCCTGGCAAAAGGTCAGAG TCGGTTGTTGCTGATCTGTC	ChIP
Defensin α 20	Mouse	TGGGACCTGCTCAGGACGACT TCATCTGCATGTTCAGTGGCGG	qRT PCR
LGR5	Human	CTCTTCCTCAAACCGTCTGC GATCGGAGGCTAAGCAACTG	qRT PCR
LGR5	Human	GTGAAGGAAAAGGGTGTCCA ATGTGCCTTCCTTCATGTCC	ChIP
Lgr5	Mouse	CCTGTCCAGGCTTTCAGAAG CTGTGGAGTCCATCAAAGCA	qRT PCR
Lysozyme	Mouse	CCTGACTCTGGGACTCCTCCTGCT CTAAACACACCCAGTCGGCCAGGC	qRT PCR
NKD1	Human	CGCCGGGATAGAAAACTACA CTGGAGCTCTGAGACCTTGG	qRT PCR
NKD1	Human	GACCTCCCCAGACAAAACAA TCAGCCAGTCTCTGGGATCT	ChIP
Nkd1	Mouse	AGGACGACTTCCCCCTAGAA TGCAGCAAGCTGGTAATGTC	qRT PCR
SP5	Human	ACTTTGCGCAGTACCAGAGC ACGTCTTCCCGTACACCTTG	qRT PCR
SP5	Human	TCCAGACCAACAAACACACC GCTTCAGGATCACCTCCAAG	ChIP
Sp5	Mouse	ACTCACTGCAGGCCTTCCT TCCAAGGGTGGAAAAGTCTG	qRT PCR
TROY	Human	CTATGGGGAGGATGCACAGT TCTCCACAAGGCACACACTC	qRT PCR
TROY	Human	TTTCATCTCCCTGCTCGTCT TGCGAAAAATGCAGTGAAAG	ChIP
Troy	Mouse	GCTCAGGATGCTCAAAGGAC CCAGACACCAAGACTGCTCA	qRT PCR
Troy locus	Mouse	TTGCATGCTGTGCAGAGACG CACCTCAGTGTGGCATCGAC	qRT PCR/genotyping
UBB	Human	GCTTTGTTGGGTGAGCTTGT TCACGAAGATCTGCATTTTGA	qRT PCR
Ubb	Mouse	ATGTGAAGGCCAAGATCCAG TAATAGCCACCCCTCAGACG	qRT PCR

The sequence of the forward primer is given in the upper line with the corresponding reverse primer in the lower line, respectively.

Supplemental Table S2. Clinical and histopathological features of colorectal cancer patients.

Clinical and histopathological features of colorectal cancer patients (n=20)	
	Total N (%)
No. of patients	20
Primary tumor duplicity	1 (5)
Total No. of tumors included in the study	21
Gender	
Male/Female	17/3
Age, Years	
median	64
range	45-86
≥ 65 years	9 (45)
Primary tumor location	
left-sided	10 (50)
right-sided	10 (50)
Metastatic disease	
synchronous	8 (40)
Location of metastasis	
liver	5 (25)
lymph nodes	8 (25)
other	1 (5)
Histological differentiation level	
G1	2 (10)
G2-3	19 (90)

Tumors were classified according to the 7th edition of AJCC Cancer Staging Manual.

Supplemental Table S3. Clinical and histopathological characteristics of patients with precancerous lesions of the colon.

Clinical and histopathological features of colorectal cancer patients (*n=20*)	
	Total N (%)
Total No. of lesions included in the study	35
Gender	
Male/Female	35/0
Age, Years	
median	66
range	38-83
≥ 65 years	21 (60)
Colon segment involved	
left-sided	29 (83)
right-sided	6 (17)
Histological examination	
Hyperplasia (H)	7 (20)
Low grade dysplasia (LGD)	12 (34)
High grade dysplasia (HGD)	16 (46)

Polyps were classified according to the Vienna Classification of gastrointestinal epithelial neoplasia (13).

Supplemental Table S4. Sequences of primers utilized to amplify selected *APC* and *β-CATENIN* exons.

Gene-specific primers			
Primer ID	***Primer sequence***	***Product size (nucleotides)***	***Amplified region***
APCex18/1T7	taatacgactcactatagTGTTACTGCATACACATTGTGACC	567	APC, exon 18 c.1959-55bp[2] – c.2433[1]
APCex18/1RP	tgaaacagctatgaccatgTGACCTATTATCATCATGTCGATTG		
APCex18/2T7	taatacgactcactatagTCCCAAGGCATCTCATCGTAG	567	APC, exon 18 c.2340 – c.2869[1]
APCex18/2RP	tgaaacagctatgaccatgTGTATTCTAATTTGGCATAAGGCATAG		
APCex18/3T7	taatacgactcactatagTGCCCATACACATTCAAACAC	552	APC, exon 18 c.2781 – c.3295[1]
APCex18/3RP	tgaaacagctatgaccatgCACATTCCTGCTGTCCAAAATG		
APCex18/4T7	taatacgactcactatagAAAGTGAGCAAAGACAATCAAGG	589	APC, exon 18 c.3185 – c.3736[1]
APCex18/4RP	tgaaacagctatgaccatgCCTTTTGAGGCTGACCACTTC		
APCex18/5T7	taatacgactcactatagCCGAACATATGTCTTCAAGCAGTG	545	APC, exon 18 c.3623 - c.4130[1]
APCex18/5RP	tgaaacagctatgaccatgACATAGTGTTCAGGTGGACTTTTGG		
APCex18/6T7	taatacgactcactatagATCAGCCAGGCACAAAGC	548	APC, exon 18 c.4035 - c.4545[1]
APCex18/6RP	tgaaacagctatgaccatgAAATGGCTCATCGAGGCTCAG		
APCex18/7T7	taatacgactcactatagAGAGGGTCCAGGTTCTTCCAG	552	APC, exon 18 c.4430 - c.4944[1]
APCex18/7RP	tgaaacagctatgaccatgAGGTGTCCCTTCAACACAATAC		
CTNNB1ex3T7	taatacgactcactatagTGCTTTTCTTGGCTGTCTTTCAG	571	CTNNB1, exon 3 including intron-exon boundaries; c.1-134bp[3] – c.241+172bp[4]
CTNNB1ex3RP	tgaaacagctatgaccatgTCCACAGTTCAGCATTTACCTAAG		
CTNNB1ex4T7	taatacgactcactatagTTGTGGTGAAGAAAAGAGAGTAATAGC	486	CTNNB1, exon 4 including intron-exon boundaries; c.242-111bp[5] – c.495+84bp[6]
CTNNB1ex4RP	tgaaacagctatgaccatgTGGTATTGGGTAGACATTCTGAAAC		

The sequence-specific portion of the primer is highlighted in capital letters; the T7 and RP adaptor primers are in small caps. [1)] Exon and nucleotide numbering according to NM_000038.5; [2)] primer sequence situated in intron 55 bps upstream of exon 18; [3)] primer sequence situated in intron 134 bps upstream of exon 3; [4)] primer sequence situated in intron 172 bps downstream of exon 3; [5)] primer sequence situated in intron 111 bps upstream of exon 4; [6)] primer sequence situated in intron 84 bps downstream of exon 4.

Supplemental Table S5. Summary of detected *APC* mutations in samples of sporadic human colorectal carcinomas.

Mutation analysis of *APC* in human sporadic colorectal carcinomas							
Stage	***Patient No.***	***Mutation in genomic DNA[1]***	***Change in APC protein***	***Mutation type***	***Mutation in genomic DNA[1]***	***Change in APC protein***	***Mutation type***
0	8	c.4666_4667insA	p.T1556fs*3	Insertion - Frameshift	c.3095C>T	p. S1032L[2]	Substitution - Missense
	10	c.2956_2963delTATTCTGA	p.Y986fs*2	Deletion - Frameshift	c.4474_4475insT	p.A1492fs*22	Insertion - Frameshift
I	12	c.3183_3187delACAAA	p.Q1062fs*1	Deletion - Frameshift	c.4326T>A	p.Pro1442=	Substitution - Silent
	15	c.3910delA	p.I1303fs*1	Deletion - Frameshift	c.4479 A/A (n.a.)[4]	---	---
	20	c.4666_4667insA	p.T1556fs*3	Insertion - Frameshift	c.4479 G/A (wt)[4]	---	---
II	1	c.4926T>A	p.Y1642*	Substitution - Nonsense	c.4479 G/A>A/A (LOH)[4]	---	---
	6	c.3095C>T	p. S1032L[2]	Substitution - Missense	c.4479 A/A (n.a.)[4]	---	---
III	2	n.d.[5]	---	---	c.4479 G/A>A/A (LOH)[4,5]	---	---
	5	c.3340C>T	p. R1114*	Substitution - Nonsense	c.3961A>G	p. S1321G[3]	Substitution - Missense
	18	n.d.[5]	---	---	c.4479 G/A>A/A (LOH)[4,5]	---	---
	14	c.4348C>T	p.R1450*	Substitution - Nonsense	c.4479 G/A (wt)[4]	---	---

The sequenced region of the *APC* exon 18 encompasses c.1959-c.4945 (NM_000038.5) including the exon 18´s 5´ intron-exon boundary. [1)] Mutation identifiers: "c" syntax indicates the type and localization of the mutation in the *APC* cDNA coding sequence (CDS; NM_000038.5); "p" syntax indicates the position and change in the protein; see the reference Forbes et al., 2010 for details (Forbes et al. 2010);[2)] according to PolyPhen prediction analysis (Ramensky et al. 2002), this substitution is scored as possibly damaging (it is supposed to affect the protein function or structure; PSIC score difference: 1.773); [3)] according to PolyPhen prediction analysis, this substitution is considered benign (no evidence for damaging effect is seen; PSIC score difference: 1.386); [4)]the presence of single nucleotide polymorphism (SNP) at *APC* c.4479 (G/A; rs41115) indicated that heterozygosity status in a tumor sample was preserved (wt; patients #20 and #14) or lost (LOH; patients #1, #2 and #18). In the absence of *APC* c.4479 polymorphism(c.4479 A/A; patients #15 and #6), the information concerning zygosity status of the *APC* locus is not available (n.a.).[5)] In cases of carcinomas that display a reduction to homozygosity at c.4479 (patients #2 and #18), the presence of the *APC* inactivating mutations is presumed although it was not detected (n.d.) in the analyzed region (see reference (Segditsas et al. 2009) for additional information). All tumors listed in Supplemental Table S5 constitute the subgroup of the APC-deficient tumors [designated as "APC mut" in downstream statistical analyses (Supplemental Figure S2 and Supplemental Table S6)].

Supplemental Table S6. Statistical analysis of tumor expression profiles (data accompanying Supplemental Figure S2).

Linear model *ΔCp ~ patient + tissue / (stage * mutation)*					**Wilcoxon's rank-sum test**	
Early stage cancer					***Early stage cancers***	
Gene	**ΔΔCp**	**FC**	**mean ΔCp**	**p-value**	**Gene**	**p-value**
APC wt tumor vs. healthy surrounding mucosa						
LGR5	-1.8	3.48	9.36	0.032	*LGR5*	0.09
TROY	0.07	0.95	11.92	0.91	*TROY*	0.82
AXIN2	-0.07	1.05	8.71	0.91	*AXIN2*	0.70
APC mut tumor vs. healthy surrounding mucosa						
LGR5	-4.33	20.05	9.36	< 0.0001	*LGR5*	0.0022
AXIN2	-2.35	5.09	8.71	0.002	*AXIN2*	0.0087
TROY	0.27	0.83	11.92	0.68	*TROY*	0.39
APC mut tumor vs. APC wt tumor						
AXIN2	-2.27	4.83	8.71	0.027	*AXIN2*	0.18
LGR5	-2.53	5.77	9.36	0.032	*LGR5*	0.041
TROY	0.19	0.87	11.92	0.83	*TROY*	0.82
Advanced stage cancer					***Advanced stage cancer***	
Gene	**ΔΔCp**	**FC**	**mean ΔCp**	**p-value**	**Gene**	**p-value**
APC wt tumor vs. healthy surrounding mucosa						
LGR5	-4.13	17.47	9.36	0.00072	*LGR5*	0.029
AXIN2	-1.17	2.26	8.71	0.22	*AXIN2*	0.11
TROY	0.26	0.83	11.92	0.77	*TROY*	0.89
APC mut tumor vs. healthy surrounding mucosa						
LGR5	-3.47	11.05	9.36	0.0049	*LGR5*	0.10
AXIN2	-2.34	5.06	8.71	0.024	*AXIN2*	0.10
TROY	0.76	0.59	11.92	0.41	*TROY*	0.70
APC mut tumor vs. APC wt tumor						
AXIN2	-1.16	2.24	8.71	0.4	*AXIN2*	0.63
LGR5	0.66	0.63	9.36	0.68	*LGR5*	0.11
TROY	0.5	0.71	11.92	0.7	*TROY*	0.63

Individual Cp values were normalized to the expression level of the *β-ACTIN* housekeeping gene to obtain relative expression levels (-ΔCp) and averaged. Biological replicates were further treated by a random effect and all HM treated as a single group (ΔΔCp). FC, fold change.

Supplemental Table S7. Statistical analysis of expression profiles of precancerous lesions of human colon (data accompanying Supplemental Figure S3).

Linear model *ΔCp ~ tissue_stage + patient*					**Wilcoxon's rank-sum test**	
Precancerous lesions of the colon						
Gene	**ΔΔCp**	**FC**	**mean ΔCp**	**p-value**	**Gene**	**p-value**
Polyp hyperplasia vs. healthy mucosa						
TROY	1.98	0.25	8.58	<0.0001	*TROY*	0.0012
AXIN2	1.09	0.47	2.75	<0.0001	*AXIN2*	0.097
LGR5	0.05	0.97	4.60	0.91	*LGR5*	0.71
Polyp low grade dysplasia vs. healthy mucosa						
AXIN2	-2.45	5.47	2.75	<0.0001	*AXIN2*	0.00057
LGR5	-2.69	6.46	4.60	< 0.0001	*LGR5*	0.0012
TROY	2.31	0.20	8.58	<0.0001	*TROY*	0.0041
Polyp high grade dysplasia vs. healthy mucosa						
AXIN2	-2.73	6.63	2.75	<0.0001	*AXIN2*	<0.0001
LGR5	-3.59	12.00	4.60	<0.0001	*LGR5*	<0.0001
TROY	1.60	0.33	8.58	<0.0001	*TROY*	<0.0001
Polyp low grade dysplasia vs. polyp hyperplasia						
AXIN2	-3.54	11.62	2.75	<0.0001	*AXIN2*	0.00058
LGR5	-2.74	6.67	4.60	<0.0001	*LGR5*	0.0023
TROY	0.33	0.80	8.58	0.50	*TROY*	0.71
Polyp high grade dysplasia vs. polyp hyperplasia						
AXIN2	-3.82	14.10	2.75	<0.0001	*AXIN2*	<0.0001
LGR5	-3.63	12.39	4.60	<0.0001	*LGR5*	<0.0001
TROY	-0.38	1.30	8.58	0.35	*TROY*	0.33
Polyp high grade dysplasia vs. polyp low grade dysplasia						
LGR5	-0.89	1.86	4.60	0.044	*LGR5*	0.19
TROY	-0.71	1.63	8.58	0.087	*TROY*	0.25
AXIN2	-0.28	1.21	2.75	0.29	*AXIN2*	0.21

Individual Cp values were normalized using geometric average of three housekeeping genes (β-*ACTIN*, *AXIN1* and *UBB*) to obtain relative expression levels (-ΔCp) and averaged. All healthy mucosae were treated as a single group (ΔΔCp). FC, fold change.

Supplemental Table S8. A complete list of genes identified by Chip-on-chip using the TCF4-specific antibody and chromatin isolated from Colo320, DLD1, LS174T and SW480 CRC cells.

To see the table, please, follow this link:
http://www.editorialmanager.com/gastro/download.aspx?id=699743&guid=27e5eb1b-e990-4396-9738-497835b15034&scheme=1

7.8.5 Supplemental References

1. Kim TH, Barrera LO, Zheng M, et al. A high-resolution map of active promoters in the human genome. Nature 2005;436:876-80.
2. Scacheri PC, Crawford GE, Davis S. Statistics for ChIP-chip and DNase hypersensitivity experiments on NimbleGen arrays. Methods Enzymol 2006;411:270-82.
3. Tamai K, Zeng X, Liu C, et al. A mechanism for Wnt coreceptor activation. Mol Cell 2004;13:149-56.
4. Ootani A, Li X, Sangiorgi E, et al. Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. Nature medicine 2009;15:701-6.
5. Sato T, van Es JH, Snippert HJ, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 2011;469:415-8.
6. Miyoshi Y, Nagase H, Ando H, et al. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Human molecular genetics 1992;1:229-33.
7. Smith G, Carey FA, Beattie J, et al. Mutations in APC, Kirsten-ras, and p53-- alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci U S A 2002;99:9433-8.
8. Lustig B, Jerchow B, Sachs M, et al. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol 2002;22:1184-93.
9. Cattaneo E, Laczko E, Buffoli F, et al. Preinvasive colorectal lesion transcriptomes correlate with endoscopic morphology (polypoid vs. nonpolypoid). EMBO Mol Med 2011;3:334-47.
10. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 2009;459:262-5.
11. Kojima T, Morikawa Y, Copeland NG, et al. TROY, a newly identified member of the tumor necrosis factor receptor superfamily, exhibits a homology with Edar and is expressed in embryonic skin and hair follicles. The Journal of biological chemistry 2000;275:20742-7.
12. Pispa J, Mikkola ML, Mustonen T, et al. Ectodysplasin, Edar and TNFRSF19 are expressed in complementary and overlapping patterns during mouse embryogenesis. Gene expression patterns 2003;3:675-9.
13. Schlemper RJ, Riddell RH, Kato Y, et al. The Vienna classification of gastrointestinal epithelial neoplasia. Gut 2000;47:251-5.
14. Forbes SA, Tang G, Bindal N, et al. COSMIC (the Catalogue of Somatic Mutations in Cancer): a resource to investigate acquired mutations in human cancer. Nucleic acids research 2010;38:D652-7.
15. Ramensky V, Bork P, Sunyaev S. Human non-synonymous SNPs: server and survey. Nucleic acids research 2002;30:3894-900.
16. Segditsas S, Rowan AJ, Howarth K, et al. APC and the three-hit hypothesis. Oncogene 2009;28:146-55.

8 General discussion

8.1 Bacterial components in the intestinal inflammation prevention and treatment

Complex ecosystem of microbes inhabiting our GIT can broadly influence our physiological functions. Chronic inflammatory disorders of GIT arise as a consequence of impaired interaction of organism with its environment in the gut lumen. Thus, aberrant host interaction with gut commensal microbiota triggers sensitive immune cells and results in inflammation. These processes are frequently supported by genetic (inherited) defects in barrier function, microbial killing, and immune responses (Baumgart and Carding 2007).

Arising from IBD pathogenesis that the disease is caused by aberrant immune response to gut microbiota, many of recent studies concern with the search for pathological agents among commensals. In accord, it was found that immune cells isolated from IBD patients produce pro-inflammatory cytokines when cultivated with their own commensal bacteria (Duchmann et al. 1995). We tested the idea if the over-reaction against commensal bacteria in IBD could be prevented by peroral exposition to the components of gut microbiota. Our experiments showed that administration of antigens from intestinal anaerobic bacteria reduced the severity of acute colitis (Verdu et al. 2000). One of recent studies revealed *Faecalibacterium prausnitzii* (*F. prausnitzii*) as bacterium with potentially beneficial effects on experimental colitis development and whose reduction in intestinal content was found in CD patients (Sokol et al. 2008). As *F. prausnitzii* produces high amounts of SCFA, one of the engaged mechanisms can include protective activity promoted by such bacterial metabolites. Similarly, our further investigation led to determination of specific commensal bacteria having preventive effect on colitis. Isolation and testing of various members of endogenous microbiota resulted in the identification of *Parabacteroides distasonis* (*P. distasonis*) as potentially effective agent. Although the effects of certain commensal bacteria on gut health are evident, the active components remain unrevealed. We showed that peroral administration of membranous fraction of the lyzed and fractionated *P. distasonis* led to the reduction of inflammatory score and pro-inflammatory cytokines production in the murine model of acute colitis.

To achieve the beneficial effects, we found that the antigens must be administered per os rather than parenterally. Only oral way of treatment was sufficient to prevent colitis. Oral

tolerance is induced by repeated contact of immune cells in mucosa with antigens in the lumen leading to acquired hypo-responsiveness. Aberrant induction of tolerance to commensal microbiota can be caused by abnormal DC priming, or antigen presentation resulting in increased aggressive effector T cells induction and/or decrease in Treg cells (Baumgart and Carding 2007). Our results indicate that functioning adaptive immune systetm is necessary for the colitis prevention. The experiments in severe combined immunodeficient (SCID) mice showed no improvement in any parameter we measured after preventive treatment by *P. distasonis* components. Development of T cells is influenced by IL-2 levels, as its deficiency leads to pro-inflammatory stimulation of Th1 immune response by IL-12 (Ludviksson et al. 1997). We found that acute colitis induced in BALB/c mice in our animal facility is also associated with an increase in IL-12 production in colonic tissue. Lots of studies showed positive correlation of disrupted Treg cells-maintained mucosal tolerance and chronic intestinal inflammation. For example IL-2 and IL-10 deficient mice developed spontaneous intestinal inflammation when kept under conventional conditions but stay healthy when reared as germ-free (Kuhn et al. 1993; Sadlack et al. 1993). Although the gut microbiota possesses great functional redundancy, the latest observations highlighted the individual role of certain bacteria in T cells maturation (Ivanov et al. 2009). Recent study by Lathrop et al. describes origin and TCR antigen specificity of thymic and colonic Treg cells. Interestingly, they found that great portion of intestinal Treg cells is induced extrathymically and their receptors recognize the antigens of commensal bacteria. TCR response was mainly stimulated by bacterial antigens associated with the Parabacteriodes/Bacteroides genera, especially by *P. distasonis* (Lathrop et al. 2011). In agreement with the important role of *P. distasonis* in the induction of immune tolerance, we found increase in the population of Treg cells in MLN after peroral administration of its components.

We conclude that peroral administration of certain components of commensal bacteria is sufficient to prevent colitis by various mechanisms including stabilization of intestinal microbiota and limitation of immune response to pro-inflammatory stimulation. Tight cooperation between intestinal microbiota and host leads to fine tuning of the homeostasis. Understanding this interaction became a crucial task that can reveal the role of certain bacteria in health and diseases where the microbiota composition is suspected of considerable contribution. Qualities of individual commensal bacterium in influencing the inflammatory processes bring new opportunities in microbe-based therapy. Using “dead” components of

endogenous microbiota suggests a safe way of alternative prevention and cure of various inflammatory diseases including IBD.

8.2 Probiotics in the intestinal inflammation prevention and treatment

Although the efficacy of probiotic bacteria in prevention or treatment of various diseases was tested for many years, inconsistent results still reduce their broader clinical application. Beneficial effects of probiotics in prevention and treatment of allergy, eczema and diarrhea as well as chronic intestinal inflammation including IBD were analyzed in controlled studies in animals and humans. Primarily different conditions like formulation, viability, dosage, indication, timing and strain or mixture of probiotic bacteria led to inhomogeneous results (Ruemmele et al. 2009; Sanders et al. 2010). For these reasons, we used well described strain of bacteria – *Lactobacillus casei* DN-114 001 (*L. casei*), which is a frequent part of dairy products made by Danone. Our study aimed on mechanisms, by which crude lysate of this probiotic promotes the resistance to chemically-induced colitis in mice.

The principal finding of our study is that non-living French-pressed-bacteria possess the similar biological activity as live bacteria. We found that peroral administration of lysate from *L. casei* prevented the mice against DSS-induced acute colitis. Although the years of probiotics consumption showed their high safety, their use in neonates, or patients with severe form of IBD, or immunocompromised ones brings the risk of adverse effects like sepsis (Sanders et al. 2010). To address this issue, it’s necessary to investigate the virulence of certain probiotic bacteria in such a complex milieu as human intestine. Unfortunately, objective safety measurements can only be done by the use of probiotics in at-risk individuals. Recent studies explore the active components of probiotic bacteria related to beneficial effects (Matsumoto et al. 2009). Furthermore, our results suggest much safer use of non-living formulation. Taken together use of "dead" lysate of probiotic bacteria reduces the risk of bacteria translocation and subsequent complications like sepsis with comparable beneficial effects on health.

In our experiments, we found an improvement in epithelial barrier function, as treated mice had lower intestinal permeability than untreated controls. Moreover, induction of colitis caused changes in tight junction proteins expression and distribution, which were found in lesser extent after *L. casei* lysate treatment. During last years, various mechanisms of

beneficial effects of probiotics treatment were observed and described. Probiotics enhance the gut barrier function, promote the production of mucin and antimicrobial peptides, and modulate immune response, cell cycle, and gut microbial community (Stephani et al. 2011). Such a broad spectrum of activities has unique effects on various disease traits, basically, administration of probiotics can influence the development (prevention), or course (treatment) of the disease. Moreover, numerous studies revealed the variability in mechanisms of action and in efficacy of certain strain or a mixture of probiotic bacteria. Thus, clarifying these differences can lead to the individualization of specific probiotic bacteria use. The issue of benefits and effectivity associated with the single-strain or mixture use was recently reviewed by Chapman et al.. They found that the use of mixture results in combined effects, which are often more effective than the single-strain administration in animal models. On the other hand, there are some effects that are more promoted after single-strain treatment when compared to multi-strain probiotics (Chapman et al. 2011).

Probiotics are known to influence the immune system reactivity. We also showed decrease in NF-κB activation and increase in Treg cells after *L. casei* lysate treatment. Modulation of signaling pathways of mucosal immune cells with the regulatory interventions to the systemic immune response is the important effect of probiotics use. Activation of NF-κB is associated with various humoral and cellular responses mainly including production of cytokines and chemokines by macrophages and intestinal epithelial cells (Thomas and Versalovic 2010). In accord, we found reduction in the production of pro-inflammatory cytokines like TNF-α and IFN-γ by colonic tissue of treated mice. Costimulation by DC and, also probably, by PRRs expressed on Treg cells can play an important role in the direct induction and promotion of Treg cells by the probiotics treatment and their further suppressive effects on intestinal inflammation (Himmel et al. 2008; Macho Fernandez et al. 2011). Therefore, probiotics use can partially interferes to the disease traits by fine tuning the immune background of the host.

Administration of probiotics is also associated with changes in the gut microbiota. Our experiments showed beneficial effect of *L. casei* lysate on the composition of gut microbiota as we found an increase in SCFA producing bacteria after the treatment. Probiotic bacteria produce various low molecular weight substances like fatty and amino acids, bacteriocines, NO, nucleases etc (Stacy et al. 2012). Moreover, Quorum sensing molecules can effectively influence local milieu and signaling pathways (Redfield 2002). The levels of SCFA comprise one of the important parameters influencing the homeostasis in the intestine, which can

decrease the severity of colitis (Aguilar-Nascimento et al. 1999). Thus, such molecules can promote the growth of the endogenous microbiota and, simultaneously, the resistance to pathogens. We suggest that our lysate includes these molecules, which possess some of the beneficial activities.

Although more basic research as well as randomized placebo-controlled trials is necessary to reveal all the mechanisms, the beneficial effects of probiotics treatment are evident. We conclude that the components of probiotic bacteria suggest the opportunity of safe and individualized approach in prevention and treatment of various diseases.

8.3 Microbiota in the colorectal cancer

Microbiota plays an important role in maintaining the balance between health and disease. The composition of commensal microbiota as well as its metabolic properties continuously influences the intestinal homeostasis. CAC is one of the disorders where microbiota substantially affects the induction, growth, and progression of the tumors.

Our experiments in germ-free animals confirmed the need of bacterial stimulation for tumor development and progression. Bacteria present a source of antigens, which can promote the inflammation and its further transition to dysplasia and neoplasia. As the germ-free animals display some mucosal barrier and immunity aberrations, their anti-tumor reactivity seems to be intact or even enhanced. Our previous studies showed induction of NK cells and T cells cytotoxicity in germ-free rats after tumor induction when compared to conventionally reared rats (Vannucci et al. 2008). Another mechanism, possibly supporting tumor progression, is the mucosal tolerance and Treg cells, which are induced by a huge amount of antigens of microbial and diet origin in the gut lumen of conventionally reared animals (Vannucci et al. 2008). Therefore, commensal microbiota is suspect to promote the tumorigenesis by enhancing immune tolerance, which simultaneously suppresses anti-tumor immunity.

We found differences in microbiota composition among samples collected during the initiation, promotion, and progression phases and at the end of the experiments suggesting that colon cancer development is associated with changed bacterial pattern. Although the changes in the microbiota composition that accompany the tumorigenesis are not jet uniformly assessed as cause or effect of the process, many studies showed differences between colorectal cancer and healthy microbiota (Shen et al. 2010; Marchesi et al. 2011).

Moreover, causative agent is no more restricted to a single microbe but there is a growing evidence-based theory of multiple-species dysbiosis that contributes to the disease development (Clemente et al. 2012). Recent studies revealed the differences between transient (luminal) and mucosa-associated commensal bacteria composition according to their further presence in tumor lesions (Shen et al. 2010). Thus, main microbial shifts associated with colorectal cancer are given by an increase in Proteobacteria and changes in Firmicutes/Bacteriodetes ratio. Detailed analysis revealed the increased abundance of strains from the genera *Coriobacteridae*, *Roseburia*, *Fusobacterium* and *Faecalibacterium*, which are important butyrate producers (Marchesi et al. 2011). SCFA can induce cell apoptosis via inhibition of histone deacetylase but this activity is limited to the early stages of tumorigenesis (Bordonaro et al. 2008). Therefore, the exact role of SCFA producing bacteria in tumorigenesis is not fully elucidated. Although new molecular approaches deepen the knowledge about the intestinal microbiota composition in the context of colorectal cancer, there still remains an important question: Are these bacteria causative agents (active players) or did they only passively move to the more favorable microenvironment?

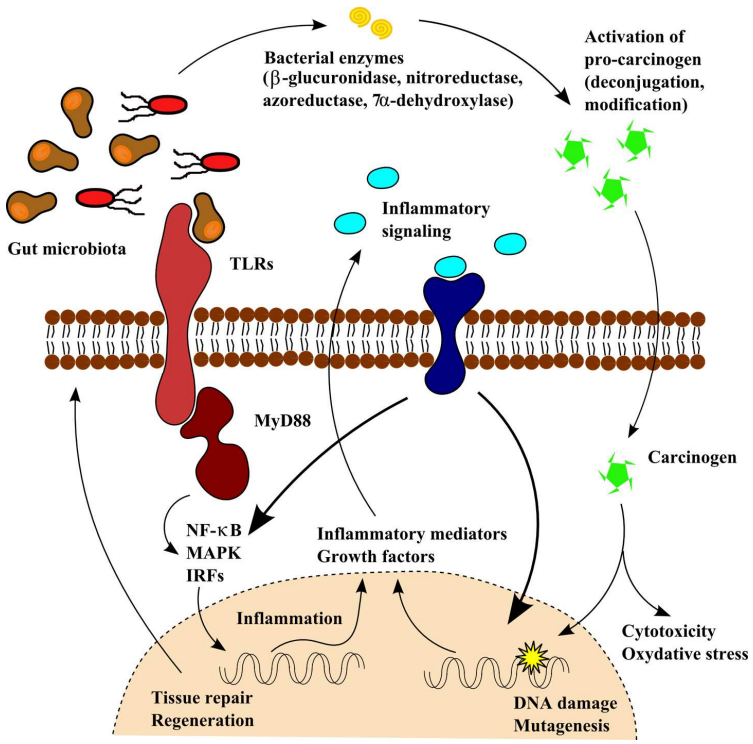
Partial answer can lie in the metabolic activity of the gut microbiota, which leads to the production and modification of various molecules affecting intestinal homeostasis as well as tumorigenesis (Azcarate-Peril et al. 2011). Our results support this idea, as we found positive correlation between tumor incidence and intestinal levels of β-glucuronidase, the enzyme, which causes the pro-carcinogen (methylazoxymethanol glucuronate) activation. Lots of exogenous and endogenous potentially harmful substances are utilized in liver to conjugates with glucuronic acid. These complexes are further excreted in bile to the intestinal lumen becoming substrates for commensal bacteria and their enzymes (Humblot et al. 2007). β-glucuronidase is one of the best known enzymes that promote intestinal tumorigenesis, the other one is 7α-dehydroxylase. Increased transformation of primary bile acids by this enzyme leads to the production of secondary bile acids, which has been linked to cholesterol gallstone disease and colorectal cancer. Secondary bile acids have many biological activities like cytotoxicity, limiting of apoptosis, and increase of oxidative stress, DNA damage, and mutagenesis (Pai et al. 2004; McGarr et al. 2005). Microbiota composition and simultaneously its metabolic capacity are directly influenced by the diet, specifically by the presence of substrates (Turnbaugh et al. 2008). Therefore, the intake of saccharides, proteins, fats and fibers as well as medicinal drugs, alcohol and other xenobiotics influence the conditions leading to the tumor development.

Moreover, not all bacteria possess such metabolic activities. Antibiotic treatment in our experiments decreased the β-glucuronidase activity and caused changes in the microbiota composition like decrease in some strains from Clostridia class and increase in Bacteroides/Prevotella group. These results are in concordance with Dabek et al. who found the β-glucuronidase enzyme in Clostridium cluster XIVa and IV, whereas no β-glucuronidase activity was detected in *Bifidobacterium* spp. and *Bacteroides thetaiotamicron* in the microbial community from human intestine (Dabek et al. 2008).

On the other hand, to address the second part of the question, tumor milieu is linked with a decrease in glucose and pyruvate, and an increase in lactate, amino acids, lipids and fatty acids, which simultaneously ensures the low pH (Marchesi et al. 2011). Thus, these conditions can attract certain strains of bacteria, which are further identified as tumor-associated ones.

Although the CAC core microbiota needs to be determined, the promoting role of bacteria on tumorigenesis was shown. Our results lead to conclusion that complex cocktail of microbiota's enzymes and their potential substrates and products substantially influence the tumor development in the GIT. The suggested idea is depicted on Figure 7.

Figure 7. How bacterial metabolism contributes to the colon carcinogenesis. Adopted from (Arthur and Jobin 2011).



8.4 Bacterial sensing and regulation in the colorectal cancer

As was previously mentioned, gut bacteria reside two compartments (lumen and mucosa) and mainly the mucosal-associated ones influence the intestinal homeostasis in health and disease. Epithelial as well as immune cells in the mucosa carry various PRRs (TLRs, NLRs) that play an important role in intestinal tumorigenesis due to promotion of pro-inflammatory response. Chronic intestinal inflammation was shown to change the number and distribution of these receptors with consequent downstream pathways modulation of apoptosis, proliferation, cell migration and inflammation.

Recent research is often aimed on the effect of PRRs in CAC development. One of the crucial roles is ascribed to adapter protein MyD88, which can be recruited by all TLRs (except TLR 3) and IL-1 family receptors. Downstream pathway includes molecules like IRAKs, TRAF6, TAK or IKK that trigger the end point transcription factor NF-κB. To address the need of MyD88 signaling in chronic colitis and CAC, MyD88 deficient mice were challenged by DSS, AOM, AOM/DSS, or crossbreed with IL-10 deficient mice or $Apc^{Min/+}$ mice. All these experiments showed two different results i) reduced or diminished tumorigenesis in $Apc^{Min/+}$ mice deficient in MyD88, and in mice deficient in both molecules IL-10 and MyD88 (Rakoff-Nahoum and Medzhitov 2007; Uronis et al. 2009) and ii) aggressive tumorigenesis in AOM/DSS-treated MyD88 deficient mice (Salcedo et al. 2010). Explanation of this discrepancy is still not fully elucidated, because TLR 4 deficient mice also showed decreased tumorigenesis (Fukata et al. 2007). TLR/IL-1R signaling regulates proliferation and healing of the epithelium after the damage of the barrier, thus, impaired tissue repair and regeneration can promote tumor growth and progression. Also, IL-10 deficient mice-treated with AOM and $Apc^{Min/+}$ mice are more likely models of spontaneous tumorigenesis. Taken together, disruption of the epithelial layer by DSS treatment seems to be a crucial activity leading to the tumor growth in MyD88 deficient mice.

Our experiments, using the IRAK-M deficient mice, confirmed the MyD88 downstream pathway as an important part of tumor development. The IRAK-M belongs to the group of MyD88-dependent signaling regulators like TOLLIP, SIGIRR, PPARγ and A20 (Shibolet and Podolsky 2007). To show their role in regulating bacterial sensing and inflammation, mice deficient in any of these inhibitors were constructed and tested in models of various diseases. Thus lack of A20 leads to colitis and arthritis (Hammer et al. 2011), and similarly SIGIRR or IRAK-M deficiencies enhance colitis or CAC (Xiao et al. 2007;

Berglund et al. 2010). Interestingly, the expression of *irak-m* is downregulated in germ-free conditions, while reaches normal levels after conventionalization as we recently showed using germ-free IRAK-M deficient mice (Biswas et al. 2011).

Moreover, we found that IRAK-M inhibition is important for tumor resistance. Wild-type mice treated with antibiotics had significantly reduced tumor development whereas IRAK-M deficient mice showed aggressive tumor growth. On the other hand, there are studies asserting the opposite. Xie and colleagues showed that IRAK-M deficient mice were resistant to growth of melanoma and fibrosarcoma cells following inoculation (Xie et al. 2007). Similarly, depletion of IRAK-M in lung macrophages led to increased anti-cancer immunity and reduced size of lung carcinoma (Standiford et al. 2011). Therefore, such confusing results suggest multiple roles of IRAK-M molecule in immune response regulation potentially associated with the cell type and localization. Inflamed gut mucosa is in direct and intensive contact with the hostile environment in the lumen, therefore, IRAK-M deficiency even more promotes the inflammation and subsequent tumorigenesis. The beneficial effects of IRAK-M deficiency observed by other researchers can be caused by different models and conditions used during the studies (e.g. chemically-induced inflammation-related tumors vs. inoculation of tumor cell lines, intestine vs. lung). Therefore, we suggest that properly activated and regulated MyD88-dependent pathway is crucial for maintaining GIT homeostasis.

8.5 Regulation of stem cell proliferation in the colorectal cancer

Disruption of physiological intracellular pathways can crucially contribute to the neoplastic transformation of the cells. Proliferation, differentiation or synthesis of various products can be affected by exogenous substances causing DNA damage, and mutation. The most frequently discussed and studied intracellular pathway linked with sporadic colorectal carcinoma development is Wnt/β-catenin signaling.

Our results confirmed that impaired degradation of β-catenin and its subsequent accumulation in cytoplasm and nucleus is associated with both spontaneous and colitis-associated colorectal cancer. Using AOM/DSS model of CAC, we found β-catenin positive staining in dysplastic and tumorous cells in the colons. Moreover, we observed the same results in $Apc^{Min/+}$ mice, whose cells lost control over the β-catenin turnover and thus were highly susceptible to spontaneous cancer. β-catenin has a dual role in the life of the cell.

Basically, under physiological conditions of adult organism, it is a part of adherent junctions bound to the catherin close to the cell membrane. On the other hand, when the Apc, Axin1 or Axin2, the main β-catenin suppressors, are blocked or mutated, β-catenin translocates into the nucleus, binds the TCF transcription factor and promotes transcription of various target genes like *c-Myc*, *Cyclin D1*, *Cox-2*, *Mdr1* etc. (Gregorieff and Clevers 2005; Cadigan and Peifer 2009). These genes interfere with cell cycle, proliferation, differentiation, inflammatory response and metabolism. Therefore, changes affecting the proteins in Wnt signaling pathway are highly predisposing to epithelial tumorigenesis.

Recent studies showed the important role of Wnt/β-catenin signalization during lineage commitment. It is suggested that tissue stem cells in the intestines trigger their cell cycle in response to Wnt ligation, which leads to differentiation into Paneth cells (Bastide et al. 2007). Furthermore, this signal is also sufficient to generate tumors from mutated stem cells localized at the bottom of the crypts. These processes are usually strictly regulated as there are plenty of inhibitors and activators of the Wnt signaling nearby. Recently, the stem cells marker Lgr5 was identified functioning as a negative regulator in normal intestinal stem cells (Barker et al. 2007). Moreover, we found a molecule, called Troy, interacting with Lgr5 and suppressing the Wnt signalization. We observed increased expression of Troy in mouse tumor tissue as well as in human cancer. Our further experiments identified as the source of Troy the fast-cycling intestinal stem cells. Moreover, Lgr5 overexpression was found to correlate with translocation of β-catenin into the nucleus in human colorectal cancer samples (Fan et al. 2010), which is in concordance with our results and suggests interactions promoting neoplastic transformation of cell. Recent study of Hashimoto and colleagues found lymphotoxin-α as a ligand interacting with Troy and triggering NF-κB transcription factor in the skin (Hashimoto et al. 2008). Although showed in the skin, certain activation of NF-κB can be associated with neoplasia also in the intestine. Further studies are needed to elucidate this interaction as possible pathway of colorectal cancer promotion. In conclusion, Troy is one of the membrane modulators of complex pathways of Wnt signaling and plays an important regulatory function in intestinal epithelial cell homeostasis and neoplastic transformation.

8.6 References

Aguilar-Nascimento, J. E., Franca-da-Silva, L. R., De-Oliveira, A. F. and Gomes-da-Silva, M. H. Enhanced mucosal re-epithelialization induced by short chain fatty acids in experimental colitis. Braz J Med Biol Res (1999) **32** (8): 961-966.

Arthur, J. C. and Jobin, C. The struggle within: microbial influences on colorectal cancer. Inflamm Bowel Dis (2011) **17** (1): 396-409.

Azcarate-Peril, M. A., Sikes, M. and Bruno-Barcena, J. M. The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer? Am J Physiol Gastrointest Liver Physiol (2011) **301** (3): G401-424.

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J. and Clevers, H. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature (2007) **449** (7165): 1003-1007.

Bastide, P., Darido, C., Pannequin, J., Kist, R., Robine, S., Marty-Double, C., Bibeau, F., Scherer, G., Joubert, D., Hollande, F., Blache, P. and Jay, P. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. J Cell Biol (2007) **178** (4): 635-648.

Baumgart, D. C. and Carding, S. R. Inflammatory bowel disease: cause and immunobiology. Lancet (2007) **369** (9573): 1627-1640.

Berglund, M., Melgar, S., Kobayashi, K. S., Flavell, R. A., Hornquist, E. H. and Hultgren, O. H. IL-1 receptor-associated kinase M downregulates DSS-induced colitis. Inflamm Bowel Dis (2010) **16** (10): 1778-1786.

Biswas, A., Wilmanski, J., Forsman, H., Hrncir, T., Hao, L., Tlaskalova-Hogenova, H. and Kobayashi, K. S. Negative regulation of Toll-like receptor signaling plays an essential role in homeostasis of the intestine. Eur J Immunol (2011) **41** (1): 182-194.

Bordonaro, M., Lazarova, D. L. and Sartorelli, A. C. Butyrate and Wnt signaling: a possible solution to the puzzle of dietary fiber and colon cancer risk? Cell Cycle (2008) **7** (9): 1178-1183.

Cadigan, K. M. and Peifer, M. Wnt signaling from development to disease: insights from model systems. Cold Spring Harb Perspect Biol (2009) **1** (2): a002881.

Clemente, J. C., Ursell, L. K., Parfrey, L. W. and Knight, R. The impact of the gut microbiota on human health: an integrative view. Cell (2012) **148** (6): 1258-1270.

Dabek, M., McCrae, S. I., Stevens, V. J., Duncan, S. H. and Louis, P. Distribution of beta-glucosidase and beta-glucuronidase activity and of beta-glucuronidase gene gus in human colonic bacteria. FEMS Microbiol Ecol (2008) **66** (3): 487-495.

Duchmann, R., Kaiser, I., Hermann, E., Mayet, W., Ewe, K. and Meyer zum Buschenfelde, K. H. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Clin Exp Immunol (1995) **102** (3): 448-455.

Fan, X. S., Wu, H. Y., Yu, H. P., Zhou, Q., Zhang, Y. F. and Huang, Q. Expression of Lgr5 in human colorectal carcinogenesis and its potential correlation with beta-catenin. Int J Colorectal Dis (2010) **25** (5): 583-590.

Fukata, M., Chen, A., Vamadevan, A. S., Cohen, J., Breglio, K., Krishnareddy, S., Hsu, D., Xu, R., Harpaz, N., Dannenberg, A. J., Subbaramaiah, K., Cooper, H. S., Itzkowitz, S. H. and Abreu, M. T. Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. Gastroenterology (2007) **133** (6): 1869-1881.

Gregorieff, A. and Clevers, H. Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev (2005) **19** (8): 877-890.

Hammer, G. E., Turer, E. E., Taylor, K. E., Fang, C. J., Advincula, R., Oshima, S., Barrera, J., Huang, E. J., Hou, B., Malynn, B. A., Reizis, B., DeFranco, A., Criswell, L. A., Nakamura, M. C. and Ma, A. Expression of A20 by dendritic cells preserves immune homeostasis and prevents colitis and spondyloarthritis. Nat Immunol (2011) **12** (12): 1184-1193.

Hashimoto, T., Schlessinger, D. and Cui, C. Y. Troy binding to lymphotoxin-alpha activates NF kappa B mediated transcription. Cell Cycle (2008) **7** (1): 106-111.

Himmel, M. E., Hardenberg, G., Piccirillo, C. A., Steiner, T. S. and Levings, M. K. The role of T-regulatory cells and Toll-like receptors in the pathogenesis of human inflammatory bowel disease. Immunology (2008) **125** (2): 145-153.

Humblot, C., Murkovic, M., Rigottier-Gois, L., Bensaada, M., Bouclet, A., Andrieux, C., Anba, J. and Rabot, S. Beta-glucuronidase in human intestinal microbiota is necessary for the colonic genotoxicity of the food-borne carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline in rats. Carcinogenesis (2007) **28** (11): 2419-2425.

Chapman, C. M., Gibson, G. R. and Rowland, I. Health benefits of probiotics: are mixtures more effective than single strains? Eur J Nutr (2011) **50** (1): 1-17.

Ivanov, II, Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K. C., Santee, C. A., Lynch, S. V., Tanoue, T., Imaoka, A., Itoh, K., Takeda, K., Umesaki, Y., Honda, K. and Littman, D. R. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell (2009) **139** (3): 485-498.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K. and Muller, W. Interleukin-10-deficient mice develop chronic enterocolitis. Cell (1993) **75** (2): 263-274.

Lathrop, S. K., Bloom, S. M., Rao, S. M., Nutsch, K., Lio, C. W., Santacruz, N., Peterson, D. A., Stappenbeck, T. S. and Hsieh, C. S. Peripheral education of the immune system by colonic commensal microbiota. Nature (2011) **478** (7368): 250-254.

Ludviksson, B. R., Gray, B., Strober, W. and Ehrhardt, R. O. Dysregulated intrathymic development in the IL-2-deficient mouse leads to colitis-inducing thymocytes. J Immunol (1997) **158** (1): 104-111.

Macho Fernandez, E., Valenti, V., Rockel, C., Hermann, C., Pot, B., Boneca, I. G. and Grangette, C. Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by NOD2-mediated recognition of a specific peptidoglycan-derived muropeptide. Gut (2011) **60** (8): 1050-1059.

Marchesi, J. R., Dutilh, B. E., Hall, N., Peters, W. H., Roelofs, R., Boleij, A. and Tjalsma, H. Towards the human colorectal cancer microbiome. PLoS One (2011) **6** (5): e20447.

Matsumoto, S., Hara, T., Nagaoka, M., Mike, A., Mitsuyama, K., Sako, T., Yamamoto, M., Kado, S. and Takada, T. A component of polysaccharide peptidoglycan complex on Lactobacillus induced an improvement of murine model of inflammatory bowel disease and colitis-associated cancer. Immunology (2009) **128** (1 Suppl): e170-180.

McGarr, S. E., Ridlon, J. M. and Hylemon, P. B. Diet, anaerobic bacterial metabolism, and colon cancer: a review of the literature. J Clin Gastroenterol (2005) **39** (2): 98-109.

Pai, R., Tarnawski, A. S. and Tran, T. Deoxycholic acid activates beta-catenin signaling pathway and increases colon cell cancer growth and invasiveness. Mol Biol Cell (2004) **15** (5): 2156-2163.

Rakoff-Nahoum, S. and Medzhitov, R. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. Science (2007) **317** (5834): 124-127.

Redfield, R. J. Is quorum sensing a side effect of diffusion sensing? Trends Microbiol (2002) **10** (8): 365-370.

Ruemmele, F. M., Bier, D., Marteau, P., Rechkemmer, G., Bourdet-Sicard, R., Walker, W. A. and Goulet, O. Clinical evidence for immunomodulatory effects of probiotic bacteria. J Pediatr Gastroenterol Nutr (2009) **48** (2): 126-141.

Sadlack, B., Merz, H., Schorle, H., Schimpl, A., Feller, A. C. and Horak, I. Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. Cell (1993) **75** (2): 253-261.

Salcedo, R., Worschech, A., Cardone, M., Jones, Y., Gyulai, Z., Dai, R. M., Wang, E., Ma, W., Haines, D., O'HUigin, C., Marincola, F. M. and Trinchieri, G. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. J Exp Med (2010) **207** (8): 1625-1636.

Sanders, M. E., Akkermans, L. M., Haller, D., Hammerman, C., Heimbach, J., Hormannsperger, G., Huys, G., Levy, D. D., Lutgendorff, F., Mack, D., Phothirath, P., Solano-Aguilar, G. and Vaughan, E. Safety assessment of probiotics for human use. Gut Microbes (2010) **1** (3): 164-185.

Shen, X. J., Rawls, J. F., Randall, T., Burcal, L., Mpande, C. N., Jenkins, N., Jovov, B., Abdo, Z., Sandler, R. S. and Keku, T. O. Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. Gut Microbes (2010) **1** (3): 138-147.

Shibolet, O. and Podolsky, D. K. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. Am J Physiol Gastrointest Liver Physiol (2007) **292** (6): G1469-1473.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L. G., Gratadoux, J. J., Blugeon, S., Bridonneau, C., Furet, J. P., Corthier, G., Grangette, C., Vasquez, N., Pochart, P., Trugnan, G., Thomas, G., Blottiere, H. M., Dore, J., Marteau, P., Seksik, P. and Langella, P. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A (2008) **105** (43): 16731-16736.

Stacy, A. R., Diggle, S. P. and Whiteley, M. Rules of engagement: defining bacterial communication. Curr Opin Microbiol (2012) **15** (2): 155-161.

Standiford, T. J., Kuick, R., Bhan, U., Chen, J., Newstead, M. and Keshamouni, V. G. TGF-beta-induced IRAK-M expression in tumor-associated macrophages regulates lung tumor growth. Oncogene (2011) **30** (21): 2475-2484.

Stephani, J., Radulovic, K. and Niess, J. H. Gut microbiota, probiotics and inflammatory bowel disease. Arch Immunol Ther Exp (Warsz) (2011) **59** (3): 161-177.

Thomas, C. M. and Versalovic, J. Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbes (2010) **1** (3): 148-163.

Turnbaugh, P. J., Backhed, F., Fulton, L. and Gordon, J. I. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe (2008) **3** (4): 213-223.

Uronis, J. M., Muhlbauer, M., Herfarth, H. H., Rubinas, T. C., Jones, G. S. and Jobin, C. Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. PLoS One (2009) **4** (6): e6026.

Vannucci, L., Stepankova, R., Kozakova, H., Fiserova, A., Rossmann, P. and Tlaskalova-Hogenova, H. Colorectal carcinogenesis in germ-free and conventionally reared rats: different intestinal environments affect the systemic immunity. Int J Oncol (2008) **32** (3): 609-617.

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. Oral administration of antigens from intestinal flora anaerobic bacteria reduces the severity of experimental acute colitis in BALB/c mice. Clin Exp Immunol (2000) **120** (1): 46-50.

Xiao, H., Gulen, M. F., Qin, J., Yao, J., Bulek, K., Kish, D., Altuntas, C. Z., Wald, D., Ma, C., Zhou, H., Tuohy, V. K., Fairchild, R. L., de la Motte, C., Cua, D., Vallance, B. A. and Li, X. The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. Immunity (2007) **26** (4): 461-475.

Xie, Q., Gan, L., Wang, J., Wilson, I. and Li, L. Loss of the innate immunity negative regulator IRAK-M leads to enhanced host immune defense against tumor growth. Mol Immunol (2007) **44** (14): 3453-3461.

9 Conclusion

In conclusion, influencing gut microenvironment by components of commensal (*Parabacteroides distasonis*) or probiotic (*Lactobacillus casei*) bacteria leads to intestinal inflammation improvement. Use of sterile bacterial lysate with similar positive results thus brings new safe strategy for colitis and also for colitis-associated cancer prevention. We confirm that intestinal microbiota has crucial role in colorectal cancer pathogenesis as well as in anti-cancer immune response. Moreover, we tested two regulatory molecules involved in maintaining the intestinal homeostasis, which can contribute to better understanding of the basic mechanisms of tumorigenesis. IRAK-M is involved in tumor resistance as a negative regulator of MyD88-dependent pathways. Troy interacts with Wnt/β-catenin pathway in intestinal stem cells influencing cell proliferation and differentiation in health and disease.

10 Appendices

10.1Abbreviations

AOM	Azoxymethane
APC	Antigen presenting cells
Apc	Adenomatous polyposis coli
ATB	Antibiotics
CAC	Colitis-associated cancer
CD	Crohn's disease
COX	Cyclooxygenase
DC	Dendritic cells
DSS, DS	Dextran Sulfate Sodium
ER	Endoplasmatic reticulum
FAE	Follicles-associated epithelium
FDC	Follicular dendritic cell
GALT	Gut-associated lymphoid tissue
GIT	Gastrointestinal tract
IBD	Inflammatory bowel diseases
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILF	Isolated lymphoid follicles
IRAK-M	IL-1 receptor-associated kinase M
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MAMP	Microbes-associated molecular patterns
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
MLN	Mesenteric lymph nodes
MyD88	Myeloid differentiation factor 88
NK	Natural killer cells
NLR	Nod-like receptors
NOD	Nucleotide-binding oligomerization domain
PP	Peyer's patches
PRR	Pattern recognition receptors
RA	Retinoic acid
RLR	Retinoic acid inducible gene I-like receptors
SCFA	Short-chain fatty acids
SFB	Segmented filamentous bacteria
SIgA	Secretory IgA
TAM	Tumor-associated macrophages
TCR	T cell receptor
Treg	Regulatory T cells
Troy	Tumor necrosis receptor family member 19
TGF	Transforming growth factor
TNBS	Trinitrobezenesulfonic acid
TNF	Tumor necrosis factor
TLR	Toll-like receptors

UC	Ulcerative colitis
ZO-1	Zonula occludens 1

10.2 Curiculum vitae

PERSONAL INFORMATION

Date of birth: 26th March 1982

Place of birth: Sternberk, Czech Republic

EDUCATION AND EMPLOYMENT

Since 2005 Ph.D. student

Charles University in Prague 2nd Faculty of Medicine, and Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Department of Immunology and Gnotobiology

Ph.D. thesis: Role of microbiota and gut inflammation in the pathogenesis of experimental colorectal cancer

Supervisor: Prof. MUDr. Helena Tlaskalova – Hogenova, Dr.Sc.

Since 2005 Histology technician

Institute of Microbiology v.v.i., Academy of Sciences of the Czech Republic, Department of Immunology and Gnotobiology

2000 – 2005 Student

Charles University in Prague Faculty of Pharmacy in Hradec Kralove (M.Sc. degree, the principal subject – Medical Analysis)

Diploma thesis: Optimizing and comparison of different methods used for the activity evaluation of human complement

CERTIFICATES AND MEMBERSHIPS

Certificate of competency according to §17 on Protection Animals against Cruelty (2005) – license for animal experiments (CZU 909/05)

Czech Immunology Society (since 2006)

Czech Society for Allergology and Clinical Immunology JEP (since 2006)

Society for Mucosal Immunology (since 2007)

European Academy of Allergology and Clinical Immunology (since 2008)

10.3 List of publications

1. **Klimesova K**, Kverka M, Zakostelska Z, Hudcovic T, Hrncir T, Stepankova R, Rossmann P, Ridl J, Kostovcik M, Mrazek J, Kopecny J, Kobayashi KS, Tlaskalova – Hogenova H. Altered gut microbiota promotes colitis-associated cancer in IL-1 receptor-associated kinase M deficient mice. *Inflamm Bowel Dis*. 2012; in press.

2. Fafilek B, Krausova M, Vojtechova M, Tumova L, Pospichalova V, Sloncova E, Huranova M, Chmelikova J, Hlavata A, Svec J, Sedlacek R, Luksan O, Oliverius M, Voska L, Jirsa M, Paces J, Kolar M, Krivjanska M, **Klimesova K**, Tlaskalova-Hogenova H and Korinek V. Troy, a Tumor Necrosis Receptor Family Member 19, Interacts with Lgr5 to Inhibit Wnt Signaling in Intestinal Stem Cells. Provisionally accepted for publication in *Gastroenterology*. 2012.

3. Pribylova J, Krausova K, Kocourkova I, Rossmann P, **Klimesova K**, Kverka M, Tlaskalova-Hogenova H. Colostrum of Healthy Mothers Contains Broad Spectrum of Secretory IgA Autoantibodies. *J Clin Immunol.* 2012 Jul 10; in press.

4. Golias J, Schwarzer M, Wallner M, Kverka M, Kozakova H, Srutkova D, **Klimesova K**, Sotkovsky P, Palova-Jelinkova L, Ferreira F, Tuckova L. Heat-Induced Structural Changes Affect OVA-Antigen Processing and Reduce Allergic Response in Mouse Model of Food Allergy. *PLoS One.* 2012; 7(5):e37156.

5. 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.

6. 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.

7. Kverka M, Rossmann P, Tlaskalova-Hogenova H, **Klimesova K**, Jharap B, de Boer NK, Vos RM, van Bodegraven AA, Lukas M, Mulder CJ. Safety and efficacy of the

immunosuppressive agent 6-tioguanine in murine model of acute and chronic colitis. *BMC Gastroenterol.* 2011 May 5; 11:47.

8. 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. Review.

9. 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.

10. Vannucci L, Stepankova R, Grobarova V, Kozakova H, Rossmann P, **Klimesova K**, Benson V, Sima P, Fiserova A, Tlaskalova-Hogenova H. Colorectal carcinoma: Importance of colonic environment for anti-cancer response and systemic immunity. *J Immunotoxicol.* 2009 Dec; 6(4):217-26. Erratum in: *J Immunotoxicol.* 2010 Mar; 7(1):76. Review.

11. Frol'ová L, Smetana K Jr, Borovská D, Kitanovicová A, **Klimesová K**, Janatková I, Malícková K, Lukás M, Drastich P, Benes Z, Tucková L, Manning JC, André S, Gabius HJ, Tlaskalová-Hogenová H. Detection of galectin-3 in patients with inflammatory bowel diseases: new serum marker of active forms of IBD? *Inflamm Res.* 2009 Aug; 58(8):503-12.

12. Frolova L, Drastich P, Rossmann P, **Klimesova K**, Tlaskalova-Hogenova H. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. *J Histochem Cytochem.* 2008 Mar; 56(3):267-74.