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Použití bakteriálních složek v prevenci a léčbě experimentálního střevního zánětu

Bacterial components in experimental intestinal inflammation prevention and therapy

Disertační práce

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Thesis summary

Although strong protective immune response is essential for preventing invasion by pathogens, equivalent responses against antigens originating from commensal bacteria can lead to chronic inflammatory diseases, such as inflammatory bowel disease (IBD). Manipulating the mucosal immune responses with microbial antigens might be an excellent tool to IBD therapy or prevention. Our aim was to gain some insight into the regulation of the intestinal inflammation and to isolate bacterial immunomodulatory components that could be used in intestinal inflammation therapy and prevention.

One particular mechanism of how healthy colon tissue regulates the inflammation during acute experimental colitis is through modulation of bioavailability of glucocorticoids (GCs) in gut mucosa. Here, we show that intestinal inflammation changes the local GC metabolism, which ultimately leads to decrease in inflammatory readiness of cells in the gut mucosa and in mesenteric lymph nodes. This pre-receptor regulation of GC function could represent an important homeostatic function of the gut mucosa.

The actual triggers of intestinal inflammation in IBD seem to be either microbial dysbiosis or microbes with special “pathogenic” abilities, which both could be rectified by feeding with probiotics. Here, we report that oral feeding with live probiotic bacteria can protect mice from severe forms of acute colitis. Furthermore, we found that even the oral treatment with lifeless lysate of one of them, *L. casei* DN-114 001 (Lc), has a similar protective effect. This treatment also prevented some typical features of intestinal inflammation, such as production of pro-inflammatory cytokines in gut tissue and increase in intestinal permeability. Moreover, the lysate also significantly changes the gut microbiota composition. We conclude that antigens from Lc can reduce the intestinal inflammation by influencing both gut microbiota and the host’s immune system.

Next, we show that oral treatment of mice with lysate of one particular commensal, *Parabacteroides distasonis*, or its membranous fraction (mPd), have similar beneficial effect as the lysate of probiotic bacterium. We demonstrated that this beneficial effect is gut-dependent and that the mechanism is executed mainly by the adaptive arm of the immune system. Interestingly, unlike the oral treatment with Lc, mPd leads to the rise of specific antibodies in serum and regulatory T cells in mesenteric lymph nodes.

Compared with the use of live bacteria, bacterial lysates seem to be a safer and more convenient approach in IBD therapy. The differences in immunomodulatory properties of different bacteria also suggest the need to individualize such therapy.

Souhrn v češtině

Zatímco silná imunitní odpověď je nezbytná pro zabránění infekce patogeny, stejná odpověď namířená proti antigenům komenzální mikroflóry může vést ke vzniku chronického zánětlivého onemocnění, jakým jsou idiopatické střevní záněty (IBD). Manipulace s imunitní odpovědí pomocí mikrobiálních antigenů představuje zajímavou metodu k prevenci a léčbě IBD. Naším cílem bylo rozšířit znalosti o regulaci střevního zánětu a izolovat bakteriální složku vhodnou k této manipulaci.

Jedním z mechanismů, jakým se střevní sliznice brání rozvoji chronického zánětu, je modulace lokálního metabolismu glukokortikoidů (GC). V našich pokusech jsme prokázali, že experimentální střevní zánět spouští zpětnovazebné mechanismy ve střevní stěně a mezenteriálních uzlinách, které snižují zánětlivou pohotovost v této oblasti a zesilují účinnek GC. Regulace účinku endogenních GC na pre-receptorové úrovni představuje významný homeostatický mechanismus v zánětem postižené střevní sliznici.

Vlastním spouštěčem střevního zánětu u IBD je buďto porucha složení střevní mikroflóry, nebo přítomnost doposud neznámých patogenních mikroorganismů. Oba tyto faktory mohou být napraveny podáváním probiotických bakterií. V našich pokusech jsme prokázali, že krmení myší probiotickými bakteriemi zvyšuje jejich rezistenci k vyvolání experimentálního střevního zánětu, a že podobného efektu lze dosáhnout i s lyzátem jedné z nich, *L. casei* DN-114 001 (Lc). Tato léčba také částečně zabránila vzniku některých změn, které jsou typicky spojované se střevním zánětem, jako je například vzestup lokální produkce prozánětlivých cytokinů, vzestup střevní propustnosti, a nebo výrazné změny ve složení střevní mikroflóry. Složky Lc dokáží ovlivnit střevní mikroorganismy i imunitní systém hostitele a tak snížit citlivost organismu ke střevnímu zánětu.

Dále jsme ukázali, že lyzát komenzální bakterie, *Parabacteroides distasonis*, a zejména její membranózní frakce (mPd), mají podobný protektivní efekt jako lyzát Lc. Pro tento efekt je nezbytné orální podávání a neporušený adaptivní imunitní systém. Na rozdíl od Lc léčba pomocí mPd vede k vzestupu specifických protilátek v séru a regulačních T lymfocytů v mesenteriálních lymfatických uzlinách.

V porovnání s živými bakteriemi je terapeutické podávání bakteriálních lyzátů jednoznačně bezpečnější a pohodlnější. Rozdíly v mechanismu účinku mezi jednotlivými bakteriemi a jejich lyzáty poukazují dále na nutnost přísné individualizace takovéto léčby.

1. GENERAL INTRODUCTION

1.1 MUCOSAL IMMUNE SYSTEM OF THE GUT

The human mucosal immune system comprises the lymphoid-associated structures of the nasal, bronchial, gastrointestinal and urogenital tracts, as well as the lacrimal, salivary and mammary glands. A significant part of this system is anatomically located in the gut. There are several structural, functional and regulatory features that set the mucosal and systemic immune systems apart. This distinction is largely due to the fact that the antigenic environment (commensal flora and dietary antigens) is vastly different from that experienced by the systemic immune system. These features include a) strongly developed mechanisms of innate defense, b) the existence of characteristic populations of unique types of lymphocytes (e.g. intraepithelial lymphocytes, lamina propria lymphocytes, and γδ T cells), c) colonization of the mucosal and exocrine glands by cells originating from the mucosal organized tissues (Common mucosal system) and d) preferential induction of inhibition of responses to innocuous antigens (Mucosal tolerance) (Tlaskalova-Hogenova et al., 2002).

Mucosal immunity is most abundantly expressed in the gut, and the intestinal mucosa of an adult contains more than 80% of the body's plasmatic cells. It is estimated that in healthy humans approx. 3 grams of IgA is transported to the gut lumen every day (Conley and Delacroix, 1987), which makes the gut the largest antibody producing organ in the body (Brandtzaeg et al., 1999).

To fulfill its digestive function, the gut has a huge surface area (approx. 300m^2), but this huge area is covered by a single layer (approx. 30 μm thick) of tightly connected enterocytes, that literally separate the organism from its environment. Despite the fact that the gut epithelium is completely shed and replaced every 2–3 days, this layer maintains its integrity.

The epithelial cells are supported in their effort of maintaining integrity of the organism by a highly specialized system of immune cells forming mucosa-associated lymphoid system (MALT). MALT consists partly of organized tissue comprising both solitary and multiple lymphatic follicles (Peyer's patches, appendix), which represents inductive site of immune system, and cells scattered in the mucosa responsible mainly for effector function. This system must be tightly regulated, discriminating between often very similar indigenous and harmful antigens (e.g. commensal and pathogenic *E. coli*).

Dysfunction or dysregulation of the gut mucosal immune responses has been associated with a number of diseases including inflammatory bowel disease (IBD), celiac disease and food allergy.

1.1.1 Barrier function

Intestinal barrier prevents viable enteric bacteria and other antigens from excessive interaction with the immune system. The intestinal barrier is, rather than a rigid anatomical structure, dynamic and actively regulated apparatus formed by several components. These components include mucus layer, secretory immunoglobulins (Ig), intestinal epithelial cells (IECs), tight junction, and mucosa associated lymphoid tissue. While the proper function of the intestinal barrier is vital for our survival, the quality of an individual's barrier function is influenced by many factors, such as the genetic background, age (especially in newborns), concurrent infection, interactions between the mast cells, nerves and neuropeptides, and the mucosa-shielding effect of SIgA produced in the gut or provided in the first months of life via breast milk (Brandtzaeg, 2009).

1.1.1.1 Mucus layer

The goblet cells, that can be found scattered in the entire gastrointestinal mucosa, produce abundantly glycosylated (up to 80% wt/wt) proteins called mucins. In humans, there are 18 types of mucins identified so far, and MUC2 is the predominant type found in the gut (Tytgat et al., 1994). The NH_2- and COOH-termini are rich in cysteine residues that allow these proteins to link together with disulfide bonds forming a thick, viscous and relatively impermeable gel layer on the apical surface of the epithelium – mucus layer (Roberts, 1976). The thickness of this layer differs in the different parts of the gut ranging from 100 μm to 800 μm, but the 30 μm-thick layer closest to the epithelial surface firmly adheres to the epithelial carbohydrates. In healthy individuals, this adherent layer is essentially bacteria free, but in areas of active inflammation, the mucus layer is weakened allowing the bacteria to reach the epithelium (Swidsinski et al., 2007). This barrier integrity is further strengthened by various antimicrobial compounds produced by gut mucosa. These compounds are concentrated in the mucus layer, preventing the proliferation of bacteria. These are, most notably, cationic antimicrobial peptides (defensins, cathelicidins and

calprotectins) produced by specialized epithelial cells – Paneth cells – and secretory antibodies.

1.1.1.2 Secretory immunoglobulins

The secretory Ig are typically present in mucosal secretions of all mammals. Their main source (local vs. distal production), isotype distribution and levels are, however, tissue and species specific (Mestecky et al., 2005). The dominant Ig isotype in the human gut is secretory IgA (SIgA) produced by lamina propria plasma cells. Other isotypes are of low importance and quantities, except for the secretory IgM (SIgM), which is of greater importance in newborns and adults with selective IgA deficiency (Brandtzaeg, 2009).

Secretory Ig are produced as polymers, SIgA mainly as a dimer and SIgM as a pentamer, with subunits joined together by a small peptide called joining (J) chain. These polymeric antibodies are than transported to the gut lumen by enterocytes, as recently reviewed by Brandtzaeg (2009). This transport starts when the polymeric Ig receptor (pIgR) expressed at the basolateral surface of the enterocyte binds polymeric Ig molecules, the complex is then transported across the cell to be secreted at the apical surface, where the pIgR is enzymaticly cleaved. Part of the pIgR stays bound to the Ig as secretory component (SC), which significantly increases Ig's stability (Crottet and Corthesy, 1998). In the lumen, these Ig are concentrated in the mucus fortifying the gut barrier function by shielding the internal body surfaces. Because the production of the Ig is stimulated by antigens that target the gut mucosa, the secretory Ig protects the gut surface against antigens found in the individual's environment.

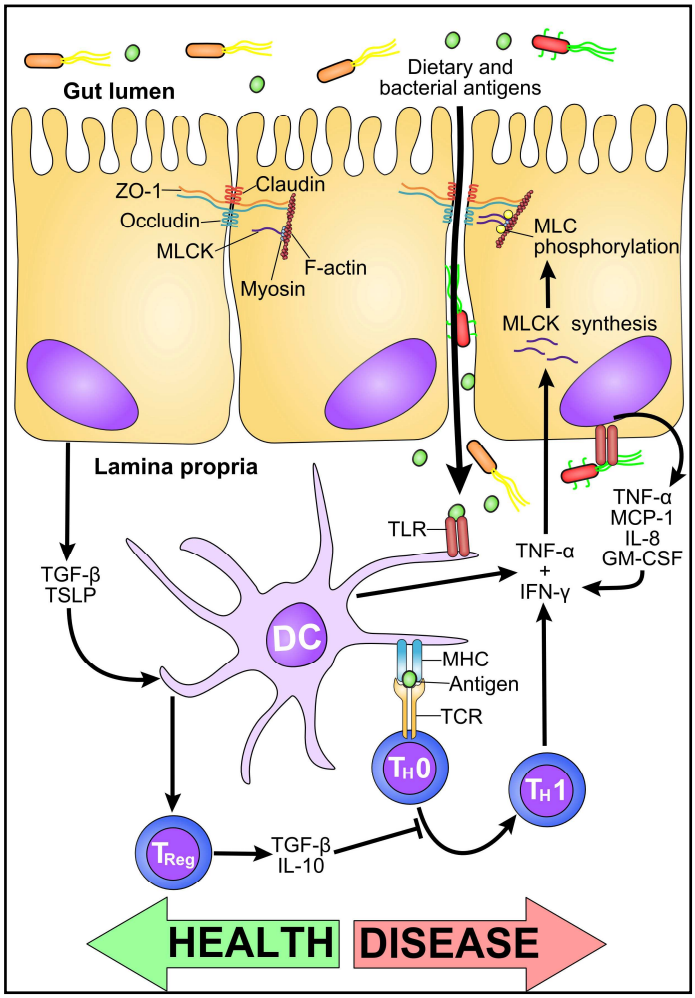
Early after delivery, the production of secretory Ig is not sufficient to protect the suckling from environmental threads, so the SIgA from the mother's milk and colostrum substitute its protective function (Dickinson et al., 1998; Arifeen et al., 2001).

1.1.1.3 Intestinal epithelial cells

The gut epithelium is an epithelium specialized for digestion and absorption of nutrients. To achieve its primary purpose it covers the considerable area of 300 m^2, with a single layer of the cells. The dominant cell type in the epithelium is absorptive enterocytes, followed by the mucin-secreting goblet cells and enteroendocrine cells. To avoid intensive translocation of the potentially dangerous antigens or microorganisms, the epithelial cells are connected together with specialized intercellular junctions – Tight junctions (TJ) – that seal the apical part of the epithelium. This seal prevents the fluid

from moving through the intercellular gaps and separates apical membranes from the basolateral ones. These junctions are formed by several proteins connected to the epithelial cytoskeleton as shown on Figure 1.1.

Figure 1.1 Schematic representation of the tight junction and its regulation by immune system in health and disease.



The paracellular space between the enterocytes is sealed just below the base of microvilli by tight junctions. This complex is formed by several proteins, including claudins, zonula occludens 1 (ZO-1) and occludin which are connected to polymeric (F-) actin and myosin. Myosin light chain kinase (MLCK) is associated with the perijunctional actomyosin ring and its activity leads to the phosphorylation of myosin light chain (MLC) which results in TJ opening. This process can be initiated by high levels of pro-inflammatory cytokines, TNF-α and IFN-γ. In healthy individuals, interaction between epithelium and immune cells in lamina propria (namely Tolerogenic Dendritic cells (DC) and regulatory T cells (T_{reg})) create anti-inflammatory millieu. Minor defects in the barrier allow immunostimmulatory bacterial/dietary antigens to cross the epithelium and enter the lamina propria, causing the production of the innflammatory mediators. If this reaction goes adrift, the tight junctions in the area start to leak, thereby allowing further stimulation of lamina proprie immune cells by bacterial and dietary antigens from the lumen, thus amplifying the cycle of inflammation.

The barrier function of the TJ can be regulated by pro-inflammatory cytokines - TNF-α and IFN-γ. (Madara and Stafford, 1989; Taylor et al., 1998). Both these cytokines have synergic effect, which seems to be mediated by the activation of myosin light chain kinase (MLCK) with subsequent phosphorylation of perijunctional myosin II regulatory light chain, which then opens TJ and enhances paracellular permeability (Wang et al., 2005).

Besides separating the host connective tissue from the external environment, epithelial cells participate in the effector function of the mucosal immune system and play

an important immunomodulatory role. Enterocytes express molecules involved in antigen presentation to the gut lymphocytes, such as the class I and class II MHC and CD1d molecule (Gorvel et al., 1984; Blumberg et al., 1991). However, since the IECs do not express costimmulatory molecules, they cannot prime naive T cells and therefore supply the mucosal cells with tolerogenic signals (Sanderson et al., 1993). Moreover, when the IECs are stressed, they express MHC class I-related molecules MICA and MICB on their surfaces. This serves as an immune surveillance mechanism for the detection and destruction of damaged, infected, or transformed IECs by intestinal γδ T cells (Groh et al., 1998).

The IECs express a wide range of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), that recognize conserved microbial motifs. These microbial components, such as the lipopolysaccharide (LPS), lipoproteins, flagellin and unmethylated CpG-containing DNA, are shared among both dangerous pathogens and harmless commensals (see Table 1.1). In general, the PRRs react to these microbial components by activation of pro-inflammatory immune response. Therefore, the proximity of billions of microbes and IECs raises the question of why the IECs do not respond by inflammation to the commensals in gut lumen. Although this question is still not completely answered, several anti-inflammatory mechanisms have been proposed. First, the distance between the PRRs and gut microbes is extended by the mucus layer, as mentioned above. Second, under steady-state conditions, IECs downregulate the expression of LPS recognising TLR4/MD-2, minimizing the recognition of bacterial LPS (Abreu et al., 2001). Third, there is a different representation of PRRs in the apical and basolateral cell membrane. So while LPS recognising TLR4 is expressed on the luminal membrane, flagellin recognising TLR5 is expressed exclusively on the basolateral membrane (Gewirtz et al., 2001). Flagellated bacteria cannot, therefore, trigger TLR5 unless they actively penetrate the mucosa. This is in agreement with the findings that IECs dampen the pro-inflammatory immune response if the bacteria trigger the luminal PRR, but react with strong pro-inflammatory response if the basolateral PRRs are stimulated by invading bacteria (Artis, 2008). Fourth, some commensal bacteria were found to downregulate the pro-inflammatory cascade by interfering with regulation of NF-κB signalling cascade (Neish et al., 2000; Kelly et al., 2004). Fifth, IECs do not express costimmulatory molecules so they may act as tollerogenic cells maintaining the survival and activity of T_{reg} (Mowat, 2003).

IECs produce a variety of cytokines that could also be responsible for the "anti-inflammatory" tuning of the gut mucosa. Stimulation of the CD1d expressed on the epithelial cells causes them to produce anti-inflammatory cytokine IL-10 and attenuate the pro-inflammatory IFN-γ signaling. This cytokine environment then downregulates the T cell response in the mucosa (Colgan et al., 1999). When challenged with some commensal bacteria, human enterocytes produce thymic stromal lymphopoietin (TSLP) and TGF-β, which cooperate in the induction of tolerogenic phenotype in the dendritic cells (DCs) (Zeuthen et al., 2008). On the other hand, when invaded by pathogenic microorganisms, they rapidly secrete a large number of cytokines and chemokines that draw inflammatory cells to the site of infection and help the organism to deal with the infection (Jung et al., 1995).

Another important effector function of IECs is executed by transporting secretory Ig from the lamina propria plasmatic cells to the gut lumen. During this transport, secretory Ig integrate SC into their molecule, as mentioned earlier in this chapter.

1.1.2 Induction of the mucosal immune response

1.1.2.1 Peyer's patches

Peyer's patches (PP), together with mucosa-draining local/regional lymph nodes, represent the inductive site of the mucosal immune system. There is growing evidence, however, that the induction of mucosal immune response could occur in the absence of PP, probably through microscopic lymphoid aggregates (Hamilton et al., 1981). Structurally, the PPs are well organized structures of special epithelium and lymphoid tissue, found in the small intestine. Unlike the epithelium found in the rest of the intestine, the follicle-associated epithelium (FAE) is cuboidal in shape and contains few or no goblet and enteroendocrine cells. Approximately 10% of PP's surface is covered by specialized microfold (M) cells. These M cells have characteristic ultrastructural features like irregular microvilli on the luminal surface and invaginations (or "pockets") on the basal surface, harboring infiltrating lymphocytes. They closely collaborate with underlying MALT by transporting the antigen into the underlying follicles (Owen, 1977). Unlike the regular villous IECs, the M cells cannot transport secretory antibodies produced by the plasmocytes, because they do not express the SC (Bjerke and Brandtzaeg, 1988).

Right under the epithelial layer, there is a mixed cell zone called dome area, where most of the antigen presentation takes place (Kelsall and Strober, 1996). This zone contains several types of cells, including $CD4^+$ T cells, plasma cells, dendritic cells (DCs) and small B cells with cleaved nuclei (centrocyte-like cells) which can infiltrate the overlying epithelium. The M cell's associated B cells have a memory phenotype and resemble germinal centre B cells, so it seems that the B cells in M cell pockets are extensions of germinal centers (Yamanaka et al., 2001). The development of the PP is dependent on the B cells, because the already differentiated epithelial cells can be transformed into M cells when co-cultured with B cells, and B cell or RAG1 deficient mice (with no T or B cells) have very reduced size and numbers of PPs and only few M cells, while mice deficient in T cells have normal numbers and sizes of PPs and M cells. (Kerneis et al., 1997; Golovkina et al., 1999)

Under the dome area, there is thymus-dependent area which contains lymphatic follicles, typically with germinal centers. The follicle center, supported by a network of follicular DCs, is populated mainly by the B cells and it is surrounded by the mantle of T cell–rich areas that merge into the mixed cell zone of the dome area. The interfollicular region contains $CD4^+$ and $CD8^+$ T cells, DCs and a dense network of high-endothelium venules that serve as entry and exit points for migrating cells (Kelsall and Strober, 1996). In the intestinal mucosa, these venules express mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), which is specifically recognized by the $\alpha_4\beta_7$ integrin on the mucosa-induced lymphocyte populations, and also, at least to some extent, by the L-selectin (CD62L) expressed on naive T cells (Bargatze et al., 1995).

1.1.2.2 Cryptopatches and isolated lymphocyte follicles

As mentioned earlier, antigen presentation to the gut mucosal immune system could also occur outside PPs, in the microscopic isolated lymphatic follicles (ILFs) and in mice also in cryptopatches (CPs).

CPs, first identified by Kanamori et al. (1996), are small lymphocyte follicles, approximately 100-150 μm in diameter, scattered in the small and large intestine of mice. Unlike the ILFs or PPs, CPs are smaller, do not contain mature T ($CD3^+$) or B ($B220^+$) cells, do not have germinal centers, and are not associated with M cells (Hamada et al., 2002). Interestingly, CPs are populated primarily by precursor lymphocytes and the fact that they are present in germ-free mice and in immunodeficient SCID and athymic nu/nu

mice suggests that they are microbiota and thymus independent source of lymphocytes (Kanamori et al., 1996)

There are numerous ILFs scattered throughout the mucosa of both small and large intestine. Similarly to the PPs, these ILFs are mainly populated by antigen producing B cells with a close contact of APCs and their surface is covered with follicle associated epithelium containing M cells (Hamada et al., 2002). ILFs are, therefore, important for the humoral response to the luminal antigens. They can be formed de novo in adult animals and although they do exist in germ-free mice, they need exogenous stimuli, including normal bacterial flora, to become fully developed (Lorenz et al., 2003).

Besides M cells, the gut lumen is also actively sampled for antigens by intestinal DCs. These APCs can reach through epithelial layer by their long dendrites. To achieve this without disturbing the barrier function of the gut epithelium, they express ZO-1, Claudin-1 and Occludin, proteins involved in the TJs formation (Rescigno et al., 2001).

1.1.3 Effector function

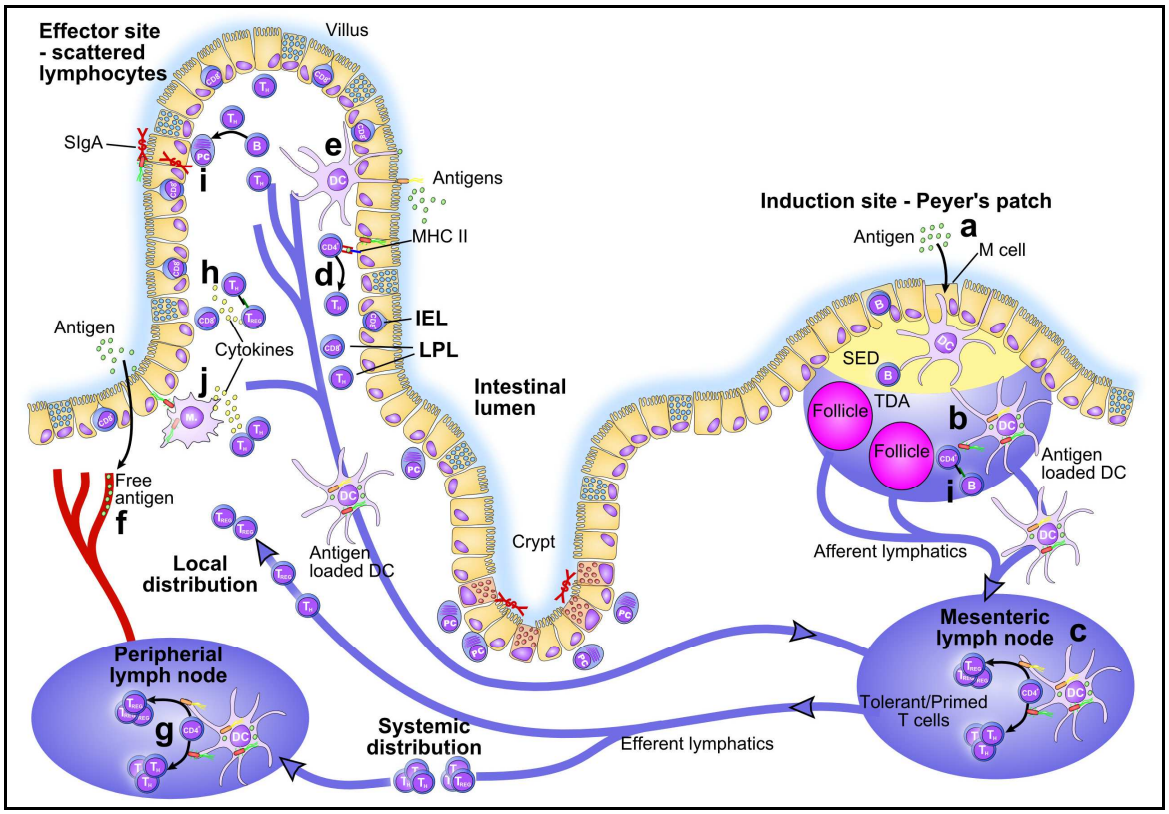
There are two main effector functions executed by the mucosal immune system of the gut, depicted on Figure 1.2. First, there is a strong inflammatory response against invading microorganisms or potentially harmful antigens protecting the integrity of the organism. Second, the mucosa is protected from being destroyed by excessive inflammatory response by active anti-inflammatory mechanisms. This system is tightly controlled and regulated in order to keep the balance between these two, partially opposing, mechanisms.

Mucosal immune system provides a first line of defense for the inner body surfaces. Most abundantly, the mucosal immunity is expressed in the gut. Effector functions of the mucosal surfaces are executed by cells in various histological compartments, most notably in the lamina propria and in the gut epithelium (Brandtzaeg and Pabst, 2004).

Although dendritic cells could enter the gut epithelium, this compartment is populated mostly by $CD8^+$ T cells, with fewer $CD4^+$ T cells. There are neither macrophages, nor B cells, nor plasma cells. These Intraepithelial lymphocytes (IEL) are phenotypicaly distinct population, they could bear either $\alpha\beta$ or $\gamma\delta$ T-cell receptor (TCR), and they are of large portion thymus independent cells (Kunisawa et al., 2007). The $\gamma\delta$ T-cells represents 10% of IELs in healthy humans, and up to 50% of IELs in rodents

(Spencer et al., 1989; Guy-Grand et al., 1991). Although they are in constant contact with extensive amount of antigenic stimuli, these T cells are counterintuitively, markedly oligoclonal (Regnault et al., 1994). The T cells in newborns have polyclonal repertoire, but soon after birth, they become increasingly restricted. This restriction is antigen driven and the resulting repertoire is unique and quite stable for every individual (Chowers et al., 1994).

Figure 1.2 Schematic representations of the main mechanisms of mucosal immune response.



Antigen may enter through the microfold (M) cells to the Peyer's patch (**a**), and after transfer to local dendritic cells (DCs), might then be presented directly to T cells (**b**). Alternatively, antigen or antigen-loaded DCs from the Peyer's patch may gain access to draining lymph, with subsequent T-cell recognition in the mesenteric lymph nodes (MLNs) (**c**). A similar process of antigen or antigen-presenting cell (APC) dissemination to MLNs may occur if antigen enters through the epithelium covering the villus lamina propria (**e**), but in this case, there is the further possibility that stimulation of the basolateral PRR of the enterocytes causes them to express MHC class II$^+$, thus acting as local APCs (**d**). In all cases, the antigen-responsive CD4$^+$ T cells acquire expression of the $\alpha 4\beta 7$ integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph (**e**) and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the lamina propria. T cells which have recognized antigen first in the MLN may also disseminate from the bloodstream throughout the peripheral immune system. Antigen may also gain direct access to the bloodstream from the gut (**f**) and interact with T cells in peripheral lymphoid tissues (**g**). Antigen specific regulatory T cells than enter the lamina propria and render the LPL unresponsive to the gut commensals (**h**) by direct contact or by producing anti-inflammatory cytokines. Antigen specific T cells stimulate the production of SIgA by plasmatic cells (**i**) or stimulates the innate immunity cells, resident in gut mucosa, to clear the pathogens or potentially harmful antigens (**j**) by pro inflammatory cytokines. Based on Mowat (2003), SED, subepithelial dome; TDA, thymus-dependent area; DC, dendritic cell; IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes; SIgA, secretory IgA.

The lamina propria (LP) is the layer of connective tissue between the epithelium and muscularis mucosa. This compartment contains a number of macrophages, dendritic cells and both T and B lymphocytes. This mixture of cells could therefore execute all functions of the mucosal immune system from antigen processing to the specific response.

LP T cells are mainly $CD4^+$ T cells bearing $\alpha\beta$ TCR, generated in the PP as a result of antigen stimuli. Interestingly, all of them are on pro-apoptotic pathway, expressing Fas, and sometimes even Fas Ligand on their surfaces (De Maria et al., 1996). The lack of direct antigen stimulation in the LP leads them to apoptosis. There are also some $CD8^+$ $\alpha E\beta_7^+$ T cells, which are probably only transiting IELs (Farstad et al., 1996). Intestinal LP is richly endowed with IgA^+ plasmocytes, which make up 30–40% of the mononuclear cells in humans. Most of the plasma cells are found in the region around the crypts (Brandtzaeg et al., 2005). The population of the LP by these cells is also antigen driven, because there are no plascmocytes in the LP at birth (Perkkio and Savilahti, 1980). There is also considerable amount of macrophages and DCs in the LP, their main function is to phagocyte the invading pathogens, to present the antigens to T cells and to modulate the immune functions. Furthermore, it has been shown that subepithelial macrophages at the top of the villi contain debris of the apoptotic IECs, which suggest that these cells take part in the gut epithelium renewal (Nagashima et al., 1996).

1.1.3.1 Defense mechanisms of the innate immunity

The mucosa contains both effector arms of the immune system, innate and adaptive, which operate in synergy. This synergy allows proper activation of the immune system against pathogens and also long-term tolerance of commensal microbiota and other harmless antigens.

Strongly developed mechanisms of innate immunity are typical for protection of mucosa. Main effector functions of the innate immunity of the gut mucosa are executed by the production of cytokines, phagocytosis of the pathogens, production of the antimicrobial factors and presenting the antigens to the cells of the adaptive immunity (Tlaskalova-Hogenova et al., 2004). The innate immunity response against microbes and their components is initiated through the PRR, as mentioned earlier. They can be expressed on the cell surface, in the intracellular compartments, or secreted into the

bloodstream and tissue fluids (Medzhitov and Janeway, 1997). These receptors can recognize conserved structures on microbes called microbe-associated molecular patterns (MAMPs) so that the host could discriminate between different groups of microbes (see Table 1.1) (Janeway, 1989; Akira et al., 2006).

These receptors are found in many cell types, including those responsible for gut mucosa protection - macrophages, DCs, neutrophils and previously mentioned IECs. Once PRRs are triggered by microbial components, the cells start to produce cytokines essential for both pathogen elimination and initiation of subsequent adaptive immune response (Tlaskalova-Hogenova et al., 2005).

Table 1.1 TLR recognition of microbial components

Receptor	Ligand	Species
TLR1/2	Triacyl lipopeptides, Glycolipids	Bacteria and mycobacteria
TLR2	Peptidoglycan, Phospholipomannan, Porins	Gram-positive bacteria, *Candida albicans*, Neisseria
TLR2/6	diacyl lipopeptides, Zymosan, Lipoteichoic acid	Group B Streptococcus, Yeast
TLR3	polyinosinic–polycytidylic acid (polyI:C), dsDNA	viruses
TLR4	Lipopolysaccharide (LPS), Mannan, Glycoinositolphospholipids and others	Gram-negative bacteria, *Candida albicans*, Trypanosoma
TLR5	Flagellin	Flagellated bacteria
TLR7 and TLR8	ssRNA, imiquimod, loxoribine, others	RNA viruses
TLR9	Unmethylated CpG-DNA	Bacteria
TLR11	Profilin-like molecules	*Toxoplasma gondii*, uropathogenic bacteria,

Adopted from Akira et al. (2006)

Microbial density in the gut lumen is controlled by the production of the antimicrobial peptides, because these small cationic peptides are concentrated in the mucus layer. These peptides are produced mainly by IECs, but also by other cell types resident to the intestinal mucosa – e.g. mastocytes (Di Nardo et al., 2003). These antimicrobial peptides are produced constitutively, but their production in IECs can be significantly increased in response to intestinal pathogens (O'Neil et al., 1999; Ayabe et al., 2000).

1.1.3.2 Defense mechanisms of the adaptive immunity

Gut mucosa is effectively protected from potentially dangerous antigens by defense mechanisms of both innate and adaptive immunity. While the innate immune system

forms the first line of defense against invasive pathogens, the adaptive immune system is usually needed for clearing the infection and for prevention of the subsequent infection with homologous or related pathogens (Hughes and Galan, 2002). Although the adaptive mechanisms are very efficient, they require previous encounter with antigen and therefore needs time to develop.

Humoral mechanisms of adaptive immune system involve mainly production of secretory Ig. Vast majority of secretory Ig protecting gut mucosa are polymeric SIgA and SIgM produced in the mucosa by resident plasma cells (Brandtzaeg, 2009). There is also a small portion of monomeric IgG produced in the mucosa and a small portion of monomeric Ig from the systemic circulation, but none of these molecules contain J chain, and therefore could not be transported through IEC in the way previously described (Brandtzaeg and Prydz, 1984). Microbes in the intestinal lumen are coated with secretory Ig. This agglutination leads to efficient neutralization of invading pathogens even before they reach the tissue and without starting the inflammatory response. This mechanism is called immune exclusion, and it is the key non-inflammatory homeostatic mechanism that controls the endogenous microbiota (Brandtzaeg, 2009). Therefore, the production of antibodies seems to be the most important mechanism for preventing infection.

Cell mediated immunity, on the other hand, is needed for clearing already established infections and protects the individual from intracellular pathogens, malignant tumors, as well as from excessive inflammatory response to harmless antigens from food and commensals. $CD8^+$ cytotoxic T cells (CTLs) provide effective protection by destroying cells infected with viruses, malignantly transformed or damaged cells. $CD4^+$ Th cells are known to provide help to B cells or CTLs, thereby augmenting the antibody production or cytotoxicity, but they also possess CTL-independent anti-viral activities *in vivo*, so they could function as antiviral effector cells (Doherty et al., 1997).

All subsets of Th cells are involved in the regulation of inflammatory response, by producing a wide spectrum of pro-inflammatory and anti-inflammatory cytokines, thus protecting the individual from both infection and excessive inflammation. Apart from already established Th1 and Th2 effector T cells, new lineage of $CD4^+$ T-cells with distinct cytokine spectrum has been recently described and referred to as Th17. (Aggarwal et al., 2003). This new T-cell lineage, characterized by production of pro-inflammatory cytokine IL-17, has been proposed to play an important role in the tissue inflammation and ultimately also in the pathogenesis of autoimmune diseases (Park et al., 2005). Another functional subset of $CD4^+$ T cells, natural regulatory T cells (T_{regs}), has

been shown to dampen the inflammation in the tissue and induce tolerance to the harmless antigens, as discussed later in this chapter.

1.1.3.3 Common mucosal immune system

Cells primed against environmental antigens in the inductive sites of mucosae have the ability to recirculate throughout the whole body, forming an interconnecting network of immune structures, called common mucosal immune system (CMIS). This system provides effective immunity to mucosal surfaces and exocrine glands and could even extend between two individuals through breast-feeding. It was originally found in rodents (McDermott and Bienenstock, 1979), but there is enough evidence that it exists also in humans (Czerkinsky et al., 1987). The exposure of the naive precursor cells to environmental antigens, and their subsequent migration to remote mucosae or glands, leads to dissemination of the adaptive immune response against these antigens throughout the body (Goldblum et al., 1975; Mazanec et al., 1993). The existence of this system could be exploited in the design of new vaccines protecting the whole organism including sites less accessible to vaccination (Mestecky et al., 1978). There is apparent compartmentalization within this system. It has been shown that after the intestinal priming with viruses, the whole respiratory tract is protected, but while the upper respiratory tract is protected mainly by the specific antibody producing plasmocytes, the lower respiratory tract is protected by CTLs (Zuercher et al., 2002). This suggests that different mechanisms protect different organs after mucosal immunization. Furthermore, there is a marked difference in the immune response profiles found in various secretions after oral versus rectal antigen administration, so the inductive sites of the CMIS seem to be compartmentalized as well (Kantele et al., 1998).

The mechanisms behind the lymphocyte homing are not completely elucidated, but the tissue specific homing receptors and chemotactic signals seem to be of critical importance (Brandtzaeg et al., 2001). For trafficking to the gut, the MAdCAM-1 addressin on the local venules and $\alpha 4\beta 7$ integrin on the cells form the key receptor-counter-receptor pair, and the chemotactic signals depend on the target cell type. It has been shown that thymus-expressed chemokine (TECK, CCL25) could attract CCR9 expressing cells including IgA-producing cells and $CD4^+$ or $CD8^+$ T cells to the small intestine (Kunkel et al., 2000; Bowman et al., 2002).

Mesenteric lymph nodes (MLNs) are the largest lymph nodes in the body. Similarly to the other lymph nodes in the body, MLNs are well designed for the immune response

induction. Circulation of the lymphocytes through the MLNs requires either α4β7 integrin (expressed on the gut-committed cells) or L-selectin (expressed on the naive cells), and could therefore act as a crossroads between the mucosal and peripheral compartments (Mowat, 2003). This mechanism explains, how orally acquired antigens can induce systemic immunity or tolerance (see later). The further fate of the lymphocytes depends on the site of their initial priming. Lymphocytes primed in the gut exit the mucosa and reside and differentiate further in the MLNs. They loose expression of L-selectin and start to express both α4β7 integrin and CCR9 which allow them to home back to the gut, while T cells primed in the periphery express P-selectin instead, and therefore cannot home to the gut (Campbell and Butcher, 2002).

1.1.3.4 Mucosal vaccination

Most pathogens invade us through our mucosal surfaces, yet the conventional ways of immunization often do not protect this area enough to prevent the pathogen invasion. Furthermore, the mucosal vaccination has also other appeals, such as easy and painless administration and reduced costs of production, storage and delivery, which makes it ideal for mass immunization in case of emergencies. There are few successful mucosal vaccines available in humans, such as the orally administered polio vaccine that was used to eliminate polio from the Americas, the Western Pacific, and Europe. There are also other mucosal vaccines available (at least in some countries), such as the nasal vaccine against influenza or oral vaccine against cholera, and many others are being developed (Boyaka et al., 2005).

In order to achieve the systemic response to the mucosal vaccine, it must be delivered to the inductive sites of mucosal immune system; e.g. GALT in the intestine, bronchi-associated lymphoid tissue (BALT) or nasopharynx-associated lymphoid tissue (NALT), otherwise it has only minor and localized effect (Boyaka et al., 2005). Unfortunately, the antigens administered to mucosa often cause unresponsiveness (mucosal tolerance) instead of protective immune response (see later). Moreover, the optimal doses of antigens are difficult to establish because of the often low and unpredictable absorption from the mucosal surfaces (e.g. intestine), interference with a vast array of other antigens or destruction of the antigen by enzymes in the mucosal secretions. To overcome these obstacles, they must be co-administered with a) suitable adjuvants that reverse the natural

unresponsiveness of the mucosa, and b) delivery systems that protect the administered antigen from proteases and mediate its preferential uptake.

The most potent mucosal adjuvants are enterotoxins (e.g cholera toxin, heat-labile toxin of *E. coli*) that strongly increase the immune response to the antigen, but their use in humans is limited due to their toxicity. Therefore, many of these enterotoxins were modified to lose their toxicity. The main mechanisms of action of mucosal adjuvants and delivery systems are described in Table 1.2.

Table 1.2. Most common mucosal adjuvants and delivery systems and their mode of action

Adjuvants	Mode of action	Reference
Cholera toxin, heat-labile toxin of *E. coli* and its derivatives	Triggering TLR, MHC class II and increases the gut permeability, increases the antigen uptake by M cells. Increases the activity of APCs, $CD4^+$ T cells and class switching in B cells.	(Elson and Ealding, 1984)
TLR ligands (LPS and immunostimulatory DNA)	Stimulation of TLR causes cytokine secretion favoring mucosal immunity, B cell proliferation and Ig synthesis. Their mechanisms of action are very complex, often opposite to each other.	(Johnson et al., 1956; Kang and Compans, 2003)
Zonula ocludens toxin (ZOT)	Increases the epithelium permeability	(Marinaro et al., 1999)
Cytokines and chemokines (IL-1, IL-12, GM-CSF, RANTES)	Many functions, depending on the cytokine, often influences the APC-T cell interaction and B cell function.	(Staats and Ennis, 1999; Lillard et al., 2001; Bradney et al., 2002)
Saponins (QS-21)	Possibly increases antigen uptake.	(Kensil et al., 1991)
Liposomes	Protects the antigen from being lysed, increase immunogenicity by the interaction of antigen with MHC inside the APC	(Gregory et al., 1986)
Biodegradable microspheres	Protects the antigen from being lysed and possibly increase the antigen uptake by M cells	(Moldoveanu et al., 1993)
Immunostimulating complexes	Protection of the antigen from being lysed and immunostimulation	(Morein, 1990)

Indeed, the protection from the infection by known pathogens is the most obvious use of mucosal vaccines. However, since yet unknown microbe/microbial component triggers the inflammation in autoimmune disorders such as IBD (see chapter 3), it seems intriguing to employ the mucosal vaccination strategies in the prevention or therapy of these chronic diseases.

1.1.3.5 "Oral (Mucosal) tolerance"

Strong protective immunity is essential to prevent invasion by pathogens, but similar responses against dietary antigens or commensal bacteria can lead to chronic

inflammatory disease. In order to maintain homeostasis, the mucosal immune system has, through evolution, developed immunosuppressive mechanisms that inhibit overreaction against innocuous luminal antigens. This is achieved by mechanism called “oral (or mucosal) tolerance”, firstly described almost hundred years ago by Wells and Osborne (1911), who found that orally administered antigens induce systemic hyporesponsiveness to the same antigen.

The nature of the antigen greatly influences its ability to induce oral tolerance. Oral administration of viable organisms, such as viruses or bacteria, more likely provoke protective immunity but the same organism induce tolerance if administered killed or inactivated (Rubin et al., 1981). Furthermore, the mice fed by killed bacteria develop tolerance to the thymus-dependent K antigen but acquire protective immunity to thymus-independent O antigen of the same organism (Stokes et al., 1979). Moreover, proteins are particularly easy to induce tolerance in native form, but often fail to do so when denaturated (Peng et al., 1995).

In order to induce reliable and clinically usable mucosal tolerance, antigens also need special delivery system and mucosal adjuvant, although the nature of adjuvants is different from the ones suitable for vaccines. Most promising results are with nontoxic B subunit of cholera toxin, which has been shown to attenuate experimental models of multiple sclerosis, type 1 diabetes, rheumatoid arthritis when administered together (linked or chemically coupled) with myelin basic protein, insulin, collagen II or low dose of allergen respectively (Bergerot et al., 1997; Tarkowski et al., 1999; Rask et al., 2000; Sun et al., 2000).

The mechanism how the tolerance is maintained depends also on the dose of the antigen. Approximately 10-100x higher doses of antigen are needed to suppress humoral immunity than cellular immunity and while high doses of antigen suppress the immune response preferentially by deletion or anergy of Th1 and Th2 clones, repeated low dose stimulates the active immune suppression by regulatory T cells (Mowat et al., 2005). Regulatory T cells are the key effector cells in the mechanisms of oral tolerance under physiological conditions.

There are several types of regulatory T cells, each equipped with the ability to dampen the protective immune response, by cell-cell interaction and by production of anti-inflammatory cytokines (Table 1.3).

Table 1.3 Regulatory T cells in mouse, their markers and their mechanisms of action

Name	Marker	Function/mechanisms	Reference
natural regulatory T cells	$CD4^+$ $CD25^+$ $FoxP3^+$	Direct contact, anti-inflammatory cytokines, maintenance of peripheral self tolerance	(Thorstenson and Khoruts, 2001; Fontenot et al., 2003)
Th3 cells	$CD4^+$ TGF-β	TGF-β, low amounts of IL-10 and IL-4	(Chen et al., 1994)
Tr1 cells	$CD4^+$ IL-10	IL-10, bystander suppression	(Groux et al., 1997)
NK-T cells	$\alpha\beta TCR^+$, $NK1.1^+$	Control immune responses via cytokines (e.g. IL-4, IFN-γ, IL-10)	(Godfrey et al., 2000)
γδ T cells	γδ-TCR	Protects from infection in the early phase (TNF-α) but also downregulate the inflammaory response (IL-10)	(Ke et al., 1997; Andrew and Carding, 2005)

Oral tolerance is mediated by $CD4^+$ T cells even if the pathogenetic mechanism is mediated by $CD8^+$ cytotoxic T cells independently of $CD4^+$ T cell help, as shown in the animal model of contact hypersensitivity (Garside et al., 1995; Dubois et al., 2003). Nevertheless, the $CD8^+$ T cells play an important down-regulatory function during the oral tolerance in the gut mucosa, because oral administration of antigen can induce systemic, but not gut-localized hyporesponsiveness in CD8-deficient mice (Grdic et al., 1998).

There are three main mechanisms, how regulatory T cells mediate their effect; bystander suppression, clonal deletion and clonal anergy. Most important mechanism of oral tolerance is bystander suppression, in which T cells specific for one antigen can suppress the function of T cells with other specifity, providing that both antigens are present at the same time (Miller et al., 1991). This effect does not require cell-cell contact and it is partially dependent on TGF-β or IL-10. This mechanism is crucial for keeping the homeostasis in the gut, because T cells in the gut mucosa indeed recognize the commensal bacteria, but their inflammatory reaction to them is in healthy individuals dampened by local regulatory T cells through IL-10 and/or TGF-β-dependent mechanism (Khoo et al., 1997). In mechanism of clonal anergy, T cells go through few divisions after encounter with the antigen but soon become unresponsive to this antigen, which is reversible by IL-2 (Whitacre et al., 1991). Clonal deletion, i.e. apoptosis of antigen specific T cell clone, is probably rare mechanism of oral tolerance; nevertheless, it has been reported after high oral dose of antigen (Chen et al., 1995).

Oral tolerance is a T cell dependent mechanism, but it is the way how and when the antigen is presented to T cells that drive the immune response towards immunity or tolerance (Mowat, 2003). The APCs involved in oral tolerance are DCs, and to a lesser

extent also MHC class II-expressing enterocytes and B cells. Their activity is greatly influenced by the local cytokine microenvironment and state of maturation. Interestingly, IECs can mediate the oral tolerance through small vesicles carrying MHC class II molecules with bound antigenic peptides sampled from the gut lumen, tolerosomes, without actually meeting the T cells (Karlsson et al., 2001). Many other APCs, such as DCs and B cell, can release similar vesicles that are capable to interact with T cells. Whether this interaction results in immunity or tolerance depends on the state of the particular APC, as mentioned earlier (Thery et al., 2002).

1.1.4 Development

1.1.4.1 Prenatal development

The prenatal development of the gut mucosal immune system is tightly bound to the formation of the gut itself. All components of the mucosal immune system are in place by 200th day of gestation, and although it needs postnatal activation by antigens to became fully mature, it is capable to mount the immune response (Gleeson and Cripps, 2004).

First true component of the mucosal immune system to appear during the human development is SC, that could be detected as early as 40th day of gestation, but it is not until one week post partum when it reaches the adult level of production and distribution (Rognum et al., 1992). Also J chain could be detected at 40th days post partum in liver, and since 110 day of gestation it could be found in small intestine (Moro et al., 1991). The maturation of the B cells starting around 100th day of gestation, when IgM/IgD/CD5-positive cells appear. At the same time, first T cells are observed in the human gut starting to organize themselves around B cells (Spencer et al., 1986). Around 120th day of gestation, gut B cells start to produce first IgA. First B cell follicles are apparent two weeks later, together with formed T cell zones, dome regions, high endothelium venules and FAE (Gleeson and Cripps, 2004). However, germinal centre formation is not seen in Peyer's patches of the fetus and only develops after antigenic exposure at birth (Bridges et al., 1959). At the end of the gestation, T cells are appearing within the epithelial layer, but at this stage, they bear predominantly γδ TCRs (Spencer et al., 1989).

1.1.4.2 Postnatal development

The newborn's mucosal immune system can successfully mount immune response, but its effectiveness is not in par with the adult one. Nevertheless, regardless the gestational age at birth, it became fully mature within the 12 month of postnatal development. The rapid maturation of mucosal immune system is essential for protection against infection and allergen exposure in the postnatal life. This maturation is driven mainly by two essential factors 1) antigen stimulation of the mucosal immune system (colonization with microbiota and oral feeding) and 2) breast-feeding (see Table 1.4.)

Table 1.4 Prenatal and postnatal factors influencing the development of the mucosal immune system in infancy

Prenatal factors
Genetic factors, particularly cytokine gene polymorphisms
Maternal nutritional status
Intrauterine infections
Toxic chemical exposure (alcohol, products of smoking cigarettes and illicit drugs)
Perinatal factors
Hormonal influences at birth
Infant feeding practices, particularly non-breast fed infants
Timing of mucosal membrane closure (exclusion of antigenic stimulation)
Bacterial colonization, particularly gut flora in response to feeding practices
Up-regulation of regulatory T cell's cytokines in response to antigenic stimuli
Postnatal factors
Composition of microbiota
Level of protective antibodies in maternal serum and breast-milk
Immunization
Exposure to new infections (family members, particularly older siblings; day-care or child-minding centers; hospitalization; commencing school)
Exposure to cigarette smoke
Inherited or induction of IgA-deficiency states
Diet and nutritional status of infant, (adequate protein, essential vitamins and minerals)
Exposure to physiological and psychological stressors

Adopted from Gleeson and Cripps (2004)

At birth, the B cells are found only in follicles and not in LP, and humans are born virtually IgA-deficient (Perkkio and Savilahti, 1980). Moreover, during the first two days of life, the mucosal epithelial barrier is not sealed, allowing the high amount of intact

macromolecules to reach systemic circulation (Bines and Walker, 1991). Speeded up by the maturation factors in the colostrum, the intestinal barrier closes, thus preventing overwhelming infection or inappropriate sensitization. During this critical period, the newborn can easily absorb any of the other important macromolecules from colostrum, which could positively influence the development of distant organ systems, such as central nervous system (Kverka et al., 2007). Furthermore, oral feeding per se provides important stimulus for mucosal immune development by influencing the gut microbiota composition and providing antigenic stimulation needed for fast population of LP with plasmocytes (Knox, 1986).

The IELs gradually expands until they reach the adult levels by 2 years of life, with preferential increase in αβ T cells over γδ T cells, ultimately leading to dominance of these T cells in IEL compartment (Cerf-Bensussan and Guy-Grand, 1991). Lack of the antigen is keeping the antigen-presenting cells in gut mucosa quiescent. Antigenic stimulation after birth drives the T cells to produce pro-inflammatory cytokines (most notably IFN-γ and IL-2) that stimulate the APCs to upregulate the MHC class II molecules (HLA-DR). This is probably the key element determining the immunologically reactive epitopes and cause of the above mentioned oligoclonality (Rognum et al., 1992; Lionetti et al., 1993; Gleeson and Cripps, 2004). In response to the bacterial colonization, the LP is started to be populated with IgM expressing plasmatic cells, shortly followed by IgA expressing plasmocytes, and since approximately 1 month after birth the latter starts to dominate (Perkkio and Savilahti, 1980).

1.2 HOST-MICROBE INTERACTIONS IN THE GUT

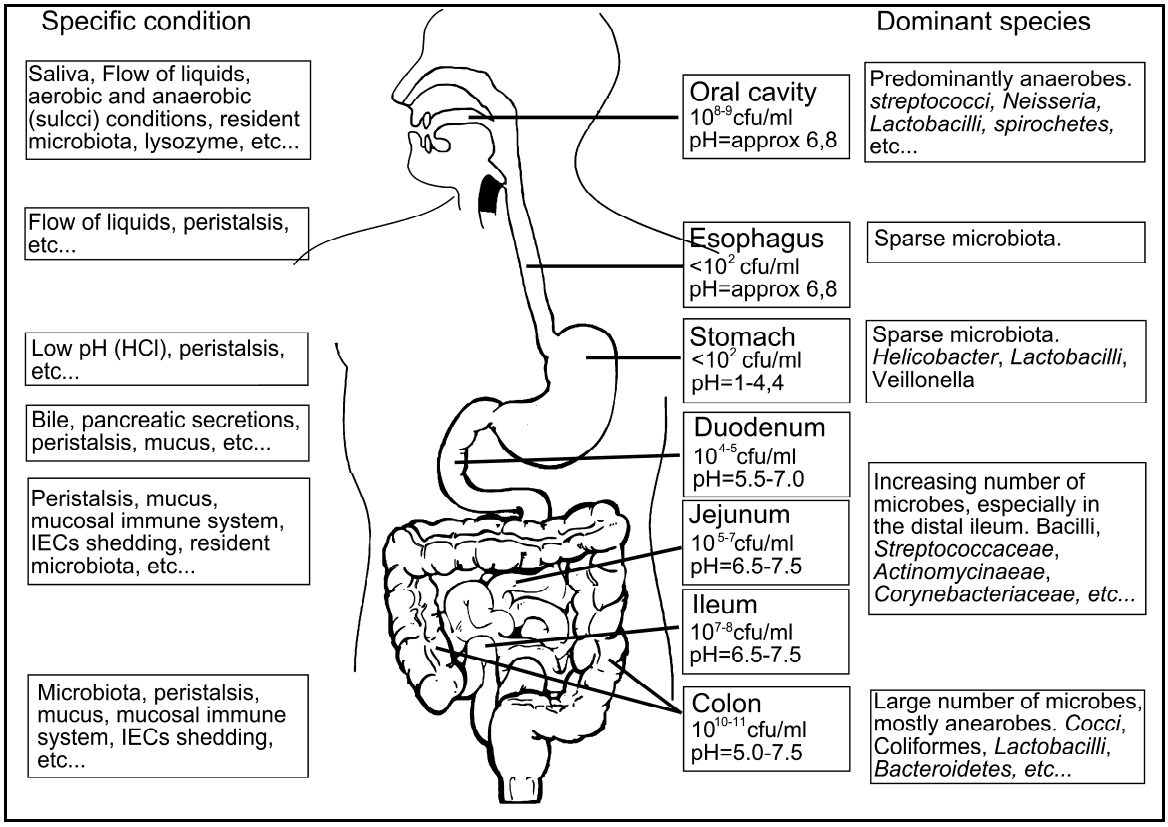
In all animals, microbiota forms a complex and open ecosystem, comprises of both resident and transiently present microbes. This ecosystem is formed by 10^{14} of microbes belonging to approximately 500 species of Eubacteria kingdom and similar number of species derived from Archibacteria and Fungi (Kuhbacher et al., 2006; Scupham et al., 2006). Other classes of microbes in this ecosystem, such as the viruses or protists have not been systematically studied so far. Vast majority (>99%) of these microbes is present in the gastrointestinal system.

1.2.1 Gut microbial community

The numbers and species distribution in various parts of the gastrointestinal tract differs, as shown on the Figure 1.3., depending on the prevailing specific conditions (Savage, 2005).

There is a consistent crosstalk between the microbiota and the host and among microbes themselves. Some bacteria can induce host gene expression to create more suitable environment for themselves. For example, when reaching critical density, common gut commensal *B. thetaiotaomicron* can enhances the synthesis of fucosylated glucoconjugates in the gut epithelium, which can than utilize as a source of energy (Bry et al., 1996). This strategy improves its chances to gather enough nutrition improving its chances to survive in the competitive ecosystem.

Figure 1.3 Specification of bacterial communities in various parts of the alimentary canal and specific local conditions that shapes them.



Adopted from Acheson and Nataro (2006)

The microbiota composition is not entirely stable throughout the life. There are two key time periods shaping the gut microbiota composition: 1) birth and 2) weaning.

Fetal gut is sterile until it is colonized by microbiota at birth. The colonization with microbiota continues during first few years of life and after this period, the individual's resident microbiota composition became relatively stable. The exact composition of the gut microbiota is shaped by the unique conditions found in the gut, but it also reflects the individual's environment, especially during the first months of life. It is strongly influenced by the individual genotype, mode of delivery, diet composition, ingestion of pathogenic and non-pathogenic microbes, hygiene levels and medication (Zoetendal et al., 2001; Mountzouris et al., 2002; Fanaro et al., 2003).

Few days after birth, the infant gut is colonized by enterobacteriacae and bifidobacteria, but they are soon influenced by feeding habits. Breast-fed infants develop microbiota rich in bifidobacteria and poor on clostridia and bacteroides, formula fed babies develop microbiota poor on bifidobacteria with wider range of anaerobes (Fanaro et al., 2003). After weaning, the microbiota became quite stable in each individual, with predominance of Firmicutes and Bacteroidetes species (Savage, 2005). It seems that the observed stability is on the species level, but not on the strain level, which could fluctuate unpredictably (McCartney et al., 1996). To some extent, the microbiota composition also vary with age, with decreased *Bifidobacterium* and increased *Bacteroides* species diversity (Hopkins et al., 2002).

1.2.2 The role of gut microbiota in human physiology

Gut microbiota has several functions important for human health. Gut microbiota contribute to the host energy recovery from the diet, which could represent 18% of total energy intake in rodents and much more in ruminants (Wostmann et al., 1983). The effectiveness of the energy recovery seems to be dependent on the microbiota composition, which could give important advantage to some individuals during the outbreaks of famine but it might be the cause of the obesity in some individuals (Turnbaugh et al., 2009).

This symbiosis between host and its microbiota goes all the way to the genome level. In a metagenomic study, Gill et al (2006) compared metabolic repertoire of gut microbiota with those of humans finding, that resident bacteria genome is significantly enriched for metabolism of amminoacids, glycans, xenobiotics and biosynthesis of vitamins and isoprenoids, while human genome is enriched for transport and metabolism

of inorganic ions and secondary metabolites. This reveal that the resident microbiota has evolved (or is regulated) to complement metabolic functions of the human gut.

Studies on gnotobiotic (germ-free; GF) animals clearly showed that microbial colonization has profound influence on the immune system development (Tlaskalova et al., 1970). Therefore, the variation in microbiota composition could be the reason for the well documented variation in immune responses (O'Hara and Shanahan, 2006). Colonization with bacteria increases the production of Ig in general and of specific antibodies, substantially changing the gut lymphocyte populations, thus improving the host's ability to fight pathogens (Weinstein and Cebra, 1991; Cebra, 1999). Some of these changes could be imitated by bacterial components in the food (Tlaskalova-Hogenova et al., 1983; Hrncir et al., 2008)

Even simple presence of the massive amount of bacteria in the gut greatly increases the host's resistance against pathogens (Zachar and Savage, 1979). This "colonization resistance" is executed by several mechanisms. Commensal bacteria compete with pathogens for limited resources and adhesion sites and interfere with pathogen‘s multiplication by decreasing the local pH via the production of organic acids and the production of specific antibacterial substances as recently reviewed by Stecher and Hardt (2008).

1.2.3 Probiotics

Food and Agriculture Organization of the United Nations and World Health Organization defined probiotics in the 2001 report as "live microorganisms which when administered in adequate amounts confer a health benefit on the host."(FAO/WHO, 2001).

There are three main mechanisms, how probiotics contribute to human health and single probiotic bacterium could possess more than one. First, probiotics may exclude or inhibit the growth of certain pathogens, as mentioned earlier. Second, they may improve the barrier function, and third, they can modulate mucosal and/or systemic immune response (Lebeer et al., 2010).

Anti-microbial properties of probiotics are probably the best known mechanism. Probiotics, and some gut commensals as well, can produce H_2O_2, organic acids,

biosurfactants and antimicrobial molecules that are capable, more or less selectively, kill other bacteria as reviewed elsewhere (Servin, 2004).

Certain probiotic bacteria may stabilize gut barrier function by increasing the production of antimicrobial peptide β-defensin 2 in the host's IECs (Wehkamp et al., 2004; Schlee et al., 2008). This mechanism could be triggered by PRR-ligands, such as flagellin, through NF-κB- and AP-1-dependent pathways (Schlee et al., 2007). Moreover, while *Lactobacillus* strain that is known to adhere to the IECs can induce mucin production *in virto* in these cells, another *Lactobacillus* strain that does not adhere cannot (Mack et al., 2003). Furthermore, *L. rhamnosus* GG produce two soluble proteins that inhibit cytokine-induced apoptosis in IECs *in vitro*, which could strengthen the intestinal barrier function even during ongoing inflammation (Yan et al., 2007).

Probiotics have been shown to promote both pro-inflammatory (IL-12) and anti-inflammatory (IL-10) mechanisms in human PBMC or monocytes *in vitro*. Their ability to induce either one is, however, strain dependent and may be still present in heat killed bacteria (Hessle et al., 1999). Furthermore, *L. reuteri* and *L. casei*, but not *L. plantarum*, are able to prime DCs to drive the development of T_{regs} by binding to their surface C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (Smits et al., 2005). Hence, the clinical utility of probiotics depends greatly on the actual health status of the macroorganism and on whether we want to improve its resistance to pathogens or dampen the pathological inflammation.

The therapeutic potential of probiotics, suggested by *in vitro* and *in vivo* studies, is very broad, but at this time, the clinical indications based on clinical studies are much narrower (Goldin and Gorbach, 2008). On one hand, probiotics have been successfully used to treat several human diseases, including antibiotic-associated diarrhea, traveler's diarrhea or ulcerative colitis (Oksanen et al., 1990; Arvola et al., 1999; Kruis et al., 2004), but in critically ill patients, live probiotic bacteria may be dangerous (Besselink et al., 2008).

1.3 ANIMAL MODELS OF IBD

The two main forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and Ulcerative colitis (UC), are both complex chronic inflammatory diseases, each associated

with a typical phenotype. The description and pathogenesis of IBD is in details reviewed in chapter 3.

These mechanisms of IBD pathogenesis were, from large part, elucidated using rodent models of IBD. Although there are significant differences among species, as mentioned above, these animal models of human diseases are still crucial for understanding the diseases and for therapy development. There are several dozens of IBD models, but most of them are based on one of two crucial steps in the IBD pathogenesis a) intestinal barrier disruption or b) impaired regulation of immune system. From the different point of view most of these models could be roughly classified as chemically induced animal models, models in genetically modified animals, spontaneous models, and adoptive transfer models (see Table 1.5).

Table 1.5 Summary of the most commonly used rodent models of IBD

Model		Description	Reference
Chemically induced models	DSS-induced colitis	Mixed Th1/2-type of colitis caused by intestinal barrier disruption.	(Okayasu et al., 1990)
	TNBS-induced colitis	Th1-type of the colitis caused by destruction of the mucosal barrier by ethanol followed by hapten-induced delayed-type hypersensitivity.	(Morris et al., 1989)
	Oxazolone-induced colitis	Atypic Th2-type of the colitis caused by destruction of the mucosal barrier by ethanol followed by hapten-induced delayed-type hypersensitivity.	(Boirivant et al., 1998)
Genetically modified animals (gene knockout or transgenic)	IL-10 KO	Intestinal inflammation (affecting small intestine and ascending colon) due to the impaired immunoregulation caused by the loss of IL-10 regulatory function.	(Kuhn et al., 1993)
	Interleukin-2 KO/IL-2 receptor (R)α KO mice	Colitis affecting distal colon caused by Impaired immunoregulation due to the defective apoptosis of activated lymphocytes in the gut.	(Sadlack et al., 1993)
	TCR mutant mice	Th2-type colitis due to the defect in antigen presentation.	(Mombaerts et al., 1993)
	HLA B27 transgenic rats	Rats transgenic for human HLA-B27 develop chronic inflammation ranging from the stomach to colon.	(Hammer et al., 1990)
Spontaneous colitis models	C3H/HejBir mice	Mice occasionally develop transient Th1 type of colitis caused by impaired regulation of microbiota sensing by Th cells.	(Cong et al., 1998)
	SAMP1/Yit mice	Spontaneously develop chronic Th1-type terminal ileitis and some even develop perianal fistulae similarily as in Crohn's disease.	(Rivera-Nieves et al., 2003)
Adoptive transfer models	$CD4^+CD45RB^{hi}$/ $CD62L^+$ to SCID mice	Th1-type of transmural colitis caused by the impaired immunoregulation caused by the shift in Effector T cell/regulatory T cell balance.	(Morrissey et al., 1993; Powrie et al., 1993; Mudter et al., 2002)

Further details and less common models were reviewed by Jurjus et al (2004)

The presence and quality of the intestinal microbiota often plays a crucial role, because the intestinal inflammation is often less severe or even fails to develop in the GF conditions (Sadlack et al., 1993; Dianda et al., 1997; Hudcovic et al., 2001). Furthermore, colonization with specific bacterial species or limited microbiota may not make mice more susceptible to disease compared to the GF conditions, therefore the presence of specific bacteria and not bacteria *per se*, is needed for the disease to develop (Sadlack et al., 1993; Rath et al., 1996; Dianda et al., 1997; Hudcovic et al., 2007; Stepankova et al., 2007). These studies show an important fact, that intestinal inflammation does not develop spontaneously and that the presence of the microbiota is often an integral part of the animal model explaining the problems to reproduce some animal models in different laboratories. In order to perform the experiments described in this thesis, we used the dextran-sulfate sodium (DSS)-induced model of intestinal inflammation. Since it was first described by Okayasu et al. (1990), the DSS colitis became one of the most commonly used animal models due to its simplicity, high reproducibility and fast induction of the inflammation. It has been successfully used in rats, hamsters, guinea pigs and many strains of mice. The clinical features of this model include weight loss, loose stools/diarrhea, and rectal bleeding. The histological evaluation of the colon shows severe ulceration, significant granulocyte and mononuclear infiltrate ranging from lamina propria to submucosa, tissue edema, and in chronic phase even dysplasia. These features resemble the ulcerative colitis.

The inflammation is induced by replacing the drinking water with 3% DSS solution of approx. 40-50kD DSS. Interestingly, almost every laboratory use own "titrated" concentration, usually ranging from 2-5%, which achieve best results. This further stress the importance of the gut microbiota composition in the animal models mentioned above. This model also could be use to mimic the chronic ulcerative colitis, if the DSS is administered in several 5-7 day long cycles followed by cycles of tap water (Okayasu et al., 1990). If it is not severe, most mice have a tendency to recover from the acute DSS-induced colitis within several weeks, but it has been reported that in C57BL/6 mice the inflammation became chronic after one cycle of DSS (Melgar et al., 2005).

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 1st day after DSS treatment by gradual decrease in tight junction protein - ZO-1 - production, which results in increase of colon permeability (Poritz et al., 2007). Furthermore, Vetsuchi et al. (2002)

reported that there is a significant increase in epithelial cell apoptosis in DSS treated rats, and that this apoptosis is soon followed by dramatically increased proliferation, suggesting that a) injury to the epithelial cell destruction leads to the barrier dysfunction and b) that abnormal and persistent epithelial hyperproliferation could lead to the development of colorectal cancers. The finding, that long-term DSS administration could produces colorectal carcinoma in mice or hamsters has been also reported earlier. (Yamada et al., 1992; Cooper et al., 1993)

As a consequence of the enhanced epithelial barrier permeability and immune cell infiltration, epithelial and immune cells produce pro-inflammatory cytokines. There is a significant increase in mucosal expression of TNF-α, IL-1β, IFN-γ, IL-10 and IL-12, as early as the 1st day of DSS treatment (Yan et al., 2009).

It had been proposed that acute DSS colitis is driven mainly by innate immunity, because it also occurred in the absence of functional T, B and NK cells (Dieleman et al., 1994; Axelsson et al., 1996). During the chronic phase, lymphocytes and macrophages attracted to the site of inflammation secrete high levels of both interferon (IFN)-γ and IL-4, which suggests that activation of both Th1 and Th2 play a pathogenic role in chronic DSS-induced colitis (Dieleman et al., 1998).

1.4 DOWNREGULATING THE INFLAMMATORY RESPONSE

Besides the immunomodulation performed by regulatory and antigen-presenting cells, as described above, the inflammation could be also downregulated directly or indirectly by glucocorticoids (GCs). GCs are steroid hormones that are able to modulate various functions of the immune system. Both natural and synthetic GCs have the capacity to dampen the inflammation and these compounds are therefore, used as anti-inflammatory agents in many immune-mediated diseases since 1940s (Hench et al., 1949). In order to transmit their message, steroids need to bind to an intracellular receptor, which in response dissociate from the anchoring multiprotein complex and translocate to the nucleus. Here it binds to specific DNA sequences of the target genes, called glucocorticoid response element, and regulates gene transcription (Pratt, 1993). In addition to these mechanisms, GCs can interact with cell membrane-associated proteins (e.g. ion channels) through physiochemical or (possibly) receptor-mediated interaction, although the membrane GC receptor has not been found in normal human cells so far

(Buttgereit et al., 1998). These interactions may cause the changes in intracellular signaling pathways tuning and cytoskeleton reorganization, resulting in the impairment of cell adhesion and locomotion. The effectiveness of these mechanisms depends on intracellular concentration of GCs, which is determined by their extracellular concentration and their local metabolism.

1.4.1 Prereceptor regulation of glucocorticoid function

The local metabolism of GCs is controlled by the intracellular enzyme 11β-hydroxysteroid dehydrogenase (11HSD), which has two isoforms with different characteristics– 11HSD1 and 11HSD2 (Table 1.6) (Draper and Stewart, 2005).

Table 1.6 Direct comparison between the characteristics of human 11HSD1 and 11HSD2 isozymes

	11HSD1	11HSD2
Chromosomal location	1q32·2	16q22
Size of the gene	30 kb, 6 exons	6·2 kb, 5 exons
Size of the protein	292 aa, 34 kDa	405 aa, 44 kDa
Main tissue expression	Liver, lung, gonads, brain, adipose tissue	Kidney, colon, salivary glands, placenta
Enzyme kinetics	Bidirectional (*in vitro*), mainly reductase (*in vivo*)	Only dehydrogenase
Cofactor	NADP(H)	NAD
Main function	Supplies cortisol to glucocorticoid receptor	Protects mineralocorticoid receptor from cortisol

Adopted from (Draper and Stewart, 2005)

The 11HSD2 isoform is a unidirectional, NAD-dependant dehydrogenase, converting the biologically active cortisol (in humans) and corticosterone (in rodents) into their inactive 11-oxo derivatives, cortisone and 11-dehydrocorticosterone respectively. Since cortisol and corticosterone, have similar affinity to mineralocorticoid receptor as natural mineralocorticoid aldosterone, and the circulating levels of GCs are several orders of magnitude higher than those of aldosterone, the ability of 11HSD2 to inactivate glucocorticoids in some tissues is therefore vital for the individual's homeostasis (Arriza et al., 1988).

11HSD1 is a low affinity NADP(H)-dependent bi-directional enzyme, capable of carrying out both 11-oxo-reductase and dehydrogenase reactions, interconverting inactive cortisone and active cortisol. *In vivo*, it predominantly catalyzes the conversion of inactive GCs to their active counterparts (Draper and Stewart, 2005).

While increasing the dosage of GCs could improve their immunosuppressive function it also brings along severe side effects. Our understanding of the pre receptor control of their function could not only increase our knowledge on natural anti-inflammatory mechanisms, but it could also lead to more effective and less toxic anti-inflammatory therapy.

1.4.2 Regulation of the immune function with glucocorticoids

GCs are major immunomodulatory agents with profound influence on the function of the immune system. They positively (e.g. TLR-2 and TLR-4) or negatively (e.g. IL-1 and CD40) regulate 21% of the genes expressed in human leukocytes (Galon et al., 2002). As immunosuppressive agents, GCs interfere with the inflammatory process on several levels. First, they influence the leukocyte development, second, they inhibit the access of leukocytes to inflammatory sites, and third, they interfere with the functions of leukocytes, endothelial cells, and fibroblasts at the site of inflammation (Ashwell et al., 2000; Webster et al., 2002).

Most of the immunosuppressive functions of GCs are mediated by repression of NF-κB, which is a key pro-inflammatory and pro-immune transcription factor, involved in the regulation of cytokines and other immune responses (McKay and Cidlowski, 1999). The mechanisms, how this inhibition is executed are not completely elucidated. It has been shown that GCs induce expression of inhibitory protein IκB that prevents translocation of NF-κB to the nucleus and that there is a direct cross-talk between GC receptor and NF-κB that prevents gene expression (Mukaida et al., 1994; Ray and Prefontaine, 1994; Scheinman et al., 1995). Another anti-inflammatory and immunosuppressive mechanism of GC action involves interaction of GC receptor with transcription factor activator protein-1 (AP-1) altering its interactions with DNA and expression of pro-inflammatory factors (Jonat et al., 1990; Adcock et al., 1994).

GCs mediate their anti-inflammatory response by modulating the transcription of many pro-inflammatory cytokines, e.g. IL-1, IL-6, IFN-γ and TNF-α (Kunicka et al.,

1993; Steer et al., 2000). This effect could be either direct, by interfering with the cytokine mRNA or indirect, mediated by other cytokines or transcription factors. It has been shown that GCs can decrease the production of IL-6 via NF-κB-dependent downregulation of TNF-α, because the TNF-α itself potentiate the production of IL-6 (Lee et al., 1988; Steer et al., 2000; Vanden Berghe et al., 2000).

GCs reduce the trafficking of leukocytes to the areas of inflammation by interfering with the expression of protein molecules involved in both attraction and adhesion of leucocytes to the vessels in that areas. Indeed, GCs have been shown to inhibit the inflammation-induced expression of several adhesion molecules on the endothelium, including intracellular adhesion molecule 1 (ICAM-1), endothelial-leukocyte adhesion molecule 1 (ELAM-1), which prevents the recruitment of leucocytes at inflammatory loci (Cronstein et al., 1992). Moreover, GCs inhibits the chemotaxis to the inflamed loci by interfering with the chemokine production (e.g. IL-8, MCP-1) by the resident cells (Miyamasu et al., 1998).

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2. SIGNIFICANCE, AIMS AND OUTLINE OF THE THESIS

2.1 SIGNIFICANCE OF THE STUDY

The human body is surrounded by enormous amounts of microbes. Most of them are carried in the gut, where the mucosal surface is exposed to both potential pathogens and beneficial commensal microorganisms. On one hand, the strong immune response against the microbes was one of the main forces that drove human evolution in the past. On the other hand, it is remarkable, how healthy humans are able to control this immune system and tolerate huge and often unpredictable load of antigens in the gut. The peaceful coexistence of the host and gut microbiota has evolved for millennia and its importance for the human physiology is well acknowledged. However, the mechanisms, how our immune system is keeping the balance are not well understood. When this carefully balanced interaction between host immune system and gut microbiota is broken, severe inflammatory disease could emerge. Better understanding of these interactions is not only important for the understanding of the pathogenetic mechanisms of those diseases, but it could also give us the tools needed for the development of new strategies for their prevention and therapy.

2.2 AIMS AND OUTLINE OF THE THESIS

This thesis has two main aims, to promote the better understanding of the host-microbe interactions during the state of inflammation and to set up the basis for the development of new therapeutic strategies based on this understanding. To achieve these aims, we have addressed several particular questions outlined in this thesis. Our hypothesis is that if live bacteria play a role in the gut homeostasis, could we achieve similar effect with their components? And if so, which mechanisms of mucosal immune response are responsible for this effect?

Chapter 1 is review of current knowledge on mucosal immune system and related topics. In **chapter 3**, we review the pathogenetic mechanisms of IBD and how to influence the natural course of the disease by probiotics and bacterial components. In **chapter 4**, we discuss how the changes in microenvironment could change some important mediators of physiological and immune functions. In particular, what is the role of local metabolism of glucocorticoid and how might the pre-receptor regulation of

endogenous corticosteroids play a role in inflammatory processes. In **chapter 5**, we address the question if the preventive administration of live probiotic bacteria could decrease the severity of acute experimental colitis. This is further discussed in the **chapter 6**, where the hot question on whether the probiotic baceteria needs to be alive or not in order to have beneficial properties is discussed. While the use of well established probiotics seems to be an obvious choice, another question arises. While the effect of probiotics is strain specific, why most healthy individuals lack it. Is it simple lack of knowledge or could these properties be achieved by some common commensal? This question, and underlined mechanisms, are addressed in **chapter 7**, where we showed that oral treatment of mice with lysate of one particular commensal, *Parabacteroides distasonis*, could have similar beneficial effect. In **chapter 8** a general discussion is presented on the impact of these novel findings by us and others on bacterial components as therapeutic agents.

3. PROBIOTICS AND BACTERIAL COMPONENTS IN INTESTINAL INFLAMMATION THERAPY AND PREVENTION.

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3.1 SUMMARY

Crohn's disease (CD) and ulcerative Colitis (UC), the two major forms of inflammatory bowel disease (IBD), are both severe chronic inflammatory disorders. Although the exact etiology and pathogenesis of both forms of IBD have yet to be completely understood, it is widely accepted that they result from a continuous microbial antigenic stimulation of pathogenic immune response in genetically predisposed individuals. Genome-wide association studies identified several defects in genes responsible for mucosal barrier function, bacterial sensing and killing and for the regulation of the inflammatory response. The changes in microbiota composition or abilities (epithelial adhesion and invasion) are supposed to be the trigger of the inflammation. These findings are further supported by conclusions of studies in both humans and experimental animals. Those studies showed that impaired host reaction to commensal microbiota, or their abundant presence in the subepithelial layer, leads to the pathological stimulation of the mucosal immune system.

Several clinical studies as well as some interventional studies on animal models demonstrated that antibiotics, probiotics and bacterial components are useful in maintaining disease remission and in disease prevention. This effect is partially due to the changes in microbiota composition and partially due to imunomodulation.
During many years of co-evolution with humans, the microbiota, indigenous as well as pathogenic, have acquired immunomodulatory mechanisms to bypass our mechanisms of protective immunity. Our aim is to isolate the immunomodulatory components from bacteria and use them in IBD therapy and prevention. Compared to use of live bacteria, this approach seems to be safer and easily applicable in practice. The differences in immunomodulatory properties of these components also suggest the need of individualized therapy. This review will focus on IBD pathogenesis and the possibility of influencing it by therapy with bacterial components.

3.2 INTRODUCTION

The two major forms of inflammatory bowel disease (IBD), Crohn’s disease and ulcerative colitis, are both severe relapsing inflammatory diseases of the intestine, each associated with a typical phenotype. Ulcerative colitis is characterized by diffuse continuous mucosal inflammation that extends proximally from the rectum to a varying

degree. Although it is located only in the large intestine, in some patients, the terminal ileum is also affected (so-called backwash ileitis). Patients typically suffer from bloody diarrhea, abdominal pain, rectal bleeding and malnutrition. Crohn's disease is characterized by segmental and transmural inflammation, fistulas and granulomas in any part of the gastrointestinal tract, most commonly in terminal ileum. Crohn's disease leads to strictures, abscesses and fistulas and the clinical manifestation depends mainly on the disease localization. The IBD usually starts to manifest in the second and third decades of life and the majority of affected individuals progress to chronic relapsing disease (Baumgart and Sandborn, 2007).

The IBD is a systemic disorder and in almost half of the patients other organs are affected by the disease as the extraintestinal disease manifestation or disease (therapy) complications. These manifestations and complications typically affect the musculosceletal system (peripheral arthritis, ankylosing spondylitis and osteoporosis), skin (erythema nodosum, pyoderma gangrenosum and fistulas), eye (iritis and uveitis) and biliary system (primary sclerotising cholangiopathy and gall stones) (Rothfuss et al., 2006).

The highest prevalence of IBD is traditionally in North America, northern Europe and the United Kingdom, with averages ranging from 100 to 300 cases per 100 000 (Ehlin et al., 2003; Loftus, 2004). Recent epidemiological studies suggest that the increase in disease incidence has probably reached its plateau in these countries, however, there is a strong increase in IBD incidence in regions with traditionally low IBD prevalence (e.g. Asia and Latin America) (Linares de la Cal et al., 1999; Lee et al., 2000; Yang et al., 2000; Yang et al., 2008).

Although the etiopathogenesis of the IBD remains obscure, IBD is thought to be a result of uncontrolled inflammatory response to indigenous intestinal microbiota in genetically predisposed individuals. The genetic predisposition is associated with genes related to host bacteria interaction, suggesting the crucial importance of intestinal microbiota in IBD pathogenesis. General intestinal dysbiosis, presence of some pathogenic bacteria or even enhanced virulence of certain commensal bacteria were all proposed to be the trigger of the IBD (Chiodini, 1989; Darfeuille-Michaud et al., 1998; Swidsinski et al., 2002; Seksik et al., 2003; Frank et al., 2007; Rabizadeh et al., 2007). The importance of intestinal microecology is further supported by the finding that manipulating intestinal microbiota using probiotics and antibiotics is effective in IBD therapy (Guslandi et al., 2000; Kruis et al., 2004; Rahimi et al., 2007).

Some strains of probiotic bacteria also possess immunomodulatory properties, so their effect in IBD therapy could be mediated by modulation of the mucosal immune system or intestinal barrier function, rather than by intestinal ecology changes (Damaskos and Kolios, 2008). Therefore, we could use bacterial lysates or isolated bacterial components to mimic the therapeutic effect that probiotics have. Use of sterile bacterial components with immunomodulatory properties seems to be a safer and more practical approach than the use of live bacteria.

3.3 IBD PATHOGENESIS

The luminal antigens can initiate the pathogenetic inflammatory cascade in the gut only after four conditions are met. First, the host's mucosal immune system must be genetically susceptible to recognize the antigens from indigenous microbiota and misinterpret them as potentially harmful. Second, the antigen must reach the gastrointestinal tract. Third, the antigen must pass through the intestinal barrier to reach the immunocompetent cells in the mucosa. And finally, the regulatory mechanisms of mucosal immune system must fail to control the inflammation.

3.3.1 Genetics of IBD

Family aggregation of IBD is a well known phenomenon; the life-time risk of developing IBD for first-degree relatives of a CD proband is 5 % and that of an UC proband 1.6% in white non-Jewish Euro-American population (8 % and 5.2% for Jews). The concordance in monozygotic twins is 28% and 16% for CD and UC respectively, and 4% for dizygotic twins for each (Halfvarson et al., 2003; Halme et al., 2006). On one hand, these data clearly indicate that genetic factors definitely contribute to IBD, but also that the environmental and developmental factors are more important in disease pathogenesis.

Genome-wide association studies for IBD susceptibility genes performed in the last few years have reported many genes that contribute to disease susceptibility. Interestingly, most of these newly identified genes are related to the epithelial barrier permeability, bacteria sensing and killing, and regulation of the immune system (Duerr et al., 2006; Rioux et al., 2007). All these findings fit well into the current concept of IBD pathogenesis and stress the importance of host-microbiota interaction.

3.3.2 Microbiota in IBD

Humans are colonized by huge number of microbes; microbial cells form 90% (10^{14}) of all cells in adult humans (Savage, 1977). This colonization starts during the birth and continue during the first few years of life. After this period, the composition of the individual microbiota seems to be relatively stable. Although there are some representatives from archaea and eukarya, as well as viruses and bacteriophages, most members of gut microorganisms belong to the domain bacteria (Breitbart et al., 2003; Eckburg et al., 2003; Curtis and Sloan, 2004). Over 98% of all gut bacteria in mammals belong to the two divisions of Bacteria - Firmicutes and Bacteroidetes (Eckburg et al., 2005). Although this uniformity probably reflects the specific conditions in the gut, the differences on a species level is striking, as it resembles an individual fingerprint. The diversity of individual microbiota is caused by many variables such as dietary habits, use of pharmaceuticals, health status and by microbial exposure of both mother and child during the child's perinatal and infant period. Introduction of probiotic bacteria during this period could result in long term colonisation and could have impact on health later in adult life (Kalliomaki et al., 2001; Lodinova-Zadnikova et al., 2003).

Although the microbiota composition has a distinctive pattern in every individual, under physiological conditions it serves important biological functions for all hosts. The microbiota prevent colonization with pathogens, providing important nutrients (vitamins and short-chain fatty acids), and modulate intestinal barrier maturation and development of the immune system (Chapman, 2001; Hooper et al., 2002; Tlaskalova-Hogenova et al., 2004). Luminal microbiota also positively influence the development and preservation of oral tolerance in a strain-dependent manner (Gaboriau-Routhiau and Moreau, 1996; Moreau and Gaboriau-Routhiau, 1996; Prioult et al., 2003).

The close link of intestinal inflammation to microbiota was proposed many years ago and later proven with animal models of IBD, which showed that intestinal inflammation is much milder or even fails to develop, if animals are reared under germ-free conditions (Sellon et al., 1998; Hudcovic et al., 2001; Stepankova et al., 2007). These findings led to closer investigation of microbiota composition in IBD patients. Many studies initially focused on searching an individual pathogen responsible for IBD. The pathological similarity of Johne's disease in cattle to Crohn's disease in humans led to a proposition that *Mycobacterium avium* subsp. paratuberculosis (MAP) is the causative agent of IBD. This hypothesis was supported by finding of MAP in inflamed tissue of CD

patients (Chiodini et al., 1984; Sanderson et al., 1992; Fidler et al., 1994). Although there were reservations about these studies, recent meta-analysis confirmed specific association of MAP with CD (Feller et al., 2007). The controlled trials for the therapy of CD with antimycobacterial drugs failed, so the question how this bacterium could influence the IBD pathogenesis remains unclear (Goodgame et al., 2001; Selby et al., 2007).

Several ecological studies of gut microbiota showed that there is a difference in microbiota composition in IBD patients compared to healthy individuals. This dysbiosis or increase in some bacterial group was proposed to cause or at least perpetuate the intestinal inflammation in IBD. These studies also identified some candidate bacteria that could be responsible for the IBD development in susceptible individuals (Seksik et al., 2003; Sokol et al., 2006). All these studies must be interpreted with caution, because the real trigger might be only transient and changes we are detecting are just secondary. There are also changes in mucosa-associated bacteria, suggesting the increased ability of bacteria to adhere to mucosa or some other changes in the bacterial metabolism could be responsible for IBD triggering (Darfeuille-Michaud et al., 1998; Swidsinski et al., 2002). To date, the question remains open whether these bacteria are introduced into the gut from the outside environment, or whether this new ability is introduced to intestinal microbial society by horizontal gene transfer or by another, yet unknown, signal.

Although we do not completely understand the natural relations between intestinal microbiota, the success of antibiotics and probiotics in IBD therapy clearly shows, that manipulation with intestinal microecology might be the future treatment of at least some forms of IBD.

3.3.3 Intestinal barrier function failure

Several studies reported that the intestinal permeability is increased in inflamed as well as in noninflamed IBD mucosa of patients and even in first degree relatives of CD patients (Jenkins et al., 1988; Katz et al., 1989). These findings clearly demonstrate the importance of the intestinal barrier function, and its genetic control, in IBD pathogenesis. Increased intestinal permeability also has been shown useful in the prediction of relapse in asymptomatic CD patients (Wyatt et al., 1993; D'Inca et al., 1999).

Several mechanisms might be involved in increased gut permeability. First, there is a defect in mucous production in IBD patients (Buisine et al., 2001). Mucus forms a

rather thick layer (approximately 100 μm in jejunum and over 800 μm in colon) on the gut epithelium (Atuma et al., 2001). This layer acts as a mechanical and antimicrobial barrier protecting the underlying epithelium. In healthy individuals, this layer contains high concentrations of secreted IgA, lysozyme and other antimicrobial components keeping the epithelial surface free of bacteria.

Defensins, antimicrobial peptides produced by the Paneth cells in the base of the Lieberkühn's crypts, are concentrated in the mucus layer protecting vulnerable epithelium from invasive bacteria, yet allowing the presence of harmless enteric microbiota (Meyer-Hoffert et al., 2008). The production of defensins is, however, significantly decreased in terminal ileum of CD patients, which may result in aberrant ileum colonisation causing the inflammation (Wehkamp et al., 2004; Wehkamp et al., 2007).

Another mechanism, capable to increase the intestinal permeability is a downregulation of tight (ZO-1 and occludin) and addherens (E-cadherin and α-catenin) junctions' proteins in the epithelium of IBD patients. The degree of this downregulation positively correlates with degree of inflammation, showing the importance of these proteins for intestinal epithelium integrity (Gassler et al., 2001).

Another explanation for the increased intestinal permeability in IBD is increase of extracellular matrix degrading endopeptidases - matrix metalloproteinases (MMP). These enzymes were found upregulated in the inflamed gut tissue of IBD patients causing mucosal degradation and ulceration (Heuschkel et al., 2000). They are produced by activated gut myofibroblasts, macrophages and resident plasma cells in the presence of proinflammatory cytokines IL-1β and TNF-α (Okuno et al., 2002; Gordon et al., 2008). Although the defect in intestinal barrier function could be the initial defect in IBD pathogenesis, the production of TNF-α and IFN-γ secondary to the inflammation perpetuates the increased intestinal permeability by reorganizing the tight junction, causing further leakage of luminal content to submucosa (Ma et al., 2005; Wang et al., 2005). This way, the vicious circle of inflammation is created.

3.3.4 Mucosal immune system disregulation

In healthy subjects, there is an immunological tolerance to intestinal flora from autologous but not heterologous intestine. This tolerance is, however, broken during intestinal inflammation (Duchmann et al., 1995).

There is an increase of activated macrophages and dendritic cells (DCs) and changes in cytokine production in the intestinal mucosa of IBD patients. The cytokine profiles are, however, unique for each form of IBD. While proinflammatory cytokines TNF-α, IL-1β and IL-6 are increased in both forms, the increase of IFN-γ, IL-12, IL-17, IL-23 and IL-27 are specific for CD and increase of IL-5 and IL-13 for UC (Fujino et al., 2003; Fuss et al., 2004; Schmidt et al., 2005). On the other hand, the deficiency in IL-10 and TGF-β signaling in the intestine might contribute to the development of IBD, because both cytokines are important in directing naïve T cell maturation to a regulatory pathway (Hahm et al., 2001). Moreover, local delivery of IL-10 with genetically engeneered bacteria, *Lactococus lactis* producing IL-10, shown good results in experimental colitis therapy (Steidler et al., 2000).

Even under normal physiological condition, the microbes in the intestine make contact with the host's immune system without inducing inflammation. It has been shown, that DCs in the lamina propria actively sample the gut lumen, but also there is active intake of IgA coated bacteria to the Peyer's patches through the M cells (Rescigno et al., 2001; Mantis et al., 2002).

There is also evidence that live commensal bacteria are shuttled by DC through the Payer's patches to mesenterial lymph nodes causing specific mucosal, but not systemic IgA response (Macpherson and Uhr, 2004).

All these observations shows that at least some intestinal microbes make contact with the mucosal immune system, but also raise the question of why this contact does not cause the inflammation as the loss of the intestinal barrier does. The answer is not known but it is thought, that the amount of antigen is too small to trigger the inflammation or it is well "guarded" by the unresponsive innate immunity cells. Once there is a big load of bacteria in the mucosa due to the barrier failure or the cells are more susceptible to the inflammatory response due to the defect in bacteria sensing, the mucosal immune cells overcome their unresponsiveness and the inflammation starts. Three immune mechanisms are involved in this process: defect in microbe sensing by the resident mucosal cells, the accumulation of the effector cell in the mucosa and a loss of the local tolerogenic signals.

3.3.4.1 Microbe sensing by the resident mucosal cells

Luminal antigens are continuously sampled by intestinal epithelium as well as by cells of innate immunity such as DCs using several evolutionarily conserved and structurally

related receptors, pattern recognition receptors (PRR). These receptors could be membrane-bound (e.g. Toll like receptor (TLR) 1, 2, 4, 5, 6, 10 or membrane bound CD14), residing in the cytoplasm (e.g. nucleotide-binding oligomerization domain containing (NOD) 2 protein, TLR 3, 7, 8 and 9) or even released from the cell (e.g. mannan-binding lectin or soluble CD14). These receptors are recognizing conserved structural motives on microbiota or microbe-associated molecular patterns such as lipopolysaccharide, peptidoglycan, lipoteichoic acide, single- and double-stranded RNA and methylated DNA (CpG-motives).

Interestingly, epithelial cells are also expressing PRRs and are able to be activated in response to microbes and produce cytokines. This way, the epithelium could deliver the inflammatory signals to underlying cells in lamina propria. In normal gut, the epithelial cells are activated by invading pathogens yet maintain the tolerance to resident bacteria (Duchmann et al., 1995).

Antigen-presenting cells (APCs; e.g. DCs and macrophages) are found in a resting (inactive) state in lamina propria and Peyer's patches of normal gut. In this state the cells do not respond to bacterial stimuli and pro-inflammatory cytokines (Smythies et al., 2005). They became activated with conserved structural motives, they migrate to the local lymph nodes and activate naive T cells. They also contribute to the T cell activation with production of IL-6, IL-12, IL-23 and TGF-β (Drakes et al., 2005). Interestingly, the expression of some of some PRR is dysregulated in the intestines of CD and UC patients. This may result in easier triggering of the inflammatory cascade in these individuals (Frolova et al., 2008). This is further stressed by the fact, that the IL-$10^{-/-}$ mice are resistant to spontaneous colitis, if they are lacking MyD88 - important adaptor protein in TLR signaling (Rakoff-Nahoum et al., 2006).

3.3.4.2 Accumulation of the effector cell in the mucosa

Pro-inflammatory molecules, responsible for tissue damage during the inflammation, are produced by effector cells that have accumulated to high numbers in the inflamed mucosa. There are two main mechanisms responsible for the accumulation of effector cells in the inflamed intestine: enhanced recruitment due to the upregulation of adhesion molecules or chemokines, and by increased cell recruitment and prolonged survival caused by decreased cellular apoptosis.

High levels of inflammatory cytokines (e.g. TNF-α, IL-1β and IL-6) in the mucosa of IBD patients increase the expression of chemokines and adhesion molecules (on endothelium as well as on circulating CD^+ cells) causing circulating leucocytes to adhere to endothelium and enter to the inflamed mucosa (Burgio et al., 1995; Garcia de Tena et al., 2006). The presence of TNF-α and IL-6 in the inflamed mucosa also mediates T-cell resistance against apoptosis causing their accumulation and further tissue damage (Atreya et al., 2000; Neurath et al., 2001).

3.3.4.3 Loss of the local tolerogenic signals

While innate immunity is activated in both forms of IBD, the T cell response differs. In CD, there is upregulation of Th1 (characterized by IFN-γ) and Th17 (characterized by IL-17) pathways. The Th1 response is iniciated by IL-12 and Th17 response is enhanced by the presence of IL-6, TGF-β and IL-23. These cytokines are produced by activated local APCs and other innate immunity cells upon bacterial colonization (Becker et al., 2003; Kamada et al., 2005). On the other hand, the UC is characterised by atypical Th2 response, with increase IL-5 and IL-13 produced by natural killer T (NK-T) cells. These NK-T cells are stimulated by APCs bearing nonclassical MHC class molecule CD1d, which is specialised in presenting the lipids (Fuss et al., 2004).

In normal gut, the inflammatory response of effector T cells is regulated by T regulatory cells. To date, there are three types of T regulatory cells identified in humans. Naturally occurring T regulatory cells (T_{reg}) originate in thymus and typically express transcription factor forkhead box P3 (FoxP3) and high levels of CD25. These cells regulate the effector cells with cell-cell contact. The other two types, regulatory T cell type 1 (Tr1) and T helper type 3 (Th3), originates in intestine and react to luminal antigens with production of IL-10 and TGF-β on bacteria or food proteins respectively. The function of T regulatory cells is under close control of APCs and local cytokine milieu as recently reviewed by *Belkaid* and *Oldenhove* (Belkaid and Oldenhove, 2008). Interestingly, the anti-inflammatory T_{REG} as well as pro-inflammatory Th17 cells could be induced locally in the intestinal mucosa from naïve T cells by TGF-β. The balance between these two functionally opposite subsets of T cells is kept with IL-6 and IL-21, because these cytokines promote the differentiation to Th17 by blocking the expression of FoxP3 in the naïve T cells (Fantini et al., 2007).

3.4 PROBIOTICS AND BACTERIAL COMPONENTS IN INTESTINAL INFLAMMATION THERAPY

Recent advances in our understanding of IBD pathogenesis and mucosal immune response regulation led to a proposal of novel strategies in intestinal inflammation therapy. As mentioned before, the indigenous microbiota is crucial for induction and perpetuation of intestinal inflammation and manipulation with microbiota composition with probiotics and antibiotics is possible approach in IBD therapy. However, each probiotic strain have unique effect on immune system, therefore deeper insight into the particular microbe-host interaction and careful choice of particular strain for particular function might be needed for successful therapy (Maassen et al., 2000).

Each strain could also have several mechanisms of action, interfering with one or more steps of IBD pathogenesis. Live probiotic bacteria could change composition of intestinal microbiota resulting in changes in host's sensitivity to inflammation. These changes could be mediated by simple competition with other microbes for limited resources and limited number of receptors or they could produce antimicrobial peptides or even increase the host antibody production against other microbes (Kaila et al., 1992; Lievin et al., 2000; Hutt et al., 2006). Moreover, probiotic *E. coli* Nissle 1917 and *Lactobacillus casei* DN-114 001 have inhibitory effect on adhesion and invasion of adherent invasive *E. coli* isolated from patients with Crohn's disease (Boudeau et al., 2003; Ingrassia et al., 2005). The mechanism of this action could also be explained by recent findings, that *L. acidophilus* secrete molecule(s) capable of downregulating expression of genes involved in attachment of enterohemorrhagic *E. coli* to gut mucosa (Medellin-Pena et al., 2007). This indicates that we might be able to convert adherent bacteria into non-adherent simply by administering this bacterial component to the gut. Furthermore, treatment with *L. casei* or mixture of probiotic bacteria VSL#3 results in an improvement of intestinal barrier integrity and thus prevents enteric antigens from excessive stimulation of lamina propria immune cells (Madsen et al., 2001; Llopis et al., 2005).

Several probiotics have shown immunomodulatory properties on basically all levels of regulation, including downregulation of the PRRs expression, NF-κB signaling and pro-inflammatory cytokine production (Matsumoto et al., 2005; Sturm et al., 2005; Grabig et al., 2006; Sougioultzis et al., 2006). Interestingly, some of these effects could be achieved by soluble factor produced by these bacteria or their lysates. Lysate of

Lactobacillus brevis and *Streptococcus thermophilus* could induce apoptosis in immune cells, which could reverse the insensitivity to apoptosis of lamina propria immune cells in IBD patients (Di Marzio et al., 2001). Oral administration of lysate prepared from normal intestinal flora containing anaerobes reduces the severity of acute experimental colitis in mice which suggests that there might be some bacterial components with special anti-inflammatory properties among commensals (Verdu et al., 2000).

Interestingly, also some well known pathogens could decrease the immune response by dampening inflammatory signals or inactivating immune cells which help them to evade host's immune system and establish infection or persistence (Ruckdeschel et al., 1997; Sansonetti and Di Santo, 2007; van der Velden et al., 2008). It would be therefore interesting to isolate the active component and to exploit it in the therapy of IBD with these components.

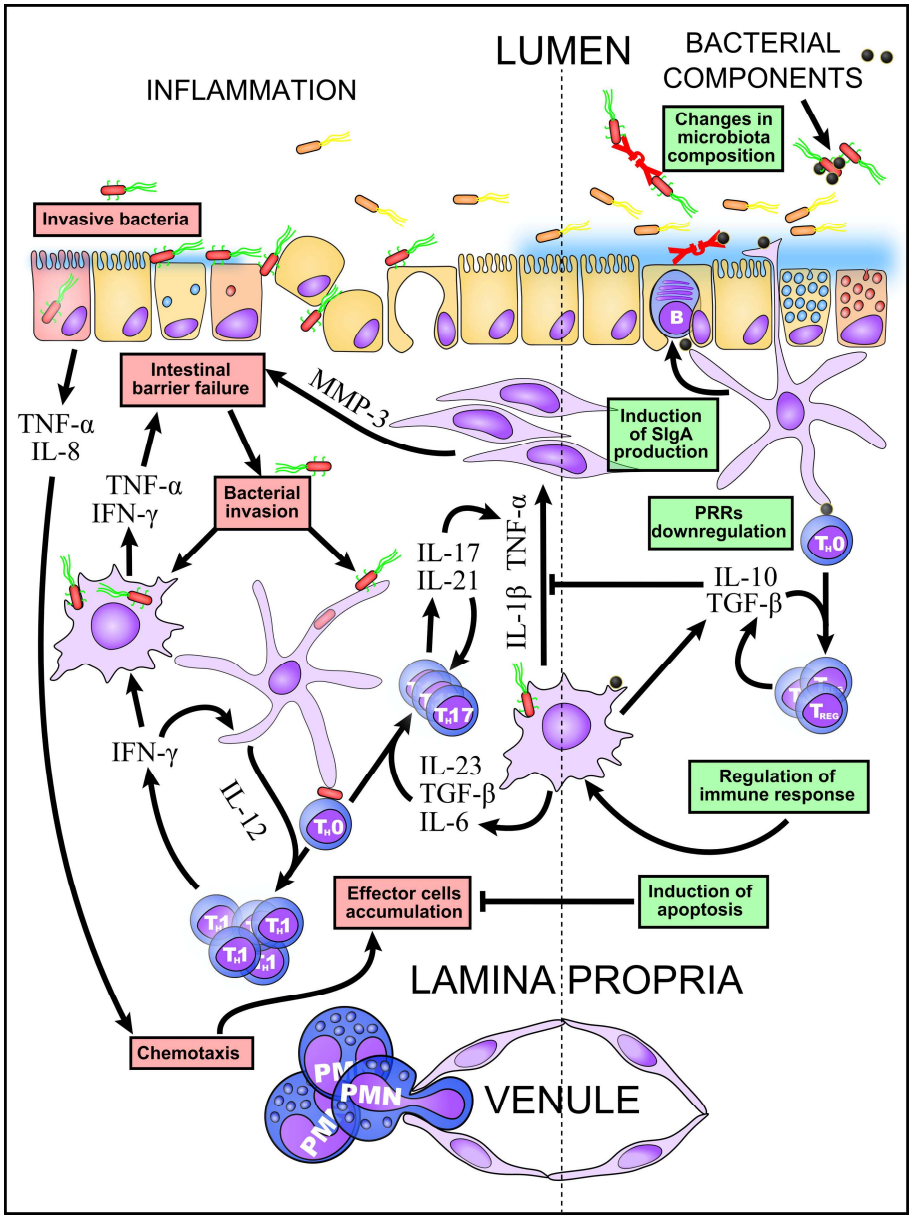
Several recent studies showed that TLR-9 ligand, an immunostimulatory bacterial oligonucleotide (CpG-ODN), ameliorates experimental colitis and decreases the production of proinflammatory cytokines by human colonic mucosa, but this effect is present only in certain CpG-ODN (Rachmilewitz et al., 2002; Rachmilewitz et al., 2006). Experiments with isolated bacterial DNA showed that intestinal epithelial cells respond to pathogenic bacterial DNA by increasing surface localization of TLR9 and production of IL-8, but remain unresponsive to DNA isolated from commensal or probiotic bacteria (Ewaschuk et al., 2007). In dextran-sulfate sodium (DSS) induced colitis, exposure to CpG-ODN during acute inflammation was found to exacerbate the disease, whereas preexposition proved to be protective (Obermeier et al., 2003). These results could explain the observed positive correlation between IBD and domestic hygiene in infancy, and they also suggest that CpG-ODN is a promising candidate for the maintenance therapy, but not for the therapy of active disease.

Another interesting approach is to think about bacteria that have been proposed as the IBD triggers as targets for vaccination. If we will identify the causative bacteria, than the protective immune response against this bacteria could prevent the IBD in otherwise susceptible individuals. Although *B distasonis* and mycobacteria were both proposed to be involveded in induction and perpetuation of intestinal inflammation, we found that introduction of *B. distasonis* lysate, its DNA or mycobacterial heat shock proteins by gavage led to decreased sensitivity of mouse to DSS colitis (Kverka et al., submitted). We still do not know whether we induce the oral tolerance to indigenous microbiota or protective immunity against some closely related potential pathogen causing the

inflammation, but this therapy promotes changes in cytokine production in the intestine. Recent findings on how could bacterial components beneficially influence the natural course of intestinal inflammation are summarized on Figure 3.1.

It is important to mention that the reaction of the immune system to the addition of the bacterial component depends on the actual tuning of mucosal immune system. As shown on animal models the introduction of the bacterial component into the inflamed condition could lead to opposite effect. Although there are still mysteries about the role of microbe-host interactions in IBD pathogenesis, our deeper understanding of these underlying mechanisms is important for new strategies in IBD therapy. It seems that the use of immunomodulatory properties of microbiota could be fundament for that purpose.

Figure 3.1 Simplified diagram of IBD pathogenesis summarizing the possible mechanisms by which bacterial components could beneficially influence the natural course of intestinal inflammation



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4. INTESTINAL INFLAMMATION MODULATES EXPRESSION OF 11β-HYDROXYSTEROID DEHYDROGENASE IN MURINE GUT

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4.1 ABSTRACT

The effect of glucocorticoids is controlled at the pre-receptor level by the activity of 11β-hydroxysteroid dehydrogenase (11HSD). The isoform 11HSD1 is an $NADP^+$-dependent oxidoreductase, usually reductase, that amplifies the action of glucocorticoids due to reduction of the biologically inactive 11-oxo derivatives cortisone and 11-dehydrocorticosterone to cortisol and corticosterone. The NAD^+-dependent isoform (11HSD2) is an oxidase that restrains the effect of hormones due to 11β-oxidation of cortisol and corticosterone to their 11-oxo derivatives. Although the immunosuppressive and anti-inflammatory effects of glucocorticoids are well known, the relationship between inflammation and local metabolism of glucocorticoids is not well understood. In this study, we demonstrated that colitis induced by dextran sulfate sodium modulates colonic 11HSD1. Experimentally induced intestinal inflammation stimulated colonic $NADP^+$-dependent but not NAD^+-dependent 11HSD activity. Colonic 11HSD1 mRNA was increased, whereas 11HSD2 mRNA was not changed. Additional parallel studies revealed a similar pattern of 11HSD1 mRNA induction in mesenteric lymph nodes and intestinal intraepithelial lymphocytes, but not in spleen and peritoneal macrophages. These data suggest that inflammation modulates local metabolism of glucocorticoid and support the notion that pre-receptor regulation of endogenous corticosteroids might play a role in inflammatory processes.

4.2 INTRODUCTION

Glucocorticoids are known to be essential modulators of immune and inflammatory processes. They influence the development and effector functions of the immune system, trafficking of immune cells through the vascular bed, and chemotaxis. At the cellular level, they modulate maturation, differentiation, proliferation, and activation of immune cells (Ashwell et al., 2000; Webster et al., 2002). One of the main effects of glucocorticoids is the downregulation of pro-inflammatory cytokines. These cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1), have been shown to link inflammation with glucocorticoid production by stimulating the hypothalamic–pituitary–adrenal axis and elevating thereby the plasma glucocorticoid concentration (Besedovsky and del Rey, 1996). However, the biological activity of glucocorticoids depends not only on their plasma concentration, the number of receptors, and the

responsiveness of the target cell, but also on the local metabolism of glucocorticoids catalyzed by 11β-hydroxysteroid dehydrogenase (11HSD), which can change the concentration of active glucocorticoids within tissues and/or target cells.

Two isoforms of 11HSD have been characterized. Isoform 2 (11HSD2) is a high-affinity NAD^+-dependent enzyme that operates exclusively as an oxidase inactivating biologically active glucocorticoids cortisol and corticosterone to their 11-oxo derivatives cortisone and 11-dehydrocorticosterone respectively (Stewart and Krozowski, 1999). In contrast, 11HSD1 is a low-affinity NADP(H)-dependent oxidoreductase whose reductase activity has been found in various intact cells (Seckl and Walker, 2001), but alterations in the $NADP^+$/NADPH redox potential governed by the metabolism of glucose-6-phosphate via hexose-6-phosphate dehydrogenase seem to determine whether 11HSD1 operates as a reductase or an oxidase (Hewitt et al., 2005). Thus, 11HSD2 decreases the local concentration of active glucocorticoids, whereas 11HSD1 increases it due to regeneration of biologically active steroids from the circulating inactive 11-oxo metabolites or decreases it due to oxidation of active glucocorticoids (Stewart and Krozowski, 1999; Seckl and Walker, 2001; Hewitt et al., 2005). Exposure to pro-inflammatory stimuli such as TNF-α and IL-1β increases 11HSD1 expression and enzymatic activity in some cells, while inducing a decrease of 11HSD2 in others (Cai et al., 2001; Heiniger et al., 2001; Thieringer et al., 2001; Tomlinson et al., 2001; Curtis and Sloan, 2004). The biological significance of this process was shown recently (Escher et al., 1997; Thieringer et al., 2001; Zhang et al., 2005).

With regard to the colon, 11HSD2 is expressed in epithelial cells, whereas 11HSD1 is localized in the cells of lamina propria (Whorwood et al., 1994). This matches with the findings of 11HSD1 in fibroblasts (Hammami and Siiteri, 1991) macrophages (Thieringer et al., 2001), and lymphocytes (Zhang et al., 2005). Consistent with the effect of TNF-α and IL-1β on 11HSD1 and 11HSD2, we have shown in a rat model of colitis that 11HSD1 mRNA expression and 11-reductase activity increased, whereas 11HSD2 mRNA expression and 11-oxidase activity decreased during intestinal inflammation (Bryndova et al., 2004). Considering that colitis is accompanied by activation of mucosal immune cells and increased recruitment of leucocytes from the vascular space (Elson et al., 1995), one can hypothesize that the link between the upregulation of colonic 11HSD1 mRNA and the increased ability of the tissue to reduce 11-dehydrocorticosterone to corticosterone might be the cells of the intestinal immune system. To address this

question, we used the dextran sulfate model of murine colitis and studied the changes of 11HSD1 in colon and immune cells during intestinal inflammation.

4.3 MATERIALS AND METHODS

4.3.1 Animals and preparation of immune cells

Female 3-month-old mice Balb/c (Velaz, Prague, Czech Republic) were used in this study. Experimental colitis was induced by adding 3% (w/v) dextran sulfate sodium (DSS, MW 36 000–50 000; ICN Biomedicals Inc., Cleveland, Ohio, USA) in drinking water (Okayasu et al., 1990). Mice were treated with DSS for 7 days and subsequently killed. Control animals received only tap water. The mice were killed by decapitation and the colon, spleen, and mesenteric lymph nodes were excised. Macrophages were collected by a peritoneal lavage and intraepithelial lymphocytes (IEL) as originally described by Lefrancois (2001) with some modifications. Briefly, the inflamed part of colon was dissected and the lumen gently flushed with cold physiological saline (4°C). The intestine was then incised longitudinally and cut into 5 mm pieces. The pieces were incubated in flasks containing RPMI-1640 medium supplemented with fetal bovine serum (12.5%) for 20 min at 37 °C with stirring. The incubation step was repeated thrice, the supernatants were combined, and IEL were separated from epithelial cells by discontinuous Percoll gradient at the interface 67%/44%. The harvested cells were washed in RPMI medium and used for further analysis.

The animal study was approved by the Animal Care and Use Review Committee of the Czech Academy of Sciences.

4.3.2 Evaluation of colitis

The clinical assessment of DSS-treated animals included body weight, colon length, evaluation of stool consistency, and the presence of blood in the stool. A clinical disease activity index representing the sum of separate scores ranging from 0 to 4 was calculated using the following parameters: body weight decrease (0, less than 5% decrease; 1, 5–10%; 2, 10–20%; 4, more than 20%), stool consistency (solid 0, loose 2, diarrhea 3), and bleeding (none 0, macroscopic in colon 2, blood adhering to the anus 4) as described previously (Bendjelloul et al., 2000).

4.3.3 Quantitative analysis of 11HSD and cytokine RNA

Total RNA from the colon was extracted by the guanidinium thiocyanate method. The isolated RNA was treated with DNase (Promega) to remove potential contamination by genomic DNA as mentioned earlier (Mazancova et al., 2003). Total RNA from the spleen, mesenteric lymph nodes, macrophages, and IEL was obtained using GeneElute Mammalian Total RNA Miniprep Kit (Sigma). cDNA was synthesized from 5 μg RNA and M-MLV Reverse Transcriptase reagents (Invitrogen GmbH). Amplification of the target cDNA was performed in the LightCycler (Roche) as previously reported (Mazancova et al., 2005) using QuantiTect Sybr Green PCR Kit (Qiagen GmbH) and the primers given in Table 4.1. Results were analyzed with LightCycler software using the second derivative maximum method to set CP. For the quantification of the target genes 11HSD1, 11HSD2, TNF-α, and IL-1β, we performed the quantitative comparison of several candidate reference genes to select the most stable genes for gene normalization. The panel of five reference genes generally used in many physiological and pathophysiological conditions, such as β-actin (ACTB), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13a (RPL13A), elongation factor 1a (EF1A), and peptidylpropyl isomerase B (cyclophilin B; PPIB), were tested (Table 4.1). Because of the high concentration of β-actin and 11HSD2, the samples were diluted 1/1000 before analyses of these RNA species. For other analyses, 1/10 prediluted cDNA was used as a template for PCR. Calibration curves were generated for each pair of primers from serial dilutions of standard cDNA. After statistical analysis of reference genes, the data of target gene expression were normalized according to the normalization factor calculated by the geNorm applet (Vandesompele et al., 2002).

The expression of 11HSD1 mRNA in IEL was determined by the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The probes and primers used for this experiment were developed as TaqMan Gene Expression Assays by Applied Biosystems. The reaction was carried out in a final volume of 20 μl using TaqMan Universal PCR Master Mix with AmpEraseUNG (Applied Biosystems) and Expression Assay. Target 11HSD1 mRNA was achieved using calibration curve method and the amount of mRNA was normalized to the level of 18S rRNA (Bas et al., 2004).

Table 4.1 Primers used for PCR

Gene	Sense (5'→3')	Antisense (5'→3')
11HSD1	GGTAGTGTCTCGCTGCCTTGAA	CACGTTGACCTTGGTTATGTAGAGTT
11HSD2	CCGGTTGTGACACTGGTTTTG	GGGGTATGGCATGTCTCCTG
TNF-α	GGACAAGGCTGCCCCGACTAC	TCTGAGCCATAATCCCCTTTC
IL-1β	TCCCAAGCAATACCCAAAGAAGAA	ATCAGAGGCAAGGAGGAAACACAG
β-actin	GAACCCTAAGGCCAACCGTGAAAAGAT	ACCGCTCGTTGCCAATAGTGATG
Ppib	AGGGGAGATGGCACAGGAGGAAAGAGC	ACCCAGCCAGGCCCGRAGTGC
EF1a	TGACAGCAAAAACGACCCACCAAT	GGGCCATCTTCCAGCTTCTTACCA
RP13A	CTCCCGAGGCCCCTACCACTT	GGCCTTTTCCTTCCGTTTCTCCTC
HPRT	GCAGTCCCAGCGTCGTG	TAATGTAATCCAGCAGGTCAGC

4.3.4 Enzyme activity assays

Colon homogenates were prepared in ice-cold buffers containing 10 mM Tris, 250 mM sucrose (pH 8.5; 11HSD2 assay) or 10 mM Tris, 5 mM EDTA, 0.5% Triton X-100 (pH 7.5; 11HSD1 assay). After centrifugation at 500 g for 15 min, the supernatant was obtained and protein concentration was measured using the Bradford technique (Bradford, 1976). 11HSD1 and 11HSD2 activities were measured as $NADP^+$- and NAD^+-dependent 11β-oxidation of corticosterone according to Livingstone & Walker (2003) and Gomez-Sanchez et al. (2003). 11HSD1 activity was measured in incubation buffer containing 50 mM Tris, 100 mM KCl, 1 mM NADPC, 480 nM corticosterone, and 20 nM 1,2,6,7- [^{3}H]corticosterone (pH 7.5). 11HSD2 activity was determined in a similar way, with 50 mM Tris, 100 mM KCl, 1 mM NADC, and 20 nM 1,2,6,7-[^{3}H]corticosterone (pH 8.5). The amounts of protein and the incubation times were determined in preliminary experiments to establish the optimal conditions, in order to work in the linear portion of the enzyme reaction. Steroids were extracted from the incubation buffer by SepPak cartridges (Waters, Milford, MA, USA) and separated by HPLC with on-line detection using a flow-cell detector (Radiomatic 150TR, Canberra Packard, Meriden, CT, USA). The separation was performed in a C18 column using a water methanol gradient (for details see Pácha et al. 2004).

4.3.5 Data analysis

All data are expressed as means±S.E.M. or medians with 25th– 75th percentile values. The distribution–fitting procedure according to Shapiro–Wilk's W-test of normality was applied and the comparison between the control animals and the mice with colitis was analyzed using unpaired Student's t-test or Mann–Whitney U-test. The values $P<0.05$ were considered statistically significant. For stability comparison of candidate reference genes and the calculation of normalization factor, the geNorm program was applied after conversion of C_P values into relative quantities (Vandesompele et al., 2002). Using this approach, the normalization factor based on two candidate reference genes was calculated. Statistical analysis was performed using the statistical software Statistica v.6 (StatSoft Inc., Tulsa, OK, USA).

4.4 RESULTS

The mice with colitis developed loose stools or diarrhea associated with blood in the stool and decreased colon length and body weight. Mortality was 10%. The index of disease activity is given in Table 4.2.

Table 4.2 Disease activity of DSS-induced colitis in colon

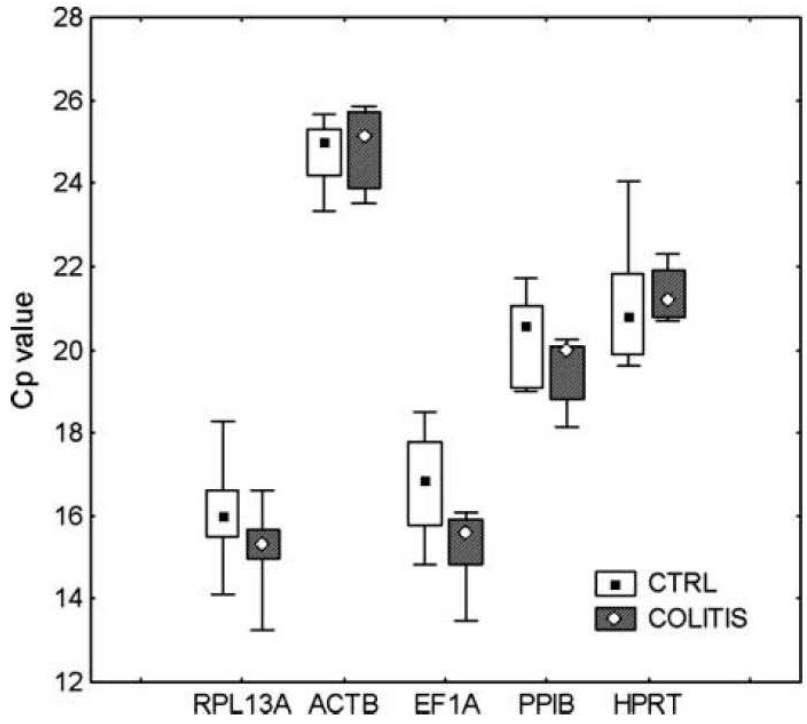
	Controls	Acute colitis
Symptom score	0.0 (0.0–0.0)	8.0 (6.0–10.0)*
Diarrhea score	0.0 (0.0–0.0)	3.0 (3.0–3.0)*
Blood score	0.0 (0.0–0.0)	4.0 (0.0–4.0)*
Body weight	2.0±0.8	-11.4±1.5*
Colon length	9.9±0.3	7.3±0.1*

The data are given as medians and 25th–75th percentile values (score values) or mean±S.E.M. (body weight change, colon length) based on records of 10 control and 9 DSS-treated animals. The score values were calculated as mentioned in Materials and Methods section, colon length is given in cm, and body weight change is given by dividing body weight at day 7 by body weight at day 0 (starting body weight) and is expressed as a percentage. *P<0.05 compared with controls.

To investigate whether metabolism of glucocorticoids is modulated in inflamed tissue, we used enzyme assay and quantitative reverse transcription (RT)-PCR. First, we evaluated the expression levels of five putative reference genes in the colon of healthy mice and in animals with colitis. These genes displayed a relatively wide range of CP (Fig. 4.1). Using the unpaired Student's t-test or Mann–Whitney U-test, significant

differences in gene expression between healthy and inflamed colon were observed for EF1A and RPL13A. The geNorm program was then used to calculate the gene expression stability measure M of the remaining genes and the normalization factor based on the geometric average of the two reference genes (Vandesompele et al., 2002). We found ACTB and PPIB to be the most convenient reference genes and thus these two genes were used for normalization of mRNA expression levels.

Figure 4.1 Expression levels of candidate reference genes in healthy and inflamed colon

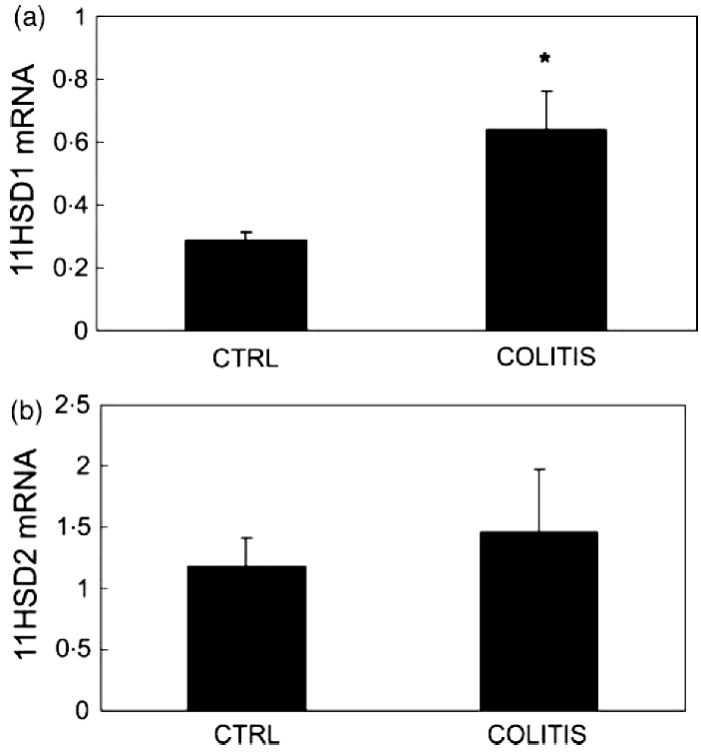


Data are shown as medians (points), 25th–75th percentiles (boxes), and ranges (whiskers). For the analyses, 1/1000 (ACTB) or 1/10 pre-diluted cDNA (RPL3A, EF1A, PPIB, and HPRT) was used as a template for PCR.

To determine whether 11HSDs are modulated during inflammation, we measured colonic 11HSD1 and 11HSD2 mRNAs in control animals and in mice with colitis. As shown in Fig. 4.2, both groups of mice were found to express 11HSD1 and 11HSD2 transcripts and the inflammation affected the expression. Colitis upregulated 11HSD1 mRNA but did not significantly modulate the levels of 11HSD2 mRNA. To test whether the changes in transcript levels reflect changes in 11HSD1 and 11HSD2 enzyme activities, we used a colonic homogenate assay to analyze the NAD^+- and $NADP^+$-dependent conversion of corticosterone to 11-dehydrocorticosterone. The colon of

controls and the mice with colitis had similar level of NAD^+-dependent 11HSD activity, but $NADP^+$-dependent 11HSD activity was significantly increased in inflamed tissue (Fig. 4.3). To verify the presence of colonic inflammation in this tissue, we analyzed gene expression of pro-inflammatory cytokines, TNF-α and IL-1β mRNAs. As shown in Table 4.3, the level of IL-1β transcript was significantly increased in inflamed colon but the level of TNF-α transcript was changed much less, in a similar way as in the study of Egger et al. (2000) and Kwon et al. (2005).

Figure 4.2 (a) Colonic 11HSD1 mRNA and (b) 11HSD2 mRNA levels in control mice and in animals with colitis.



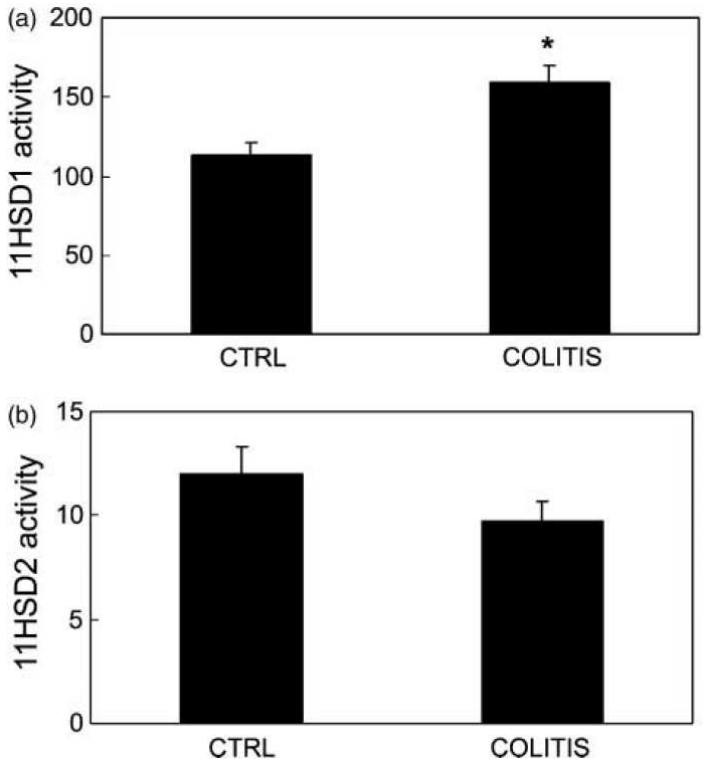
The relative expression levels were normalized against normalization factor from the two most stable reference genes provided by geNorm (for details see the text). The data are given as means±S.E.M. (control (CTRL), n=10; colitis, n=9). *P<0.05 compared with controls.

Table 4.3 Expression of TNF-α and IL-1β mRNAs in colon

	Controls	Acute colitis
TNF-α mRNA	0.202±0.063	0.258±0.61
IL-1β mRNA	0.023±0.002	0.356±0.183*

The data are given as means±S.E.M. of 10 control and 9 DSS-treated animals.The expression of cytokines is given in arbitrary units. *P<0.05 compared with controls.

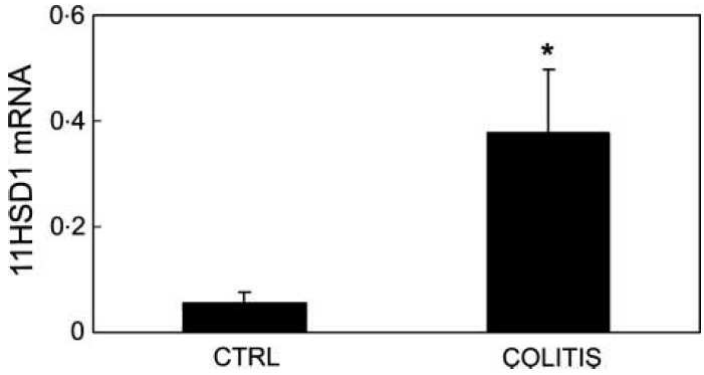
Figure 4.3 (a) NAD+- and (b) NADP+-dependent 11HSD activity in colonic homogenates of control mice and animals with colitis.



Values are means±S.E.M. (control (CTRL), n=10; colitis, n=10). 11HSD activity is given in picomoles of corticosterone per hour per mg protein. *P<0.05 compared with controls.

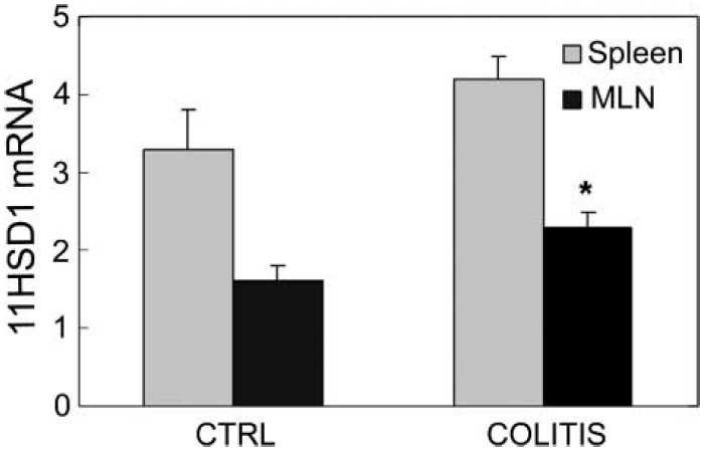
Next, we evaluated the changes in the level of 11HSD1 mRNA in the cells of gut-associated lymphatic tissue. There was a significant increase in 11HSD1 mRNA in IEL isolated from animals with colitis (Fig. 4.3). Consistent with upregulation of 11HSD1 mRNA in IEL, the lymphatic mesenteric nodes that contain large numbers of lymphocytes coming from the intestine demonstrated upregulation of 11HSD1 mRNA (Fig. 4.5), whereas 11HSD2 transcript was undetectable in the same nodes. In contrast to mesenteric lymph nodes, 11HSD1 transcript in spleen was not changed during colitis. Finally, to establish the potential contribution of macrophages to upregulation of colonic 11HSD1 during inflammation, the peritoneal macrophage 11HSD1 transcript was quantified. 11HSD1 mRNA was detectable in macrophages, but this transcript was not affected by colitis (controls: 0.56±0.05 (n=10); colitis: 0.48±0.04 (9)). Taken together, these findings demonstrate similar changes in 11HSD1 mRNA in the colon, mesenteric lymphatic nodes, and IEL, but not in the spleen and peritoneal macrophages.

Figure 4.4 The relative expression levels of 11HSD1 mRNA in murine intestinal intraepithelial lymphocytes during DSS-induced colitis.



Quantification of 11HSD1 gene expression was performed relative to the expression of 18S rRNA. *P<0.05 compared with controls (control (CTRL), n=5; colitis, n=9).

Figure 4.5 Expression of 11HSD1 in mesenteric lymph nodes (MLN) and spleen.



The values were expressed as mentioned in Fig. 4.2. *P<0.05 compared with controls (control, n=10; colitis, n=9).

4.5 DISCUSSION

The present study revealed that inflammation is associated with changes in 11HSD1 mRNA expression and enzyme activity in colon. The inflamed colon upregulated 11HSD1 transcript and $NADP^+$-dependent 11HSD activity without any significant changes in 11HSD2 transcript and NAD^+- dependent 11HSD activity. As 11HSD2 is expressed only in epithelium, whereas 11HSD1 is expressed in the submucosal layer (Whorwood et al., 1994), it is likely that changes in 11HSD1 proceed in the intestinal compartment of lamina propria or gut-associated lymphatic tissue and not in the epithelium. 11HSD1 was found in macrophages (Thieringer et al., 2001), T cells (Zhang

et al., 2005), and fibroblasts (Hammami and Siiteri, 1991), and we have found an increased level of 11HSD1 mRNA in IEL and mesenteric lymphatic nodes of mice with colitis. The question is what is the direction of 11HSD1 reaction in intact cells *in vivo*? This direction (11β-reduction or 11β-oxidation) depends on the ratio of NADPH/ $NADP^+$, which is determined by hexose-6-phosphate dehydrogenase and pentose-phosphate pathway (Atanasov et al., 2004; McCormick et al., 2006). Thus, it is difficult to anticipate the reaction direction of 11HSD1 in inflamed colon. Considering that 11HSD1 operates as a reductase in activated macrophages (Gilmour et al., 2006), lymphocytes (Zhang et al., 2005), and dendritic cells (Freeman et al., 2005), it is likely that colonic 11HSD1 is increased by a shift in favor of the reductase activity. Further studies are needed to clarify the role of 11HSD isoforms of colonic wall in vivo by cellular distribution of 11HSDs in cell types of colonic mucosa and submucosa during inflammation and the effect of NADPH depletion during oxidative stress on the direction of the reaction catalyzed by 11HSD1 in immune cells.

The exact mechanism of inflammation induced by DSS is not yet fully elucidated, but the pathogenesis seems to depend on the interaction between local immune reaction and environmental factors because animal models of inflammatory bowel disease reared in germfree conditions did not develop the disease (Tlaskalová-Hogenová, 1997; Hudcovic et al., 2001; Elson and Cong, 2002). Drinking of DSS generates in murine colon the upregulation of pro-inflammatory cytokines as well as reactive oxygen and nitrogen species and infiltration by polymorphonuclear and mononuclear cells (Okayasu et al., 1990; Kojouharoff et al., 1997; Arai et al., 1998). Previous data and our findings suggest that these processes are accompanied by upregulation of 11HSD1 in the cells of gut associated lymphatic tissue. First, activated macrophages and lymphocytes acquire increased capacity of glucocorticoid reactivation via 11HSD1 (Zhang et al., 2005; Gilmour et al., 2006). Secondly, our data show that $NADP^+$-dependent but not NAD^+-dependent activity is increased in inflamed colon. Thirdly, the level of 11HSD1 transcript is increased not only in inflamed colon but also in IEL and in mesenteric lymphatic nodes. This upregulation of 11HSD1 is presumably induced by the pro-inflammatory cytokines, whose levels of transcript and protein are increased in colon of DSS-treated mice (Arai et al., 1998; Egger et al., 2000; Obermeier et al., 2002; Kwon et al., 2005). The cytokines TNF-α and IL-1β are known to increase 11HSD1 mRNA and 11-reductase activity in various cell types, such as glomerular mesangial cells (Escher et al., 1997),

osteoblasts (Cooper et al., 2001), adipocytes (Tomlinson et al., 2001), and aortic smooth muscle cells (Cai et al., 2001). In addition, pro-inflammatory cytokines are important inducers of nitric oxide generation in macrophages and intestinal epithelial cells, and the interaction between the NO system and the 11HSDs has recently been demonstrated (Kolios et al., 1996; Saito and Nakano, 1996; Sun et al., 1997; Ruschitzka et al., 2001).

It is well recognized that glucocorticoids effectively modulate a number of immunological processes, including positive or negative regulation of cytokine production (Hennebold et al., 1996; Ashwell et al., 2000). Therefore, changes in the metabolism of glucocorticoids within peripheral lymphatic organs and in immune cells could modulate not only the suppression of cell activation by proinflammatory cytokines but also other immunomodulatory processes (Elenkov and Chrousos, 1999; McKay and Cidlowski, 1999). Using glomerular mesangial cells exposed to IL-1β and TNF-α, it was demonstrated that the release of phospholipase A2, a key enzyme producing inflammatory mediators, is decreased by 11HSD1 activity (Escher et al., 1997). Similarly, the pharmacological inhibition of 11HSD in vivo greatly enhanced the susceptibility to progressive bacterial diseases and these changes in resistance following 11HSD inhibition correlated with changes in the patterns of inducible cytokines in lymphocytes and macrophages (Hennebold et al., 1997). Given the central role of 11HSD1 in glucocorticoid action, we can speculate that the increased expression of this enzyme may serve to enhance the exposure of immune cells to active glucocorticoids via the paracrine and/or intracrine pathway.

In summary, our observations are consistent with the notion that inflammation is associated with changes in 11HSD1. Although the mechanism and accurate function of 11HSD1 upregulation is equivocal, the findings suggest that the bioavailability of glucocorticoids in inflamed colon differs from the healthy tissue.

4.6 ACKNOWLEDGEMENTS

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Muricová. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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5. ORAL ADMINISTRATION OF PROBIOTIC BAKTERIA (*E. COLI* NISSLE, *E. COLI* O83, *LACTOBACILLUS CASEI*) INFLUENCES THE SEVERITY OF DEXTRAN SODIUM SULFATE-INDUCED COLITIS

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5.1 ABSTRACT

Our study examined whether repeated preventive oral administration of live probiotic bacterial strains *Escherichia coli* O83:K24:H31 (Ec O83), *Escherichia coli* Nissle 1917 O6:K5:H1 (Ec Nis) and *Lactobacillus casei* DN 114001 (Lc) can protect mice against dextran sodium sulfate (DSS)-induced colitis. A significant decrease in average symptom score was observed in Ec O83-, Ec Nis- and Lc-pretreated group ($p < 0.05$). Significant differences in body mass loss between Lc pretreated mice with DSS-induced colitis were found when compared with nontreated mice ($p < 0.05$). PBS pretreated mice had a significantly shorter colon than Ec O83-, Ec Nis- and Lc-pretreated mice ($p < 0.05$). Administration of Lc significantly decreased the severity of DSS induced histological marks of inflammation ($p < 0.05$). A significant difference ($p < 0.05$) was also found in specific IgA level against given probiotic in enteral fluid between colitic mice and healthy mice pretreated with Ec 083 and Ec Nis.

5.2 INTRODUCTION

The pathogenesis of IBD represents the outcomes of 3 essential interactive cofactors: host susceptibility, enteric microflora and mucosal immunity (Shanahan, 2001). There is increasing evidence that IBD results from an abnormal immune response to normal intestinal flora, with a break of tolerance towards non-pathogenic bacteria (Duchmann et al., 1995; Tlaskalová-Hogenová et al., 2004). Normal flora profoundly influences the host mucosal structure and function as well as the development of the whole immune system (Tlaskalová-Hogenová, 1997; Vancikova et al., 2003; Tlaskalová-Hogenová et al., 2004; Cebra et al., 2005).

Manipulation of the resident microflora provides an opportunity to influence mucosal cytokine signaling. This is best appreciated when comparing genetically engineered immunodeficient mouse models reared under germ-free and conventional conditions (Tlaskalova-Hogenova et al., 2004). When genes encoding T-cell immunoregulatory functions are deleted, spontaneous chronic inflammation of the gut mucosa develops in conventionally reared mice. For example, TCR α-chain-deficient mice, TCR β-chain-deficient mice and MHC class II-deficient mice, cytokine knockout mice lacking IL-2, IL-10 and TGF-β develop chronic inflammation (Strober and Ehrhardt, 1993). Another experimental model of IBD directly evidencing the role of T-

cell subpopulations in gut immunoregulation has been described in SCID mice. Restored with CD4 CD45 RB^{high} T cell subpopulation from conventional BALB/c mice they develop colitis whereas simultaneous transfer of the CD4 CD45 RB^{low} T-cell subpopulation prevented colitis (Powrie, 1995; Tlaskalova-Hogenova et al., 1998).

SCID mice also remained healthy after transfer of a pathogenic T-cell subpopulation when reared in germ-free conditions (Singh et al., 2001).

The finding that there is an abnormal T-cell responsiveness to indigenous microflora in human and experimental models awakened interest in the possibility that commensals may initiate and maintain IBD lesions. The use of animal models has provided a new insight into the influence of environmental, genetic and immunoregulatory factors involved in the development of IBD. In previous studies, we induced colitis by DSS in immunocompetent and immunodeficient mouse models. SCID and BALB/c mice reared in conventional conditions (colonized by microflora) developed signs of colitis (colonic bleeding, epithelial erosions, increase in the number of inflammatory cells in lamina propria and submucosa, reduction of the number of goblet cells) after 7 d feeding with DSS. Both strains of mice reared in germ-free conditions showed only minor changes after DSS feeding. These experiments suggest that in DSS-induced colitis the presence of microflora augments the development of the inflammatory reaction which occurs even in the absence of specific components of the immune system (Hudcovic et al., 2001).

Here we addressed the question whether manipulation of intestinal microflora by probiotics could affect experimentally induced intestinal inflammation by examining whether repeated preventive oral administration of live probiotic strains *Escherichia coli* O83:K24:H31 (Ec O83), *E. coli* Nissle 1917 O6:K5:H1 (Ec Nis) and *Lactobacillus casei* DN 114001 (Lc) could protect mice against DSS-induced colitis.

5.3 MATERIALS AND METHODS

5.3.1 Preparation of bacteria.

Strains were cultivated to the stationary growth phase. *E. coli* strains used by Cukrowska et al. (2002) as probiotics, i.e. Ec O83 and Ec Nis were cultured on tryptan-soya agar (Oxoid), *L. casei* (Lc) (provided by the Danone Institute) on MRS agar (Oxoid). The cells were harvested, washed 3× and diluted in PBS and their concen tration was

measured spectrophotometrically at 600 nm, final concentration being 1 CFU/pL (i.e. 10^9 CFU/mL).

5.3.2 Application of probiotics and induction of colitis.

BALB/c mice aged 17 weeks (n = 60) were fed daily for 14 d by gastric lavage with 100 µL of live bacterial suspension (n = 20 in the group for 1 bacterial strain), i.e. each of these mice received daily 10^8 CFU of live bacteria. Mice in the control group (n = 15) were fed daily with 100 µL of PBS. DSS colitis was induced by administering 5 % DSS (diluted in distilled sterile water) in drinking water for 7 d similarly as described by Bendjelloul et al. (2000) and Hudcovic et al. (2001) to 10 mice in every bacterial group and to 10 mice in the control group. Five control mice and 10 mice in each bacterial group drank water without DSS. After 6 d the mice were sacrificed and the severity of colitis was evaluated. There were 10 mice in every probiotic and DSS group, 10 mice in probiotic and water group, 10 mice in PBS and DSS group and 5 mice in PBS and water control group. The planned experiments were approved by the Commission for Experimental Animal Studies of the Academy of Sciences of the Czech Republic.

5.3.3 Evaluation of colitis.

The presence of rectal prolapse, rectal and colonic bleeding and mass loss was recorded. A symptom score (0–4) was constructed attributing 1 point to each of the following events: rectal prolapse, rectal bleeding, colonic bleeding and death. The length of the colon was measured and colonic samples were obtained for histopathology. The slides were reviewed by a pathologist unaware of the treatment schedule of the animals. The severity of the colitis was scored, attributing between 0 and 3 points to each of the following parameters: polymorphonuclear infiltrate, mononuclear infiltrate, edema, erosions and ulcerations, crypt abscess, crypt destruction and distribution of the inflammation (mucosa = 1, mucosa and submucosa = 2, transmural inflammation = 3) (Verdu et al., 2000). Photomicrographs of H&E stained paraffin sections of a representative sigmoid colon of a mouse from group PBS and water group, a mouse from PBS and DSS group and a mouse from Ec O83 and DSS group was taken.

5.3.4 Antibody levels.

Sera and enteral fluid obtained by using 2 mL of PBS wash containing a mixture of proteinase inhibitors (Sigma) were collected. Px conjugated anti Ig serum was used. Concentration of specific antibodies against probiotic bacteria was evaluated by ELISA using bacterial sonicates similarly to Lodinová-Žádníková et al. (1991).

The results are mean values with 95 % CI, statistical analysis was performed by Student's t-test, $p < 0.05$ (vs. PBS) being considered statistically significant.

5.4 RESULTS

5.4.1 Clinical and histological changes in DSS-induced colitis.

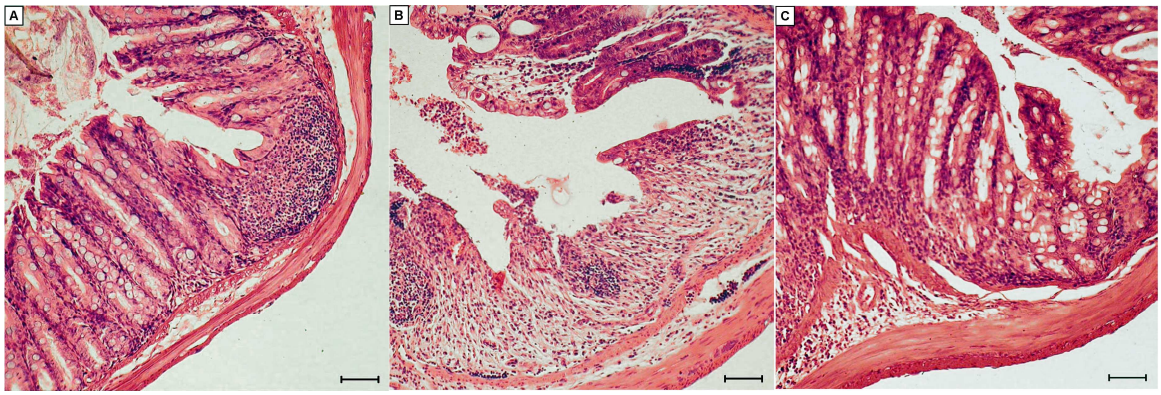
Relative to animals from PBS and DSS group, colitic mice fed with probiotic strains exhibited a decrease in the symptom score (Table 5.1) and had significantly longer colon at the end of experiment. There was also significant decrease in mass loss of colitic Lc mice. While severe inflammation, crypt destruction and ulceration of the mucosa were observed in the PBS-fed group by histology evaluation (Fig. 5.1B), significantly less inflammation was observed in the Lc-fed group. Mice exposed to both strains of E. coli had the inflammation histology score comparable to PBS-fed colitic controls. Mice drinking water instead of DSS had no colonic inflammation regardless of the probiotic strain they had previously received, indicating that the administration of the probiotic strains had no intrinsic harmful effect on the colonic mucosa (data not shown).

5.4.2 Antibody levels.

There was a significant difference in IgA Ab content between Ec O83- and Ec Nis-DSS-induced colitis mice and healthy colonized mice (Table 5.2). Ec Nis and Lc pretreatment therefore stimulated IgA Abs production in DSS colitic mice while Ec O83 stimulated intestinal IgA production in healthy mice.

No difference in specific IgM Ab production between mice with DSS-induced colitis and control animals was found (Table 5.2). There was a significant difference in specific IgG Ab production against Lc in DSS-induced colitis and healthy Lc-colonized mice.

Figure 5.1 Histopatological findings: photomicrographs of H&E-stained paraffin sections.



A: Through a healthy sigmoid colon; well-preserved mucosal crypts; sparse focal cellular infiltration limited to the basal layer of tunica propria (*cower center* and *top right*); focal depletion of lymphocytes from the follicle (right); tunica submucosa is devoid of cellular infiltration. **B**: Through severely inflammated sigmoid colon in a mouse fed PBS before induction of DSS colitis showing edema of the submucosa and extensive inflammation and ulceration of the mucosal layer with subtotal destruction of the crypts and infiltrate in lamina propria (H&E); top right: remains of mucosal crypts with marked epithelial dysplasia; thickening of tunica propria with massive mixed cellular infiltration of granulocytes and mononuclear elements; purulent exudates in the lumen (*center* and *top left*), edema and mixed cellular infiltration of tunica submucosa (*bottom*); inflammatory grade 1.5. **C**: Transversal section through sigmoid colon – moderate DSS colitis in a mouse pretreated with probiotic Ec O83 strain showing well-preserved mucosa and normal crypts; mixed (mainly mononuclear) infiltration involving the deep layer of tunica propria, focally spreading to the middle and superficial areas (*upper center*); focal edema and cellular infiltration of tunica submucosa (*lower center*); inflammatory grade 1. *bars* correspond to 50 µm.

Inflammation scores in colonic samples of DSS-treated mice:

treatment	score
Ec O83	0.98 ± 0.27
EC Nis	1.03 ± 0.24
LC	0.86 ± 0.23
PBS	1.23 ± 0.17
none	0.35 ± 0.23

Inflammation was maximal in mice pretreated with PBS instead of bacterial strains; administration of Lc significantly decreased the severity of DSS-induced inflammation ($p < 0.05$ vs. PBS); average measure with 95 % CI; no differences between healthy control mice and DSS-untreated mice pretreated with bacterial strains (*data not shown*).

Table 5.1 Clinical signs of colitis (Ec O83, Ec Nis, Lc and PBS groups):

Symptom scores, mass loss (g) and length of the colon (mm)[a]

Quantity	Ec O83	Ec Nis	Lc	PBS	H[b]
Symptom score	1.44±0.33	0.85±0.54	0.65±0.32	2.2±0.18	–
Mass loss	1.7±0.34	1.7±0.32	1.09±0.24	2.17±0.36	0.8 2±0.11
Colon length	82.7±3.3	82.1±3.3	82.8± 3.7	72.1±1.4	1 08.0± 4.3

[a]Mean ± CI (n = 10); for further details see *Materials and Methods*. [b]Healthy (control mice).

Table 5.2 Antibody levels:

Specific IgA Abs in enteral content, specific IgM Abs in serum and total specific IgG Abs in serum against Ec O83, Ec Nis, and Lc in DSS-treated mice (first rows) and healthy mice (non-DSS mice; second rows) pretreated with Ec O83, Ec Nis and Lca

Ig	Ec O83	Ec Nis	Lc	PBS
IgA	12.0±2.3	18.0±3.4	20.0±2.0	8.0±1.50
	18.5±3.5	11.5±3.4	15.0±1.50	10.0±0.35
IgM	23.5±1.57	11.5±1.55	16.0±5.6	14.5±2.3
	25.0±2.6	12.0±2.4	21.5±4.6	12.5±0.75
IgG	14.5±1.96	6.0±1.86	50.0±2.7	41.5±5.6
	16.0±1.86	7.0±1.58	45.0±3.7	36.0±1.31

[a]Mean ± CI; for further details see *Materials and Methods*.

5.5 DISCUSSION

Probiotic microorganisms are defined as viable nutritional agents conferring benefits to the health of the human host (Reid et al., 2003). In human disease, the beneficial effects of various probiotics were demonstrated and used for the treatment of a variety of disorders such as infectious rotavirus-induced diarrhea in infants, amelioration of the side effects of antibiotic therapy and prevention of atopic disease. Comparative studies of probiotics trying to select proper bacterial probiotic strains useful in specific conditions are still scarce (Strompfova et al., 2004). Clinical trials have also suggested a potential role of different probiotic preparations in the treatment of inflammatory bowel disease (Schultz et al., 2003b). Interesting data exist for Ec Nis in the maintenance of remission of ulcerative colitis. Treatment with Ec Nis demonstrated beneficial effects equal to those of mesalazine (Rembacken et al., 1999; Kruis et al., 2004).

However, the mechanisms by which Ec Nis or other probiotics mediate their effect are not fully understood. Changes in the intestinal microflora composition (Tannock et al., 2000; Mego et al., 2005) and other effects including local and systemic immunomodulation (Mack et al., 1999; Lammers et al., 2002) or barrier enhancement (Madsen et al., 1999; Schultz et al., 2003a; Bujnakova et al., 2004) have been described. Moreover, lactic acid bacteria secrete anti-inflammatory metabolites (Menard et al., 2004); lipoteichoic acids from *Lactobacillus johnsonii* strain La1 and *L. acidophilus* strain La 10 antagonize the responsiveness of human intestinal epithelial cells to lipopolysaccharide (Vidal et al., 2002)

The gnotobiotic animal model is a useful tool for evaluating the role of bacterial species or strains in the development of intestinal inflammation (Kozakova et al., 2001).

DSS-induced colitis is a well established model. Affected animals manifest body mass loss, bloody diarrhea, and a hunched posture, and the histological phenotypes (patchy mucosal damage, including focal crypt loss, followed by acute transmural infiltration with inflammatory cells) are similar to those of human IBD (Tsuchiya et al., 2003). The mechanism by which DSS induces colitis is not well defined, but seems to result from an alteration of colonic epithelial cells, and from an alteration of neither T nor B cell responses, because DSS colitis develops in SCID mice (Dieleman et al., 1994). However, DSS colitis also seems to be dependent on the intestinal microflora (Rath et al., 2001). This suggests that DSS modifies either the nature or the sampling of luminal antigens (Cooper et al., 1993). Antibiotics and probiotics have been used to prevent and ameliorate experimental colitis as well as IBD by changing the equilibrium of commensal bacteria in the intestinal flora (Madsen et al., 2001).

Our results demonstrate that manipulation of intestinal microflora could affect DSS-induced intestinal inflammation. Ec O83, Ec Nis and Lc caused amelioration of the clinical signs of intestinal inflammation, as was demonstrated by a lower symptom score, healthier clinical appearance of the animals and improving in colon length. Lc-precolonized mice had also a significantly lower mass loss at the end of the experiment and lower histological inflammation score in comparison with untreated mice with DSS-induced colitis. No differences were observed between probiotics-colonized healthy animals and healthy uncolonized controls (data not shown).

A significant difference between probiotic-colonized mice with DSS-induced colitis and healthy probiotic-colonized mice was found in the level of specific IgA against bacterial strains in enteral contents. In the Ec Nis-pretreated group, the IgA concentration was significantly higher in DSS mice, while an opposite situation was found in the Ec O83-treated group. A difference in total specific IgG in serum between DSS-colitic and healthy mice was found in the Lc-pretreated mice. This difference could be caused by a higher ability o f Lc to translocate during inflammation

Our data corroborate the evidence that nonpathogenic luminal bacteria participate in the pathogenesis of chronic intestinal inflammation. Administration of probiotic bacteria influencing the composition of intestinal flora may be relevant to the development of novel strategy in the prevention and management of IBD patients.

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6. ORAL TREATMENT WITH LYSATE OF PROBIOTIC *LACTOBACILLUS CASEI* DN-114 001 AMELIORATES EXPERIMENTAL COLITIS BY STRENGTHENING THE GUT BARRIER FUNCTION AND BY CHANGING GUT MICROENVIRONMENT.

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6.1 ABSTRACT

Probiotic bacteria can be used to treat human inflammatory diseases including inflammatory bowel disease. However, the nature of active component and exact mechanisms of this activity are not yet fully elucidated. Our aim was to investigate if lysate of probiotic bacterium *L. casei* DN-114 001 (Lc) and its fractions could influence the development of acute dextran sodium sulfate (DSS) colitis.

BALB/c mice received lysate orally, in four weekly doses. Seven days after last dose, the acute colitis was induced by 3% DSS dissolved in drinking water for one week. Numbers of $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{regs}) were measured by FACS analysis and cytokine production in various parts of the gut was estimated by tissue fragment culture with subsequent ELISA. Intestinal permeability for macromolecules was determined *in vivo* using a FITC-labeled dextran method and changes in microbiota composition in the stool was assessed by 16S rDNA denaturing gradient gel electrophoresis.

Oral treatment with Lc and its membranous fraction (mLc) significantly reduces the severity of acute DSS colitis and decrease intestinal permeability for macromolecules. Neither Lc nor mLc treatment changed the number of T_{regs} in Peyer's patches, mesenteric lymph nodes or spleen. Oral Lc lysate decrease production of proinflammatory cytokines TNF-α and IFN-γ, and anti-inflammatory IL-10 in Peyer's patches and large intestine, and changes the gut microbiota composition.

Our study provided evidence that even nonliving probiotic bacteria can alleviate the development of colitis by strengthening integrity of intestinal barrier and stabilization of gut microenvironment.

6.2 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 is not fully understood, it is generally accepted, that the inflammation results from an aberrant immune response to antigens of resident gut microbiota in genetically susceptible individuals (Sartor, 2006). Dysbiosis, an imbalance in the intestinal bacterial ecosystem, has been found in IBD and linked to its pathogenesis (Packey and Sartor, 2009). It has been suggested, that this microbial imbalances and an aberrant immune response could be restored by oral feeding with certain beneficial bacterial species, probiotics (Sheil et al., 2007).

Probiotics, defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001), have been successfully used in treatment of IBD (Guslandi et al., 2000; Kruis et al., 2004; Bibiloni et al., 2005). Using animal models of IBD, three main mechanisms of how these beneficial microbes protects from intestinal inflammation have been described and single probiotic bacterium could possess more than one depending on its unique specific metabolic activities and cellular structures (Lebeer et al., 2010). First, probiotics may exclude or inhibit the growth of certain pathogens (Servin, 2004), second, they may improve the gut barrier function (Gupta et al., 2000), and third, they can modulate mucosal and/or systemic immune response or metabolic functions (Reiff et al., 2009). The outcome of probiotic therapy also depends on stage of the disease and other and overall health status of the patient. Despite of the generaly safety profile of the probiotic therapy, use of live microorganisms always bring a possibility of severe infections and represent considerable risk especially in severely ill patients (Cannon et al., 2005; Besselink et al., 2008). There is increasing evidence, that similar beneficial effects could be achieved with microbial components isolated from probiotics or even commensals (Verdu et al., 2000; Rachmilewitz et al., 2004; Okada et al., 2006; Sokol et al., 2008).

One of the most intensively studied and used probiotic are lactobacilli (Kleerebezem and Vaughan, 2009). 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 (Pedone et al., 2000; Agarwal and Bhasin, 2002; Merenstein et al., 2010). These studies clearly shows the benefitial 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 not been established. In our previous studies we have shown that preventive treatment with well known probiotic bacterium *L. casei* DN-114001 protects mice from subsequent acute DSS-induced colitis (Kokesova et al., 2006). In the present study, we investigated if the preventive oral treatment with sterile lysate of this bacterium has similar effect and what is the underlined mechanism.

6.3 MATERIALS AND METHODS

6.3.1 Preparation of bacterial lysates

Lactobacillus casei DN-114 001 (Danone Institute, France), *Lactobacillus plantarum* (strain 185; Microbe collection of Institute of Animal Physiology and Genetics ASCR, Czech Republic), were grown in anaerobic chamber in De Man, Rogosa, and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) at 37°C until the 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 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; mLc) and cytoplasmic (soluble; cLc). The lysate and its fractions were lyophilised 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 cultivation before administration.

6.3.2 Animals

Female BALB/c mice (8-12 weeks old) were obtained from a breeding colony at the Institute of Physiology (Academy of Sciences of the Czech Republic, Prague, Czech Republic) and reared under conventional conditions at the Institute of Microbiology AS CR. All animal experiments were approved by the Animal Care and Use Committee of the Institute of Microbiology AS CR.

6.3.3 Study design and DSS colitis

We administered 1.5 mg of different bacterial components (whole lysate, membranous or cytoplasmic fraction) or 200 µg of DNA in 50 µl of sterile PBS, 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 number of 4 doses (on days 0, 7, 14 and 21). Acute colitis was induced 7 days later by 3% (wt/v) dextran sulfate sodium (DSS; mol wt = 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 clinical activity score, colon length, and histological scoring system as described previously (Kverka et al., *in press*).

6.3.4 Intestinal permeability *in vivo*

We measured intestinal permeability by determining the amount of fluorescein isothiocyanate conjugated dextran (FITC-dextran) in blood after it was orally administered as described previously (Wang et al., 2001). Briefly, each mouse received 440 mg/kg of body weight of FITC-dextran (MW 4,400; 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., Switzerland) with an excitation wavelength of 483 nm and an emission wavelength of 525 nm using serially diluted samples of the marker as standard.

6.3.5 Evaluation of microbiota changes with PCR-DGGE

We collected stool samples from five mice from the PBS and Lc-treated groups on day 0, 28 (just before DSS administration) and 35 (the last day), and isolated total DNA with ZR Fecal DNA Kit^{TM} (Zymo Research Corp., Orange, CA) according to the manufacturer's recommendation. Bacterial 16S rRNA genes were amplified from total bacterial DNA by following protocol: 1 µl of DNA template, primers fD1 (AGA GTT TGA TCC TGG CTC AG) + rP2 (ACG GCT ACC TTG TTA CGA CTT) (each 0.5 µM final concentration), 10

µl of Sigma RedTaq Ready mix and sterile dH2O to final volume 20 µl. PCR program was as follows: initial denaturation 4 minutes at 94C, 35 cycles (94°C 1 m, 52.5°C 1m, 72°C 2min) and final elongation 7 minutes at 72°C (Weisburg et al., 1991). PCR products were then purified by QIAquick PCR Purification kit (QIAGEN GmbH, Hilden, Germany) and used for second PCR with primers 338GC and RP534 (Muyzer et al., 1993).

PCR products were separated and analyzed on the DCode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). 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 (Invitrogen)dye for 30 minutes and observed in digital imaging system (Vilber Lourmat, Marne la Vallee, France) 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 (Nei and Li, 1979) using FreeTree software FreeTree (Pavlicek et al., 1999). This distance matrix was then used to calculate the similarity between the DGGE profiles from the same mouse at different time points using the 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.

6.3.6 Gut tissue culture and measurement of cytokines

We took sections of the distinct parts of the intestine from every mouse (Peyer's patches, jejunum, ileum, caecum and colon). The intestines were then cut open 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 (Kverka et al., *in press*). We then used commercially available ELISA sets 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.

6.3.7 Determination of specific antibodies

We collected sera and small intestine (gut) washings for specific antibody evaluation. Gut washings were obtained by flushing the content of prepared 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. To assess specific antibody response against Lc lysate in serum (IgG, IgM and IgA) and gut washings (secretory IgA; SIgA), we used indirect ELISA, optimized in our laboratory as previously described (Kverka et al., *in press*). Briefly, Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific Inc., Rochester, NY, USA) coated overnight with Lc (100 μl/well at 10 mg/l in PBS) and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich) in PBS were incubated for 2 h with 1:50 serum samples or 1:10 gut washing samples, both diluted in 1% BSA. 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 1:2000 rabbit anti-mouse secretory IgA (SIgA) (Uscn Life Science Inc., China), 1:2000 horseradish peroxidase (HRP)-labeled anti-mouse IgG (The Binding Site Ltd, Birmingham, UK), 1:500 HRP-labeled anti-mouse IgM (The Binding Site Ltd) all three diluted in 1% BSA, or biotinylated anti-mouse IgA (Sigma-Aldrich) diluted 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.) diluted 1:200 in 1% BSA into the IgA plate or 1:500 in 1% BSA HPR-labeled anti-rabbit IgG (Cell Signaling Technology Inc., Danvers, MA, USA) into SIgA 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.

6.3.8 Flow cytometry

We prepared single-cell suspensions of spleens, mesenteric lymph nodes and Peyer's patches and stained them for regulatory T cells (T_{regs}) using FoxP3 Staining Buffer 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. Flow cytometric analysis was performed on LSRII (BD Biosciences), and data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

6.3.9 Anti-inflammatory properties of Lc *in vitro*

To test the anti-inflammatory effect of selected bacterial components, we cultured the LPS-activated macrophage cell line (RAW 264.7; ATCC TIB-71) in the presence of different concentrations of bacterial lysate, as previously described (Kverka et al., *in press*). The cells were then cultured for 24h (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 a100 mg/l streptomycin (Sigma-Aldrich). The cells were cultured together with Lc, *L. plantarum* (Lp) or sterile PBS in the presence or absence of LPS (*Salmonella typhimurium*, 1 mg/l, Sigma-Aldrich). After the cultivation, we measured the concentration of TNF-α in the supernatant 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 a TransAM NF-κB family transcription factor assay kit (Active Motif). In this experiment, we used only the concentrations of bacterial components with the strongest immunomodulatory properties, i.e. 10 pg/l of Lc and 100 pg/l mLc. All assays were performed according to the manufacturer's recommendation.

6.3.10 Statistical analysis

One-way analysis of variance (ANOVA) with Dunnett'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. The data is presented as the mean ± standard deviation (SD) 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.

6.4 RESULTS

6.4.1 Components of *L. casei* attenuate acute colitis in BALB/c mice

In our previous study. we showed that oral treatment with probiotics attenuates the severity of acute experimental colitis (Kokesova et al., 2006). To test if the lifeless bacterial have similar activity, we pretreated mice with four weekly oral doses of lysate or component from Lc and induced the colitis by DSS. We report here, that oral administration of either Lc or mLc, is effective in preventing acute DSS colitis in BALB/c mice, improving clinical and morphological markers of colitis (Table 6.1), the former being slightly superior to the latter. Oral cLc had only minor protective effect, improving only disease activity index (Table 6.1).

Table 6.1 Pretreatment with Lc and mLc significantly improves the severity of DSS-induced colitis.

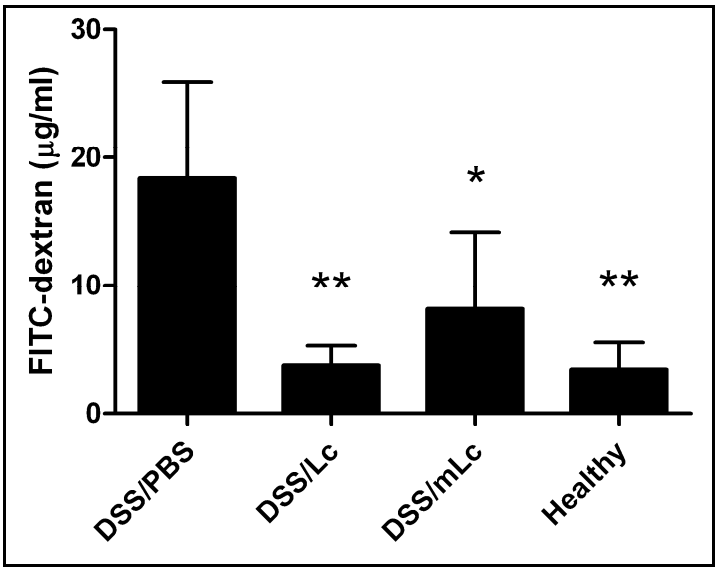
Experimental group	Colon length (cm)	Disease activity index	Histological grade
DSS/PBS	6.35 ± 0.62	2.80 ± 0.68	1.59 ± 0.54
DSS/Lc	7.14 ± 0.34***	1.67 ± 1.09**	1.20 ± 0.51*
DSS/mLc	6.82 ± 0.38**	2.15 ± 0.84	1.15 ± 0.41*
DSS/cLc	6.63 ± 0.51	1.97 ± 0.93*	1.54 ± 0.54

Values are expressed as means ± standard deviations (20 mice per group) of one representative experiment out 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 PBS-treated control group (*P<0.05, **P<0.01, ***P<0.001).

6.4.2 Components of *L. casei* prevents increase in intestinal permeability in acute colitis

Increased intestinal permeability caused by impairment in gut barrier function drives the pathogenesis of intestinal inflammation in both DSS-induced colitis and human IBD (Munkholm et al., 1994; Kitajima et al., 1999). To investigate the effect of Lc and mLc on gut barrier function in acute DSS-induced colitis, we administered single dose of FITC–dextran by gavage and measure the intensity of fluorescence in the mouse serum 5h later. Oral pretreatment with Lc or mLc significantly decreased the intestinal permeability to macromolecules at the last day of DSS (day 35) to the same extent as found in healthy mice (Figure 6.1).

Figure 6.1 Oral treatment with DSS/Lc or DSS/mLc strengthen the gut barrier function as compared to DSS/PBS control mice.



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). Data represent means (bar) ± standard deviations (whisker) of five mice of one representative experiment out of two independent experiments.

6.4.3 Oral treatment with Lc changes the gut microbial ecology

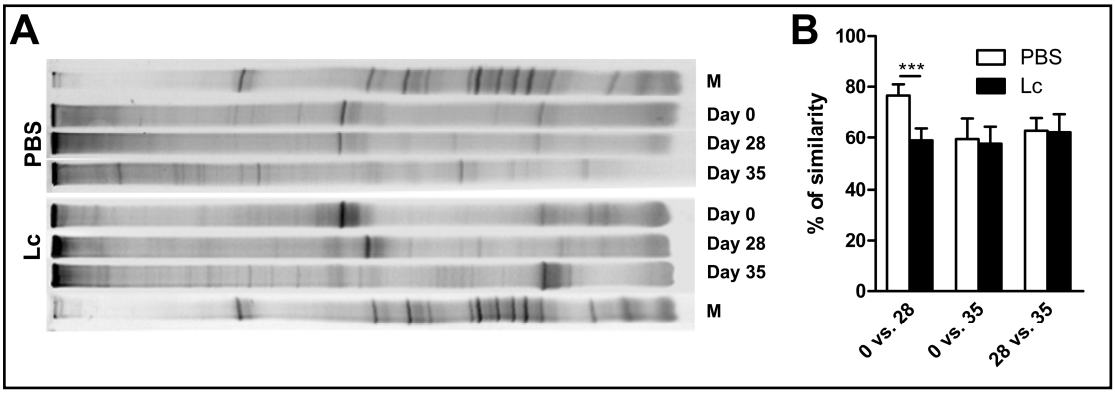
To specify the impact of oral treatment with Lc on the intestinal microbiota, we collected feces before the treatment (day 0), before colitis induction (day 28), and at the end of the experiment (day 35). We found that oral treatment with Lc induced significant changes in intestinal microbial ecology (Figure 6.2). Marked changes in microbiota composition of similar magnitude were observed during DSS administration in both Lc-treated and PBS-treated control group.

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

The changes in cytokine microenvironment in gut mucosa can change the mucosal immune response to luminal antigens leading to the decrease in intestinal inflammation. We, therefore, investigated whether the protective effect of Lc lysates is associated with modifications in inflammatory response in the key compartments of the gut. So, we cultivated five distinct parts of the gut from either DSS/PBS- or DSS/Lc-treated mice for 48h and then measure the cytokines in the supernatants by ELISA. We found that

pretreatment with DSS/Lc decreases the production of pro-inflammatory cytokines (IL-6, IFN-γ) and anti-inflammatory cytokine IL-10 in Peyer's patches, cecum and colon as compared with DSS/PBS treated mice (Table 6.2).

Figure 6.2 Oral treatment with Lc changes the intestinal microbiota composition.



(A) Example of fecal microbiota changes of one PBS and one Lc-treated mouse before the treatment (Day 0), before colitis induction (Day 28) and at the end of the experiment (Day 35) as measured by 16S rRNA gene PCR-DGGE. (B) Comparison of changes in DGGE profiles between PBS and Lc-treated mice at different time points. The data are expressed as mean (bar) and SD (whisker) and the differences between PBS- and Lc-treated mice was calculated by unpaired Student's t-test (***P < 0.001; n = 5 per group).

Table 6.2 Pretreatment with Lc changes cytokine production (pg/mg of tissue) in different parts of the gut as measured by ELISA.

Group	Part of the gut	Cytokine concentration (pg/mg of tissue)				
		IL-10	TNF-α	IL-6	TGF-β	IFN-γ
Control (DSS/PBS)	Peyer's patches	13.7 ± 7.5	3.1 ± 4.4	17.3 ± 7.8	8.6 ± 6.3	10.3 ± 9.7
	jejunum	2.4 ± 2.4	0.3 ± 0.5	0.2 ± 0.2	0.7 ± 0.8	1.27 ± 1.8
	ileum	6.4 ± 3.7	1.7 ± 2.5	1.5 ± 1.3	2.1 ± 1.3	2.3 ± 3.0
	cecum	6.9 ± 5.7	1.5 ± 0.8	4.8 ± 3.1	1.3 ± 1.5	3.5 ± 3.1
	colon	3.8 ± 3.0	2.5 ± 2.1	12.3 ± 14.2	1.0 ± 1.3	3.5 ± 2.0
Treated (DSS/Lc)	Peyer's patches	4.3 ± 2.7*	2.5 ± 2.8	1.7 ± 1.7*	5.6 ± 5.9	2.8 ± 4.0*
	jejunum	1.9 ± 1.1	0.8 ± 0.8	2.9 ± 3.7	0.9 ± 0.7	1.0 ± 2.0
	ileum	3.3 ± 3.4	2.4 ± 3.6	4.7 ± 5.8	2.4 ± 3.9	1.7 ± 2.6
	cecum	2.8 ± 1.2*	1.9 ± 1.0	5.0 ± 3.3	1.2 ± 1.1	1.1 ± 1.0*
	colon	2.3 ± 3.0	2.5 ± 1.5	5.1 ± 4.8*	1.4 ± 1.8	1.5 ± 1.4*

*P < 0.05 between DSS/PBS- and DSS/Lc-treated mice in the same part of the gut was compared by unpaired Student's t-test (n = 5 per group).

6.4.5 Oral administration of Lc does not change the specific antibody response in serum or in gut washings.

Oral administration of microbial antigens can change the local and systemic antibody production. To examine the effect of oral Lc on humoral immune responses, we measured levels of Lc-specific antibodies of IgA, IgG and IgM isotype in serum and specific secretory IgA in gut washings. We found that oral treatment with Lc does not change serum titers of anti-Lc antibodies; as compared with PBS-treated control group (Table 6.3). The levels of Lc-specific SIgA were generally around the detection limit of ELISA without any significant differences between DSS/PBS- and DSS/Lc-treated group.

Table 6.3 Treatment with Lc did not induce specific antibody response in serum and gut washings.

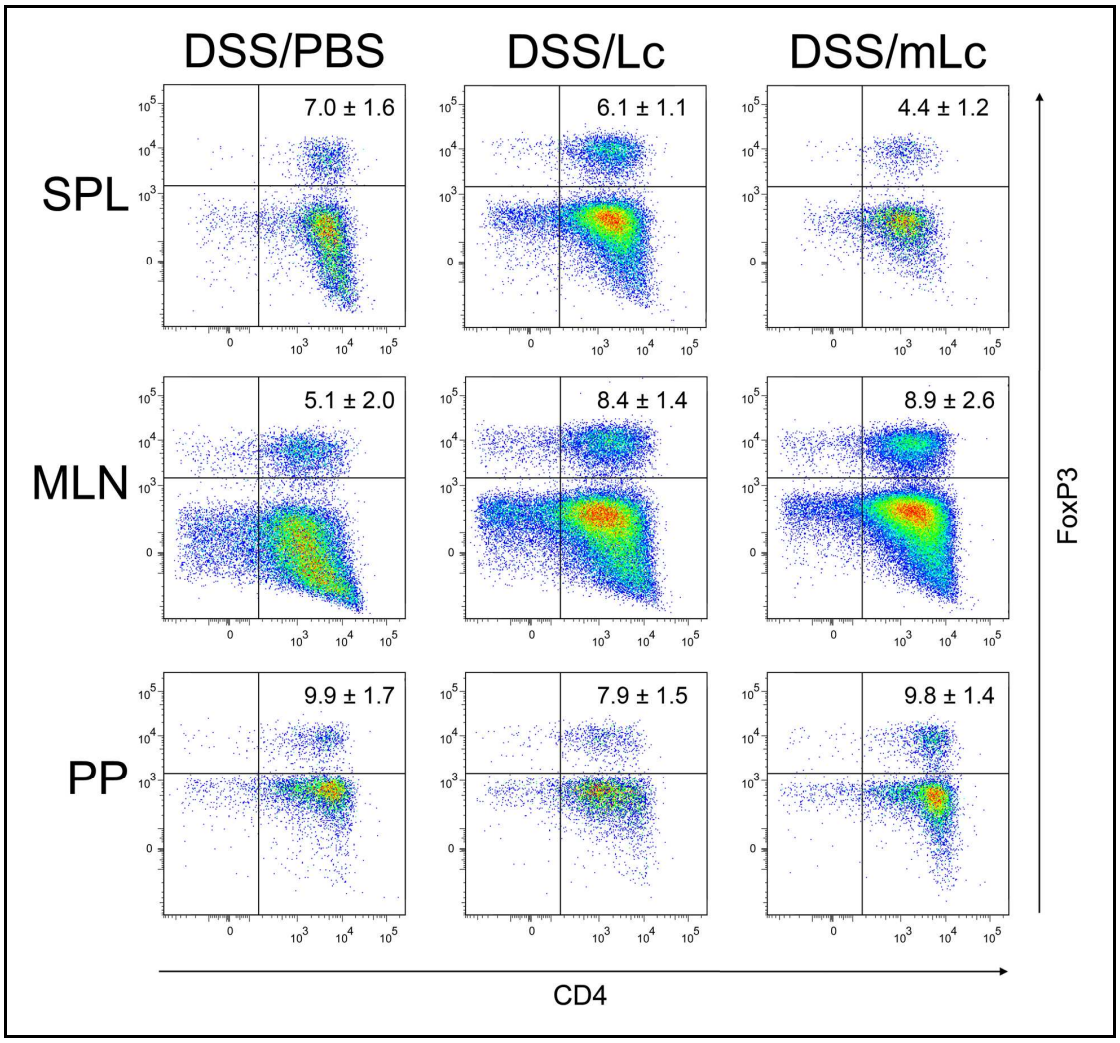
Isotype[a]	DSS/PBS	DSS/Lc	Reference serum[b]
IgA	0.07 ± 0.06	0.05 ± 0.06	0.05 ± 0.01
IgG	0.01 ± 0.02	0.01 ± 0.02	0.01 ± 0.00
IgM	0.16 ± 0.06	0.17 ± 0.05	0.06 ± 0.01
SIgA	0.01 ± 0.01	0.02 ± 0.02	*not done*

Values are means ± standard deviations of OD. There were no significantly significant differences between antibody titers in DSS/PBS and DSS/Lc-treated mice as compared by Student's t-test (n=10 per group). [a]Secretory IgA (SIgA) measured in enteral washings and other isotypes in serum. [b]Normal Reference Serum was purchased from Bethyl Laboratories.

6.4.6 Neither Lc nor mLc increased number of regulatory T cells

Since the intestinal inflammation in acute DSS-induced colitis is triggered by microbial antigens (Hudcovic et al., 2001), the induction of oral tolerance to microbiota could be the potential mechanism of protective effect of Lc. The oral tolerance is maintained at the periphery by regulatory T cells, so we analyzed the changes in $CD4^+FoxP3^+$ T_{regs} in spleen, mesenteric lymph nodes or Peyer's patches of DSS/PBS, DSS/Lc and DSS/mLc treated mice. We did not find any statistically significant changes between these groups in any compartment we tested (Figure 6.3).

Figure 6.3 Oral treatment with Lc or mLc does not change the number of $CD4^+FoxP3^+$ regulatory T cells in spleen (SPL), mesenteric lymph nodes (MLN) or Peyer's patches (PP) as analysed by flow cytometry.



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 two independent experiments (3-5 mice per group). The numbers of $CD4^+FoxP3^+$ cells between DSS/Lc, DSS/mLc, or DSS/PBS group are not statistically significantly different in any organ as calculated by one-way ANOVA with Dunnett's multiple comparison test.

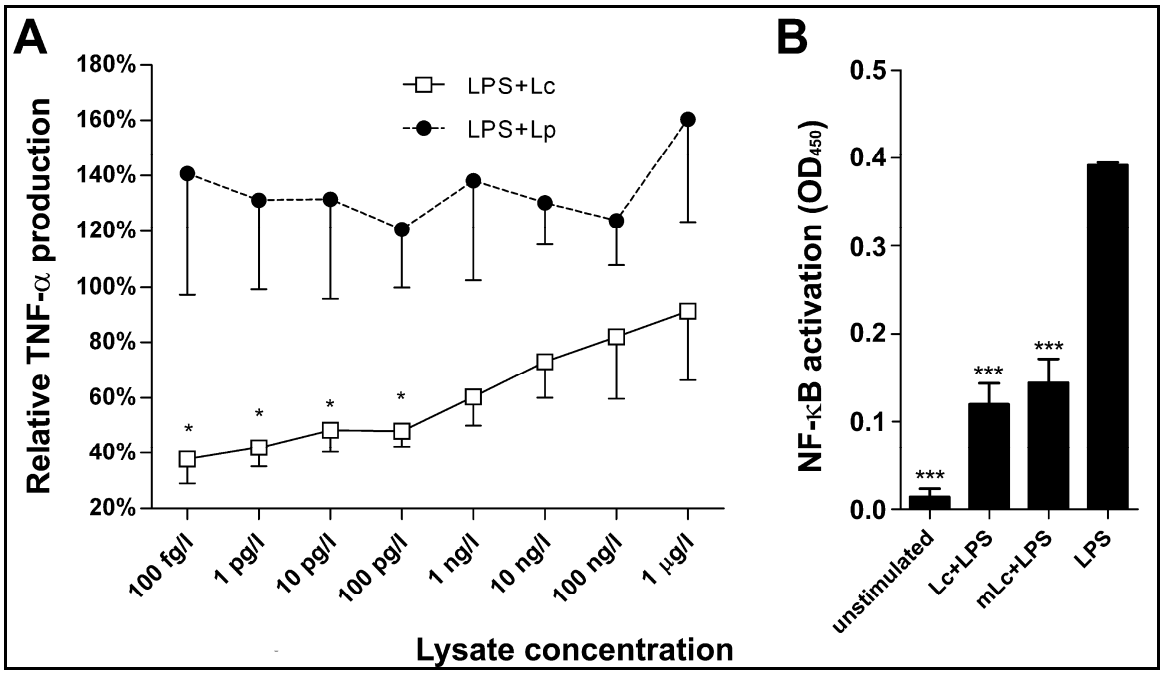
6.4.7 Lysate of *L. casei*, but not *L. plantarum*, decreases the production of TNF-α in LPS activated macrophages *in vitro*.

Because probiotics have an immunomodulatory effect on cells of innate immunity (Mileti et al., 2009) and because the macrophages play role in pathogenesis of DSS-induced colitis (Okayasu et al., 1990), we analyzed the anti-inflammatory effect of Lc in LPS-activated macrophages *in vitro*. We found that doses below 100pg/l significantly

decreases the production of production of TNF-α by LPS-stimulated RAW 264,7 cells *in vitro*, while similarly prepared lysate of *L. plantarum* (Lp) did not (Figure 6.4A). Treatment with either bacterial lysate in the absence of LPS did not change the TNF-α production (data not shown).

This 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 either Lc or mLc and measured the activity of the NF-κB signaling pathway. Both Lc and mLc significantly decreased the NF-κB/DNA binding activity of p65 subunit as compared to the LPS-only treated cells (Figure 6.4B).

Figure 6.4 The lysate of Lc have anti-inflammatory effect on LPS-activated macrophage cell line RAW 264.7 *in vitro*.



(A) Lc decreases the production of TNF-α in LPS-activated macrophages while lysate of the *L. plantarum* does not. TNF-α production when cells are stimulated 1 mg/l of LPS is set as 100% and data are means ± standard deviation 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 components on NF-κB binding activity in LPS-stimulated RAW 264.7 cells. Lc and mLc were co-cultured with LPS-activated cells for 24 hours, and then the binding activity of NF-κB subunit p65 was analyzed by colorimetric assay. Lc and mLc lysate downregulate the activation of NF-κB subunit p65 as compared to LPS only activated RAW 264.7 cells. Data are 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<0.001).

6.5 DISCUSSION

Oral treatment with probiotic bacteria has recently emerged as a potentially useful therapeutic strategy for human IBD (Kruis et al., 2004; Bibiloni et al., 2005). 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 previously shown that repeated oral administration of probiotic bacterium *L. casei* DN-114 001 protects mice from severe forms of acute intestinal inflammation. In this study we showed that not only live probiotics, but also the lifeless lysate of *L. casei* DN-114 001 (Lc) and its membranous fraction (mLc) can protect mice from severe forms of DSS-induced inflammation. This therapy significantly changes the gut microbiota composition, the immune response of gut mucosa, and strengthens the gut barrier function.

Several studies showed that there is marked difference in microbiota composition in IBD patients compared to healthy individuals. This dysbiosis or increase in some bacterial group is proposed to cause or at least perpetuate the intestinal inflammation in IBD (Seksik et al., 2003; Sokol et al., 2006). Here, we report that oral treatment with Lc significantly changes the composition of gut microbiota. Similar effects have been already described as mechanisms how probiotics protects from intestinal inflammation (Tannock et al., 2000; Schultz et al., 2003; Ukena et al., 2007). Some of them are attributed to the fact, that probiotics can grow and colonize the gut, which could not be achieved with the bacterial lysate. Moreover, bacterial lysates are not probably present in the gut during the intestinal inflammation induction. The clear protective effect of bacterial lysates 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 employed to explain this effectivity. 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 (Boyer and Wisniewski-Dye, 2009). These molecules could be presented in the bacterial lysates and, quite selectively, modify the bacterial populations as showed by others (Jamuna and Jeevaratnam, 2004). 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 (Mondel et al., 2009). Both these mechanisms could explain the changes in bacterial populations during therapy we observed during the treatment with Lc. More favorable gut microbiota composition at the beginning of inflammation induction could then slow down the subsequent inflammatory response. We are currently trying to describe these microbiota changes in more details by high-throughput sequencing of the whole gut microbiota populations. It is important to note, that we observed significant changes in the gut microbiota composition in both PBS and Lc-treated group during colitis induction. Although the changes were of similar magnitude in both groups, the banding pattern was different, suggesting that the actual differences in microbiota composition were unique for each group. These differences could be, however, also caused indirectly, by decreased inflammation in Lc-treated mice caused by different mechanism.

Intestinal barrier prevents viable enteric bacteria and other antigens from excessive interaction with the immune system. The disruption of gut barrier function has been identified as one of the crucial steps in IBD pathogenesis, causing this excessive interaction during the initial phases of the IBD (Munkholm et al., 1994). Protection of the gut barrier from disruption by induction of changes in expression and distribution of tight junction proteins was proposed as a key mechanism of probiotic function (Ukena et al., 2007; Chen et al., 2010). Here we report that oral treatment with Lc or mLc significantly strengthen the gut barrier function during acute DSS inflammation. Elucidation of this mechanism on the molecular level is a subject of our ongoing studies

Induction of oral tolerance is crucial for keeping the homeostasis in the gut. This mechanism is mediated on the periphery mainly by regulatory T cells (T_{regs}) whose protective role in intestinal inflammation has been clearly established (Singh et al., 2001). Although the co-administration of live *L. casei* with collagen can potentiate the oral tolerance and led to increase in T_{regs} in collagen induced arthritis model, oral treatment with live *L. casei* alone cannot (So et al., 2008; Chiba et al., 2010). Since oral tolerance is easier to achieve with killed or inactivated mirobes rather than live ones (Rubin et al., 1981), we decided to measure the changes in T_{reg} numbers after the treatment with Lc. We did not find any differences in T_{reg} numbers neither in spleen, mesenteric lymph nodes, or Peyer's patches of the mLc- or Lc-treated mice as compared to controls. This would suggest that the stimulation of oral tolerance does not play significant role in Lc-induced protective effect, we observed *in vivo*. We observed, however, tendency for redeployment

of T_{regs} from spleen to MLN in treated mice. This finding needs to be verified on larger groups of mice.

The cytokines produced in the gut mucosa microenvironment greatly influences the direction of the immune response. Production of the anti-inflammatory cytokines induces the mucosal unresponsiveness and tolerance and high levels of proinflammatory cytokines induce protective immune response and inflammation (MacDonald et al., 2000). Here, we report that Lc treatment decrease the production of proinflammatory cytokines IL-6 and IFN-γ as well as anti-inflammatory cytokine IL-10 in both PP and large intestine. This suggests that Lc can influence both the induction and effector functions of mucosal immune system. Decrease in IL-10 in DSS/Lc-treated mice could be simply secondary to decreased "need" of the inflammation control as compared to clearly more diseased control mice. Interestingly, since IFN-γ has been shown to increase the gut permeability (Madara and Stafford, 1989), decrease in its local production can lead be responsible for strengthening of gut barrier function we found in Lc-treated mice. On the other hand, lower stimulation of cells in lamina propria by luminal bacterial antigens caused by gut barrier failure may lead to decrease in inflammatory response to luminal antigens. This is with agreement with findings that live *L. casei* can downregulate the proinflammatory mediators in the lamina propria of inflamed mucosa from Crohn's disease patients during *ex vivo* cultivation (Carol et al., 2006; Llopis et al., 2009).

Acute DSS colitis is believed to be driven initially by innate immunity mechanisms and, in particular, the role of macrophages has been suggested (Okayasu et al., 1990; Dieleman et al., 1994; Murano et al., 2000; Hudcovic et al., 2001). We therefore tested the ability of bacterial lysates to decrease the inflammatory response of LPS-activated macrophages *in vitro*. We found that Lc, but not Lp, decrease the production of TNF-α, and the activation of NF-κB cascade, suggesting a possible direct effect of these lysates on the cells of innate immunity. This mechanism may contribute to the attenuation of acute DSS injury observed *in vivo*.

In conclusion, our data provide evidence that even lifeless components of probiotic bacterium can protect from induction of intestinal inflammation, thus confer a health benefit for the host. This is achieved by mechanisms that comprise a) correcting the dysbiosis, b) modulation of the mucosal immune response and c) improving the gut barrier function. These immunomodulatory lysates may lead to the development of new therapeutic approaches for treatment of chronic intestinal inflammation. Moreover, oral

administration of sterile bacterial components, in contrast to live bacteria, may be safer in severely ill or immunocompromised patients.

6.6 ACKNOWLEDGEMENTS

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7. ORAL ADMINISTRATION OF *PARABACTEROIDES DISTASONIS* ANTIGENS ATTENUATES EXPERIMENTAL MURINE COLITIS THROUGH MODULATION OF IMMUNITY AND MICROBIOTA COMPOSITION

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7.1 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.

7.2 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 (Sartor, 2008).

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 (Seksik et al., 2006). Secondly, manipulation of luminal content using selective antibiotics, or fecal stream diversion, improves inflammation in IBD patients (Rutgeerts et al., 1991; Prantera and Scribano, 2009). Thirdly, in some models of IBD intestinal inflammation is attenuated, or fails to develop, if the animals are maintained under germ-free conditions (Sadlack et al., 1993; Taurog et al., 1994; Rath et al., 1996; Hudcovic et al., 2001; Stepankova et al., 2007). 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 (Seksik et al., 2003) and decreased number of commensal bacteria with reduction in the Firmicutes phylum (Scanlan et al., 2006).

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 (Van Assche et al., 2006). 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 (Mahida and Rolfe, 2004). Some randomized, placebo-controlled studies, using *Escherichia coli* Nissle 1917 and a combination of eight probiotic strains, have demonstrated a beneficial effect in IBD (Kruis et al., 2004; Mimura et al., 2004; Bibiloni et al., 2005). 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 (Seksik et al., 2008). 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 (Obermeier et al., 2003; Rachmilewitz et al., 2004; Kokesova et al., 2006). Our previous results suggest that orally administered lysates from anaerobic microbiota decrease the severity of experimental colitis (Verdu et al., 2000). 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.

7.3 MATERIALS AND METHODS

7.3.1 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.

7.3.2 Identification of candidate anaerobic 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 S7.4 and S7.5). 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 (Westphal et al., 1952; Ali et al., 2005).

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

Because macrophages have been proposed to play a role in acute intestinal inflammation (Okayasu et al., 1990; Murano et al., 2000), 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).

7.3.4 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 (Okayasu et al., 1990). 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*. (Cooper et al., 1993), 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.

7.3.5 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.

7.3.6 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).

7.3.7 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 S7.2). 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).

7.3.8 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).

7.3.9 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).

7.3.10 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.

7.4 RESULTS

7.4.1 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 7.1 and Supplementary Table S7.4).

Table 7.1 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).

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. S7.5). Neither cytoplasmic fraction of *P. distasonis* lysate (cPd) nor DNA decreased TNF-α production *in vitro* (data not shown).

7.4.2 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 7.1). This effect was seen only with oral administration and was not observed with intraperitoneal or subcutaneous administration of mPd (see Supplementary information; Table S7.6). In contrast to mPd and DNA treatments, oral administration of cPd did not have a protective effect (Table 7.1). Orally administered mPd did not prevent colitis in SCID mice (Table 7.1), 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 7.2).

Table 7.2 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.

7.4.3 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 7.3).

Table 7.3 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*

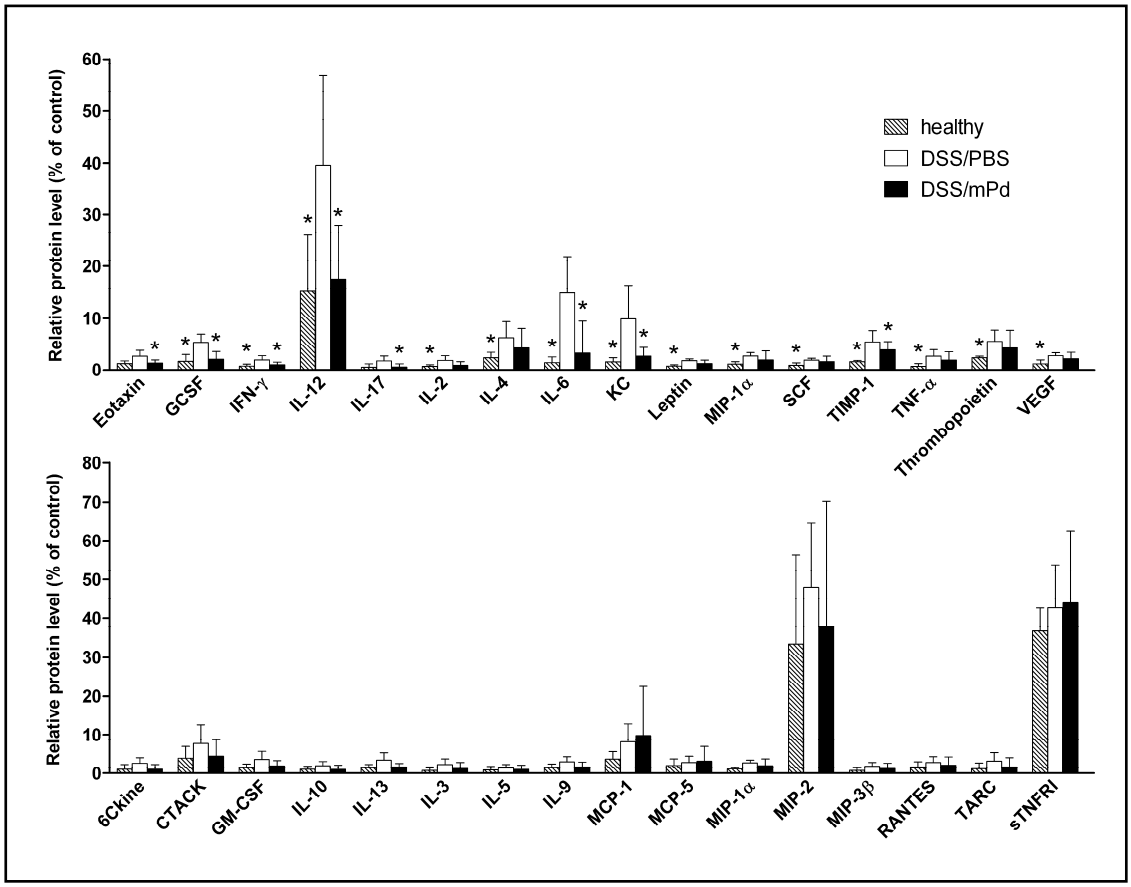
Data are representative of one experiment. Similar results were obtained from three independent experiments. Values are expressed as means ± standard deviations (five mice per group). An unpaired Student's t-test was used to calculate the significance of differences between the mPd-treated group and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

7.4.4 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. 7.1). 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. 7.2), as measured by ELISA. No significant differences in cytokine production were found in the ileum,

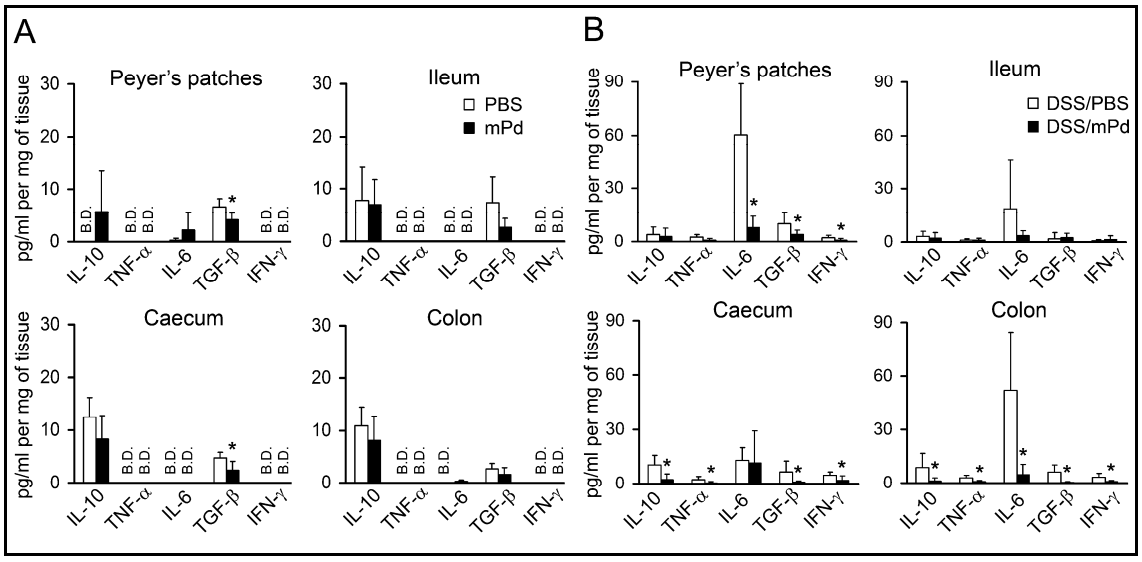
jejunum mucosa (data not shown) or spleen cells (data not shown). mPd treatment did not change the production of cytokines in SCID mice except for an increase in IL-10 production in the colon (see Supplementary information; Fig. S7.6).

Figure 7.1 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 7.2 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.

7.4.5 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. S7.3). There were no differences between mPd-treated and control groups in T_{regs} numbers in spleen or Peyer's patches (data not shown).

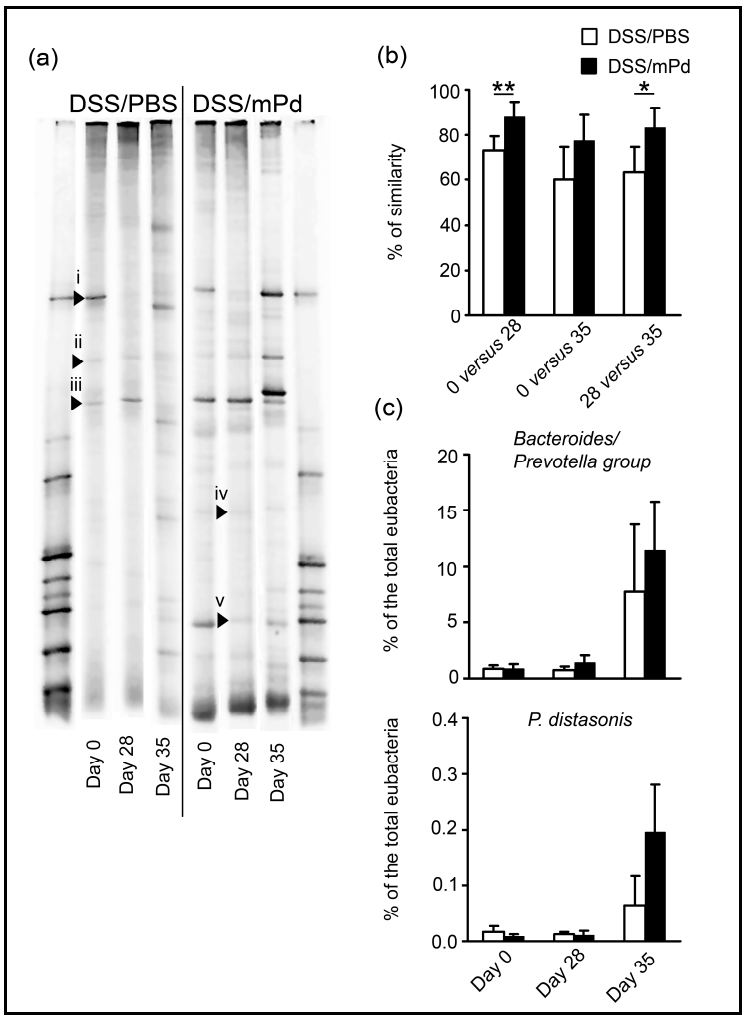
7.4.6 Oral treatment with mPd does not affect the *P. distasonis* or *Bacteroides/Prevotela* group stool content but stabilizes the gut microbial ecology

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. 7.3a). In contrast, marked changes in microbiota composition were observed during DSS administration in the PBS-treated control group, as demonstrated in Fig. 7.3b.

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. 7.3c).

Figure 7.3 Oral treatment with membranous fraction of *Parabacteroides distasonis* (mPd) stabilize 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

7.5 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 (Kruis et al., 2004; Mimura et al., 2004; Bibiloni et al., 2005; Prantera and Scribano, 2009). 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 (Verdu et al., 2000). 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 (Hudcovic et al., 2001; Tlaskalova-Hogenova et al., 2004; Monteleone et al., 2006; Williams et al., 2006; Sartor, 2008). Moreover, it has been proposed that microbiota composition can be regulated by the host's immune system (Williams et al., 2006; Petnicki-Ocwieja et al., 2009; Salzman et al., 2010).

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 (Obermeier et al., 2003). 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 (Okayasu et al., 1990; Dieleman et al., 1994; Murano et al., 2000;

Hudcovic et al., 2001). 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 (Rachmilewitz et al., 2004; Grabig et al., 2006), 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 (Atreya et al., 2000; Wang et al., 2005). 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 (Grabig et al., 2006).

It is known that some components of the indigenous microbiota have immunomodulatory properties and affect cytokine production (Rachmilewitz et al., 2004; Okada et al., 2006; Hrncir et al., 2008; Sokol et al., 2008). 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 (Mazmanian et al., 2008). 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 (Papadakis and Targan, 2000; Siddiqui and Scott, 2005).

The presence of serum antibodies directed against commensals in IBD patients suggests that some patients may exhibit systemic priming against microbiota (Adams et al., 2008). 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 (Slack et al., 2009). 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 (Adams et al., 2008), 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 (Dieleman et al., 1994; Hudcovic et al., 2001), 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 (Keilbaugh et al., 2005). The innate and adaptive immune systems work in synergy to mount appropriate immune responses to the commensal microbiota and maintain homeostasis (Slack et al., 2009). 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 T_{regs} 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 T_{regs} 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 T_{reg} activity (Singh et al., 2001; Hrncir et al., 2008). 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 (Okayasu et al., 1990), 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* (Okada et al., 2006; Sokol et al., 2008).

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 T_{regs}. 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.

7.6 ACKNOWLEDGEMENTS

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7.7 DISCLOSURE

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

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7.9 SUPPLEMENTARY MATERIALS AND METHODS

7.9.1 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* (Westphal et al., 1952). DNA from *P. distasonis* was isolated using a shortened version of the cetyltrimethylammonium bromide (CTAB) DNA isolation method described previously (Ali et al., 2005). Sterility of all components was verified by both aerobic and anaerobic cultivation before administration.

7.9.2 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* (Cooper et al., 1993).

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 S7.1 and Fig. S7.1) 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 S7.6.

Figure S7.1 Histological examples of different grades of mucosal damage in DSS treated mice (H&E stained colon descendens; magnification, ×40). See table S7.1 for detailed description.

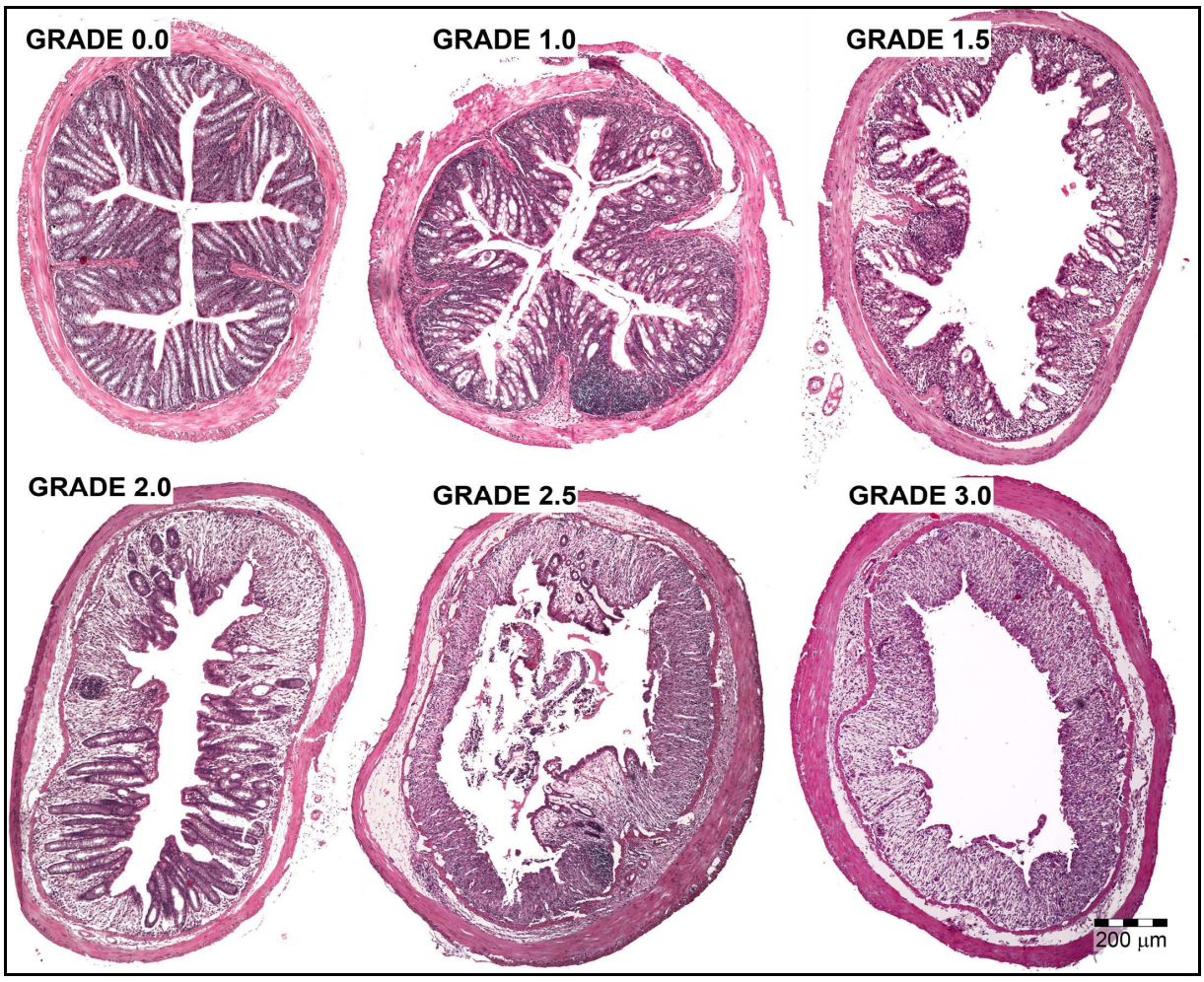


Table S7.1. Detailed description of individual histological grades.

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.

7.9.3 Determination of serum and faecal antibodies

Faecal pellets were collected and processed as previously described (deVos and Dick, 1991). 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.

7.9.4 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.

7.9.5 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. S7.2).

7.9.6 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.

7.9.7 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 S7.2 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 (Kverka et al., 2007). 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 S7.2 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 S7.2 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.

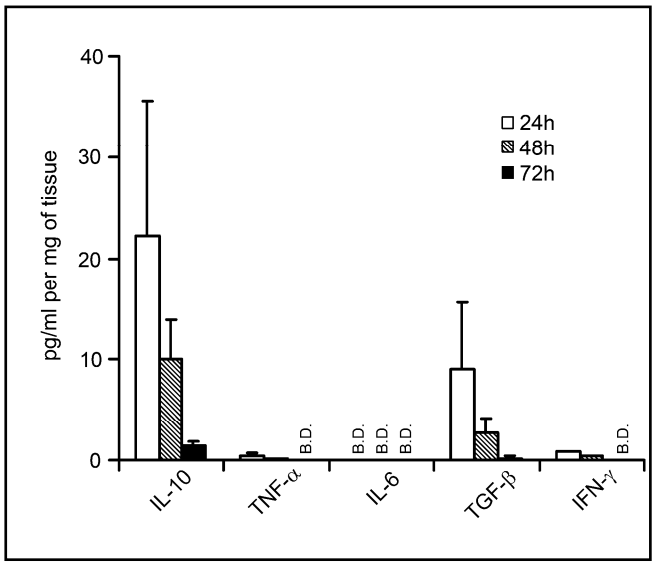
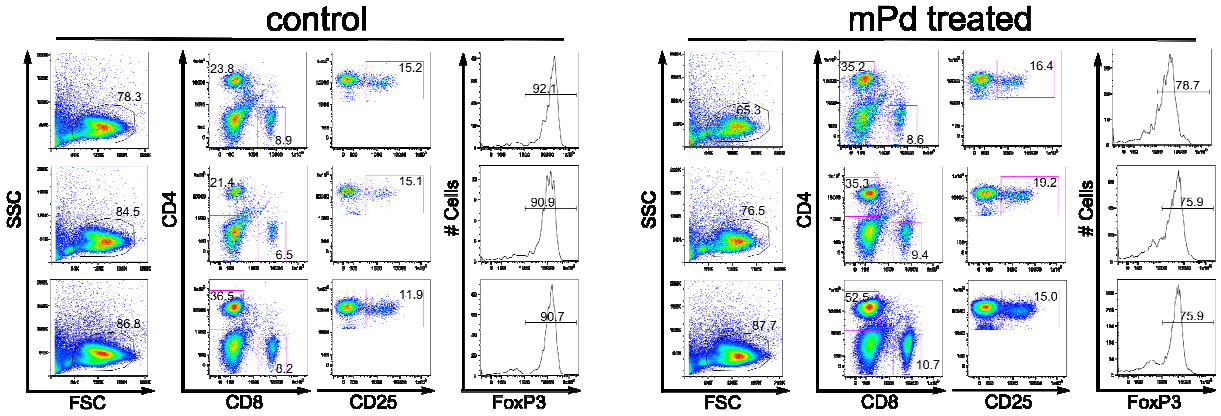


Figure S7.3 Showing gating strategy in 3 mice (rows) from DSS/PBS and 3 mice from DSS/mPd treated group.



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.

7.9.8 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 (Muyzer et al., 1993).

PCR products were separated and analysed on the DCode™ 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 (Nei and Li, 1979) and a dendrogram comparing all 30 samples was obtained with UPGMA (unweighted pair-group method with arithmetic averages) using FreeTree software (Pavlicek et al., 1999).

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).

7.9.9 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) (Bartosch et al., 2004), *Bacteroides-Prevotella* group (primers Bac303F+Bac708R) (Bartosch et al., 2004) and *P. distasonis* (Bd180F+Uni797R) (Kreader, 1995). 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 S7.3), 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 S7.3 PCR primer sets used in the study.

Target organism	Primer set	Sequence (5'-3')	Product size (bp)	Annealing temp (℃)	Reference
All eubacteria (PCR-DGGE)	338GC	CGCCCGCCGC GCCCCGCGCC CGGCCCGCCG CCGCCGCCGC ACTCCTACGG GAGGCAGCAG	196	58	(Muyzer et al., 1993)
	RP534	ATTACCGCGG CTGCTGG			(Muyzer et al., 1993)
All eubacteria (qPCR)	Uni331F	TCCTACGGGAGGCAGCAGT	466	58	(Bartosch et al., 2004)
	Uni797R	GGACTACCAGGGTATCTATCCTGTT			
Bacteroides-Prevotella group	Bac303F	GAAGGTCCCCCACATTG	418	56	
	Bac708R	CAATCGGAGTTCTTCGTG			
Parabacteroides distasonis	Bd180F	AAT ACC GCA TGA AGC AGG	617	62	(Kreader, 1995)
	Uni797R	GGACTACCAGGGTATCTATCCTGTT			(Bartosch et al., 2004)

7.10 SUPPLEMENTARY TABLES

Table S7.4 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 S7.5 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 S7.6 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).

7.11 SUPPLEMENTARY FIGURES

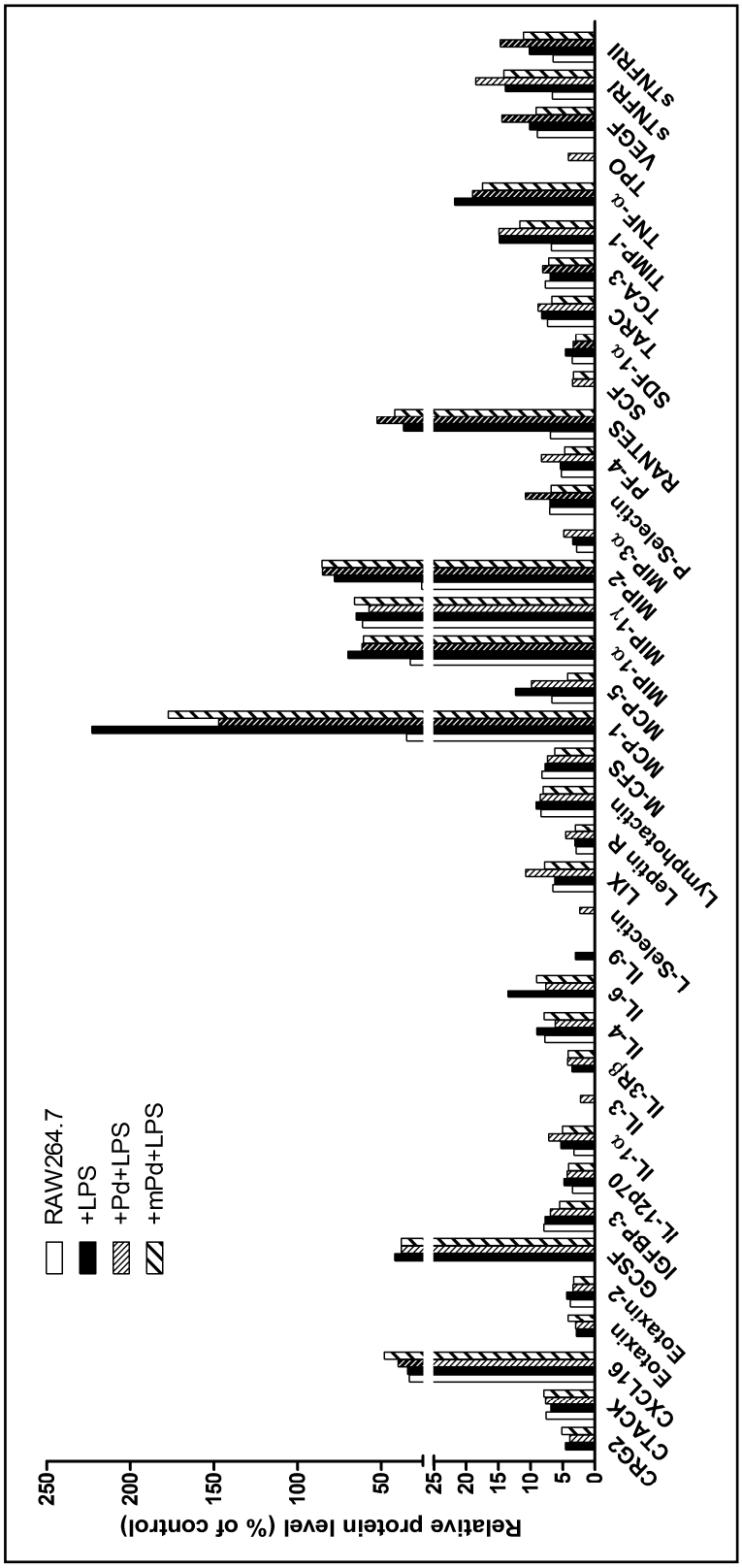


Figure S7.4 Cytokine profiling of the supernatants after the cultivation of untreated RAW264.7 cells or cells after treatment with LPS, Pd+LPS or mPd+LPS, as measured by RayBio Mouse Cytokine Antibody Array 3. Only cytokines positive at least in one sample are shown. As compared with LPS-activated cells, the Pd and mPd decrease TNF-α, IL-6, MCP-1 and MCP-5, and increase in CXCL16.

Figure S7.5 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.

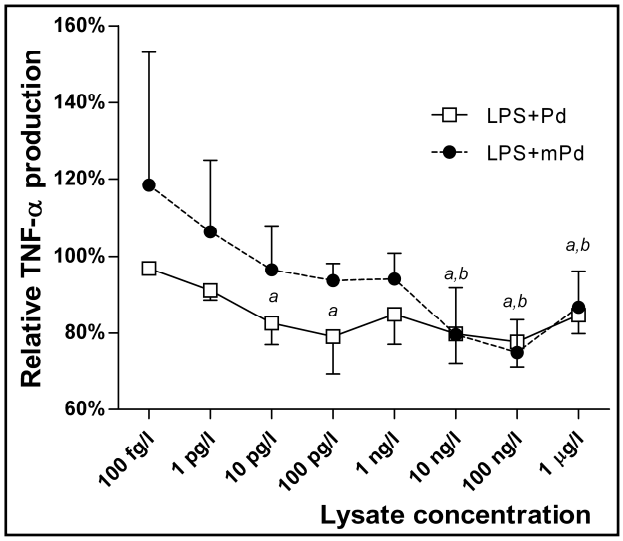
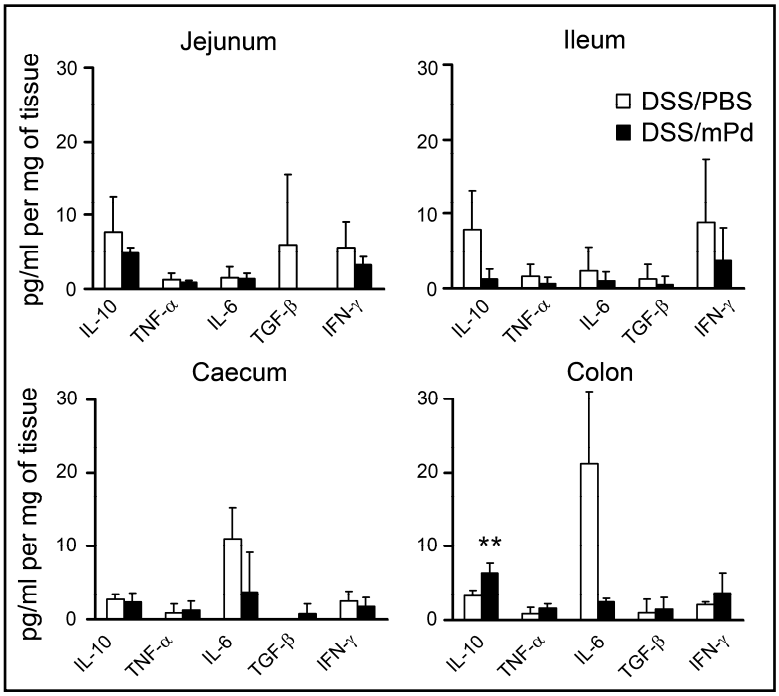


Figure S7.6 Pretreatment with mPd decreases cytokine production (pg/mg of tissue) in different parts of the gut in orally treated SCID mice as measured by ELISA.



**P<0.01: DSS/mPd versus DSS/PBS-treated mice; n = 5 mice per group.

7.12 SUPPLEMENTARY REFERENCES

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8. GENERAL DISCUSSION

The studies presented in this thesis provide novel information about the mechanisms of intestinal inflammation and possibilities to manipulate it with bacterial components. Since the main points are already discussed in individual chapters, I will focus on common mechanisms of immunomodulation.

8.1 TARGETING THE GCS EFFECTS ON PRE-RECEPTOR LEVEL: HIDDEN OPPORTUNITIES FOR IBD THERAPY?

In the study presented in chapter 4, we reported that there is a marked increase in 11HSD1 mRNA expression and enzyme activity in inflamed colon, as compared with healthy tissue. These changes were also present in colonic IELs and in MLNs, but not in the spleen, suggesting compartmentalization of this immunomodulatory mechanism. Therefore, it might be possible to use this mechanism in therapy without the general (systemic) anti-inflammatory adverse effects of GCs.

Since the 11HSD1 amplifies the effect of endogenous GC *in vivo*, the higher the 11HSD1/11HSD2 ratio is, the stronger anti-inflammatory effect the endogenous GCs have (Paterson et al., 2005). Regulation of substrate availability by the changes in the isoenzymes synthesis thus modifies receptor activation and effect of endogenous GCs. Similar mechanism preventing an overshoot of immune response during local inflammation has been recently reported also in TNBS-induced colitis and in adjuvant arthritis, suggesting that it is a common regulatory mechanism (Ergang et al., 2008; Ergang et al., 2010). Enhancing this natural defense mechanism with substances that are capable to counteract pro-inflammatory signals could be used in therapy of inflammatory diseases. It has been found that oral administration of glycyrrhizic and glycyrrhetinic acids isolated from licorice can change the 11HSD mRNA and potentiate the GCs immunomodulatory activities in rats (Whorwood et al., 1993).

8.2 HOST-BACTERIAL RELATIONSHIPS IN THE GUT

Human gut is colonized by a vast community of symbiotic, commensal bacteria that have important effects on immune function, nutrient processing, and a broad range of other physiological functions. Despite the current revolution in our knowledge about this

community, powered mostly by rapid advancements in technology, our understanding of host-microbe and microbe-microbe interactions in the gut is still limited.

It is estimated that human microbiome is composed of at least 1000 distinct bacterial species, out of which almost 50% cannot be cultivated *ex vivo* and therefore properly studied (Hooper and Gordon, 2001). Moreover, the gut microbiome, with around 150times the number of genes in our genome, imparts to us new functional features that we have not had to evolve ourselves (Qin et al., 2010). Furthermore, using selective antibiotics and pre- or probiotics and by dietary interventions, we can manipulate with gut microbiome, hence selectively introduce, enhance or reduce some of these functional features. To do so in a controlled manner, we need to unravel the key mechanisms of host-microbiota interactions and answer a few fundamental questions:

1) How do gut microbiota contribute to our normal physiology and how functionally redundant are the members of the human gut microbiota?

2) How do they contribute to pathologic states?

3) How can they be exploited to develop new therapeutic strategies?

Host–microbe interactions involve the complex interactions of various microbial molecules with various host receptors and adaptor molecules, and the final host response is therefore determined by the coordinated action of many different cell types (Lebeer et al., 2010). The nature of this action depends mainly on which receptors are triggered, the tuning of the cells in the microenvironment and the existence of tolerance or immunity to the particular microbe.

Several physiological functions of gut microbiota are briefly reviewed in the first chapter of this thesis. The question of how the gut microbiota shape intestinal immune responses can be elegantly answered by comparing the immune responses of GF animals with those reared under conventional conditions. Known defects in mucosal immune system of GF mice are summarized in Table 8.1.

Certain bacteria were found to actively shape a healthy immune system. The importance of these microbes to the normal development of the immune system (and other physiological functions) supports the notion that the total information encoded by the mammalian genome is not sufficient to maintain health, and that microbial products are crucial for protection from various diseases (Zaneveld et al., 2008). This suggests that diseases could result from the absence of certain beneficial microbial molecules and that we might be able to treat them by supplementing these molecules. The molecules can

manipulate the immune system directly or indirectly, by influencing another bacterial species already present in the gut.

Table 8.1 Intestinal immunological defects in germ-free mice

Immunological defect	Site	Phenotype in germ-free mice compared with conventionally housed mice
Development of small intestine	Peyer's patches	Fewer and less cellular
	Lamina propria	Thinner and less cellular
	Germinal centers	Fewer plasma cells
	ILF	Smaller and less cellular
Development of MLN	Germinal centers	Smaller, less cellular and with fewer plasma cells
$CD8^+$ T cells	IEL	Fewer cells and with reduced cytotoxicity
$CD4^+$ T cells	Lamina propria	Fewer cells; decreased Th17 cells in the small intestine but increased Th17 cells in the colon
$CD4^+CD25^+$ T cells	MLN	Reduced expression of FoxP3 and reduced suppressive capacity
Expression of angiogenin 4	Paneth cells	Reduced
Expression of REG3γ	Paneth cells	Reduced
Production of SIgA	B cells	Reduced
Levels of ATP	Intestine	Reduced
Expression of MHC class II molecules	IECs	Reduced
Expression of TLR9	IECs	Reduced
Levels of IL-25	IECs	Reduced

Adopted from Round and Mazmanian (2009)

8.3 THERAPEUTIC MANIPULATION WITH GUT MICROBIOTA

Gut microenvironment is greatly influenced by microbiota populations and immune state of the mucosa. Dysbiosis, an imbalance in the intestinal bacterial ecosystem, is a common finding in patients with IBD, but it is difficult to say whether it is the cause or consequence of the inflammation (Frank et al., 2007). It has been reported that there are significant changes in the gut microbiota composition during DSS-induced colitis (Okayasu et al., 1990). In the study presented in chapter 5, we tested if repeated oral administration of well known probiotics could reduce the intestinal inflammation in the acute DSS colitis. All three probiotic bacteria we tested, *L. casei* DN-114 001 (Lc), *E. coli* Nissle 1917 (Ec Nis) and *E. coli* O83 (Ec O83), decreased the severity of acute DSS-induced colitis. We did not observe any differences between the probiotic-colonized healthy mice and healthy non-treated mice.

Bacterial populations in a complex ecosystem can act co-operatively, keeping the growth of own as well as other bacterial species in balance. They 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 (Boyer and Wisniewski-Dye, 2009). These molecules can be produced by probiotic bacteria or be presented in the bacterial lysates and modify the bacterial populations. *L. casei* isolated from traditional Indian fermented vegetable pickle have anti-microbial activities against many microbes with the exception of *L. plantarum*. This effect is present even after autoclaving and it is attributed to bacteriocins produced also by other lactobacilli. Interestingly, even bacteriocins from closely related species, such as *L. casei* and *L. acidophilus*, have unique anti-bacterial spectra (Jamuna and Jeevaratnam, 2004). This selective anti-microbial activity, presented in both live and dead bacteria, could be responsible for changes in gut microbiota composition during Lc therapy we reported in chapter 6. This could be the key to correcting dysbiosis or decreasing the number of potentially pathogenic bacterial species, thus reducing the subsequent inflammation.

Certain bacterial components can induce production of antimicrobial peptides or cytokines in the host mucosa, both of which often have immunomodulatory as well as selective antibiotic properties (Yang et al., 2003; Guani-Guerra et al., 2010). Moreover, cytokines produced in the gut can drive bacteria to change their gene expression, increasing their pathogenicity. For example, large amounts of IFN-γ produced by gut mucosa during inflammation can be recognised by *P. aeruginosa* and stimulate the production of factors capable of disrupting epithelial cell function in the bacterium, thus mounting effective countermeasure to immune activation by their host (Wu et al., 2005).

Interestingly, another probiotic bacterium we have used in our experiments – Ec Nis, has been recently found to increase the production of anti-microbial peptide in humans. The levels of β-defensin 2 (hBD-2) were significantly increased in stool samples from orally treated humans even 9 weeks after this therapy has ended (Mondel et al., 2009). In their previous work, the authors found that, unlike other 40 strains of *E. coli*, Ec Nis or certain lactobacilli significantly increase the hBD-2 expression in human epithelial cells *in vitro*. Interestingly, while one isolate of *L. acidophilus* did induce remarkably high expression of hBD-2, other isolate of the same strain did not induce hBD-2 at all (Wehkamp et al., 2004). These results collectively show that a) probiotics could have

anti-microbial properties, b) these properties could be direct or indirect, and c) there could be significant difference in this activity even within one bacterial species.

The effect of bacterial lysates used in our experiments seems to be rather indirect. Unlike the live bacteria, e.g. both Ec Nis and Ec O83, which can colonize the gut and become resident members of gut microbe community (Lodinova-Zadnikova et al., 1991; Lodinova-Zadnikova and Sonnenborn, 1997), lifeless bacterial lysates are probably not present in the gut during the intestinal inflammation induction. This points to the fact that bacterial lysates contain some "signal molecules" capable of tuning the gut microenvironment. These molecules can either interact with gut microbiota or with mucosal immune system or both, thus correcting the dysbiosis, inhibiting the growth of yet unknown pathogen or promote the growth of beneficial bacteria.

8.4 IMMUNOMODULATORY ACTIVITIES OF BACTERIAL COMPONENTS

Microbial components interact with mucosal immune system on several levels. Here, we showed that oral treatment with live probiotics or lifeless bacterial components induces a broad array of immunomodulatory mechanisms. Apart from the above mentioned changes in microbial communities, these mechanisms could improve intestinal inflammation by a) induction of active anti-infection immunity against immunologically related pathogens, b) induction of oral tolerance to immunologically related microbe or c) rendering the mucosa less responsive to the pro-inflammatory stimuli.

In chapter 5, we have tested the changes in specific IgA response in the gut after the DSS-induced gut barrier breach. While specific IgA against Lc were not changed, gut barrier failure led to a significant increase or decrease in microbe-specific IgA in the gut washings after Ec O83 or Ec Nis treatment respectively. This suggests that the mucosal immune system handles each of these probiotic bacteria in a different way. It seems that there is an established mucosal tolerance to Lc and Ec Nis that could not be changed even if the microbial antigens had (in theory) unrestricted access to LP. This is in agreement with known fact, that lactobacilli are generally well tolerated by the mucosal immune system *in vivo* (Tannock, 2004). The increase in IgA response against Ec O83 after the DSS-induced gut barrier disruption clearly shows that it is possible to mount a strong response against this particular bacterium. Interestingly, this bacterium has been shown to boost mucosal defense against pathogens, decreasing the number of nosocomial

infections and mortality in pre-term infants (Lodinova-Zadnikova et al., 1991; Lodinova-Zadnikova and Sonnenborn, 1997; Cukrowska et al., 2002), and leads to decreased number of repeated infections and allergies later in life (Lodinova-Zadnikova et al., 2003).

Not only live probiotic bacteria, but also bacterial lysates and components have immunomodulatory properties. Here, we report that oral pretreatment with either lysate of *L. casei* DN-114 001 (chapter 6) or *Parabacteroides distasonis* (Pd) (chapter 7) changes numerous immune responses as detected both *in vitro* and *in vivo* methods. The above mentioned question of whether there is an active anti-infection immunity against immunologically related pathogens is especially interesting in regard to oral treatment with Pd, because the members of bacteroidetes phylum were proposed to play a role in IBD pathogenesis (Rath et al., 1996; Saitoh et al., 2002; Swidsinski et al., 2002). We showed in chapter 7, that oral treatment with Pd lysate, and especially with its membranous fraction (mPd), protects mice from DSS-induced colitis. Interestingly, neither of the lysates from related commensals we tested (*B. ovatus, B. vulgatus, B. stercoris, B. thetaiotamicron*) showed this activity. Moreover, this effect is gut-dependent, because neither intraperitoneal, nor subcutaneous administration protected mice from severe forms of intestinal inflammation.

All three isotypes of anti-mPd antibodies we have measured, IgA, IgM and IgG, are markedly increased in the serum of mPd treated mice. The intensity of this antibody response after oral treatment is as high as when the mPd is administered subcutaneously with adjuvants. Nevertheless, we could not detect the mPd-specific antibodies in intestinal washings, suggesting that the antigen exclusion does not play a significant part in the protective effect. Oral treatment with Lc lysate did not lead to increase in specific serum antibodies. This might suggest that the anti-bacteroides antibodies can protect the mucosa during the destruction of its barrier function from flood of microbes from the gut. Pathogen-specific serum antibodies can protect the host from the microbes already present in the mucosa (Underdown, 2005). Since the members of the bacteroidetes phylum represent the dominant antigenic stimulus during IBD (Saitoh et al., 2002), this mechanism could help with clearing the flood of microbial antigens from gut mucosa after gut barrier failure, thus interrupting the vicious circle of inflammation. This idea is further supported by our findings that this therapy does not work in mice lacking adaptive immunity (SCID) and that the effect of the therapy could be transferred between immunocompetent mice via serum. Similar results might be, however, obtained even if

the actual protective signal is transferred from the tolerized cells in the gut mucosa to lymphocytes via tolerosomes, since these particles can pass through 0.22 μm filter like antibodies (Karlsson et al., 2001; Thery et al., 2002).

Another key mechanism by which microbial components could dampen the intestinal inflammation is induction of oral (mucosal) tolerance to immunologically related antigen. If there is an aberrant immune response to gut commensals in IBD, induction of oral tolerance to these components might be a useful tool to modulate the mucosal immune system. Inducing oral tolerance is an attractive approach for treatment of other autoimmune and inflammatory diseases, such as allergy, diabetes, multiple sclerosis, and graft rejection, as reviewed by Faria and Weiner (2005). Induction of oral tolerance in vivo is dependent on the function of regulatory T cells. We found a significant increase in T_{regs} in MLN of mPd treated animals, but there were no changes in T_{regs} numbers in the spleen or Peyer's patches. It has been shown recently that both gut microbiota and the sterile diet contaminated with microbial components could induce the expansion of T_{regs} in MLNs (Hrncir et al., 2008), however, it had not been precisely determined which part of the microbe is responsible. Unlike with mPd, we have not found any changes in T_{regs} in MLN, spleen or Peyer's patches after treatment with Lc or mLc. These results point to the possible induction of oral tolerance in the gut compartment of mPd, but not Lc or mLc treated mice. Interestingly, So et al. (2008) found that oral co-administration of live *L. casei* with collagen significantly augmented oral tolerance to collagen in rat model of collagen-induced arthritis, increased number of T_{regs} and expression of TGF-β and IL-10 in isolated $CD4^+$ cells *in vitro*. However, although administration of *L. casei* or collagen alone also decreased the severity of arthritis, this effect was less pronounced and the increase in T_{reg} was not statistically significant in either case. This is in agreement with what we found in Lc and mLc lysates, and suggests that *L. casei* could potentiate the induction of oral tolerance through bystander suppression, but only when specific conditions are met.

The fine tuning of the gut mucosa with cytokines is of utmost importance for oral tolerance induction as well as for keeping the anti-inflammatory programming of the cells of innate immunity. Since the microbial components show marked influence on mucosal immune response, they may act as special "tolerizing adjuvants". We showed that Lc, mLc, Pd and mPd decrease the activation of NF-κB cascade and the production of TNF-α by LPS activated macrophages *in vitro*. This direct effect on macrophages could lead to

decrease of pro-inflammatory readiness in gut mucosa, rendering it less responsive to other pro-inflammatory stimuli. These anti-inflammatory changes were not observed with another bacterial lysates, such as *L. plantarum* or *E. coli* O83 (data not shown). In the similar *in vitro* model, Pena et al (2004) found that *L. johnsonii*, the only lactobacillus isolated from the gut of colitic IL-10KO mice, does not decrease the TNF-α production as does *L. reuteri* or *L. murinus*, both isolated from the gut of healthy C57/BL6 mice. The striking differences among the individual isolates further support the notion that the fundamental probiotic unit should be the bacterial clone and not genus or species, as discussed earlier.

Our experiments with *ex vivo* tissue fragments cultivation showed, that there are pronounced changes in the cytokine production after treatment with bacterial lysates. We showed that there is an overall decrease in cytokine production (in both pro- and anti-inflammatory) after the mPd treatment in the key compartments of gut mucosal immune system – PP and large intestine. Using protein array, we found that this general decrease in cytokine production covers also growth factors (e.g. GCSF) and enzyme inhibitors (e.g. TIMP-1) in colon tissue. Global decrease in cytokine production may be the result of lower degree of pro-inflammatory and compensatory anti-inflammatory mechanisms caused by a different mechanism, e.g. elimination of the antigen by antibodies. Nevertheless, the decrease in TGF-β after the mPd treatment was apparent even in mice without DSS, suggesting that all these changes were not related to the immune response to the inflammation. Moreover, we found that the changes in cytokine production are different after Lc as compared to mPd treatment, but all these changes were in the same gut compartments (PP and large intestine), suggesting that both inductive and effector site of the immune system play an important role in therapeutic effects of both lysates. The anti-inflammatory phenotype is more apparent after Lc treatment, with the decrease in IL-6, IFN-γ and IL-10. Different molecules included in these two different lysates probably interact with the different cells of the mucosal immune system, which could explain the different response of the gut mucosa after Lc and mPd therapy. Nevertheless, we could not say with enough confidence, whether these changes are caused directly by therapy or simply by lower amount of pro-inflammatory stimuli. The therapy could improve the gut barrier function, decreasing the amount of pro-inflammatory stimuli and thus decreasing the severity of the inflammation. This is indeed supported by our findings that oral treatment with Lc or mLc significantly improves the gut barrier function during acute

DSS inflammation. There is yet another chicken-egg paradox, because the lower intestinal permeability could be caused by decrease in IFN-γ, cytokine known to increase gut epithelium permeability (Madara and Stafford, 1989). Indeed, probiotic or commensal microbes can change the gut barrier integrity *in vivo*, but both increase and decrease in the barrier integrity is possible (Garcia-Lafuente et al., 2001). Furthermore, oral treatment with either Ec Nis or *L. plantarum* has been found to enhance mucosal integrity, by modulating the TJs proteins, and this mechanism was proposed as a key mechanism of probiotic function (Ukena et al., 2007; Chen et al., 2010).

8.5 FUTURE PERSPECTIVES

Human microbiota has been indicated as a new player in ethiopathogenesis of many human diseases, including IBD. The studies presented in this thesis suggest several possibilities for further investigations into the mechanisms of host-microbe and microbe-microbe interactions during the intestinal inflammation.

Overall, numerous studies regarding both the beneficial and deleterious role of particular microbe have recently led to many important discoveries, explaining their role in disease ethiology and pathogenesis. Several pathogens have been identified, such as Adhesive-invasive *E. coli* (AIEC) or *M. avium* subspecies *paratuberculosis* (MAP), as mentioned in chapter 3. Now, there is a growing interest in how to decrease the human exposition to these noxious microbes. We believe that development of mucosal vaccine and vaccination of the subjects at risk against these microbes might be a good strategy. There are, however, many questions regarding the antigen and suitable adjuvants and safety of this measure. The vaccine composed of recombinant MAP HSP70 has already been successfully used as a subunit vaccine against bovine paratuberculosis, a disease with pathological findings similar to human CD (Koets et al., 2006). Moreover, although the role of MAP in CD pathogenesis is still debated (Mendoza et al., 2009), minimizing human exposure to MAP by controlling the disease in cattle, might actually have the same effect as vaccinating humans. This is an open field that should be addressed once the causative agent of IBD is identified.

While the role of microbiota in various diseases is starting to be appreciated, more scientists are trying to characterize the gut microbiota and to understand its role in health and disease, using high-throughput metagenomic and metabolomic methods (Backhed

and Crawford, 2010; Booijink et al., 2010). These advanced methods can not only analyze the composition of gut microbiota in health and disease, but they can also bring an insight into the functional changes of this complex microbial community. Other approaches, such as gnotobiology, must be chosen to address the questions regarding the interactions between host and particular microbe (Tlaskalová-Hogenová, 1997).

Pro- and pre-biotics are now starting to be used in therapy of several diseases, and their beneficial properties have been showed in numerous experimental models (Petrof, 2009). However, the use of live bacteria should be considered with caution, because they can cause severe complications in immunocompromised patients (Besselink et al., 2008). This clearly shows, that we need to develop a better understanding of microbes intended to be used as probiotic and that bacterial components that could exert all the beneficial properties of the probiotics might be a safer alternative to probiotics.

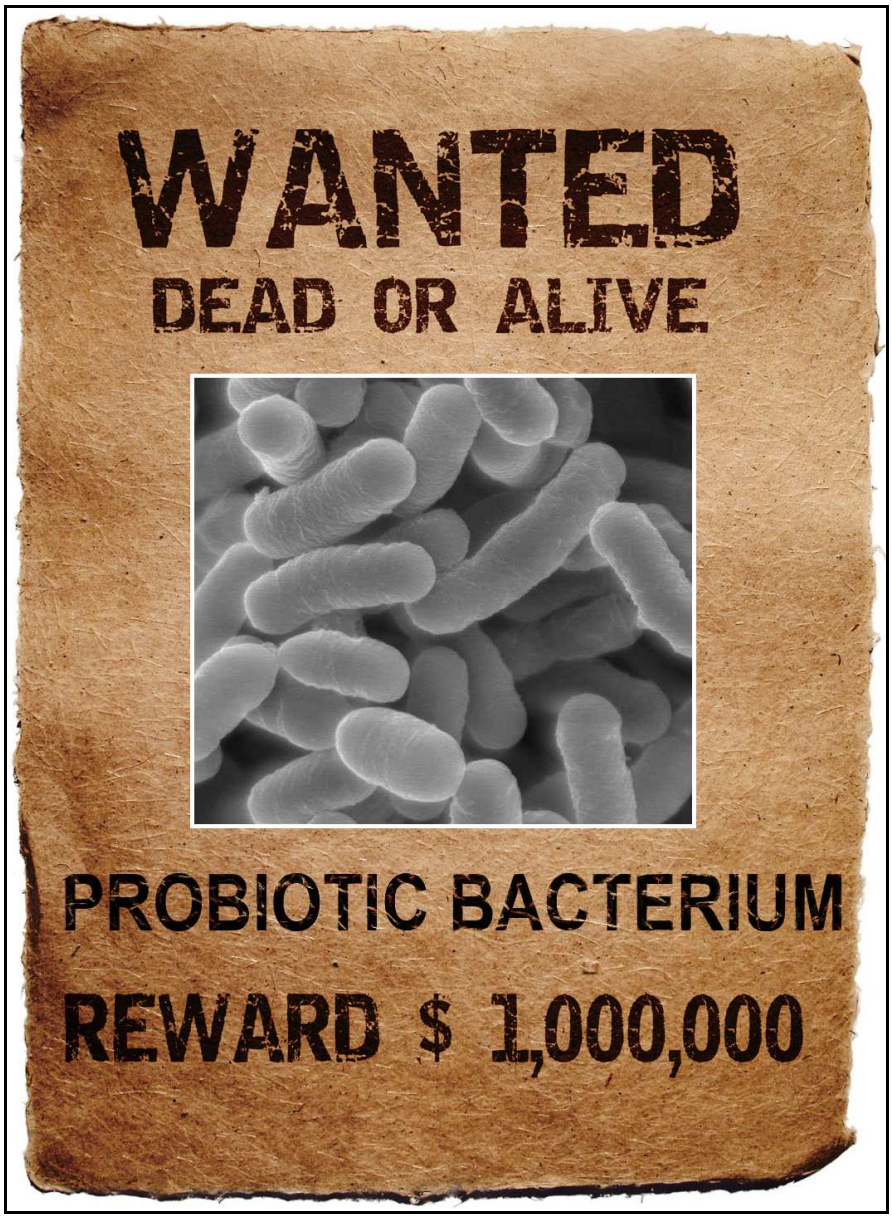
Here, we show that local GC metabolism is changed during intestinal inflammation. Interestingly, there are no data on whether probiotics or some microbial component could enhance this natural protective mechanism by directly interfering with 11HSDs. After all, this could be the mechanism by which some probiotics exert their immunomodulatory properties. This is another goal worth pursuing, because knowledge of the molecular mechanisms of probiotics is still insufficient, yet it holds the key to therapeutic use of microbial components. Therefore, our further effort will be focused on the detailed characterization of the anti-inflammatory mechanisms of our bacterial lysates, and on isolation of the active immunomodulatory component.

8.6 CONCLUSIONS

The severity of intestinal inflammation can be decreased using certain live bacteria or their components. This is achieved by different mechanisms; each could be unique for a particular bacterial component. These mechanisms comprise a) correcting the dysbiosis, b) modulation of the mucosal immune response and c) improving the gut barrier function. It is clear from the results presented in this thesis, that certain bacterial components can trigger these mechanisms, and that the spectrum of these anti-inflammatory mechanisms is strain, or even clone dependent. Oral treatment with these “immunomodulatory” bacterial components can be a good strategy for therapy, or even prevention, of severe inflammatory diseases, such as IBD. The striking differences between anti-inflammatory

properties of two different microbial components presented here suggest that their utility in the IBD therapy will depend on the stage of the disease and which mechanisms the component should trigger in a particular patient. Our findings presented here enhance the basic knowledge on how microbial components can modulate the immune system, which might help to individualize the therapy. However, we still need much deeper understanding of the underlying mechanisms of host-microbe and microbe-microbe interactions, before the fruits of this fascinating research can be used for the benefit of all patients.

Figure 8.1 Manifesto...



"Ce sont les microbes qui auront le dernier mot" (Louis Pasteur)

8.7 REFERENCES

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Appendix A – Abbreviations

11HSD – 11β-hydroxysteroid dehydrogenase

Ab – Antibody

AP-1 – Transcription factor Activator protein-1

APCs – Antigen presenting cells

BALT – Bronchus-associated lymphoid tissue

CD – Crohn's disease

cPd – cytoplasmic fraction of *P. distasonis* lysate

CPs – Cryptopatches

CTAB – Cetyltrimethylammonium bromide

CTLs – Cytotoxic T cells

DCs – Dendritic cells

DSS – Dextran-sulfate sodium

Ec Nis – *Escherichia coli* Nissle 1917 O6:K5:H1

Ec O83 – *Escherichia coli* O83:K24:H31

ELAM-1 – endothelial-leukocyte adhesion molecule 1

ELISA – Enzyme-linked Immunosorbent assay

FAE – Follicle-associated epithelium

FITC – Fluorescein-iso-thiocyanate

FoxP3 – Forkhead box P3

GC – Glucocorticoid (GC)

GF – Germ-free

H&E – Hematoxylin and eosin

HRP – Horse radish peroxidase

IBD – Inflammatory bowel disease

ICAM-1 – Intracellular adhesion molecule 1

IECs – Intestinal epithelial cells

IEL – Intraepithelial lymphocytes

IFA – Incomplete Freund’s adjuvant

IFN-γ – Interferon gamma

Ig – Immunoglobulin

IL – Interleukin

Lc – *Lactobacillus casei* DN-114 001

Lp – Lamina propria

LPL – Lamina propria lymphocytes

LPS – Lipopolysaccharide

MAdCAM-1 – Mucosal vascular addressin cell adhesion molecule 1

MALT – Mucosa-associated lymphoid system

MAP – Mycobacterium avium subsp. paratuberculosis

MCP-1 – Monocyte chemotactic protein 1 (CCL2)

MHC – Major histocompatibility complex

MICA – MHC class I-related molecule A

MICB – MHC class I-related molecule B

MLCK – Myosin light chain kinase

MLNs – Mesenteric lymph nodes

mLc – Membranous fraction of *Lactobacillus casei* DN-114 001

mPd – Membranous fraction of *Parabacteroides distasonis*

MyD88 – Myeloid differentiation primary response gene (88)

NAD(H) – Nikotinamid adenin dinukleotid

NADP(H) – Nikotinamid adenin dinukleotid fosfát

NF-κB – Transcription factor Nuclear factor kappa B

NOD – Nucleotide-binding oligomerization domain containing

OD – Optical density

MAMP – Microbe-associated molecular patterns

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PCR-DGGE – Polymerase chain reaction-denaturing gradient gel electrophoresis

Pd – *Parabacteroides distasonis*

pIgR – Polymeric Ig receptor

PP – Peyer's patches

PRR – Pattern recognition receptor

qPCR – Quantitative PCR

SBTI – Soybean trypsin inhibitor

SC – Secretory component

SCID – Severe combined immunodeficient

SED – Subepithelial dome

SIgA – secretory IgA

SIgM – secretory IgM

TCR – T-cell receptor

TDA – Thymus-dependent area

TECK – Thymus-expressed chemokine (CCL25)

TGF-β – Transforming growth factor

Th – T helper

TJ – Tight junction

TLR – Toll-like receptor

TMB – 3,3',5,5'-tetramethylbenzidine

TNF-α – Tumor necrosis factor

T_{reg} – Natural regulatory T cells

TSLP – Thymic stromal lymphopoietin

UC – Ulcerative Colitis

UPGMA – Unweighted pair-group method with arithmetic averages

ZOT – Zonula ocludens toxin

Appendix B – Curriculum vitae

Born: September 13, 1978, Prague, Czech Republic

Education and Employment:

1997 – 2003 3rd Faculty of Medicine, Charles University in Prague

2003 – now Institute of Microbiology of the AS CR, v.v.i. Department of Immunology and Gnotobiology, Prague PhD project – Interaction of commensal bacteria with mucosal immunity and its regulation. Supervisor: Prof. Helena Tlaskalová-Hogenová, MD, DSc.

Memberships, Honors, Committees and Public Service:

2003 Member of Czech Immunological Society

2003 Member of Czech Society of Gastroenterology

2003 Member of Czech Society of Allergology and Clinical Immunology

2005 Member of Society for Mucosal Immunology

2005 Member of European Academy of Allergology and Clinical Immunology

2006 Member of Czech Society for Probiotics and Prebiotics

2007 Associate Member of the Faculty 1000 Medicine

2008 Prize for the best paper by a young immunologist in 2007 awarded by the Czech Immunological Society

2008 Prize for the best poster at the 6th European Mucosal Immunology Group Meeting

2008 Member of The laboratory animal care and use committee of Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i.

Stays:

2006 and 2007 Department of Cell Biology, University Medical Center Groningen, Groningen, The Netherlands

Projects:

2010 **RayBiotech, Inc.** Biomarker Discovery Research Pilot Grants 2010. Principal Investigator: Miloslav Kverka

2009 – 2011 **The Grant Agency of the Academy of Sciences of Czech Republic.** KJB500200904 - Analysis of antiinflammatory properties of selected substances of bacterial and plant origin in experimental colitis therapy and prevention. Principal Investigator: Miloslav Kverka

2006 – 2007 **Danone – Institute**. Effects of probiotic bacteria on mucosal immunity: Analysis of anti-inflammatory propertis of probiotic bacteria components.

Publications:

Total: 13

Sum of citations: 68

H-index: 5 (WoS)

Other professional activities:

2004 – now Lecturer in the Basic course in Clinical Immunology organized for Faculty of Science, Charles University in Prague.

Appendix C – Publications

Zákostelská Z, **Kverka M**, Klimesova K, Hudcovic T, Hrncir T, Rossmann P, Mrazek J, Jan Kopecny J and Tlaskalova-Hogenova H. Lysate of the probiotic bacteria Lactobacillus casei have antiinflammatory properties *in vitro* and *in vivo*. Manuscript in preparation

Cinova J, De Palma G, Stepankova R, Kofronova O, **Kverka M**, Sanz Y, and Tuckova L Role of Intestinal Bacteria in Gliadin-Induced Changes in Intestinal Mucosa : Study in Germ-Free Rats. Plos ONE, Manuscript submitted

Kverka M, Klimesova K, Zakostelska Z, Sokol D, Hudcovic T, Hrncir T, Rossmann P, Mrazek J, Jan Kopecny J, Verdu EF, and Tlaskalova-Hogenova H. Oral administration of *Parabacteroides distasonis* antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. Clin Exp Immunol., Manuscript in press, doi:10.1111/j.1365-2249.2010.04286.x

Tlaskalová-Hogenová H, Štěpánková 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, Šrůtková D, Zídek Z, Schwarzer M, Drastich P, Funda DP. Gut microbiota (commensal bacteria) and mucosal barrier in pathogenesis of inflammatory, autoimmune diseases and cancer: Contribution of germ-free and gnotobiotic models of human diseases. Cell. Mol. Immunol., in press

Kverka M, Klimešová K, Zákostelská Z, Froľová L, Hudcovic T, Hrnčíř T, Rossmann P, and Tlaskalová-Hogenová H. Probiotics and bacterial components in intestinal inflammation therapy and prevention In: Biological consequences of host-microbe interactions (Eds.: Heidt, PJ, Hanson, LÅ, Tlaskalová-Hogenová H, Rusch, V.) Herborn litterae, Herborn, 2009,75-92

Arakelyan A, Kriegova E, Kubistova Z, Mrazek F, **Kverka M**, du Bois RM, Kolek V, Petrek M. Protein levels of CC chemokine ligand (CCL)15, CCL16 and macrophage stimulating protein in patients with sarcoidosis. Clin Exp Immunol. 2009;155:457-65.

Junek R, **Kverka M**, Jandera A, Panajotová V, Satinský D, Macháček M, Kuchař M. Antileukotrienic phenethylamido derivatives of arylalkanoic acids in the treatment of ulcerative colitis. European Journal of Medicinal Chemistry. Eur J Med Chem. 2009;44:332-44.

Strnádel J, **Kverka M**, Reisnerová H, Hlučilová J, Usvald D, Plánská D, Váňa P, Jílek F, Vannucci L, Horák V. Protein Chips in Cytokine Expression Study on Tumour Model: Optimization of Sample Preparation. Chem. Listy 2008;102, 269-273 (article in Slovak)

Zizka J, **Kverka M**, Novotná O, Stanková I, Lodinová-Zádníková R, Kocourková I, Sterzl I, Prokesová L. Perinatal period cytokines related to increased risk of future allergy development. Folia Microbiol (Praha). 2007;52:549-55.

Strnádel J, **Kverka M**, Horák V, Vannucci L, Usvald D, Hlucilová J, Plánská D, Vána P, Reisnerová H, Jílek F. Multiplex analysis of cytokines involved in tumour growth and spontaneous regression in a rat sarcoma model. Folia Biol (Praha). 2007;53:216-9.

Vrabelova Z, Kolouskova S, Bohmova K, Faresjo MK, Sumnik Z, Pechova M, **Kverka M**, Chudoba D, Zacharovova K, Stadlerova G, Pithova P, Hladikova M, Stechova K. Protein microarray analysis as a tool for monitoring cellular autoreactivity in type 1 diabetes patients and their relatives. Pediatr Diabetes. 2007;8:252-60.

Junek R, Brunova B, **Kverka M**, Panajotova V, Jandera A, Kuchar M. Antileukotrienic N-arylethyl-2-arylacetamides in the treatment of ulcerative colitis. Eur. J. Med. Chem. 2007;42:1084-94.

Kverka M, Burianova J, Lodinova-Zadnikova R, Kocourkova I, Cinova J, Tuckova L, Tlaskalova-Hogenova H. Cytokine profiling in human colostrum and milk by protein array. Clin Chem. 2007;53:955-62.

Kokešová A, Frolová L, **Kverka M**, Sokol D, Rossmann P, Bártová J, Tlaskalová-Hogenová H, Pácha J. Oral administration of probiotic bakteria (*E. coli* Nissle, *E. coli* O83, *Lactobacillus casei*) influences the severity of dextran sodium sulfate-induced colitis. Folia Microbiol (Praha). 2006;51:478–84

Vagnerová K, **Kverka M**, Klusoňová P, Ergang P, Mikšík I, Tlaskalová-Hogenová H, Pácha J. Intestinal inflammation modulates expression of 11β-hydroxysteroid dehydrogenase in murine gut. J Endocrinol. 2006;191:497–503

Tlaskalova-Hogenova H, Tuckova L, Mestecky J, Kolinska J, Rossmann P, Stepankova R, Kozakova H, Hudcovic T, Hrncir T, Frolova L, **Kverka M**. Interaction of mucosal microbiota with the innate immune system. Scand J Immunol. 2005;62 Suppl 1:106-13.

Stechova K, Kalouskova S, Sumnik Z, Cinek O, **Kverka M**, Karlsson Faresjö, Chudoba D, Dovolilova E, Pechova M, Vrabelova Z, Böhmova K, Janecek L, Saudek F, Vavrinec J. Anti-GAD65 reactive peripherial blood mononuclear cells in the pathogenesis of cystic fibrosis related diabetes mellitus. Autoimmunity. 2005;38:319-323

Tlaskalova-Hogenova H, Tuckova L, Stepankova R, Hudcovic T, Palova-Jelinkova L, Kozakova H, Rossmann P, Sanchez D, Cinova J, Hrncir T, **Kverka M**, Frolova L, Uhlig H, Powrie F, Bland P. Involvement of innate immunity in the development of inflammatory and autoimmune diseases. Ann N Y Acad Sci. 2005;1051:787-98.

Kverka M, Malý M, Dlouhý P, Kučera P. Evaluation of risk factors for Allergic diseases in group of Prague schoolchildren. Alergie. 2003;3:178-182. (article in Czech)