

Interactions of commensal bacteria with the host immune system

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Interactions of commensal bacteria with the host immune system

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Chapter 1

General Introduction

Host microbe interactions in the mammalian intestine

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Abbreviations

GIT	Gastrointestinal tract
SI	Small intestine
LP	Lamina propria
M cells	Microfold cells
GALT	Gut-associated lymphoid tissue
DSS	Dextran sodium sulphate
TFF3	Trefoil factor 3
IECs	Intestinal epithelial cells
APCs	Antigen presenting cells
TJ	Tight junction
EPEC	Enteropathogenic <i>Escherichia coli</i>
PRR	Pattern recognition receptor
HD	Human α -defensin
MMP	Matrix metalloprotease
HBD-1	Human- β -defensin-1
MBD-2	Murine β -defensin 2
DC	Dendritic cell
slgA	Secretory immunoglobulin A
MLN	Mesenteric lymph node
IL	Interleukin
TGF- β	Transforming growth factor- β
RA	Retinoic acid
J	'joining' chain
plg	Polymeric-immunoglobulin
VH	Variable region
BAFF	B cell-activating factor
APRIL	B cell proliferation-inducing ligand
LPS	Lipopolysaccharide
PRRs	Pattern recognition receptors
MAMPs	Microbe-associated molecular patterns
TLRs	Toll-like receptors
NLRs	Nod-like receptors
LRR	Leucine-rich repeats
TIR	Toll-interleukin 1 receptor
CARD	Caspase recruitment domain
NOD	Nucleotide-binding oligomerisation domain
PG	Peptidoglycan
MyD88	Myeloid differentiation primary response gene 88
MAPK	Mitogen-activated protein kinase
NF- κ B	Nuclear factor κ B
IKK	I κ B kinase
IFN	Interferon
TNF	Tumor necrosis factor
MCP-1 or CCL-2	Monocyte chemotactic protein-1
PPAR γ	Peroxisome proliferator-activated receptor- γ
TOLLIP	Toll-interacting protein
TSLP	Thymic stromal lymphopoietin
IDO	Indoleamine 2,3 dioxygenase
IBD	Inflammatory bowel disease
CD	Crohn's disease
UC	Ulcerative colitis
Tregs	Regulatory T cells
Th	T helper

1. Mutualistic host-microbe relationships

The mammalian gastrointestinal tract (GI-tract) is specialized in the digestion and absorption of nutrients and is colonized by a myriad of symbiotic microorganisms collectively known as the microbiota. In the course of evolution the symbiotic relationship between the host and the microbiota has become largely mutualistic, benefiting both partners. The concentration of bacteria forms a gradient from the stomach, and small intestine (SI) to the colon where densities reach around 10^{12} bacteria per gram of luminal content (Whitman, Coleman et al. 1998). Colonic bacteria can ferment complex carbohydrates, not digested in the upper GI-tract, and provide energy to their host. In humans, this accounts for 6-9% of the total energy requirement whereas it can be as high as 44% for ruminants (McNeil 1984; Hume, Nisbet et al. 1997). Bacterial fermentation produces short chain fatty acids (SCFA), vitamins and other nutrients. Butyrate, one of the SCFA produced in the intestinal lumen by bacterial fermentation, is the main energy source for colonic epithelial cells. Other functions of the microbiota include the competitive exclusion of pathogens and the contribution to the development of the host immune system. Compared to conventional mice, germ-free mice have an underdeveloped mucosal immune system. Germ-free mice have smaller Peyer's patches (PP) and mesenteric lymph nodes (MLN), fewer intraepithelial and lamina propria (LP) lymphocytes and lymphoid follicles compared to mice housed in specific pathogen free (SPF) conditions (Hooper and Macpherson 2010). Bacterial colonization of germ-free mice induces major changes in epithelial morphology and function as well as the development of the mucosal-associated lymphoid tissue. In addition to these developmental aspects, the microbiota contributes to homeostasis of tolerance and immunity through interactions of microbial metabolites and other components with the innate and adaptive immune systems. The role of specific organisms or groups of bacteria in intestinal homeostasis is just beginning to be unravelled and some key examples are discussed in the following sections.

2. A delicate balancing act

The coevolution of a mutualistic relationship between the host and the microbiota required the mucosal immune system to develop the ability to defend the body against infections of pathogenic microorganisms while avoiding excessive inflammatory responses to harmless antigens and commensal bacteria.

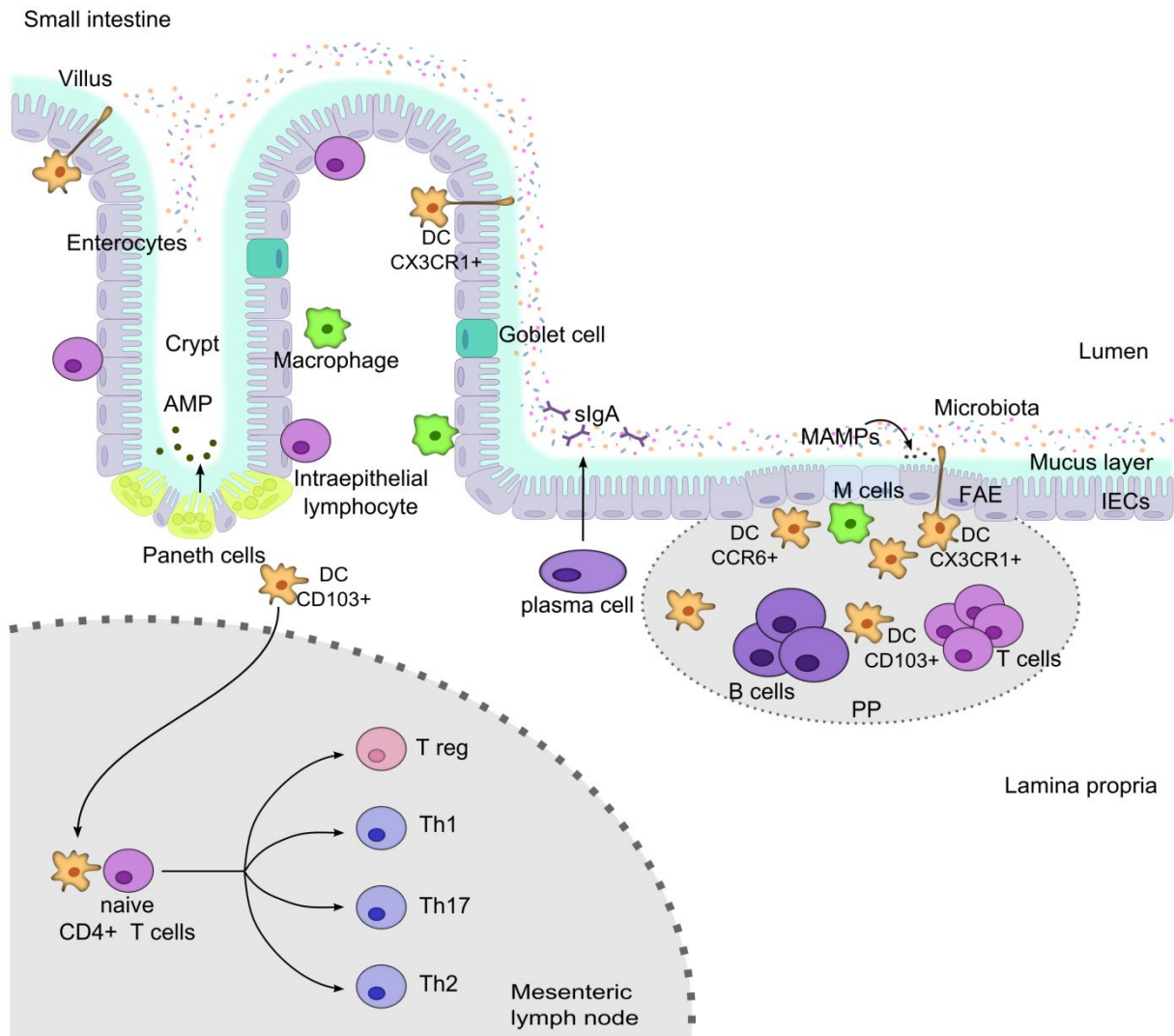


Figure 1: The mucosal epithelium, lamina propria cells and gut-associated lymphoid tissue in the small intestine. Intestinal homeostasis is maintained by several mechanisms involving epithelial cells, connective tissue and immune cells. A single layer of intestinal epithelial cells separates the luminal contents from the lamina propria. In the basolateral space between epithelial cells are positioned intraepithelial lymphocytes. The epithelium is covered by a thick layer of mucus produced by goblet cells. Enterocytes and specialized Paneth cells in the crypts are able to secrete antimicrobial peptides (AMP) preventing bacterial growth in proximity to the epithelium. Plasma cells in the lamina propria produce secretory IgA (sIgA) that mediates bacterial exclusion. The luminal content is continuously sampled by specialized epithelial cells and immune cells. In the follicle associated epithelium (FAE) of the Peyer’s patch (PP) or isolated lymphoid follicles, M cells transcytose bacteria and antigens. In the LP and PP, CX3CR1⁺ macrophages and DCs sample microbes and antigens through the epithelium to support inflammatory responses. DCs, macrophages and M cells deliver antigens and bacteria to antigen presenting cells (APCs). Activated APCs then move to the follicular area of the PP or they migrate to the mesenteric lymph nodes through the lymphatic system and present the antigen to naïve lymphocytes. Mature DCs drive the differentiation of naïve CD4⁺ T lymphocytes into effector T cells, helper T cells (Th1, Th2, Th17) or regulatory T cells (Tregs).

Inflammation, the acute response to harmful stimuli including microorganisms, is a crucial component of this host defence. In part, the protective consequences of triggering inflammation result from increased vascular permeability and attraction of phagocytes to engulf and kill invading microorganisms. The interaction of antigen presenting phagocytes with the adaptive immune system then leads to protracted cellular and humoral responses and immunological memory against “non-self” antigens. However, if the host is not immunologically tolerant to commensal microbes it can cause damage to host tissues as seen in inflammatory bowel disease. The host must also be able to elicit a localized inflammatory response to an invading pathogen while, at the same time, preserving homeostasis in the uninfected tissues. It is this delicate act of balancing opposing functions that we focus on in this chapter. In the intestine, homeostasis of inflammation is maintained by a complex network of microbe and host cell interactions involving numerous negative feedback and feed-forward controls. The interacting cell types include epithelial cells, connective tissue cells and both innate and adaptive immune cells (macrophages, dendritic cells and lymphocytes) residing in the LP (Figure 1). The LP is a layer of loose connective tissue between the epithelium and the muscularis mucosa, containing lymphatic ducts and blood vessels. The main homeostatic mechanisms involve the compartmentalization of bacteria by physical and chemical barriers (discussed in section 3), the continuous surveillance of the microbial content by innate receptors (discussed in section 4), the induction of secretory antibody responses and a state of immunological tolerance to commensals and food antigens (discussed in section 5).

Failure of intestinal immune homeostasis is clearly implicated in inflammatory bowel diseases (discussed in the section below), where several immunity gene defects interact with environmental factors including the microbiota to cause chronic intestinal inflammation.

2.1. Loss of tolerance to the microbiota in inflammatory bowel disease

Inflammatory bowel disease (IBD) is a collective term for Crohn’s disease (CD) and ulcerative colitis (UC) that are chronic intestinal inflammatory disorders caused by failure of intestinal homeostasis and inappropriate immune responses to the microbiota (Janowitz, Croen et al. 1998). CD is characterized by transmural inflammation in the ileum and colon while in UC the inflammation affects only the colonic and rectal mucosa. The origin of IBD is multifactorial, involving genetic predisposition, environmental factors and an imbalance between beneficial

and potentially harmful species in the microbiota. Genome-wide association studies have implicated several genetic polymorphisms in IBD (Wellcome Trust Case Control 2007). The genes identified to date are all involved in innate immunity or intestinal barrier functions. In monozygotic twins with known genetic risk factors for IBD, the concurrence of disease is relatively low demonstrating the importance of environmental factors including the microbiota (Wellcome Trust Case Control 2007).

Compared with healthy individuals, IBD patients show a reduction in the abundance and diversity of Firmicutes and Bacteroidetes (Frank, St Amand et al. 2007) and CD patients have increased numbers of adherent-invasive *Escherichia coli* (Barnich, Carvalho et al. 2007). Despite the above observations, a causal relationship between specific microorganisms and the disease remains unproven. On the other hand, several studies have shown a protective effect of specific commensal bacteria or their components against colitis in different mouse models, highlighting the importance of commensal bacteria in the maintenance of homeostasis and tolerance. For example, the human commensal *Faecalibacterium prausnitzii*, which is reduced in CD patients, protects mice from 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis and induces high IL-10 and low IL-12 secretion in human peripheral blood mononuclear cells *in vitro* (Sokol, Pigneur et al. 2008). Another example is *Bacteroides fragilis* that protects mice from *Helicobacter hepaticus*-induced colitis (Mazmanian, Round et al. 2008). *B. fragilis* also induces IL-10 secretion in CD4⁺ T cells and prevents the expansion of Th17 cells in mono-associated mice, these effects are mediated by bacterial polysaccharide A (PSA) (Round, Lee et al. 2011). Furthermore, colonization of germ-free mice with a cocktail of 46 strains of mouse commensal *Clostridium* induces an accumulation of regulatory T cells (Tregs) in the colon LP (Atarashi, Tanoue et al. 2011). These findings suggest that not only the bacteria themselves but also their products play a role in the maintenance of homeostasis and can have beneficial effects.

In the following sections, we discuss in more detail how homeostasis is maintained in the SI and colon, which are permanently colonized by commensal bacteria and sometimes opportunistic or obligate pathogens.

3. Keeping microbes at a distance

At the tissue level, antimicrobial factors, secretory antibodies and physical barriers limit exposure of the mucosal tissue to microorganisms. The mucus layer covering the epithelium and the epithelium itself constitute the two major physical barriers (Figure 1).

3.1. Physical barriers, the mucus layer

The first physical barrier encountered by the luminal microbes is the mucus, a thick layer of mucin glycoprotein secreted by specialized goblet cells in the epithelium. The mucus is constantly produced and removed by peristaltic flushing. In the colon, the mucus layer extends about 150 μm from the epithelial cell surface and can be divided in two structurally different layers, an outer mucus layer containing bacteria and a firmly attached inner layer that is almost devoid of bacteria (Johansson, Phillipson et al. 2008) (Figure 2). However, enteric pathogens such as *Campylobacter jejuni*, *Samonella enterica* and *Vibrio cholerae* are able to penetrate the mucus using flagella, which allow movement towards or away from a chemical stimulus. The spiral-shape and secreted proteases of these pathogens are also thought to facilitate movement through viscous mucus (Lee, O'Rourke et al. 1986; Takata, Fujimoto et al. 1992; Guerry 2007). Flagella are not only a feature of pathogenic bacteria and some prominent members of the microbiota of animals and humans such as *Roseburia* sp. and *Wolinella succinogenes*, a close relative of *C. jejuni*, are flagellated. In contrast to the colon, the mucus layer in the SI is thinner and possibly more penetrable to the contents of the lumen (Johansson, Larsson et al. 2011).

Mice lacking MUC2, the gene encoding the major mucin in the human intestine, spontaneously develop colonic inflammation, highlighting the protective role of the mucus in limiting direct contact between bacteria and epithelial cells (Van der Sluis, De Koning et al. 2006; Johansson, Larsson et al. 2011). Furthermore, in the development of dextran sodium sulphate (DSS)-induced colitis, the inner mucus layer becomes permeable to bacteria, which then contact epithelial cells and trigger an inflammatory response (Johansson, Gustafsson et al. 2010).

The outer layer of the mucus is home to many commensal mucin-degrading bacteria that derive nutrients from enzymatic breakdown of the glycan structures. Nevertheless very few mucin dwelling bacteria have been isolated and characterized except for *Akkermansia muciniphila* (Derrien, Vaughan et al. 2004). Interestingly, SCFA have been shown to

modulate the expression of MUC2 in a human goblet cell-like cell line. This highlights a potentially important regulatory link between metabolites produced by fermentative colonic bacteria and the host defenses (Burger-van Paassen, Vincent et al. 2009).

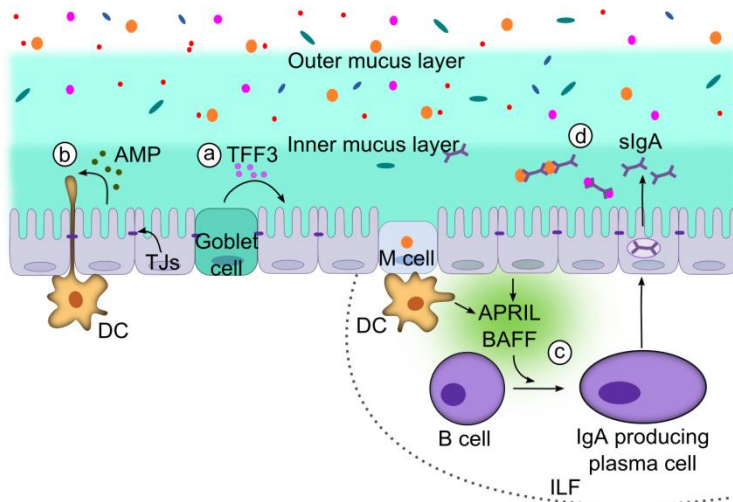


Figure 2: Physical and chemical defenses. Multiple chemical and physical barriers are in place to separate the luminal content from the intestinal epithelium. Intestinal epithelial cells are covered by a layer of mucus that consists of mucin glycoproteins secreted by goblet cells. The mucus layer forms an outer layer permeable to bacteria and an inner layer which is relatively devoid of bacteria. a) Goblet cells also produce trefoil factor 3 (TFF3) in response to TLR2 signalling which mediates intestinal epithelial repair. b) Enterocytes, Paneth cells and goblet cells secrete constitutive and inducible antimicrobial peptides (AMP) that modulate immune functions. c) In isolated lymphoid follicles (ILFs), DC- and epithelial cell- derived factors such as the B cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL), drive B cell differentiation into plasma cells that produce secretory immunoglobulin A (sIgA). d) sIgA is transported through IEC to the lumen where it can bind to bacteria to prevent their attachment or invasion and neutralize toxic molecules.

Goblet cells also facilitate mucosal protection and epithelial repair through the production of trefoil factor 3 (TFF3) which induces migration and anti-apoptotic pathways in IECs (Figure 2) (Mashimo, Wu et al. 1996). Goblet cells appear to regulate TFF3 via a Toll-like receptor 2 (TLR2)-dependent pathway (described in section 4) (Podolsky, Gerken et al. 2009). Thus, TLR2 knockout mice are deficient in TFF3 and have an increased susceptibility to DSS-induced colitis. This provides another example of how the sensing of bacteria by the host intestinal epithelium regulates the expression of homeostatic factors.

It is not known why commensal bacteria can colonize the outer mucus layer but not the inner layer of the colonic mucus. We also need more knowledge about the diffusion of bacterial TLR ligands through the mucus, mucus degrading bacteria and the mucus structure and function in the SI.

3.2. *Physical barriers, the intestinal epithelium*

The most important physical barrier is the intestinal epithelium, a 30 μm single layer of IECs that separates the lumen from the underlying LP and the inside of the body. IECs are polarized having an apical and a basolateral pole each with a distinct composition of membrane proteins, lipids and receptors. The apical pole of mature enterocytes contains microvilli, finger-like membrane protrusions that increase the surface area for exchange of nutrients and water. The basolateral pole is attached to a matrix of loose connective tissue in the LP. The intestinal epithelium is renewed approximately every 5 days in humans due to proliferation and differentiation of multipotential Lgr5-expressing stem cells located in the crypts of Lieberkühn (Sato, Vries et al. 2009). Terminally differentiated cells undergo apoptosis and exfoliation at the tips of small intestinal villi or the equivalent epithelial surface in the colon. This dynamic renewal process also serves as a protective mechanism to remove cells that may have been damaged or invaded by pathogens. As the cells migrate upwards from the crypt, they differentiate into three different cell lineages; enterocytes, enteroendocrine cells and mucus producing goblet cells (Yen and Wright 2006). In the SI, the stem cells migrating to the bottom of the crypt differentiate into Paneth cells which produce a range of antimicrobial factors to protect the crypt cells from infection with microorganisms (Garabedian, Roberts et al. 1997; Salzman, Underwood et al. 2007). Remarkably, the process of continuous epithelial replacement occurs without compromising the integrity of cell-to-cell junctions and intestinal permeability. The importance of maintaining junctional integrity is evident from studies in mice which have molecular defects in tight junction (TJ) proteins, such as JAM-A (Laukoetter, Nava et al. 2007), or regulatory mechanisms affecting TJ properties (Su, Shen et al. 2009). TJs are the most apical structures of cell-to-cell junctions and are composed of transmembrane proteins (such as occludins and claudins) and intracellular scaffold proteins (such as ZO-1, -2 and -3). TJs seal the intercellular space between adjacent cells thus creating a selective barrier keeping out microorganisms and macromolecules while allowing diffusion of fluids, electrolytes and small molecules through the paracellular space. TJs are crucial for the development and maintenance of epithelial polarity as they also prevent diffusion of plasma membrane components from the apical to the basolateral pole and vice versa. In mouse models and humans, increased intestinal epithelial permeability may precede the development of colitis (Olson, Reuter et al. 2006; John, Fromm et al. 2011; van Schaik, Oldenburg et al. 2012) and is characteristic of chronic

intestinal inflammation in humans and animals due to the effect of inflammatory cytokines on TJ regulation (Ye, Ma et al. 2006).

Many enteric pathogens have evolved ways to disrupt the TJs of epithelial cells as a means to gain nutrients or to cross the epithelial barrier. For example, this can involve proteolytic degradation of specific TJs, e.g. with enterotoxin/fragilysin producing strains of *Bacteroides fragilis*. Disruption of TJs may also occur by phosphorylation or dephosphorylation of TJ proteins because of the injection of specific effectors into the host cell by a type III secretion system. For example, during infection with enteropathogenic *E. coli* (EPEC) a type III secretion system delivers several effectors into the host cytoplasm (McDaniel, Jarvis et al. 1995), leading to rearrangements of the cytoskeleton, disruption of the cell membrane and apoptosis of IECs. This induces the effacement of microvilli and the formation of a cell protrusion known as a pedestal. In addition to these morphological and physiological changes, EPEC also causes the dephosphorylation of occludin (Simonovic, Rosenberg et al. 2000) and altered distribution of ZO-1 (Philpott, McKay et al. 1996).

In contrast to enteric pathogens, different species of commensal lactobacilli have been reported to enhance barrier function and/or protect against barrier disruption by pathogens *in vitro* (Wells, Kostantinov et al. 2008). Recently, *Lactobacillus plantarum* was shown to modulate TJ composition in human volunteers. In this landmark study, administration of *L. plantarum* to human volunteers increased the localization (immunofluorescent staining) of occludin and ZO-1 in the TJs in duodenal tissue biopsies. *In vitro*, TJ modification was mediated by TLR2 ligands and conferred protection against disruption of the TJs by phorbol ester (Karczewski, Troost et al. 2010). These results are in agreement with recent studies in mice showing protection from colitis by administration of TLR2 ligands and growing evidence that TLR signalling in the intestinal epithelium is crucial for homeostasis (Cario, Gerken et al. 2007).

Apart from serving as a semi-permeable physical barrier, the epithelium plays a key role in secreting antimicrobial factors to kill pathogens and microbes in proximity to the apical surface, which is discussed in the following section.

3.3. Homeostatic effects of epithelial antimicrobial peptides and polypeptides

Antimicrobial peptides (AMPs) and polypeptides provide the first line of chemical protection against pathogenic or commensal organisms that penetrate the mucosal barrier. In this

section, we specifically review the contribution of the enterocyte and Paneth cell produced antimicrobial factors to homeostasis. There have been excellent reviews covering other aspects such as structure and mechanism of action, including also immune cell defensins (Klotman and Chang 2006; Lai and Gallo 2009). Paneth cells are localized in the crypts of Lieberkühn throughout the SI of most mammals. A characteristic feature of Paneth cells is the extensive Golgi network that directs large electron dense secretory granules to the apical membrane for release into the crypt. The granules contain several antimicrobial factors including α -defensins, angiogenin-4, secretory phospholipases, lysozyme, Reg3- γ as well as pro-inflammatory cytokines and pattern recognition receptor (PRR) co-receptors (Stappenbeck 2009). In the mouse, six α -defensins have been characterized but genetic evidence suggests there may be as many as 17 defensin encoding mRNA expressed in the crypt. In humans, Paneth cells express α -defensins 5 and 6 (human α -defensin, HD). The α -defensins are secreted as a precursor form which is activated by trypsin (or matrix metalloprotease (MMP) 7 in the mouse) to confer antimicrobial activity.

Evidence for the role of Paneth cell defensins in protection of the epithelial barrier was demonstrated by expression of human HD-5 in transgenic mice. As a result, these mice were much more resistant to challenge with *Salmonella typhimurium* (Salzman, Ghosh et al. 2003) than the wild type mice strain. Mice deficient for MMP7 were used to show that the activity of α -defensins against the normal microbiota is important in shaping its composition (Salzman, Hung et al. 2010). This may be due to differences in the sensitivity of different microorganisms to α -defensins, their location and inter-dependencies in the intestinal environment. Moreover, it has important implications for IBD because changes in defensin production may contribute to the pathophysiology of the disease by altering the composition and thus the symbiotic or antagonistic activities of the microbiota. Notably, NOD2 polymorphisms in CD patients are associated with reduced expression of AMPs and loss of immune tolerance to the microbiota (Hugot, Chamaillard et al. 2001; Maeda, Hsu et al. 2005; Wehkamp, Salzman et al. 2005). Whereas, some components of Paneth cell granules are expressed in equal amounts in germ-free and colonized mice, others are induced by microbial exposure (e.g. Reg3- γ and angiogenin-4) (Hooper, Stappenbeck et al. 2003; Cash, Whitham et al. 2006). Using a transgenic mouse model in which MyD88 was expressed in Paneth cells in a MyD88^{-/-} background, it was shown that TLR signalling by commensal bacteria induces expression of several antimicrobial factors including Reg3- γ and

Reg3- β that inhibit growth of bacterial pathogens and commensals in the crypts of the SI (Vaishnava, Behrendt et al. 2008). Loss of MyD88-dependent TLR signalling did not influence the density of luminal bacteria emphasizing the key role for Paneth cells in protecting the crypt from colonization with commensal or pathogenic bacteria.

Intestinal enterocytes have also been shown to express antimicrobial factors including β -defensins and Reg3 proteins, which are evolutionarily conserved in mammals. In humans, beta-defensin-1 (HBD-1) is constitutively expressed, while HBD-2, HBD-3, and HBD-4 are inducible (Pazgier, Hoover et al. 2006). Although HBD-1 is highly expressed in all human epithelia it was suggested to have only minor antimicrobial activity compared to other defensins. However, recently it was shown that reducing the disulphide bonds in HBD-1, mimicking the reducing potential found in the anaerobic environment of the intestine, unmasks a potent antimicrobial activity (Schroeder, Wu et al. 2011). HBD-2 and HBD-3 are induced by microbial products and inflammatory cytokines (Garcia, Jaumann et al. 2001; Harder, Bartels et al. 2001; Liu, Destoumieux et al. 2002; Sorensen, Thapa et al. 2005). Inducible expression of HBD-2 and Reg3 proteins in enterocytes depends on TLR and MyD88-dependent signalling (Vora, Youdim et al. 2004; Vaishnava, Behrendt et al. 2008).

In addition to antimicrobial activities, HBD-2 and HBD-3 have been proposed to serve as a chemo-attractants for a broad spectrum of leukocytes in a CCR6- and CCR2-dependent manner (Biragyn, Surenhu et al. 2001; Rohrl, Yang et al. 2010). Murine β -defensin 2 (MBD-2) and HBD-3 have also been shown to have immunomodulatory effects on DC function by acting as an endogenous ligand for TLR4 and TLR1/2 heterodimers respectively (Figure 2) (Biragyn, Ruffini et al. 2002; Funderburg, Lederman et al. 2007). Additionally, HBD-2 has been shown to stimulate epithelial restitution and barrier repair in an autocrine fashion by binding to CCR6 expressed on the apical membrane of epithelial cells (Figure 2) (Vongsa, Zimmerman et al. 2009). Taken together, these findings suggest a dual or even multifunctional role for certain intestinal defensins in intestinal homeostasis. The homeostatic mechanisms of AMPs include epithelial restitution, immunomodulation and the innate killing of microorganisms.

3.4. Secretory antibodies and the “mucosal firewall”

Mammals produce much larger amounts of secretory immunoglobulin A (sIgA) in the mucosa than any other class of immunoglobulin in the body, reflecting its central role in

immunity and protection against microorganisms in the intestine. B cell activation and proliferation leading to sIgA production occurs in the organized follicular lymphoid structures of the gut associated lymphoid tissue (GALT). The lymphoid structures are present as isolated follicles in the intestinal cell wall or as large aggregates in the PP of the SI. The FAE overlaying PP contains specialized M cells, which transport antigens and microorganisms to APCs in close proximity to the basal membrane (Figure 1). Upon activation, APCs move from the sub-epithelial dome to the follicular area and present the antigens to naïve lymphocytes; alternatively, they migrate to the MLN through the efferent lymphatic ducts (Figure 1). The engagement of B cells with antigens in the GALT causes them to migrate to the interface between the B and T cell zones where they become fully activated as a result of interaction with CD4⁺ T cells and DCs. In the GALT and MLN, class switching of B cells from IgM to IgA is driven by a subset of DCs which release interleukin-10 (IL-10), transforming growth factor- β (TGF- β), IL-6 and retinoic acid (RA), a hormone-like growth factor for epithelial and other cell types (Iwasaki and Kelsall 2001; Sato, Hashiguchi et al. 2003; Mora, Iwata et al. 2006). Activated B cells then migrate to the LP from the lymph or blood, or directly from isolated lymphoid follicles. In the LP, IgA⁺ B cells differentiate into plasma cells which secrete IgA that is transcytosed across the epithelium to the apical surface (Macpherson, Gatto et al. 2000). The conventional T cell dependent pathway for high affinity IgA antibody production described above is associated with B-2 cells.

In addition to the T cell-dependent pathway for IgA production, there is a faster mechanism for generating IgA responses to highly conserved antigenic determinants on commensal bacteria and pathogens (Macpherson, McCoy et al. 2008; Suzuki and Fagarasan 2008). In mice, this involves a specialized subset of B-1 cells that can rapidly produce secretory IgA in the absence of help from CD4⁺ T cells (Fagarasan and Honjo 2000). Much of this secretory antibody appears to be polyreactive, binding several antigens and is commonly known as 'natural' antibody. The repertoire of 'natural IgA' is restricted and affinity maturation is limited, although the heavy-chain variable region genes used by IgA-producing plasma cells in the gut are somatically hyper-mutated, to diversify antibody specificity (Macpherson, McCoy et al. 2008). Humans lack canonical B-1 cells but it has been suggested that they may have functionally equivalent cells that can colonize the lymphoid tissues (Weller, Braun et al. 2004). T cell-independent secretory antibody responses are generated in the isolated lymphoid follicles by the direct presentation of antigen to B cells by DCs and M cells (Figure

2) (Batista, Iber et al. 2001; Bergtold, Desai et al. 2005; Tsuji, Suzuki et al. 2008; Batista and Harwood 2009). IECs also participate in B cell responses by releasing B cell-activating factor (BAFF) and a B cell proliferation-inducing ligand (APRIL) which play roles in IgA class-switching and B cell survival (reviewed in Wells et al., 2010).

B cells and T cells activated in the GALT and MLN acquire homing receptors that target them back to mucosal tissues and this ensures that the immune responses occur selectively and locally in the mucosa. Furthermore, DCs activated by bacterial interaction in the PP are retained in the MLN and PP (Macpherson and Uhr 2004). This has led to the “mucosal firewall” concept that proposes that productive mucosal sIgA responses to intestinal bacteria can develop without inducing unnecessary systemic immunity (Macpherson and Uhr 2004).

Much of the IgA produced in the gut appears not to be reactive to the commensal microbiota. Nevertheless, in germ-free mice IgA is present at very low levels in the gut and soon after colonization with bacteria the number of IgA positive B cells increases in the lymphoid tissue followed by the appearance of sIgA secreting plasma cells in the intestinal LP (Shroff, Meslin et al. 1995). At the epithelial surface, sIgA can bind to viruses and bacteria to prevent or inhibit their attachment to and/or invasion of epithelial cells, a process known as immune exclusion (Macpherson, Hunziker et al. 2001). Just as important may be the fact that sIgA can also interact with antigens presented by intracellular pathogens in endosomes during the pIgR-mediated transport through epithelial cells. These interactions have been shown to inhibit key steps in viral assembly and play an important role in immunity to viral infection (Mazanec, Coudret et al. 1995). Recent studies suggest that immune exclusion also helps shape the bacterial community through immunological selection (Sonnenburg, Angenent et al. 2004; Peterson, McNulty et al. 2007; Suzuki and Fagarasan 2008). Furthermore, when mice lacking a functional adaptive immune system are colonized with bacteria, they display a more robust innate immune response than their immunocompetent wild-type counterparts (Keilbaugh, Shin et al. 2005; Cash, Whitham et al. 2006). This indicates that in normal mice the adaptive immune response plays a critical role in minimizing activation of the innate immune system by the gut microbiota.

Other homeostatic mechanisms for sIgA include the removal of antigen complexes formed in the LP thereby reducing inflammatory reactions and systemic responses (Mestecky, Russell et al. 1999). This concept is supported by the finding that individuals carrying secretory antibody deficiencies are more prone to autoimmune diseases, such as celiac disease.

Secretory IgA also helps to prevent inflammatory reactions by neutralizing intracellular pro-inflammatory components such as lipopolysaccharide (LPS) (Fernandez, Pedron et al. 2003). Moreover, in contrast to IgG and IgM, IgA does not activate the classical complement cascade leading to the production of C5a that is chemotactic for phagocytes.

To avoid immune exclusion, many pathogens that colonize the mucosal surface produce IgA proteases that cleave IgA1 in the hinge region. IgA proteases are produced by opportunistic pathogens that colonize mucosal surfaces of the respiratory tract, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis*, or the genital tract such as *N. gonorrhoeae*. As the IgA1 proteases of human pathogens only cleave the IgA1 subclass found in humans and closely related primates it has not been easy to prove their role in virulence. However, IgA1 protease has been shown to compromise IgA-mediated killing of *S. pneumoniae* both *in vitro* and *in vivo* (Kadioglu, Weiser et al. 2008).

In summary, it is clear that IgAs are critical to mucosal protection against pathogens but it is only recently that we began to appreciate its homeostatic roles in shaping the intestinal microbiota, preventing mucosal inflammation by immune exclusion, removal of antigen-antibody complexes in the LP and the neutralization of inflammatory mediators.

4. Detecting bacterial incursions

Apart from its critical role as a physical barrier, the intestinal epithelium provides an early warning system for sensing microbes. Through the expression of pattern recognition receptors (PRRs), the epithelium senses the microbial content of the lumen and participates in the innate immune response by secreting a potent array of cytokines and chemokines. PRRs recognize microbe-associated molecular patterns (MAMPs), consisting of conserved structures found both on pathogenic and non-pathogenic microorganisms. PRRs are expressed along the entire GI-tract by enterocytes (Cario and Podolsky 2000), Paneth cells (Rumio, Besusso et al. 2004) and immune cells such as DCs (Hart, Al-Hassi et al. 2005). The innate sensing mediated by PRRs is essential to detect pathogens and initiate immune responses, but needs to be tightly regulated to maintain immune homeostasis towards the trillions of microbes that inhabit the GI-tract.

4.1. *Gut innate sensors and microbial interactions*

IECs have the capacity to express a range of PRRs including members of the well-characterized families of Toll-like receptors (TLRs), and intracellular Nod-like receptors (NLRs). Several TLRs are expressed in IECs in human and mice, while among the NLRs only NOD1 and NOD2 have been well characterized in the intestine. Ten TLRs have been identified in humans and 12 in mice. TLRs are type I transmembrane glycoproteins, that share a conserved structure, with an extracellular domain that contains multiple leucine-rich repeats (LRR), a short trans-membrane domain and a highly conserved intracellular domain, the Toll-interleukin 1 receptor (TIR) domain. The extracellular LRR domains are responsible for the binding to MAMPs and contribute to the homo- or hetero-dimerization of the receptors. Dimerization of the intracellular TIR domains leads to the recruitment of adaptor molecules (e.g. MyD88, TIRAP and TRIF) to initiate the signalling cascade. Each PRR recognizes a specific molecular pattern and can be expressed on the cell surface, in intracellular compartments or in the cytosol (Table 1). TLR1, 2, 4, 5, 6 and 11 recognize mainly microbial membrane components and are expressed on the cell surface, TLR3, 7, 8 and 9 recognize microbial nucleic acids and are expressed in intracellular compartments such as the endoplasmic reticulum, endosomes, lysosomes and endolysosome (Table 1). NOD1 and NOD2 are intracellular proteins that contain an N-terminal caspase recruitment domain (CARD), a nucleotide-binding oligomerization domain (NOD) and a C-terminal regulatory domain with multiple LRRs that bind to peptidoglycan (PG) fragments in the cytosol. NOD1 senses Meso-diaminopimelic acid from the PG of Gram-negative bacteria, while NOD2 recognizes muramyl dipeptides that are common to the PG of both Gram-negative and Gram-positive bacteria (Chamaillard, Hashimoto et al. 2003; Girardin, Boneca et al. 2003)(Table 1). TLR2 can form heterodimers with TLR1 or TLR6 leading to the recognition of distinct ligands. The heterodimers TLR2/1 and TLR2/6, bind triacylated and diacylated lipoproteins from Gram-positive and Gram-negative bacteria respectively. Additionally, TLR2/6 can bind lipoteichoic acid, a lipidated glycan polymer present in the cell wall of Gram-positive bacteria. In all cases, two lipid chains present on each ligand insert into a hydrophobic pocket in TLR2 whereas the dimerization with TLR1 or 6 is determined by specific interactions with other parts of the ligands. The highly conserved lipid A of LPS, a component of the outer membrane of Gram-negative bacteria, is bound to MD-2 in a

complex with TLR4. Presentation of LPS to MD-2 is facilitated by CD14 and LPS binding protein.

Table 1: PRRs, ligands and subcellular localization

Receptor	Subcellular localization	Ligand	Origin of the ligand
TLR2	Cell surface	Lipopeptide PG LTA	Bacteria G+ bacteria G+ bacteria
TLR2/1	Cell surface	Triacylated lipopeptide	G- bacteria, mycoplasma
TLR2/6	Cell surface	Diacylated lipopeptide	G+ bacteria, mycoplasma
TLR3	Cellular compartment	dsRNA	Viruses, virus infected cells
TLR4/MD2	Cell surface	LPS	G- bacteria
TLR5	Cell surface	Flagellin protein	Bacteria
TLR7	Cellular compartment	ssRNA	ssRNA viruses
TLR9	Cellular compartment, cell surface	DNA	DNA viruses, bacteria
TLR11	Cell surface	Uropathogenic components	bacterial Uropathogenic bacteria
NOD1	Cell cytoplasm	Meso-DAP	PG from G-, some G+, mycobacterium
NOD2	Cell cytoplasm	MDP	PG from G-, G+ bacteria, mycobacterium

PG, peptidoglycan; LTA, lipoteichoic acid; LPS, lipopolysaccharide; DAP, diaminopimelic acid; MDP, muramyl dipeptide; G+, Gram positive; G-, Gram negative; ssRNA, single-stranded RNA; dsRNA, double-stranded RNA.

Another important bacterial MAMP is flagellin, a protein subunit of bacterial flagella that is typically recognized by TLR5. TLR signalling pathways have been reviewed in detail recently (Kawai and Akira 2010). Essentially, after ligand binding, TLRs undergo dimerization and conformational changes that result in the recruitment of TIR-containing adaptor proteins.

All TLRs, except TLR3, recruit the myeloid differentiation primary response gene 88 (MyD88), which in turn activates the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor κ B (NF- κ B) pathway signalling cascades (Janssens and Beyaert 2002).

TLR3 and TLR4 can signal also through a different pathway via the recruitment of the adaptor protein TRIF, leading to the expression of type 1 interferons. Similarly, NLRs activate the MAPK pathway and the canonical NF- κ B pathway signalling cascades. These signalling cascades lead to the activation of transcription factors, such as NF- κ B, that, after translocation to the nucleus, stimulate the transcription of genes encoding AMPs, pro-inflammatory cytokines and chemokines, ultimately initiating the inflammatory response. In an inactivated state, NF- κ B is located in the cytosol as a protein complex with the inhibitory

protein $\text{I}\kappa\text{B}\alpha$. Activation of NF- κ B is triggered by different stimuli (e.g. TLRs, cytokines, NLRs, stress inducers) that lead to the phosphorylation of $\text{I}\kappa\text{B}\alpha$ by the enzyme $\text{I}\kappa\text{B}$ kinase (IKK). Subsequently, $\text{I}\kappa\text{B}\alpha$ is ubiquitinated and degraded by the cell proteasome, leading to its dissociation from NF- κ B. Activated NF- κ B then translocates into the nucleus and induces the transcription of specific genes. In IECs, activated NF- κ B can induce the production of a broad range of chemokines and cytokines including interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs) and growth factors. In case of microbial colonization or invasion, the most abundant cytokine secreted by IECs is IL-8. IL-8 forms a gradient that, together with others factors, acts as chemoattractant for neutrophils that are recruited at the site of infection. IECs can also produce the DC chemokine CCL20 and the monocyte chemotactic protein-1 (MCP-1 or CCL-2), inducing recruitment of monocytes and basophils.

The importance of PRR-mediated recognition in the induction of inflammatory responses to pathogens is evident from infection studies in PRR-deficient mice. Mice lacking TLR4, NOD1 or the common adaptor protein MyD88 have impaired immunity to infectious agents (Schilling, Martin et al. 2003; Viala, Chaput et al. 2004; Brandl, Plitas et al. 2007; Lebeis, Bommarius et al. 2007). It is not surprising therefore, that pathogens have evolved different strategies to evade the innate recognition. For example, they can target TLR signalling in order to suppress the host immune responses to benefit their survival and persistence. *Brucella* species are highly infectious intracellular pathogens, this bacteria express a TIR domain-containing protein that, mimicking the human adaptor protein TIRAP, blocks TLR2- and TLR4- mediated NF- κ B activation (Radhakrishnan, Yu et al. 2009). The NF- κ B pathway can also be a target for pathogens. For example, the pathogen *S. typhimurium* can inject virulence effectors into the host cells through a type III secretion system. One of these effector proteins, AvrA, inhibits the NF- κ B pathway and induces apoptosis in IECs (Collier-Hyams, Zeng et al. 2002). Some pathogens express MAMPs that are not recognized by the PRRs, for instance *Campylobacter jejuni* and *Helicobacter pylori* produce subclasses of flagellin that do not activate TLR5 (Andersen-Nissen, Smith et al. 2005). Interestingly LPS from a commensal *Bacteroidetes* species, has also been found to have a low endotoxicity (Weintraub, Zahringer et al. 1989). The lipid A part of LPS is pentacylated and consequently a weak activator of TLR4 compared with the hexacylated lipid A of other commensals and pathogens. This indicates that commensals may have also evolved to avoid detection by innate receptors as a means to favour their persistence in the intestinal tract.

4.2. Regulation of innate sensing

The section above clearly illustrates the requirement for TLR signalling and inflammatory responses to protect the host from infection with pathogens. However, to peacefully coexist with the commensal microbiota and avoid perpetual inflammation, PRR signalling needs to be carefully regulated. Mice lacking TLR9, 4, 2 and MyD88 become more susceptible to DSS-induced colitis (Rakoff-Nahoum, Paglino et al. 2004; Lee, Rachmilewitz et al. 2006). In TLR2 and 4 deficient mice or mice depleted of bacteria with multiple antibiotics, the severity of colitis and mortality levels are worse than in wild type mice, suggesting an additional role for NF- κ B in homeostasis and the epithelial repair and renewal processes (Rakoff-Nahoum, Paglino et al. 2004). This concept is also supported by the finding that mice lacking TLR5 have the tendency to develop spontaneous colitis (Vijay-Kumar, Sanders et al. 2007). Further evidence for the crucial role of the NF- κ B regulator in intestinal homeostasis came from studies in mice with a conditional knockout of the intestinal epithelial IKK γ , or both IKK α and IKK β . IKK knockout mice lose the capacity to activate NF- κ B signalling and develop severe chronic intestinal inflammation (Nenci, Becker et al. 2007). The chemical and physical barriers described in the preceding sections, play a key role in keeping the microbes at a distance but in addition, several other sophisticated regulatory mechanisms have evolved to avoid excessive immune responses to non-pathogens. These mechanisms include (i) the regulation of TLR expression, (ii). TLR localization, (iii) differential apical and basolateral TLR signalling, (iv) negative feedback regulation of the NF- κ B pathway and (v) the attenuation of NF- κ B activation by commensal bacteria.

In the absence of inflammation, TLRs are expressed at low levels on the epithelial surface and their expression is highest in the intestinal crypts to guarantee the protection of stem cells. For example, TLR2 and TLR4 are expressed at low levels in human colonic epithelium and mainly in crypt cells (Cario and Podolsky 2000; Abreu, Vora et al. 2001; Furrie, Macfarlane et al. 2005), TLR5 is expressed mainly in the colon (Cario and Podolsky 2000). In the mouse, TLR2, 4, 5 and 9 are expressed in villus and crypt epithelial cells and in the follicle associated epithelium (FAE). Expression of TLRs may also be polarized in intestinal epithelial cells and located on either the apical or the basolateral membranes. For example in villi and crypt, TLR2 is located on the apical side of epithelial cells. In the FAE, TLR4 and TLR5 are located at the apical pole, while TLR2 and TLR9 are located on both the apical and the basolateral side (Chabot, Wagner et al. 2006). In polarized human colonic epithelial cell lines,

TLR9 has been shown to be expressed on both the apical and the basolateral poles but activation leads to distinct responses depending on the location of the stimulus (Figure 3b) (Lee, Mo et al. 2006). Basolateral TLR9 stimulation activated the NF- κ B pathway while apical stimulation prevented NF- κ B activation conferring tolerance to the chronic TLR challenges represented by the commensal microbiota (Figure 3b) (Lee, Mo et al. 2006). TLR5 has been reported to be expressed on the basolateral surface in the human colonic epithelial cell line T84, such that it would only respond to flagella on pathogens that have crossed the epithelium (Gewirtz, Navas et al. 2001). However, this appears not to be the case for human intestinal Caco2 and HT29 cells (unpublished data, Chapter 2) and in the mouse, TLR5 is present on both apical and basal membranes of the villus epithelium (Chabot, Wagner et al. 2006) bringing into question the validity of this model in humans and mice *in vivo*.

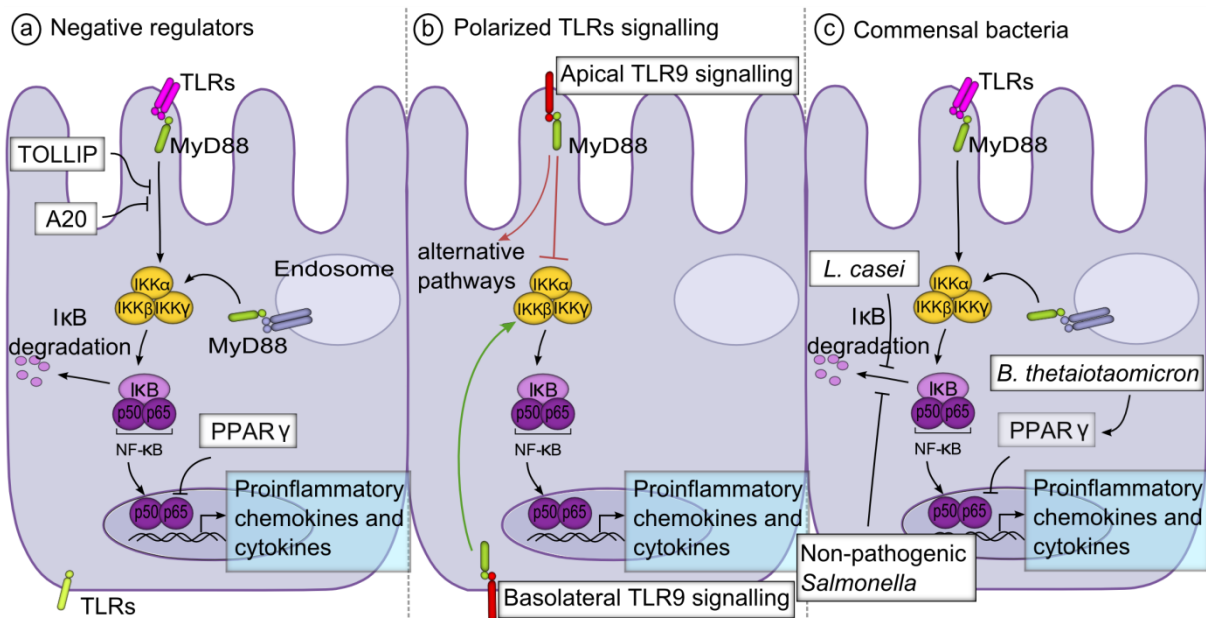


Figure 3: Homeostasis of innate sensing in intestinal epithelial cells. Toll-like receptor (TLR) sensing and signalling is regulated at multiple levels via interacting pathways that contribute to the maintenance of intestinal homeostasis. a) Negative regulators of pro-inflammatory nuclear factor κ B (NF- κ B) signalling pathway: NF- κ B activation pathway can be inhibited at different levels by NF- κ B inhibitory regulators such as the peroxisome proliferator-activated receptor- γ (PPAR γ), the Toll-interacting protein (TOLLIP) and the cytoplasmic protein A20. PPAR γ competes with the p65 subunit of NF- κ B and induces its nuclear export, thus limiting the activation of NF- κ B. TOLLIP and A20 interfere with adaptor proteins essential for the signal transduction. b) Polarized TLR signalling: Basolateral TLR9 stimulation (in green) induces NF- κ B activation and transcription of proinflammatory genes. Whereas apical stimulation (in red), can suppress the inflammatory response and induces alternative signalling pathways c) TLR signalling can be modulated by commensal bacteria. *B. thetaiotaomicron* stimulates the expression of PPAR γ , promoting the export of the p65 subunit of NF- κ B from the nucleus. *L. casei* and non-pathogenic strains of *Salmonella* inhibit the degradation of I κ B.

Several intracellular negative regulators can inhibit TLR signalling in IECs, these include the peroxisome proliferator-activated receptor- γ (PPAR γ), the Toll-interacting protein (TOLLIP) and the cytoplasmic protein A20 (Figure 3a).

Commensal bacteria can also actively participate in the regulation of TLR signalling. The Gram-negative genus *Bacteroidetes* is one of the dominant taxa of the gut microbiota. The commensal *Bacteroides thetaiotaomicron* can induce the expression of PPAR γ , promoting the export of the p65 subunit of NF- κ B from the nucleus, thus preventing the transcription of proinflammatory genes (Figure 3c) (Kelly, Campbell et al. 2004). Non-virulent *Salmonella* strains can inhibit the NF- κ B pathway blocking the degradation of I κ B α , thus promoting the attenuation of inflammatory responses elicited by proinflammatory stimuli (Figure 3c) (Neish, Gewirtz et al. 2000). A similar mechanism is used by *Lactobacillus casei*, which was reported to down-regulate inflammation induced by an entero-invasive pathogen, protecting I κ B from proteolytic degradation and blocking NF- κ B translocation to the nucleus (Figure 3c) (Tien, Girardin et al. 2006).

5. Immune cell responses to microbes

Besides the recognition by PRRs, the microbial content of the lumen is constantly sampled by specialized cells and lymphoid structures along the gut. DCs and macrophages can directly sample the luminal antigens across the mucosa or encounter bacteria/antigens sampled by M cells in the GALT. Once activated, a subset of LP DCs migrates to the MLN where they induce T cell responses. APCs in the PP also interact with T cells in the inter-follicular regions driving their clonal expansion and differentiation. Macrophages, DCs and other immune cells are also responsible for the detection and phagocytic killing of invading bacteria. The crosstalk between IECs and immune cells is essential in driving the immune response against pathogens and in preserving the intestinal homeostasis (Wells, Rossi et al. 2010).

5.1. Mucosal dendritic cells

DCs are specialized APCs that regulate both innate and adaptive immunity (recently reviewed (Rescigno 2010)). They are found in the LP of both the small and large intestine and in the GALT, which includes PP and draining lymph nodes. Typically, DCs migrate from the peripheral tissues to the lymphoid tissue where they present antigens to T cells. Most tissue resident DCs are immature and poorly immunogenic due to the low expression of

MHC molecules and co-stimulatory molecules. Immature DCs undergo maturation when they sense MAMPs or “danger”-associated molecular patterns released by damaged or stressed cells. Mature DCs express high levels of MHC, co-stimulatory molecules and cytokines for antigen presentation and immune signalling. In lymphoid tissues, mature DCs interact with naïve CD4⁺ T lymphocytes inducing their activation, clonal expansion and differentiation. Depending on the phenotype of the DC and the cytokine environment, T cells will differentiate into different subsets of effector and regulatory T cells. The intestinal mucosa contains numerous DCs specialized in different functions depending on their location and subtype. In the following sections, we describe their main characteristics and roles in regulating immunity and tolerance.

5.2. Lamina propria dendritic cells

In the LP, CD11c⁺ DCs are divided in two major subsets depending on the expression of the CD103 receptor. These two subsets are derived from two different blood cell precursors (Bogunovic, Ginhoux et al. 2009; Varol, Vallon-Eberhard et al. 2009) and have different functions. The CD103⁻ DCs differentiate from blood monocytes, they have features of macrophages and are typically in close contact with the epithelium. They can also be distinguished from the CD103⁺ DC subset for the expression of CX3CR1, the receptor for fractalkine, which is expressed on the basal membrane of polarized epithelial cells. CX3CR1⁺ DCs can sample luminal antigens and microorganisms directly by extending dendrites through epithelial cell junctions to gain access to the luminal content (Rescigno, Urbano et al. 2001; Niess, Brand et al. 2005). Mice deficient for CX3CR1 are more susceptible to the entero-invasive pathogen *S. typhimurium* (Niess, Brand et al. 2005). The formation of trans-epithelial dendrites is dependent on the presence of the microbiota and on TLR signalling in epithelial cells (Niess, Brand et al. 2005; Chieppa, Rescigno et al. 2006). CX3CR1⁺ DCs also express CD70 and can promote Th17 differentiation from naïve CD4⁺ T cells in response to bacterial adenosine 5'-triphosphate (ATP) or TLR5 signalling triggered by flagellin (Uematsu, Jang et al. 2006; Atarashi, Nishimura et al. 2008). This is in agreement with the finding that germ-free mice lack Th17 cells and that colonization by segmented filamentous bacteria (SFB) or administration of bacterial ATP induces Th17 cells (Atarashi, Nishimura et al. 2008; Gaboriau-Routhiau, Rakotobe et al. 2009).

The other major subset of LP DCs is the CD103⁺ CX3CR1⁻ cells that promote the conversion of naïve CD4⁺ T cells into inducible Foxp3⁺ Tregs and support natural CD4⁺ Foxp3⁺ Tregs (Powrie, Read et al. 2003; Coombes, Siddiqui et al. 2007). In the mouse SI, goblet cells acquire small soluble antigens from the lumen and translocate them to CD103⁺ DCs in the PP (McDole, Wheeler et al. 2012). Migratory CD103⁺ DCs found in the MLNs express the integrin CCR7 while LP CD103⁺ DCs are CCR7 negative. Tregs generated in the MLNs up-regulate the expression of the homing receptor CCR9 and the alpha4 beta7 integrin required for their homing cells back to mucosal tissues. The tolerogenic property of CD103⁺ LP DCs is determined by their local tissue environment and in particular by epithelial derived TGF- β , RA and in humans, thymic stromal lymphopoietin (TSLP) (Rimoldi, Chieppa et al. 2005; Iliev, Spadoni et al. 2009). Interestingly, these factors are induced in IECs after apical interaction with bacteria *in vitro*. This suggests that luminal contact with bacteria or bacterial factors can potentially modulate DC function in the LP. CD103⁺ DCs also express the enzyme indoleamine 2,3 dioxygenase (IDO) which is required for their tolerogenic function (Matteoli et al. 2010). Although these models are based on mechanistic studies in mice, the CD103⁺ DC subset is also found in the mucosa of humans. Furthermore, these DCs also produce IDO suggesting that this tolerogenic mechanism is conserved in humans and mice (Matteoli, Mazzini et al. 2010).

5.3. Peyer's Patch dendritic cells

The DC subsets are present in the mouse PP perform different functions and are distinguished by the expression of CX3CR1 and the chemokine receptors CCR6 and CCR7, which direct their migration into the lymphoid tissues. In the dome area of the PP, CX3CR1⁺ DCs are found associated with or in close proximity to the follicular epithelium. These cells can transcytose antigens and were previously suggested not to induce T cell responses (Salazar-Gonzalez, Niess et al. 2006). However, a CD103⁻ DC subset was recently identified that can prime IFN- γ and IL-17 producing T cells with gut homing properties (Cerovic, Houston et al. 2012).

DCs expressing CCR6, the receptor for CCL20, are found only in the PP. They are required for localized CD4⁺ T cell activation and defence against intestinal pathogens. This was elegantly shown using CCR6 knockout mice, GFP-CCR6 reporter mice and *S. typhimurium* flagellin-specific T cell receptor transgenic mice to identify the DC subset responsible for T cell

activation in the PP (Salazar-Gonzalez, Niess et al. 2006). The results were consistent with a model where CCR6⁺ DCs migrate towards the FAE during infection to engulf transcytosed bacteria. Mature DCs then activate specific effector T cells to mediate defence against entero-invasive pathogens. The CCR6⁺ DC subset is unique to the PP, despite the fact that IECs also produce CCL20.

The PP has an important role in IgA isotype switching (discussed above) and this is mediated by a CD11b⁺ subset of DCs which release IL-10, TGF- β , IL-6 and RA (Iwasaki and Kelsall 2001; Sato, Hashiguchi et al. 2003; Mora, Iwata et al. 2006). PP DCs are also involved in the T cell-dependent IgA class switching through TNF- α and inducible nitric oxide synthase (Tezuka, Abe et al. 2007).

5.4. Effector and regulatory T cells in the gut

The balance between pro-inflammatory effector T helper (Th) cells, such as Th1, Th2 and Th17 cells and anti-inflammatory Tregs is essential in maintaining immune homeostasis and tolerance in the GI-tract. CD4⁺ T cells subsets include T helper cells, Th1, Th2 and Th17, and regulatory T cells, Tregs (Figure 1). Each subset is characterized by a specific cytokine secretion profile to facilitate a different type of immune response. Th1 cells are important in promoting cell-mediated immunity against intracellular pathogens and secrete mainly, IFN- γ and TNF- α . Th2 cells produce IL-4, IL-5 and IL-13 to promote B cell development and antibody production (Mosmann and Coffman 1989) and are required for humoral immunity against helminths and extracellular pathogens. Th17 cells respond to extracellular bacteria and fungi and secrete mainly IL-17, IL-22 and IL-23 (Bettelli, Korn et al. 2007). The pro-inflammatory responses mediated by effector T cells are regulated by Tregs.

Different subsets of Tregs have been described, natural Tregs (nTregs) and inducible Tregs (iTregs). Natural Tregs originate in the thymus and are CD4⁺, CD25⁺ and transcription factor Foxp3⁺. iTregs are induced in peripheral lymphoid tissues such as GALT and MLN by interaction with APCs and dietary antigens, these cells are CD4⁺, CD25⁻ and can be Foxp3⁺ or Foxp3⁻.

CD4⁺ Foxp3⁻ Tregs subsets are T regulatory 1 (Tr1) cells, characterized by the production of IL-10, and Th3 characterized by the production of TGF- β . In the colon LP, the majority of the IL-10 producing cells are Foxp3⁺ Tregs, while in the SI IL-10 is produced by CD4⁺ Foxp3⁻ intraepithelial lymphocytes and in the LP by Tr1 cells (Maynard et al., 2007). Commensal

bacteria individually or in groups can influence T cell polarization. In SPF mice, more than 30 % of CD4⁺ cells in the colon LP and around 27 % in the SI are Foxp3⁺ and conventionalization of germ-free mice induces an increase of Foxp3⁺ CD4⁺ T cells in the colon LP but not in the SI (Atarashi, Tanoue et al. 2011). Mono-colonization of mice with the mouse commensal SFB increases the number of CD4⁺ T cells, in particular Th1, Th17 and Foxp3⁺ Tregs in the SI and colon (Gaboriau-Routhiau, Rakotobe et al. 2009). Moreover, mice colonized with a cocktail of 46 strains from Clostridium species isolated from mouse faeces, have increased amounts of Foxp3⁺ Tregs expressing IL-10 in the colon compared with germ-free mice (Atarashi, Tanoue et al. 2011). In conventional mice administration of Bifidobacterium species has been shown to induce IL-10 producing Tregs in the colon (Jeon, Kayama et al. 2012). Taken together these results highlight the essential role of intestinal bacteria in shaping T cells responses, in particular in the induction of Tregs in the colon.

5.5. Gut macrophages

Mucosal tissues contain the largest pool of tissue macrophages of the human body. They derive from blood monocytes that migrate into the tissues from blood capillaries. Despite their potent capacity for phagocytosis and pathogen killing, mucosal macrophages are relatively attenuated for proliferation and chemotactic activity compared to those found in other tissues (Smith, Ochsenbauer-Jambor et al. 2005). Additionally, in mucosal macrophages innate signalling via TLRs or NLRs does not induce potent inflammatory responses as in the case of other tissue macrophages. Intestinal macrophages are also involved in the maintenance of immunological tolerance via the induction of Tregs (Coombes, Siddiqui et al. 2007). This is attributed to a population of CD11b⁺ F4/80⁺ CD11c⁻ macrophages that constitutively produce high levels of IL-10 and express retinol dehydrogenases that convert retinol into RA. This population may also negatively regulate the ability of CX3CR1⁺ DCs to drive inflammatory Th17 responses (Denning, Wang et al. 2007).

Thesis outline and aims

The intestinal microbiota plays role in intestinal homeostasis via interactions with the epithelium and innate and adaptive immune mechanisms of the gut thereby profoundly shaping mammalian mucosal immunity and tolerance. However, in some diseases, such as IBD, the microbial community may assume a pathologic character accentuating the damage to the host. Despite the recent advances in our understanding of the microbiota structure at different stages of life and the perturbations associated with disease, our understanding of its functional impact and the contribution of individual microbial components on health is still in its infancy. The aim of this thesis was to contribute to our knowledge and understanding of several aspects of the interactions between gut commensal bacteria and host, focusing on one of the most abundant colonic microbes, *F. prausnitzii*, for the detailed mechanistic studies.

In **Chapter 2**, we investigated whether polarized IECs regulate inflammatory responses to microbes by secreting IL-8 in a vectorial fashion depending on the location of the TLR stimulus. In the Caco-2 model of polarized epithelium, apical stimulation with TLR2 and TLR5 ligands resulted in the apical secretion of IL-8. The CXCR1 receptor for IL-8 was expressed only on the apical membrane of Caco-2 cells and differentiated epithelial cells in the human SI and colon suggesting that IL-8 has an autocrine function. Transcriptome analyses revealed that Caco-2 cells respond to stimulation with IL-8, supporting the hypothesis of autocrine signalling via CXCR1.

In **Chapter 3**, mono-associated and conventional mice were used to gain new insights into the interaction of bacteria with the host mucosa and mucosal-associated lymphoid tissue. The vast majority of the bacteria are kept at a distance by the host physical and chemical barriers except for SFB which has a unique and specialized physical interaction with the epithelium.

Chapter 4, 5 and 6 focused on the immunoregulatory mechanisms of the human commensal *F. prausnitzii*, which has been proposed to have anti-inflammatory properties and is present in lower numbers in IBD patients with active disease.

In **Chapter 4**, we compared the effects of *F. prausnitzii* and other commensal bacterial strains on human monocyte-derived DCs (hDCs). *F. prausnitzii* strain A2-165 induced a very different cytokine profile in hDCs compared with four other *F. prausnitzii* strains and eight

other commensal strains, this was due to the high levels of secreted IL-10 and low levels of IL-12.

The effects of *F. prausnitzii* A2-165 and another commensal *Clostridium hathewayi* 82-B on the induction of mucosal T cell responses to the model antigen ovalbumin (OVA) *in vitro* and *in vivo* were studied in **Chapter 5**. Here we demonstrated that *F. prausnitzii* A2-165 had immunoregulatory properties *in vitro* which may be related to its strong capacity to induce IL-10 by both DCs and T cells. *In vivo*, these effects translated into enhanced OVA-specific T cell proliferation in the nose-draining cervical lymph nodes (CLNs) after combined nasal application of *F. prausnitzii* A2-165 and OVA. Strikingly, this was associated with a reduction in the differentiation of IFN- γ secreting T cells in the CLNs. In contrast, *Clostridium hathewayi* 82-B significantly decreased the percentage of dividing OVA-specific T cells in the CLNs and spleen.

In **Chapter 6** we investigated the immunomodulatory effects of *F. prausnitzii* strains A2-615, and HTF-F, which forms a biofilm in liquid culture, as well as the extracellular polymeric matrix (EPM) purified from strain HTF-F. The protective capacity of the two strains and the EPM were investigated in the DSS-induced colitis model. The *F. prausnitzii* strains had protective effects, the biofilm producing strain HTF-F conferred an increased protection compared to strain A2-165 and this was partly due to the immunomodulatory properties of the EPM.

Finally, **Chapter 7** summarizes and discusses the results of the thesis in the context of wider literature on host-microbe interactions and mucosal immunology. Furthermore, the implications of our work on intestinal health and the possible directions for future research are discussed.

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Chapter 2

Vectorial secretion of interleukin-8 mediates autocrine signalling in intestinal epithelial cells via apically located CXCR1

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Abstract

In the intestinal mucosa, several adaptations of TLR signalling have evolved to avoid chronic inflammatory responses to the presence of commensal microbes. Here we investigated whether polarized intestinal epithelial cells might regulate inflammatory responses by secreting IL-8 in a vectorial fashion (i.e. apical versus basolateral) depending on the location of the TLR stimulus. In the Caco-2 model of polarized villus-like epithelium, apical stimulation with TLR2 and TLR5 ligands resulted in the apical secretion of IL-8. The CXCR1 receptor for IL-8 was expressed only on the apical membrane of Caco-2 cells and differentiated epithelial cells in the human small intestine and colon suggesting that IL-8 has an autocrine function. Transcriptome analyses revealed that Caco-2 cells respond to stimulation with IL-8 supporting the hypothesis that IL-8 induces G protein-coupled receptor signalling. Autocrine signalling by IL-8 did not alter the permeability of an intact epithelial monolayer. These results expand our knowledge on the role of IL-8 in the intestinal mucosa.

The microarray data and the raw cel files were deposited at GEO, platform number GSE30364. This information can be accessed via the following link:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ddafvqiigumcupm&acc=GSE30364>

The supplementary materials can be accessed via the following links:

[http://pbaarlen.home.xs4all.nl/oriana/Rossi et al Supplementary QC Report.pdf](http://pbaarlen.home.xs4all.nl/oriana/Rossi_et_al_Supplementary_QC_Report.pdf)

[http://pbaarlen.home.xs4all.nl/oriana/Rossi et al Supplementary Table 1.xls](http://pbaarlen.home.xs4all.nl/oriana/Rossi_et_al_Supplementary_Table_1.xls)

[http://pbaarlen.home.xs4all.nl/oriana/Rossi et al Supplementary Table 2.xls](http://pbaarlen.home.xs4all.nl/oriana/Rossi_et_al_Supplementary_Table_2.xls)

Introduction

Intestinal epithelial cells (IECs) play a key role in the inflammatory response to colonizing or invading microorganisms via the secretion of interleukin-8 (IL-8). The chemokine IL-8 recruits neutrophils from the vasculature to sites of infection or tissue injury (Arnott, Drummond et al. 2001). IL-8 can signal through two receptors, CXCR1 and CXCR2 both of which are members of the G-protein-coupled receptor (GPCR) family (Baggiolini, Dewald et al. 1997). In neutrophils, IL-8 binding to CXCR1 triggers G protein-coupled signalling and formation of second messengers that mediate cellular migration, exocytosis of effectors and a respiratory burst to facilitate oxygen-dependent killing of phagocytosed microorganisms (Baggiolini, Dewald et al. 1997). CXCR1 is expressed in the human IEC line Caco-2 (Sturm, Baumgart et al. 2005), which is in agreement with our own microarray data using Caco-2 cell mRNA. Additionally, IL-8 has been shown to induce Caco-2 cell migration after wounding in a CXCR1-dependent manner (Sturm, Baumgart et al. 2005). The intestinal epithelium is structurally and functionally polarized, with tight junctions (TJs) preventing the diffusion of receptors, transporters and enzymes between the apical and basolateral membranes (Ulluwishewa, Anderson et al. 2011). Several cell receptors are differentially expressed on the apical and basolateral membranes of polarized IECs including Toll-like receptors (TLRs), cytokine receptors and chemokine receptors (Chabot, Wagner et al. 2006; Lee, Mo et al. 2006). In the human intestinal epithelium, TLR signalling is known to induce secretion of IL-8 in a nuclear factor (NF)- κ B-dependent manner and TLR2, TLR5 and TLR9 are expressed on the apical poles of IECs depending on their location and differentiation status (Cario and Podolsky 2000; Bambou, Giraud et al. 2004; Chabot, Wagner et al. 2006; Lee, Mo et al. 2006). Interestingly, IL-8 has been measured in the colon milieu of healthy volunteers using a dialysis bag into the rectum (Keshavarzian, Fusunyan et al. 1999) suggesting that it might be apically secreted by colonic epithelial cells. Furthermore, IL-8 is present in significant amounts in breast milk (Maheshwari, Lu et al. 2002). However, it is not known if CXCR1 is expressed on the apical or basolateral poles in polarized Caco-2 cells or human intestinal tissue or whether the direction of TLR signalling influences the direction of IL-8 secretion. Here we investigated IL-8 secretion in response to TLR signalling using model IEC lines grown in the Transwell system. In this established model, Caco-2 and HT-29 monolayers differentiate into enterocytes (Chantret, Barbat et al. 1988) possessing intact TJs and distinct

apical and basolateral membranes. We investigated whether the location (i.e. apical or basolateral) of TLR agonists influenced the production and direction of IL-8 secretion. Additionally, we determined the localization of the CXCR1 receptor in Caco-2 cells and human intestinal tissue samples and investigated the role of IL-8 autocrine signalling using transcriptomics.

Results

Secretion of IL-8 in polarised Caco-2 and HT-29 cell monolayers

Stimulation of polarized Caco-2 cells with Pam2CSK4 and Pam3CSK4, agonists for TLR2/6, and TLR2/1 from the apical or basolateral sides induced secretion of IL-8 predominantly into the apical compartment regardless of the location of the stimulus, although basolateral stimulation induced smaller amounts of IL-8 compared to apical stimulation. In response to apical stimulation with the TLR5 agonist flagellin, IL-8 was secreted into the apical compartment while basolateral stimulation induced very small amounts of IL-8 only in the basolateral compartment (Figure 1a).

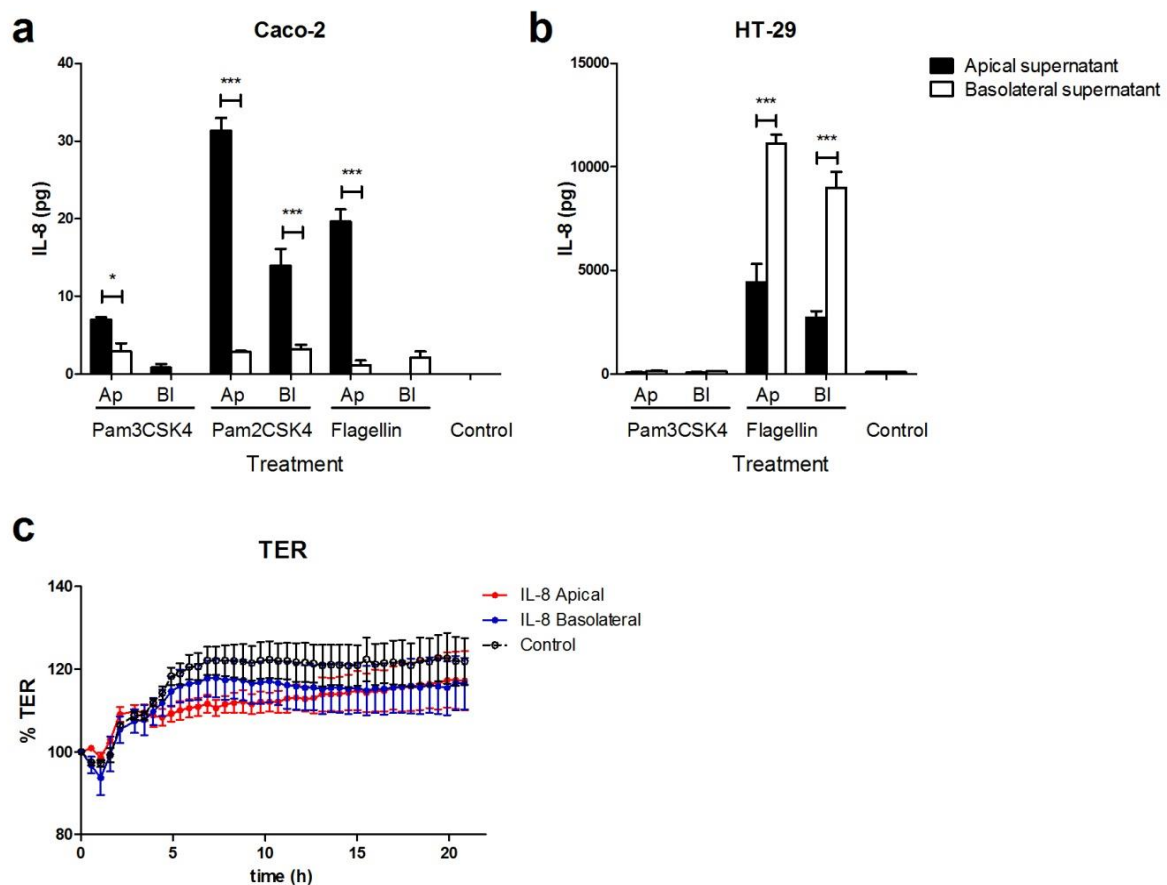


Figure 1: Secretion of IL-8 in polarized Caco-2 and HT-29 intestinal epithelial cells in response to Toll-like receptor ligands. Caco-2 (a) and HT-29 (b) monolayers were stimulated for 24 hours from the apical (Ap treatment) or the basolateral compartment (BI treatment) with: Pam3CSK4 (20 μ g/ml), Pam2CSK4 (10 μ g/ml) and flagellin (2 μ g/ml). Amounts of IL-8 secreted in the apical (black bars) and basolateral (white bars) compartments are normalized to the volume of supernatant in each chamber. Error bars represent SEM (n=3). (c) IL-8 does not alter trans-epithelial electrical resistance (TER) in Caco-2 monolayers. TER of Caco-2 polarized monolayers untreated (black) or incubated with 100 pg/ml IL-8 on the apical (blue) or the basolateral side (red) for 20 hours. TER values were normalized to the initial TER value (100%). Error bars represent SEM (n=4); * p <0.05, ** p <0.01, *** p <0.001.

HT-29 cell monolayers constitutively secreted relatively high amounts of IL-8 compared to Caco-2 cells. Stimulation of polarized HT-29 cells with the TLR5 agonist induced high amounts of IL-8 in both apical and basolateral compartments. In contrast to Caco-2, there was no significant IL-8 response to the TLR2/1 agonist (Figure 1b). We ruled out the possibility that IL-8 could diffuse across the cell monolayers by adding it either to the apical or basolateral compartment and determining the concentration in each compartment after 24 hours. In the absence of cell monolayer, IL-8 rapidly equilibrated to an equal concentration in both the apical and basolateral chambers (not shown). Additionally, we showed that the TJs remained intact throughout the experiment (not shown) and after apical or basolateral addition of IL-8 to Caco-2 monolayers by measuring the trans-epithelial electrical resistance (TER, Figure 1c).

CXCR1 is expressed on the apical membrane of polarized Caco-2 cells

Given our observations on the apical secretion of IL-8 in IEC lines, we hypothesized that the localization of CXCR1 in polarized epithelium might be an important factor regulating IL-8-mediated autocrine signalling.

CXCR1 was localized to the apical pole of polarized Caco-2 cells by confocal microscopy (Figure 2a and b). The speckled or 'patchy' staining for CXCR1 is suggestive of its localization in (macro)-domains possibly microvilli. Other GPCRs and their effector proteins have shown patchy staining in cytoplasmic membrane domains of epithelial cells (Ostrom and Insel 2004; Yang, Ogawa et al. 2005). No CXCR1 staining was observed using the secondary antibody alone as a control, indicating that fluorescence was not due to non-specific antibody binding.

In human duodenal and colonic tissues, CXCR1 is expressed on the apical surface of differentiated epithelial cells and is absent in the crypts

To investigate the localization of CXCR1 in the human intestine, frozen tissue samples of human colon and duodenum were collected from healthy volunteers, sectioned at low temperature, processed and stained.

The absorptive enterocytes forming the epithelium and the enterocytes lining the crypts were revealed by staining for the TJ protein occludin (red) and nuclear DNA (blue, Figure 3a-d). In the duodenum, CXCR1 was detected on the apical membrane of villus epithelial cells and not in crypt epithelium (green, Figure 3a and b). Similarly, in human colonic tissue CXCR1

was detected on the top of the epithelium and not in crypt enterocytes (green, Figure 3c and d). The surface of immune cells present in the lamina propria in colonic and duodenal tissues also stained positive for CXCR1 (Figure 3a and c).

These results are in agreement with the known surface expression of CXCR1 in immune cells such as macrophages, dendritic cells, T cells and natural killer cells and our results with Caco-2 cells. No CXCR1 staining was observed using the secondary antibody alone as a control (not shown)

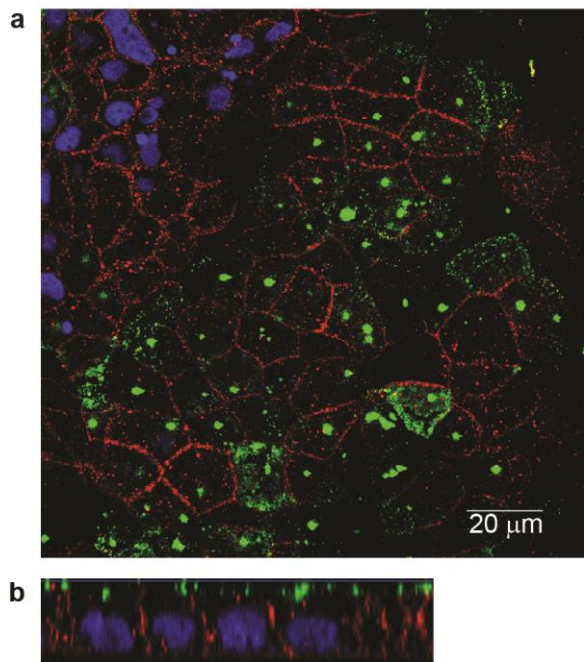


Figure 2: Caco-2 monolayers express CXCR1 on the apical surface. The cellular localization of CXCR1 in Caco-2 polarized monolayers visualized by immunofluorescent detection and confocal microscopy; anti-occludin (red), DRAQ5 (blue) and anti-CXCR1 (green). CXCR1 was expressed on the apical side of Caco-2 monolayers. (x-y (a) and x-z (b) sections).

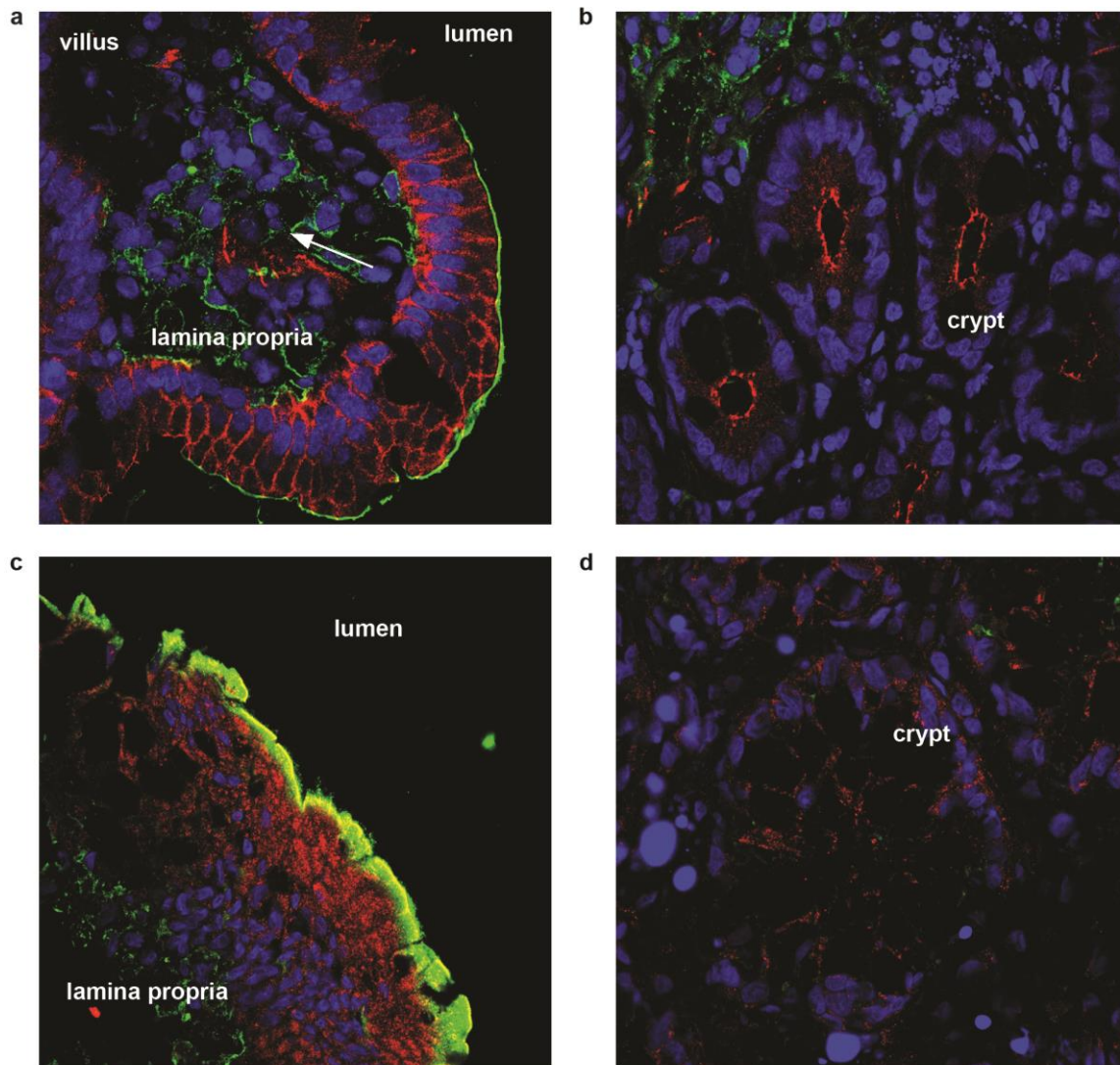


Figure 3: In human duodenal and colonic tissues, CXCR1 is expressed on the apical surface of villus enterocytes and is absent in the crypts. Duodenum and colon biopsies stained with anti-occludin (red), DRAQ5 (blue) and anti-CXCR1 (green). In villus enterocytes, CXCR1 is located on the apical membrane (sections of duodenum (a) and colon (c)), and surrounding the nuclei of cells in the lamina propria (a, arrow). CXCR1 is absent in the enterocytes lining the crypts of duodenum (b) and colon (c).

IL-8 autocrine signalling results in the differential expression of 859 genes

The autocrine role of IL-8 on IECs was investigated by measuring genome-wide transcriptome responses of Caco-2 cells to IL-8 using the Affymetrix human whole genome expression microarrays. Polarized Caco-2 cell monolayers were stimulated on the apical surface with IL-8 and, after 6 hours, total RNA was isolated for hybridization to whole genome expression microarrays. Quality control of the hybridisations and primary data analysis were performed according to strict criteria (Supplementary Quality Control Report) to ensure that the array data were of the highest possible quality.

Stimulation of Caco-2 cells with IL-8 resulted in the differential expression of 859 genes ($P < 0.02$; Supplementary table 1). As gene set comparisons and pathway reconstruction from differentially expressed genes are far more informative than their tabulated up- or down-regulation (van Baarlen, van Esse et al. 2008), four complementary *in silico* approaches were employed to deduce the biological significance of the array data set. These approaches relate changes in gene expression to cellular pathways and processes modulated by transcriptional networks, and show how these are interacting towards common cellular processes.

Gene ontology enrichment analysis and protein-protein interaction network of differentially expressed genes

To identify the strongest transcriptome changes of Caco-2 cells in response to IL-8, we first performed Gene Ontology (GO) enrichment analysis using the software tool ErmineJ. This software identifies functional groups in which the differentially expressed genes are statistically overrepresented. Caco-2 genes involved in lipoprotein biosynthesis, protein transport and secretion, response to virus, regulation of transcription and the cell cycle showed the strongest changes upon stimulation by IL-8 (Supplementary table 2). To identify the gene regulatory networks driving these changes, a protein-protein interaction network was generated using the software platform Cytoscape (Cline, Smoot et al. 2007) and overlaid with expression data using the differentially expressed genes as input. In this protein-protein interaction network, the nodes represent proteins encoded by genes that were differentially expressed. Inspection of this network (Figure 4) showed that the genes with the higher number of interactions were as follows: cAMP responsive element binding (CREB) binding protein (CREBBP), E1A-binding protein p300 (EP300), histone deacetylase 1 (HDAC1), chromobox homologue 5 (CBX5), epithelial E-cadherin (CDH1), ezrin (EZR), beta-actin (ACTB) and cell division cycle 42 (CDC42).

The genes CREBBP, EP300, CBX5, and HDAC1 encode coactivators and chromatin modification enzymes required for remodelling of tissue-specific gene loci and the activities of key transcriptional regulators during cell growth and differentiation (Wong, Pickard et al. 2010; Thomsen, Christensen et al. 2011). CREBBP was up-regulated (red, Figure 4) in response to IL-8 and its overexpression in primary cultures of smooth muscle cells inhibits cell cycle progression (Klemm, Watson et al. 2001). The genes CDC42 and EZR were down-

regulated in IL-8 treated cells suggesting decreased proliferation (Liu, Cao et al. 2010; Ou-Yang, Liu et al. 2011). The genes CDH1, PTPRM (protein tyrosine phosphatase, receptor type, M), EZR and ACTB encode structural proteins that mediate cell-cell contact (CDH1 and PTPRM), define cell shape (ACTB) or act as intermediates between plasma membrane and actin cytoskeleton (EZR). Decreased EZR expression (discussed above) was previously linked to up-regulation of E-cadherin (CDH1) as observed in this study. HOXA1, a downstream effector of E-cadherin-directed signalling, was up-regulated by IL-8 (red, Figure 4), presumably due increased E-cadherin ligation. Taken together, the protein network responses suggest that IL-8 signalling is involved in the regulation of cell differentiation (structural proteins and cell-cell contact signalling) and lipid metabolism but not proliferation.

Gene ontology categories network of differentially transcribed genes

To characterize further the processes that are regulated via these protein-protein interaction networks, we performed a GO enrichment analysis of all differentially regulated genes and visualized the interconnections between GO categories (Figure 5). The GO enrichment analysis identified three interconnected networks. The two smallest networks included GO categories involved in positive regulation of signal transduction and intracellular protein kinase cascade (top centre in Figure 5) and GO categories involved in cell morphogenesis and development (centre right in Figure 5). The largest network consisted of a central region that included GO categories involved in lipid biosynthetic processes including lipid kinase activity. This central region was connected to sub-networks consisting of GO categories involved in cyclin-dependent protein kinase activity and MAP kinase activity (top left in Figure 5), protein secretion and epithelial morphogenesis (bottom left/centre in Figure 5) and activation of phospholipase C (PLC) activity (bottom centre/right in Figure 5) which results from GPCR signalling. The latter was expected, as it is known that CXCR1 is a GPCR. These GO categories correlated well with the enriched categories found using ErmineJ

Figure 4: Network showing interactions between proteins encoded by genes differentially expressed after stimulation of Caco-2 cell monolayers with IL-8. This network includes only differentially expressed genes that encode for proteins interacting with other proteins. Up-regulated genes are shown in shades of red and down-regulated genes in shades of blue; the fold-intensity reflecting the change. Note that the genes encoding the most connected proteins in the central part of the network (CREBBP, EP300, PPARD and HDAC1) were all up-regulated; these genes encode transcriptional regulators involved in lipid metabolism and cell differentiation. The network illustrates the core transcriptome changes modulating downstream processes including cell migration, signalling and differentiation.

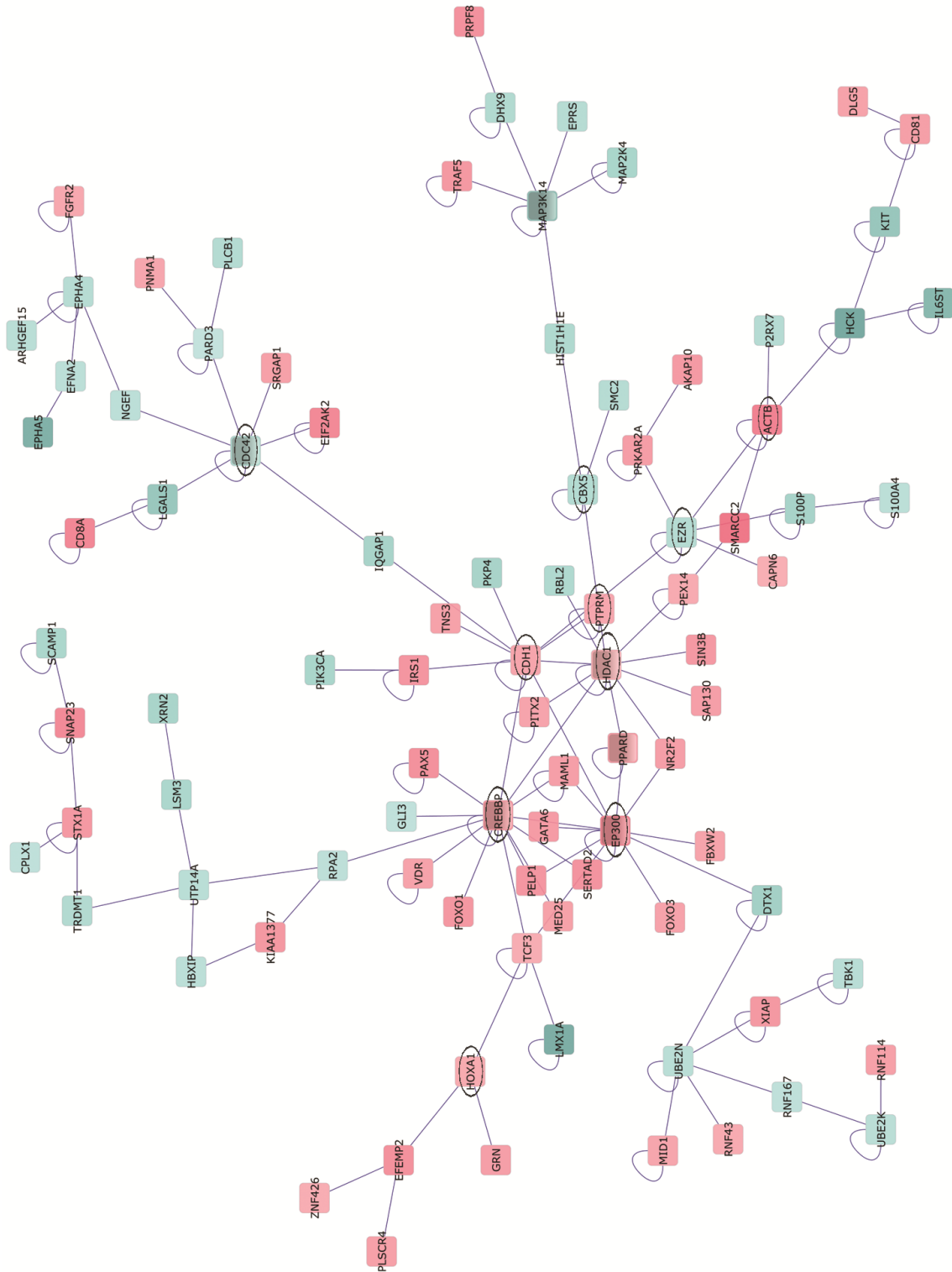
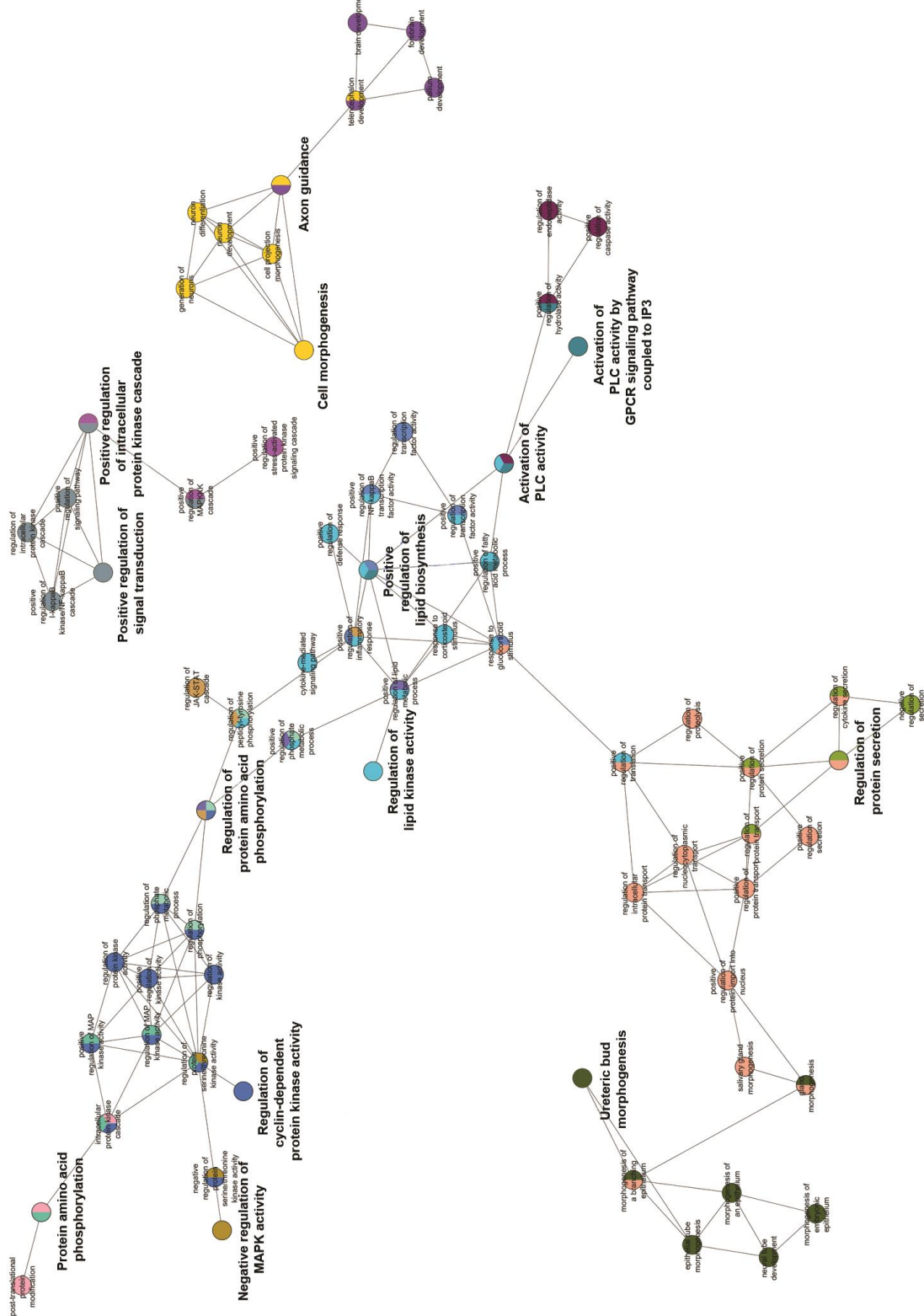


Figure 5: Network showing linked gene ontology (GO) categories representing the functional annotation of the genes that were differentially transcribed in Caco-2 cells after stimulation with IL-8. GO terms make up a formal vocabulary to describe gene functions. This network illustrates the biological processes corresponding to the transcriptome changes. Note that these GO categories exemplify cell migration, signalling and differentiation and therefore correlate well with the core protein-protein interaction network depicted in Figure 4 (see text for details). MAPK, MAP kinase; PLC, phospholipase C; GPCR, G protein-coupled receptor; IP3, inositol 1,4,5-trisphosphate.



Discussion

In IECs, IL-8 expression is known to be induced by proinflammatory cytokines, TLR signalling and cellular stress (Baggiolini, Dewald et al. 1997; Schroder and Tschopp 2010). Basolateral secretion of IL-8 plays a role in the recruitment of neutrophils from the vasculature to sites of infection or tissue injury (Arnott, Drummond et al. 2001). IL-8 can be measured in rectal dialysates of healthy subjects (Keshavarzian, Fusunyan et al. 1999) suggesting that it can be apically secreted in the intestine. The polarized secretion of IL-8, IL-1 and IL-6 has been reported in epithelial cell lines of different tissue origin (Cudicini, Kercret et al. 1997; Holtkamp, Van Rossem et al. 1998; Sonnier, Bailey et al. 2010). However, it is not known if TLR signalling can induce vectorial secretion of IL-8 in IECs. To investigate whether IECs secrete IL-8 apically in response to TLR signalling, we stimulated polarised Caco-2 and HT-29 cell monolayers grown in the Transwell system with TLR agonists applied from the apical or basolateral side and we measured the concentration of IL-8 in the two compartments. Caco-2 cells were most responsive to TLR2/1, TLR2/6 and TLR5 agonists applied to the apical membrane (Figure 1a) which is compatible with the apical localization of TLR2 and TLR5 in humans (Cario and Podolsky 2000; Bambou, Giraud et al. 2004). An exception is the IEC line T84 that expresses TLR5 on the basolateral pole (Gewirtz, Navas et al. 2001). Additionally, in Caco-2 cells, most of the IL-8 secreted in response to TLR agonists was located in the apical compartment (Figure 1a).

In contrast to Caco-2, HT-29 cells constitutively secreted a relatively high level of IL-8 in both compartments and did not respond to a TLR2/1 agonist. In response to TLR5 stimulation, HT-29 cells secreted IL-8 bidirectionally but in larger amounts in the basolateral compartment (Figure 1b). The differences between HT-29 and Caco-2 may be due to the fact that HT-29 does not polarize and differentiate into mature enterocyte-like cells to the same extent as Caco-2. In particular, the TER values measured for HT-29 monolayers were around 200-300 Ohms/cm² whereas Caco-2 monolayers reached TER values of approximately 600-800 Ohms/cm². A high TER reflects the tightness and integrity of the epithelial monolayer, the low values measured using HT-29 cells indicate that the monolayer is more leaky compared with Caco-2 cells.

These results show that Caco-2 and HT-29 cells can secrete IL-8 apically in response to TLR2 and TLR5 signalling. We speculate that *in vivo* the steady-state activation of TLRs by microbe

associated molecular patterns of commensal bacteria may account for the luminal IL-8 measured in healthy subjects (Keshavarzian, Fusunyan et al. 1999). Further support for this hypothesis comes from our data showing apical staining of the IL-8 receptor CXCR1 in Caco-2 cells and human intestinal tissue (Figure 2 and 3). Interestingly, we found CXCR1 expression in the adult intestine localized to non-proliferating and differentiated epithelial cells and not in crypt epithelial cells. In the intestine, apically secreted IL-8 might have an autocrine function. To gain more insights into the role of IL-8 autocrine signalling, we performed a microarray analysis of Caco-2 cells treated with IL-8 and in total 859 genes were differentially expressed. The transcriptome data were analysed using GO enrichment analysis to identify the functional categories of genes that were significantly altered by IL-8 signalling. Additionally, a protein-protein interaction network and GO categories network of differentially expressed genes was used to visualize the interactions between different processes altered by IL-8 treatment. The microarray data support the hypothesis that IL-8 induces GPCR signalling in Caco-2 cells. The GO categories network indicates activation of PLC, formation of the second messenger inositol 1,4,5-trisphosphate, activation of phosphatidylinositol 3-kinase and consequent generation of phosphatidylinositol 3-phosphate (bottom right of GO categories network, Figure 5). The microarray analyses suggested that IL-8 induces an increase in cell-cell contact, cell adhesion and cell survival. In particular the gene network involved in E-cadherin regulation may play a role in the formation of epithelial cell-cell junctions and differentiation of epithelial cells after migration (Taupin and Podolsky 2003). The E-cadherin–catenin complex has been shown to be a crucial to the regulation of cell adhesion, polarity, differentiation, migration, proliferation, and survival of IECs (Zbar, Simopoulos et al. 2004).

The microarray data also suggests that IL-8 is involved in the regulation of cell differentiation rather than proliferation. In this respect, it is also relevant that CXCR1 was expressed only on the differentiated and non-proliferative epithelial cells in the small intestine and colon and not in crypt enterocytes (Figure 3). Thus, IL-8 secreted in the lumen, for example in response to commensal antigens, would not have an effect on the differentiating cells that migrate upwards from the bottom of the crypts. Previously, IL-8 was reported to induce proliferation, differentiation and CXCR1-dependent migration in Caco-2 cells (Maheshwari, Lu et al. 2002; Sturm, Baumgart et al. 2005). Although the effect on of IL-8 on epithelial cell proliferation is controversial (Sturm, Baumgart et al. 2005) and was not confirmed by our own microarray data, overall the published data and our own work suggests a role for IL-8 in epithelial

restitution. Our observation on the apical location of CXCR1 in human intestinal tissues and our microarray data lend support to the hypothesis that IL-8 has an autocrine function *in vivo*. We speculate that apical secretion of IL-8 would help to initiate pathway responses in restitution prior to any potential loss of epithelial integrity, e.g. because of bacterial invasion or toxin production. In this respect, it is relevant that addition of IL-8 to the apical or basolateral side of an intact epithelial barrier had no significant effect on TER over a period of at least 20 hours (Figure 1c). This suggests that IL-8 mediated autocrine signalling would not affect the viability, permeability or integrity of an intact epithelium.

Interestingly, the effects of IL-8 on proliferation, differentiation and migration have also been shown using fetal IECs (Maheshwari, Lu et al. 2002). As significant concentrations of IL-8 are found in milk and amniotic fluid, it was suggested that IL-8 might have an additional role in the developing intestine (Maheshwari, Lu et al. 2002).

A similar autocrine role has been proposed for CCL20, which is also induced by inflammatory pathways in epithelial cells. CCL20 binds to the CCR6 receptor on the apical pole of differentiated epithelial cells (Yang, Ogawa et al. 2005) and induces epithelial cell migration *in vitro* (Vongsa, Zimmerman et al. 2009).

In summary, we show for the first time that the IL-8 receptor CXCR1 is expressed on the apical membrane of differentiated epithelial cells in the human small intestine and colon. Furthermore, our data demonstrate that IL-8 is secreted from the apical pole of IEC lines in response to TLR signalling and suggest that polarized secretion of IL-8 may occur in differentiated epithelial cells of the villi in response to apical stimulation with microbe-associated molecular patterns.

Materials and methods

For space reason the microarray data and the supplementary materials were not included in the chapter. The microarray data and the raw cel files were deposited at GEO, platform number GSE30364 and can be accessed via the following link:

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=ddafvqiigumcupm&acc=GSE30364>

The supplementary materials can be accessed via the following links:

[http://pbaarlen.home.xs4all.nl/oriana/Rossi et al Supplementary QC Report.pdf](http://pbaarlen.home.xs4all.nl/oriana/Rossi%20et%20al%20Supplementary%20QC%20Report.pdf)

[http://pbaarlen.home.xs4all.nl/oriana/Rossi et al Supplementary Table 1.xls](http://pbaarlen.home.xs4all.nl/oriana/Rossi%20et%20al%20Supplementary%20Table%201.xls)

[http://pbaarlen.home.xs4all.nl/oriana/Rossi et al Supplementary Table 2.xls](http://pbaarlen.home.xs4all.nl/oriana/Rossi%20et%20al%20Supplementary%20Table%202.xls)

Human materials

Human duodenum biopsies derive from the study of Troost et al., 2008 (Troost, van Baarlen et al. 2008), this study was approved by the University Hospital Maastricht Ethical Committee, Colon biopsies were obtained from healthy subjects at the University Hospital Örebro, according to the study protocol from Brummer et al: "Characterising intestinal microbiota and immune response in IBD, microscopic colitis and IBS". The University Hospital Örebro Ethical committee (Dnr 2010/261) approved this study. Studies were conducted in full accordance with the principles of the 'Declaration of Helsinki' (52nd WMA General Assembly, Edinburgh, Scotland, October 2000). All subjects gave their written informed consent prior to their inclusion into the study.

Cell culture

The Caco-2 BBE cell line (CRL 2102; American Type Culture Center, Manassas, VA) was maintained at 37°C in a humidified 5% CO₂/95% O₂ atmosphere. The HT-29.cl19A cell-line was obtained from Augeron and Laboisse (INSERM, Paris, France). Cell lines were grown in DMEM (Invitrogen, Paisley, UK) containing Glutamax and supplemented with 10% fetal bovine serum (PAA laboratories, Colbe, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO). Cells (between passage 55 and 74) were seeded at a density of 2.6 x 10⁵ cells/cm² and grown for 14 days until they differentiated into polarized monolayers. After 14 days, the TER reached 600 to 800 Ohms/cm² for Caco-2 and 200 to 300 Ohms/cm² for HT-29 (Volt/Ohm-meter, World Precision Instruments, Sarasota, FL).

Electrical resistance measurements in monolayer cell cultures

To measure the effects of IL-8 on epithelial permeability, polarized Caco-2 monolayers grown in Transwell filter inserts were treated with 100 pg/ml recombinant human IL-8 (rhIL-8, R&D Systems) added to the apical or basolateral compartment and the TER was measured using a Cellscope (Nanoanalytics) which allows the continuous and automated measurement of 24 individual filter inserts under cell culture conditions.

IL-8 secretion

DMEM containing TLR ligands was added to cell monolayers on day 14 either in the apical or in the basolateral compartment. The stimuli were used at the following concentrations: 20 µg/ml Pam3CSK4, 10 µg/ml Pam2CSK4, 2 µg/ml flagellin (InvivoGen, San Diego, CA). After 24 hours, 75 µl samples of apical or basolateral supernatant was taken and the concentration of IL-8 was determined using a cytometric bead array (BD, San Diego, CA) following the manufacturer instruction and flow cytometry (FACS Cantoll, BD). Similar findings were obtained in triplicate experiments.

Confocal microscopy

Caco-2 cell monolayers grown in transparent Transwell inserts for 14 days were fixed in 4% (weight/vol) paraformaldehyde and permeabilized with PBS containing 0.2% (vol/vol) Triton X100, 1% (vol/vol) normal goat serum and 0.1% (weight/vol) sodium azide. Samples were then incubated with primary antibodies, 1:250 of anti-occludin polyclonal antibody (Zymed, Invitrogen) and 1:100 dilution of monoclonal anti-human IL-8RA (i.e. CXCR1, R&D Systems) were added overnight at 4°C followed by a 2 hours incubation with Cy3- and Alexa 488-labeled secondary antibodies at room temperature. Nuclei were stained for 20 minutes using a dilution 1:500 of DRAQ5 (Enzo Life Sciences BVBA, Zandhoven, Belgium). Confocal images were obtained using a Zeiss LSM 510 system consisting of a Zeiss Axioskop with Plan Neofluar ×63 NA 1.3 oil objectives. Human duodenum and colon tissue sections (20 µm thick) were collected at -18°C on superfrost object glasses (Menzel-Glazer, Braunschweig, Germany) then processed and stained as described for Caco-2 cells.

RNA isolation and quality control

Caco-2 monolayers grown for 14 days were treated with none or 100 pg/ml rhIL-8. After 6

hours treatment, total RNA was extracted from Caco-2 cells using Trizol reagent (Invitrogen), purified using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) and treated with DNase set (Qiagen) following the manufacturer's instructions. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips according to the manufacturer's instructions. Only RNA samples showing intact bands corresponding to the 18S and 28S ribosomal RNA subunits, no chromosomal peaks or RNA degradation products, and an RNA integrity number (RIN) above 8.0 was used for microarray hybridization.

Affymetrix GeneChip oligoarray hybridization and scanning

Total RNA, extracted from Caco-2 cells was amplified and labelled using the GeneChip 3' IVT Express kit (cat. no. 901229) according to the manufacturer's specifications. Three independent experimental replicates per condition were used for total RNA extraction. The correspondingly labelled RNA was hybridised to a GeneChip Hu133 Plus2 array. Detailed methods for the labelling and subsequent hybridisations to the arrays are described in the eukaryotic section of the GeneChip Expression Analysis Technical Manual, Revision 3, from Affymetrix (Affimetrix, Santa Clara, CA), and are available upon request.

Statistical and functional analyses of microarray data

Transcriptome datasets underwent rigorous checks for quality control before the pathway and network analyses. These software packages are part of Bioconductor project (www.bioconductor.org) and integrated in the automated MADMAX pipeline (<https://madmax.bioinformatics.nl>) as described in previous publications (van Baarlen, Troost et al. 2009). Using the same statistical approaches we showed a good correlation between microarray and quantitative PCR (qPCR) data (van Baarlen, van Esse et al. 2008). Thus, we did not perform qPCR analyses on these samples.

Four complementary methods were used for the functional analysis of microarray expression data: ErmineJ (GO annotation enrichment or overrepresentation; (Lee, Braynen et al. 2005)), Gene Set Enrichment Analysis (Subramanian, Tamayo et al. 2005), Ingenuity Pathway Analysis (IPA; www.ingenuity.com) and gene regulatory network reconstruction and analysis via Cytoscape (Cline, Smoot et al. 2007). Using these software tools, we performed: (i) identification of statistically supported overrepresentation of functional GO annotation

(Subramanian, Tamayo et al. 2005), (ii) mapping of expression data onto pathways to determine their up- or down-regulation in a statistical meaningful way (IPA); (iii) projection of transcript fold-change values of co-expressed genes onto interaction maps of the corresponding proteins, and (iv) reconstruction of biologically meaningful cellular pathways and their mode of modulation (IPA and Cytoscape). Our approach of combining data outputs from one bioinformatics package with another to strengthen the analysis has been described previously (van Baarlen, Troost et al. 2009).

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Chapter 3

Compartmentalisation and immune sampling of bacteria in the small intestine and colon of gnotobiotic and conventional mice

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In preparation

Abstract

The aim of this study was to obtain a better understanding of the mucosal interactions with orally administered bacteria in the small intestine and colon of gnotobiotic and conventional mice. This study focused on the small intestinal lymphoid tissue including Peyer's patches (PPs) and mesenteric lymph nodes (MLNs). *Lactobacillus plantarum* was chosen as model commensal and was administered to germ-free (GF) mice. Lymphoid and mucosal tissues were collected after 20 h or after 21 days of colonization. Sections of the ileal and colonic tissue were fixed or cryopreserved for histochemistry and immunofluorescent microscopy. Additionally, immune cells were isolated from the resected PPs and MLNs for enumeration of lactobacilli. For comparison, tissue and organ samples were also collected from conventional mice of the same genetic background as the GF mice.

We showed that the secreted mucus presents a formidable physical barrier to the direct interaction of bacteria with the epithelial surface with the exception of segmented filamentous bacteria which were seen below the mucus layer in the ileum. CFU of lactobacilli in cells isolated from the PPs and MLNs was highly variable between mice (up to 1 per 236 immune cells) which may have been influenced by the location of the PPs or MLNs. Higher numbers of bacteria per cell were recovered from mouse PPs than MLNs. The colony counts of lactobacilli are an underestimate of the number of bacteria sampled by antigen presenting cells as *L. plantarum* is killed rapidly in the phagosome. In conclusion, uptake of commensal bacteria in the organized lymphoid structures such as PPs or isolated lymphoid follicles seems to be the predominant mechanism for immune sampling in the gut.

Introduction

Commensal and probiotic bacteria can have beneficial effects on host immunity. The beneficial effects may be strain specific and influenced by the capacity of the bacteria to signal through pattern recognition receptors (PRRs) and elicit innate responses in intestinal epithelial cells (IECs) and antigen presenting cells (APCs) such as dendritic cells (DCs) (Wells, Rossi et al. 2010; Rossi, van Baarlen et al. 2011). Endocrine cells, goblet cells, and enterocytes of the intestinal epithelium express a range of PRRs to sense the presence of microbes. PRR signalling in Paneth cells and enterocytes increases the production of defensins and other anti-microbial factors. Thereby, limiting the number of mucosa-associated bacteria and preventing bacterial penetration of host tissues. Additionally, PRR signalling in IECs plays a key role in modulating the activities of immune cells in the lamina propria via the induction of tolerogenic signals such as TSLP, BAFF and APRIL (reviewed by (Wells, Rossi et al. 2010)). In the lamina propria, DCs exposed to TGF- β and retinoic acid (RA) produced by IECs take on a tolerogenic phenotype characterised by the expression CD103⁺ and CCR7 the integrin required for migration to the lymph nodes and induction of regulatory T cells (Tregs). PRR signalling and maturation of conditioned CD103⁺ DCs induce the expression of high levels of IL-10 and low levels of the pro-inflammatory cytokines IL-12 and TNF- α , favouring the induction of Foxp3 expressing regulatory T cells (Tregs). Specific strains of bacteria have been shown to induce tolerogenic DCs and to expand the population of Foxp3⁺ Tregs in mice (Kwon, Lee et al. 2010) and IL-10 producing Tr1 regulatory T cells that that are Foxp3⁻ (Jeon, Kayama et al. 2012). However, it remains unclear to what extent the immunomodulatory effects of commensal bacteria are mediated through soluble microbe-associated molecular patterns (MAMPs) inducing PRR signalling in IECs and immune cells, or through the direct interaction of bacteria with the epithelium and immune cells in the intestine.

Bacteria on the epithelial surface may be sampled directly by epithelial-associated APCs expressing dendritic and macrophage markers that can extend protrusions through the epithelial tight junctions to the luminal compartment (Rescigno, Urbano et al. 2001) in a TLR/MyD88-dependent fashion. The frequency of these events is dependent on the location and is increased by infection or MyD88/TLR epithelial signalling (Chieppa, Rescigno et al. 2006; Arques, Hautefort et al. 2009).

The exposure of the mucosal tissue to microorganisms is limited by the presence of a layer of mucus secreted by goblet cells in the epithelium. The mucus layer in the intestinal tract is composed of Muc2, a large protein of around 5,200 amino acids that is heavily glycosylated and assembled into huge net-like structures (Hansson and Johansson 2010). In the mouse, it was recently shown that the colonic mucus consists of two layers extending 150 μm above the epithelial surface with a similar protein composition (Johansson, Phillipson et al. 2008). The inner mucus layer is densely packed and devoid of bacteria and the outer mucus layer is less dense and colonized by bacteria (Johansson, Phillipson et al. 2008). In the small intestine, the inner mucus layer is thinner (Atuma, Strugala et al. 2001). However, the follicular epithelium covering the mucosal associated lymphoid tissue of the Peyer's patches (PPs) in the small intestine is reported to be more accessible to antigens and bacteria present in the luminal compartment due to the absence of a mucus layer over the follicular epithelium. The follicular epithelium overlaying the lymphoid tissue contains specialized transport cells (M cells) which are the major route for the transfer of antigen, including intact bacteria and viruses, from the gut lumen to underlying DCs (Neutra, Mantis et al. 1999; Iwasaki and Kelsall 2001). M-cell mediated uptake of bacteria may also occur in the isolated lymphoid follicles associated with the colonic epithelium. Recent studies suggest that M cells also exist in villi where they may contribute to the transfer of organisms from bowel lumen to lamina propria DCs (Vallon-Eberhard, Landsman et al. 2006). These antigen-presenting DCs can interact with associated T and B lymphocytes to selectively induce IgA, which helps protect against mucosal penetration by commensals. The commensal-loaded DCs in the PPs can prime T cell responses in the inter-follicular region or in the draining lymph node after migration through the lymphatics (Macpherson and Uhr 2004).

Immune sampling of fluorescently labelled *Enterobacter cloacae* by DCs in PPs was previously demonstrated following intragastric (i.g.) challenge of mice with 1×10^9 colony forming units (CFU) of bacteria expressing green fluorescent protein (Macpherson and Uhr 2004). *E. cloacae* was recovered from DCs in the washed mesenteric lymph nodes (MLNs) for up to 60 hours after administration, but viable bacteria were not recovered from splenocytes or other systemic tissues. Intestinal DCs carrying *E. cloacae* were restricted to the mucosal tissues and MLNs. Retinoic acid, synthesized by the intestinal epithelium and gut-associated DCs, induces expression of the specific mucosal homing receptors integrin $\alpha 4\beta 7$ and chemokine receptor CCR9 in T cells ensuring their homing to the mucosa. This ensures that immune responses to

commensal bacteria are induced locally, without potentially damaging systemic immune responses (Iwata, Hirakiyama et al. 2004). The compartmentalisation of mucosal immune responses is one of the mechanisms by which pathological responses to the intestinal microbiota are avoided.

The aim of this study was to obtain a better understanding of the mucosal interactions with orally administered bacteria in the small intestine and colon of gnotobiotic and conventional mice with a focus on the small intestinal lymphoid tissue including PPs and MLNs. *Lactobacillus plantarum* was chosen as model commensal since lactobacilli are relatively abundant in the small intestine of mice. *L. plantarum* were administered to germ-free (GF) mice. The lymphoid and mucosal tissues were collected after 20 h or after 21 days of colonization when the mucosal immune system had reached maturity. Sections of the ileal and colonic tissue were fixed or cryopreserved for histology and immunofluorescent microscopy. Immune cells were isolated from the resected PPs and MLNs and washed prior to enumeration of viable lactobacilli. For comparison, tissue and organ samples were also collected from conventional mice of the same genetic background as the GF mice.

Results

In gnotobiotic and conventional mice, bacteria are compartmentalized in the lumen and loose mucus

These experiments were performed using *L. plantarum* harbouring the pTv-mCherry plasmid. This fluorescent protein was selected because it could be visualized both by flow cytometry and by confocal microscopy (Excitation max: 587 nm, Emission max: 610 nm). *L. plantarum* pTv-mCherry stably expressed the mCherry protein *in vitro* in culture. However, we could not observe fluorescent bacteria in the tissue sections. For this reason, we decided to detect the bacteria using fluorescence *in situ* hybridization (FISH). For the sake of brevity, we will refer to *L. plantarum* pTv-mCherry as *L. plantarum* in the remaining parts of this manuscript.

To investigate the interactions of *L. plantarum* with the intestinal epithelium, GF and conventional mice were colonized for 20 h or at day 1 and 21 after which they were sacrificed for tissue collection and isolation of immune cells from MLNs and PPs in the small intestine (Figure 1).

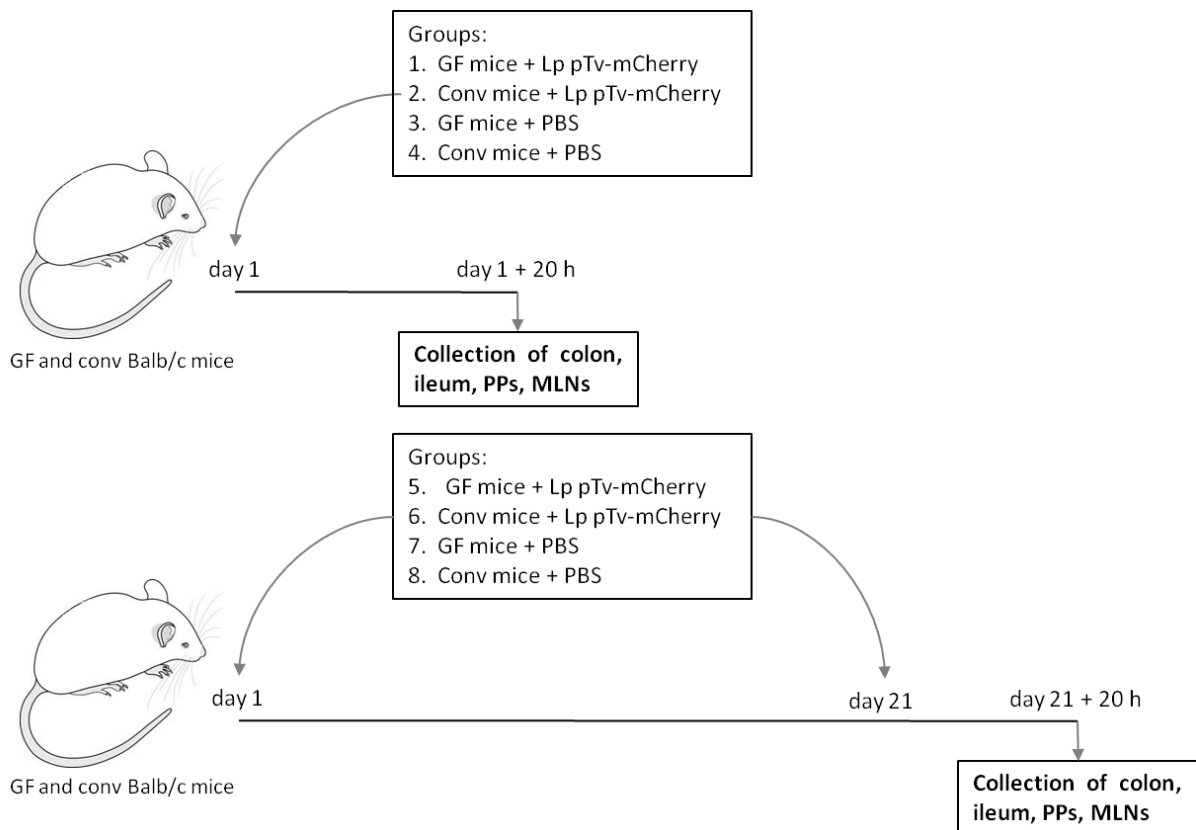


Figure 1: Outline of the *in vivo* experiments performed on germ free (GF) and conventional (conv) Balb/c mice; Lp, *L. plantarum*.

To visualize the full complement of acid and neutral proteoglycans present in the tissue, sections from colon and ileum were stained using the combined PAS and Alcian blue methods. Colonic tissue from all mice revealed a relatively firm layer of secreted mucus covering the epithelium and mucus filled goblet cells in the crypts (Figure 2a). In GF mice, the firm mucus layer in the colon was thinner suggesting less mucus production (Figure 2b) but after 21 days of colonization the thickness was similar to that of conventional mice (Figure 2c). The mucus layer was also thinner in GF mice colonised with *L. plantarum* for 20 h than in mice which had been colonized with *L. plantarum* for 21 days (not shown). There was notably less epithelial-associated mucus in the ileum than in the colon (Figure 2d, e and f). In the ileum the mucus resides between the villi and surrounding the ileal content. The darker purple staining on the surface of the epithelium most likely represents the acidic membrane bound mucins (Figure 2f).

Crossmon staining was used to visualize *L. plantarum* in monocolonized mice and microbiota in conventional mice. In the colon of conventional mice, the microbiota reside on top of the firm mucus layer covering the epithelium and within the loose mucus and faecal material as previously described by Johansson (Figure 3a and b) (Johansson, Phillipson et al. 2008). A few particles can be seen in the loose mucus in GF mice but they do not resemble the bacteria observed in conventional mice (Figure 3c). Evidently, there are much less bacteria in the ileum than in the colon and the majority appear to be attached to the loose mucus the exception being segmented filamentous bacteria (SFB) which are known to colonize the ileum of wild-type mice where they form stable interactions with the villous epithelium (Figure 4a and b) (Ivanov, Atarashi et al. 2009). As expected, no bacteria were seen in the ileum or colon of GF mice even at higher magnification (Figure 4c). In gnotobiotic mice colonized with *L. plantarum* there were fewer bacteria present at 20 h than after 21 days and these were present throughout the intestine (Figure 5a and b).

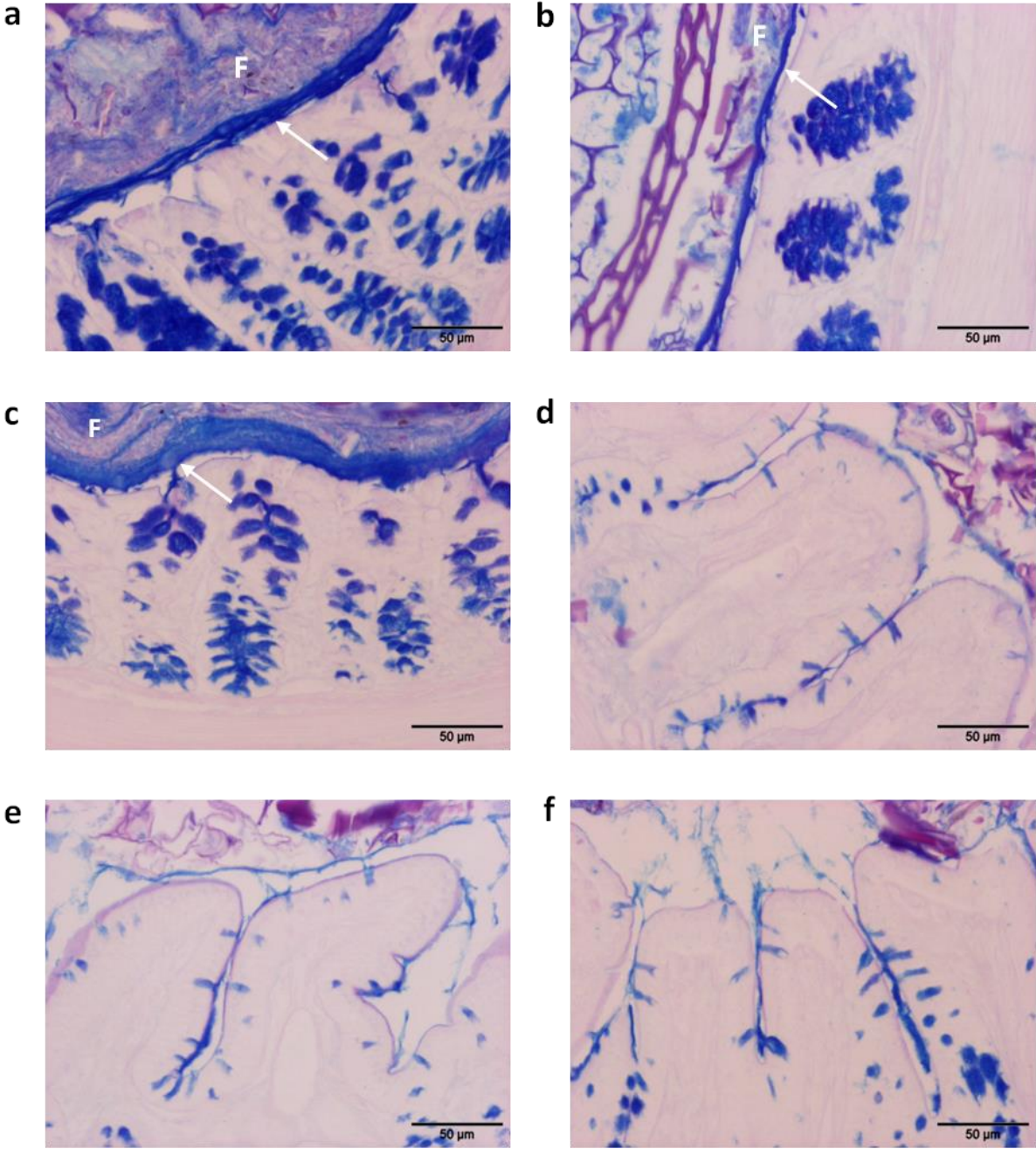


Figure 2: Sections of colon (a, b and c) and ileum (d, e and f) from conventional mice (a, d), germ free mice (GF, b, e) and GF administered *L. plantarum* (c, f) on day 1 and 21. Sections were stained using the combined Alcian blue/PAS methods. White arrows in a, b and c indicate the dark blue stained firm mucus layer. Blue stained goblet cells are seen in the colon crypts (a, b, c) and villus epithelium (d, e, f). F, faecal material in the colon.

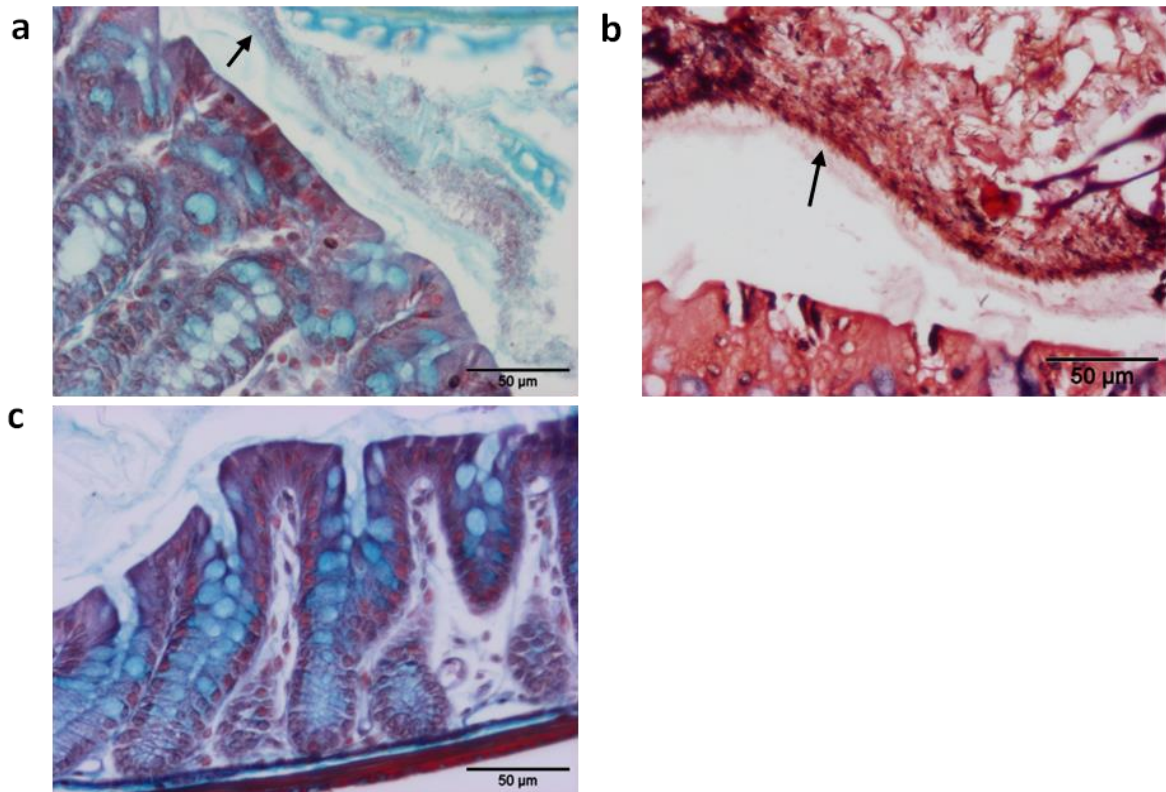


Figure 3: Sections of colon from conventional mice (a and b) and germ-free mice (c) were stained using Crossmon's method to visualize cells and mucus (a and c) and Gram staining to visualize bacteria, mucus and cells (b). Arrows indicate bacteria sitting on top of the firm mucus layer.

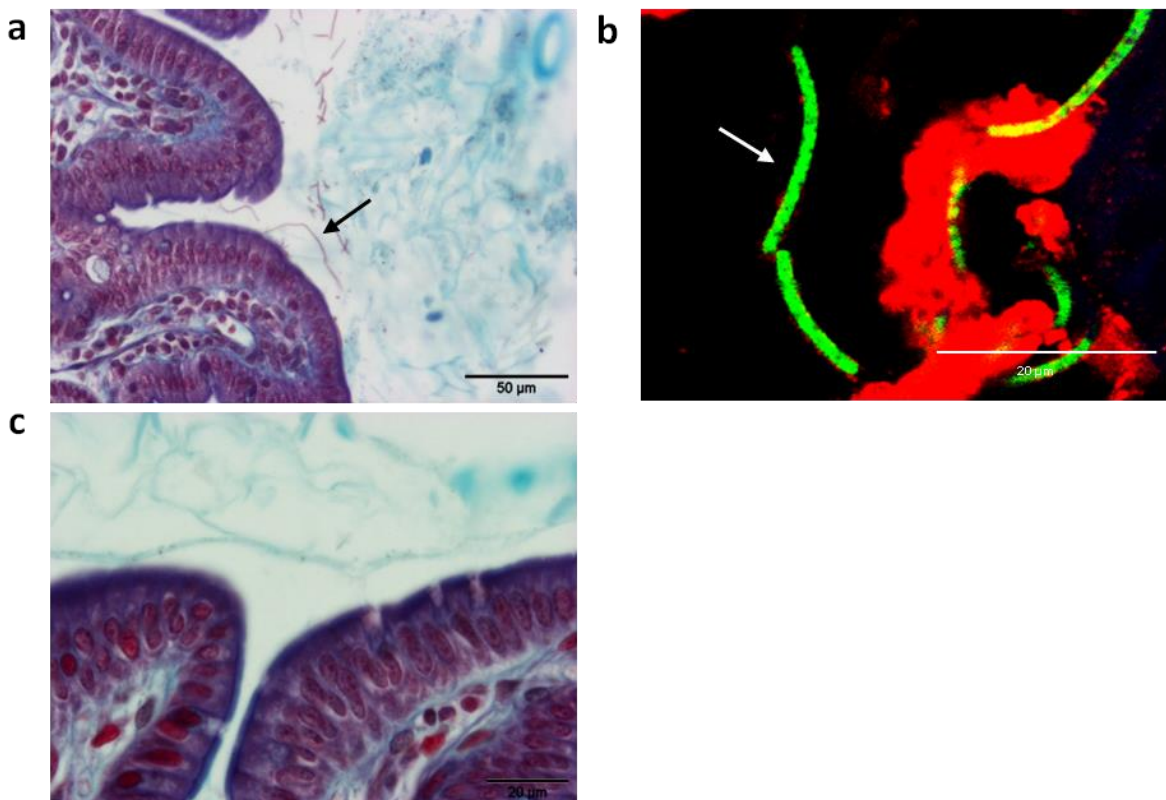


Figure 4: Sections of ileum from conventional mice stained using Crossmon's method (a) and fluorescence *in situ* hybridisation with Eubacterial probe EU388 (b) and GF mice stained using Crossmon's method (c). Arrows indicate segmented filamentous bacteria.

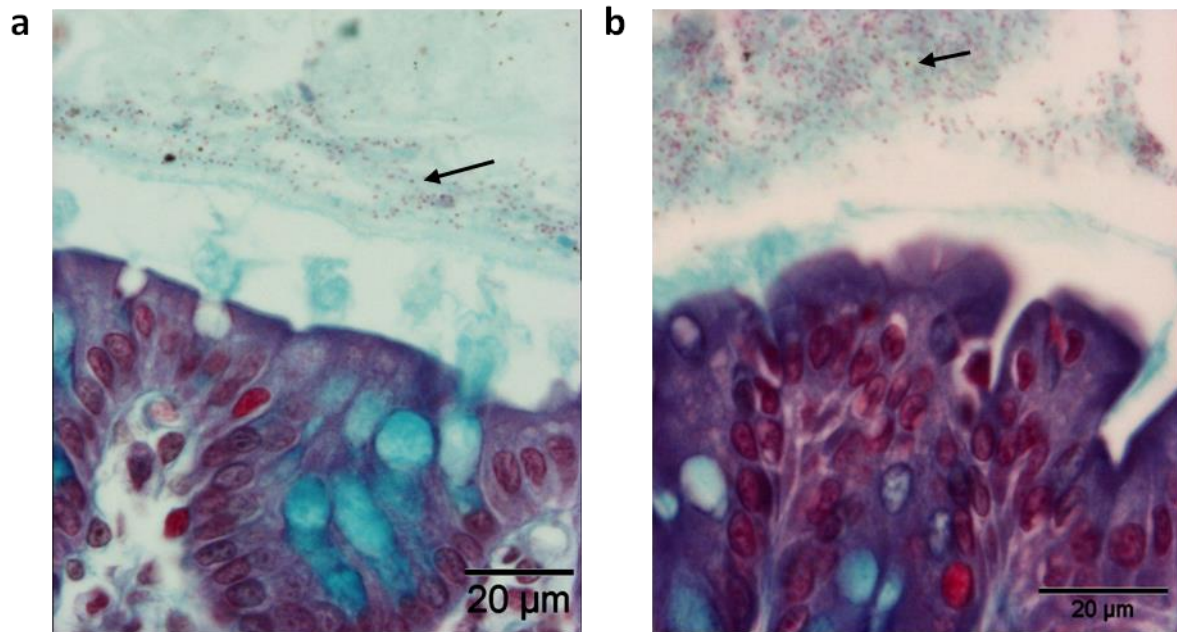


Figure 5: Sections of colon from mice mono-colonized with *L. plantarum* after 20 h (a) or 21 days (b) from colonization. Sections were stained using Crossmon's method to visualize the cells, mucus and bacteria. Arrows indicate the bacteria.

Viable L. plantarum can be recovered from immune cells within the PP and MLNs

Resected PPs and MLNs from both groups of mice were used to generate immune cell suspensions that were counted and then gently lysed and plated on agar plates to enumerate bacterial colony forming units (CFU). As expected, no bacteria were recovered from the PPs of GF mice but bacteria were recovered from PPs of all the mice colonized by *L. plantarum* (Table 1). The CFU of bacteria recovered per host immune cell was highly variable between mice and in the range of 10 to 100 CFU per million immune cells at 20 h and 21 days, although the CFU/cell tended to be higher in the group of mice colonized with *L. plantarum* for 21 days (Table 1). These counts most likely underestimate the number of CFU of *L. plantarum* in PPs and MLNs cells because bacteria would be killed by phagocytes such as macrophages and DCs. Previously, we determined that *L. plantarum* is rapidly killed in human monocyte derived DCs (estimated 1-2 h) (Meijerink et al., unpublished).

In mice colonized by *L. plantarum*, the bacterium was recovered from MLN cells presumably due to phagocytosis by DCs in the PP or lamina propria that can migrate to the draining lymph nodes and induce T cell responses. There was a large variation in CFU counts and for some mice no bacteria were detected, although only some of the MLNs were used for this experiment. *L. plantarum* was recovered from the splenic cells of only one mouse although this was probably not due to bacteraemia as the mouse appeared to be normal. Isolation of

bacteria from the spleen may have be due to *L. plantarum* carrying antigen presenting cells entering the blood circulation from the gut-associated lymphoid tissue.

Table 1: CFU of *L. plantarum* recovered from isolated lymphoid cells

Inoculation	CFU/10 ⁶ cell PP 20 h	CFU/10 ⁶ cell MLN 20 h	CFU/10 ⁶ cell PP day 21	CFU/10 ⁶ cell MLN day 21	CFU/10 ⁶ cell Spleen day 21
<i>L. plantarum</i>	97.1	62.2	1330	21.7	zero
<i>L. plantarum</i>	No PP	zero	144	186	20.8
<i>L. plantarum</i>	27.2	37.6	4230	1.26	zero
<i>L. plantarum</i>	814	zero	163	Zero	zero
<i>L. plantarum</i>	1080	25	12.2	Zero	zero
<i>L. plantarum</i>	10.5	0.909	1050	6.69	zero
<i>L. plantarum</i>	97.1	62.2	1330	21.7	zero
<i>PBS</i>	zero	zero	zero	Zero	zero
<i>PBS</i>	zero	zero	zero	Zero	zero
<i>PBS</i>	zero	zero	zero	Zero	zero

CFU, colony forming units; PP, Peyer's patches; MLN, mesenteric lymph nodes.

Discussion

Recent studies in mice have shown that in the colon, the secreted mucus forms a firmly adherent layer on top of the surface of the epithelium that is devoid of bacteria and an outer 'loose' mucus layer that is contact with bacteria and faecal material (Hansson and Johansson 2010). The firm mucus layer stains appears stratified and more intensely stained than the loose mucus using Alcian blue/PAS staining suggesting this layer contains a higher concentration of mucin glycoprotein (Figure 2). Although the thickness varied between tissue sections, the firm mucus was notably thinner in GF mice but after colonization with *L. plantarum* for 21 days, it was similar to the conventional group (Figure 2). This is consistent with published observations of reduced mucus production in GF animals and with the concept that metabolites and other components of the microflora are key regulators of colonic mucus secretion (Hill, Cowley et al. 1990; Schwerbrock, Makkink et al. 2004). The appearance of both firm and loose mucus in GF mice suggests that the conversion of firm mucus layers into loose mucus does not depend on the presence of bacteria. By combining Crossmon's staining method with the Carnoy fixation we were able to visualize the compartmentalization of the microbiota to the loose outer mucus layer in the colon (Figure 3) as previously described using immunofluorescent microscopy (Johansson, Phillipson et al. 2008). Although microscopy techniques cannot exclude the presence of small numbers of bacteria in the firm mucus, it is evident that these events are rare. Previously Johansson et al., showed using semi-quantitative qPCR of the bacterial 16S gene that there was bacterial DNA present in the firm mucus but in much less amounts than in the loose mucus (Johansson, Phillipson et al. 2008). The mucus layer in the ileum appears to be thinner, as previously demonstrated using different techniques (Atuma, Strugala et al. 2001) and has the appearance of densely stained strands residing between the villi and in-between the tops of the villi and the ileal content (Figure 3). In conventional mice no bacteria are seen to be in contact with the epithelium with the exception of the long filamentous structures characteristics of SFB (Figure 4). SFB are known to appear in the small intestine of Taconic mice after weaning. The most intriguing feature of SFB is their apparent intimate association with epithelial cells in the terminal ileum and their capacity to stimulate Th17 effector cell differentiation which contributes to protection against infection (Ivanov, Atarashi et al. 2009). The secreted mucus barrier in the colon and ileum prevents epithelial contact with *L. plantarum* in mono-colonized mice

although bacteria appear to be abundant in the loose mucus and lumen after 21 days (Figure 4). The spatial segregation of bacteria does not rule out the possibility that microbial metabolites and other components can diffuse through the firm mucus layer to trigger epithelial signalling and crosstalk to the immune cells in the lamina propria.

Live *L. plantarum* could be recovered from immune cells isolated from the PPs and MLNs of mono-colonized mice. The counts were highly variable between mice which may have been influenced by the location of the PPs or MLNs but overall, higher numbers of bacteria per cell (up to 1 in 236 total cells) were recovered from mouse PPs after 21 days colonization than after 20 h colonisation (Table 1). This is likely to be an underestimate of the number of bacteria sampled by APCs, as *L. plantarum* is killed in the phagosome of DCs after 1 - 2 hours. However, our attempts to enumerate cells containing *L. plantarum* expressing fluorescent mCherry were unsuccessful despite the fact that it was stably produced by *L. plantarum in vitro* and could be detected by flow cytometry (not shown). The reasons for this are not clear but most likely due to the instability of mCherry expression *in vivo*. DCs are estimated to be around 2% of the total PP cell population depending on the gender of the mice (Duriancik and Hoag 2009). MLNs were also found to contain viable *L. plantarum* but the numbers were lower than in PPs. As *L. plantarum* were not seen in contact with the epithelium in the ileum, it seems most likely that bacteria-laden DCs in the PPs or ILFs migrated through the lymphatics to the draining lymph nodes. Future experiments in CCR7^{-/-} mice will be used to test this hypothesis as this receptor is required for migration of DC103⁺ DC from the LP to the lymph nodes. The lower CFU recovered from the MLNs may be due to the variability of counts in the resected MLNs and/or the extended time of *L. plantarum* within APCs recovered from the MLNs.

The preliminary study described in this chapter focused on PPs and antigen sampling. However, the colon also contains an additional type of lymphoid tissue, the solitary intestinal lymphoid tissues (SILT) which can develop in mature isolated lymphoid follicles (ILFs) (Hamada, Hiroi et al. 2002; Lorenz, Chaplin et al. 2003; Pabst, Herbrand et al. 2006). Like the PPs, ILFs can also function as inductive sites for mucosal immune responses (Lorenz, Chaplin et al. 2003).

In summary, these results clearly demonstrate that the mucus presents a formidable barrier to the immune sampling of bacteria in the intestine. Nevertheless, intact and viable intestinal bacteria are found intracellularly in the PP and MLN cells (most likely within DCs). As contact

of intact bacteria with the small intestinal epithelium was not observed, uptake in the PPs or ILFs seems to be the predominant mechanism for immune sampling of commensal bacteria. Our future aims are to confirm the reported lack of mucus over the follicular epithelium of the PPs and to stain for both *L. plantarum* and DCs in these tissue sections of the ileum, colon and PPs. Additionally, we aim to generate *L. plantarum* strains that stably express fluorescent proteins *in vivo* so that bacteria can be more easily localized with specific types of immune cells for e.g. in transgenic mice expressing green fluorescent protein in specific subsets of mucosal DCs.

Materials and Methods

Mice

This study was conducted under the oversight of the Crown Research Institute Animal Ethics Committee (Palmerston North, New Zealand) according to the New Zealand Animal Welfare Act 1999, Animal Ethics application number 12501.

Conventional Balb/c mice (CLEA Japan, INC. Tokyo, Japan) were housed under specific pathogen-free conditions. All mice were fed AIN-93M diet (Research Diets, New Jersey, US) and were housed with 12 hour light/dark cycles and at an environmental temperature of 21-22°C. Germ-free (GF) Balb/c mice from the same supplier were housed in individual cages within gnotobiotic isolators and given the same diet sterilised by gamma irradiation (25 kGy), and autoclaved sterile water. All bedding material was sterilized by gamma irradiation (25 kGy). Access to food and water was provided *ad libitum*, and food intake was measured weekly.

Bacteria

L. plantarum harbouring pTmv-mCherry was cultured at 37°C in deMan, Rogosa Sharpe (MRS) medium containing 5 µg/ml erythromycin overnight and stored in aliquots in MRS plus 10% glycerol at -80°C. On the day of administration, one aliquot was thawed and grown in MRS plus 5 mg/ml erythromycin to an OD at 600nm of 1-1.5, then harvested by centrifugation, washed in PBS and resuspended in sterile PBS prior to inoculation.

In vivo experiment and collection of tissue samples

Both GF and conventional mice were divided in 2 groups which received different treatments and 2 control groups (Figure 1). GF mice from group 1 (n=6) and conventional mice from group 2 (n=6) received 10^8 CFU/100µl of *L. plantarum* pTv-mCherry by oral gavage on day 1 and were euthanized 20 h later (n=6); GF mice from group 3 (n=4) and conventional mice from group 4 (n=4) received 100 µl of PBS by oral gavage on day 1 and were euthanized 20 h later; GF mice from group 5 (n=6) and conventional mice from group 6 (n=6) received 10^8 CFU/100µl of *L. plantarum* pTv-mCherry by oral gavage on day 1 and day 21 and were euthanized 20 h after day 21 and GF mice from group 7 (n=4) and conventional mice from group 8 (n=4)

received 100 µl of PBS by oral gavage on day 1 and day 21 and were euthanized 20 h after day 21.

For splenocytes isolation, spleens were gently pushed through 100 µm gauge cell strainers. The cell suspension was made up to 10 ml with PBS (4°C), and then centrifuged at 300 g (4°C) for 5 minutes following which the supernatant was discarded. Red blood cells were lysed by adding 2 ml of ACK lysis buffer (150 mM NH₄Cl, 1mM KHCO₃, 0.1 mM Na₂EDTA pH 7.3). Cells were then incubated for 5 minutes at room temperature. Lysis was stopped by adding 10 ml of PBS (4°C) to each tube, which were then mixed by inversion, followed by centrifugation at 300 g (4°C) for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 1 ml of PBS (4°C).

Mesenteric lymph nodes (MLNs) were removed *in situ* from mesenteric tissue, the fat removed from the lymph nodes before the MLN were dispersed by pushing through 100 µm gauge cell strainers. The cell suspension was washed by adding 10 ml of PBS (4°C), followed by centrifugation at 300 X g (4°C) for 5 minutes. The supernatant was discarded and the cells resuspended in 1 ml of PBS (4°C).

Peyer's patches (PPs) were dissected from the ileum. PPs were pierced with a syringe needle, following which they were incubated in 3 ml of Rosewell Park Memorial Institute (RPMI) medium with collagenase type IV (Sigma C5138, 100 U/ml) in a shaking incubator at 15 min at 37°C. The collagenase was then neutralised by adding 9 ml of RPMI plus 10 ml of FCS. The cell suspension was filtered by passing through a 100 µm gauge cell strainer. The cells were then centrifuged at 300 g (4°C) for 5 minutes. The cells were washed once more by adding 10 ml PBS (4°C), followed by centrifugation at 300 X g (4°C) for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml of PBS (4°C).

To enumerate live bacteria, serial dilutions of PBS suspension with single cells isolated from spleens, MLNs and PPs of mice were plated on MRS agar.

Histology and FISH

Segments of colon and ileum from mice from group 1 to 8 and conventional mice were fixed in Carnoy's fixative to preserve the mucus layer as described by Johansson et al., (Johansson and Hansson 2012). Carnoy fixed tissue was embedded in paraffin and sectioned at 5 µm. Sections were stained using the combined Alcian blue/periodic acid Schiff (PAS) methods, Gram stain, or Crossmon's method.

Segments of colon, ileum and PPs were also cryopreserved for fluorescent *in situ* hybridization (FISH). FISH was performed using the method described by Johansson et al., (Johansson and Hansson 2012). Bacteria were stained with the universal probe EUB 338 probe (Isogen-bioscience 26-2016-1/1) and the mucus with the rabbit anti mouse anti-Muc2C3 (1 : 500, Kindly gifted by Prof. Gunnar Hansson, Department of Medical Biochemistry, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden) and the secondary antibody Goat-anti-rabbit Cy3 (Jackson ImmunoResearch, lot number : 111-165-144).

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Chapter 4

Differential cytokine induction and TLR signalling by *Faecalibacterium prausnitzii* and other commensal intestinal bacteria

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Abstract

A reduction of *Faecalibacterium prausnitzii* has been associated with risk of Crohn's disease recurrence. Additionally, *F. prausnitzii* strain A2-165 has been reported to exert anti-inflammatory effects both *in vitro* and *in vivo*. The supernatant of *F. prausnitzii* cultures attenuated NF- κ B signalling in epithelial cells and both the bacterium and its supernatant reduce the severity of TNBS induced colitis *in vivo*. As dendritic cells (DCs) are the most important antigen presenting cells with the unique ability to activate naïve T cells at mucosal sites, here we investigated the immune stimulating properties of 5 *F. prausnitzii* strains and 8 other abundant intestinal commensals bacteria on DCs function *in vitro*. All bacteria induced efficient maturation and activation of DCs. *F. prausnitzii* A2-165 induced the largest amounts of IL-10, TNF- α and IL-1 β and was among the weakest inducers of IL-12p70. Principal component analysis revealed that the immune response to *F. prausnitzii* strain A2-165 is distinct from the other strains, due to the high levels of IL-10. The TLR signalling capacity of the strains was also measured using NF- κ B reporter cell lines expressing human TLRs. In general, high TLR signalling activity was associated with the ability of commensal bacteria to induce cytokines in the DC assays.

This research has implications for the exploitation of *F. prausnitzii* as a therapeutic because all protection studies to date have been performed using strain A2-165 which elicits a distinct immune response in human immune cells.

Introduction

The human gastrointestinal tract is colonized by more than 3000 different species of commensal bacteria, referred to as the microbiota, which reaches densities of 10^{12} bacteria per gram of luminal content in the colon (Whitman, Coleman et al. 1998). The dominant phyla of commensal bacteria are Firmicutes, Bacteroidetes and Actinobacteria (Tap, Mondot et al. 2009). In the healthy intestine, commensal bacteria are predominantly compartmentalized to the lumen, but can interact with immune cells associated with the epithelium or in lymphoid tissues of the small intestine. The microbiota or components of the microbiota can influence immunity and tolerance in the gastrointestinal tract through their recognition by pattern recognition receptors (PRRs) expressed on intestinal epithelial cells and immune cells (Wells, Rossi et al. 2010; Rossi, van Baarlen et al. 2011). Changes in the microbiota are associated with several intestinal-related disorders including irritable bowel syndrome, autoimmune disease and diabetes and have a profound impact on the pathophysiology of inflammatory bowel disease (IBD) which includes Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are driven by an aberrant inflammatory T cell response to intestinal microbiota in a genetically susceptible host. Both diseases are associated with loss of intestinal epithelial integrity resulting in increased bacterial translocation into the mucosa and leading to increased interactions of bacteria with immune cells. In this context, it is important to understand the direct effects of commensal bacteria on immune cells.

A decrease in the abundance and biodiversity of bacteria belonging to the Firmicutes phylum has been observed in IBD patients (Seksik, Rigottier-Gois et al. 2003; Macfarlane, Blackett et al. 2009). In particular, a reduction of a major member of the Firmicutes phylum, *Faecalibacterium prausnitzii* has been associated with risk of disease recurrence in CD patients (Sokol, Pigneur et al. 2008). In addition, certain commensal strains have been demonstrated to have strong effects on the mucosal immune system *in vitro* and *in vivo* (Gaboriau-Routhiau, Rakotobe et al. 2009; Atarashi, Tanoue et al. 2011). For example, the prominent commensal *Bacteroides fragilis* has been shown to protect against experimental colitis in mice (Mazmanian, Liu et al. 2005; Mazmanian, Round et al. 2008). Moreover, *F. prausnitzii* and its culture supernatant have been shown to reduce the severity of 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis in mice (Sokol, Pigneur et al. 2008).

The effects of commensal bacteria on the host mucosal immune system are partly mediated by their interaction with antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs) (Rossi, van Baarlen et al. 2011). In the intestinal lamina propria and Peyer's patches, immature DCs are activated by contact with bacteria or bacterial microbe-associated molecular patterns (MAMPs) via innate PRRs, such as Toll like receptors (TLRs). After activation, DCs undergo maturation and migrate from the mucosal tissue to the lymphoid tissue where they interact with naive T cells inducing their activation, clonal expansion and differentiation. Different subsets of DCs in the mucosal and lymphoid tissues are involved in the induction of regulatory T cells (Tregs, (Powrie, Read et al. 2003)) and effector T cells via distinct profiles of cytokine secretion and surface co-stimulatory molecules expression.

IL-10 is pivotal for the maintenance of homeostasis in the intestine (Geuking, Cahenzli et al. 2011; Veenbergen and Samsom 2012). It is secreted by DCs as well as Foxp3⁺ and Foxp3⁻ T cells in the lamina propria. Secretion of IL-10 by DCs is important for the maintenance of functional Foxp3⁺ Tregs during intestinal inflammation (Murai, Turovskaya et al. 2009). IL-10 also inhibits the production of pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-6 and IL-12. Moreover, IL-10 was shown to play a role in controlling pro-inflammatory responses to translocated microbes by abrogating IL-23 production (Manuzak, Dillon et al. 2012).

In vitro assays are considered as useful approaches to select strains for *in vivo* studies based on their immunomodulatory properties (Foligne, Nutten et al. 2007; Kwon, Lee et al. 2010; Snel, Vissers et al. 2011; Meijerink, Wells et al. 2012). For example, *in vitro* in culture with human peripheral blood mononuclear cells (PBMCs), *F. prausnitzii* induces high levels of IL-10 and low levels of IL-12p70 and IFN- γ which correlates with its protective effect in TNBS induced colitis *in vivo* (Sokol, Pigneur et al. 2008).

Given the important role of DCs in regulating tolerance and immunity in the intestinal mucosa, our aim was to investigate the immunomodulatory properties of *F. prausnitzii* and other commensal bacteria on human DCs *in vitro* and to assess the contribution of different TLR signalling in immune activation. Furthermore, little is known about the immunomodulatory properties of commensal bacteria as most studies have been performed on traditional probiotic species such as *Lactobacillus* and *Bifidobacterium* (Bron, van Baarlen et al. 2012).

The 13 bacterial strains selected for this study belong to the Firmicutes and Actinobacteria phyla, they include 5 isolates of *F. prausnitzii* and *Lactobacillus plantarum* WCFS1. The immune responses of DCs to the commensal bacteria were assessed measuring cytokine

secretion and surface marker expression after incubation of DCs with the bacteria. Immature DCs were differentiated *in vitro* from CD14⁺ monocytes isolated from human blood (Sallusto and Lanzavecchia 1994; Zhou and Tedder 1996). Additionally, the TLR signalling capacity of the strains was measured using NF- κ B reporter cell lines expressing TLRs.

Results

Immune responses of human PBMCs to F. prausnitzii

As *F. prausnitzii* was previously shown to induce relatively high IL-10 to IL-12p70 and IFN- γ cytokine ratio in culture with PBMCs, we compared cytokine production of PBMCs incubated with *F. prausnitzii* A2-165, *L. plantarum* WCFS1 or *L. salivarius* HMI001 at a bacterium to cell ratio of 10 to 1. *L. salivarius* HMI001 was selected as it is was previously shown to be a relatively high inducer of IL-10 compared to other *Lactobacilli* (Meijerink, Wells et al. 2012) and *L. plantarum* WCFS1, a high inducer of IL-12p70 (Meijerink, van Hemert et al. 2010).

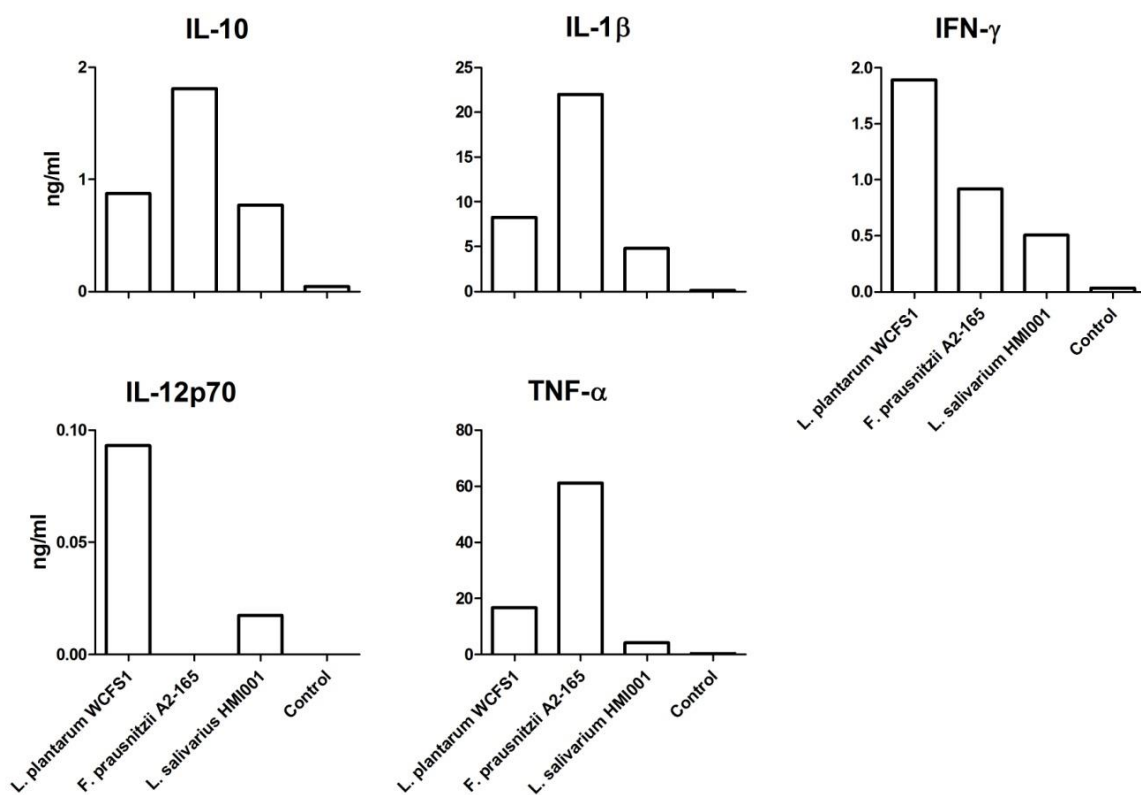


Figure 1: Cytokine secretion by PBMCs. IL-10, IL-12p70, TNF- α , IL-1 β and IFN- γ were measured in the supernatant of PBMCs from one donor after 24 hours of incubation with bacteria (bacterium: PBMC, 10:1).

PBMCs stimulated with *F. prausnitzii* A2-165 released the largest amount of IL-10, the smallest amount of IL-12p70 and small amount of IFN- γ in comparison with the other strains (Figure 1). Interestingly, *F. prausnitzii* A2-165 also induced the largest secretion of the pro-inflammatory cytokines IL-1 β and TNF- α by PBMCs when compared to the lactobacilli strains (Figure 1).

Surface activation markers are up-regulated in DCs in response to commensal bacteria

Human monocyte-derived immature DCs (DCs) were incubated with selected commensal anaerobic bacteria (listed in Table 1) at a bacterium to cell ratio of 10 to 1. Incubation with the different bacteria did not significantly alter DC viability compared to the control (typically 60-80% viability, not shown). Surface expression of the maturation marker CD83 and the co-stimulatory molecule CD86 were determined to assess the activation and maturation status of DCs *in vitro* after stimulation with the bacteria. All the bacteria tested induced increased surface expression of CD83 and CD86 when compared with the control immature non-stimulated DCs. The degrees of surface marker expression induced by the bacteria were similar, indicating that they all induced DC maturation and activation (Figure 2a and b).

Commensal bacteria induce differential cytokine responses in DCs

The immune activation of DCs was assessed by measuring the levels of IL-10, IL-12p70, IL-6, IL-1 β and TNF- α in the culture supernatant after incubation of the bacteria with DCs from three to four different donors (Figure 3). The largest amounts of IL-10 were induced by the *F. prausnitzii* strains, *Eubacterium hallii* L2-7 and *Megamonas rupellensis* Mag1B. In addition, these strains induced the largest amounts of TNF- α , as observed for *F. prausnitzii* A2-165 with PBMCs, and of IL-6. The *F. prausnitzii* strains, *E. hallii* L2-7 and *E. rectale* A1-86 also induced relatively large amounts of IL-1 β . *L. plantarum* WCFS1, *M. rupellensis* Mag1B and *E. hallii* L2-7 were among the strongest inducers of IL-12p70, whereas the *F. prausnitzii* strains were among the poorer inducers (Figure 3). Surprisingly, the strains *Ruminococcus bromii* L2-63, *Clostridium hathewayi* 82-B and *C. xylanovorans* Lac1D induced small or undetectable amounts of some cytokines (Figure 3) despite having effectively induced DC maturation (Figure 2a and b).

Bacteria and DCs were incubated in the presence of antibiotics to prevent bacterial overgrowth. However, soluble factors released by the bacteria during growth may have immunomodulatory properties as shown previously for the culture supernatant of *F. prausnitzii* A2-165 which had a protective effect against TNBS-induced colitis (Sokol, Pigneur et al. 2008). To investigate this, DCs were stimulated with *F. prausnitzii* A2-165 cell free culture supernatants, bacterial medium or butyrate at concentrations similar to that measured in the bacterial culture supernatant. In an initial experiment, the bacterial supernatants were found to be toxic for the DCs at a concentration of 10% v/v (data not

shown). This toxicity can be attributed to the high concentration of butyrate produced during bacterial fermentation (Wang, Morinobu et al. 2008). *F. prausnitzii* A2-165 culture supernatant (1.25 and 2.5 % v/v) and butyrate (25 to 500 μ M) did not affect cell viability or cytokine secretion by DCs compared to un-stimulated cells (data not shown). This demonstrates that soluble MAMPs, butyrate or other factors secreted by *F. prausnitzii* did not induce cytokine responses in DCs.

Table 1: Phylum, family and phylogenetic cluster of the bacteria used

Bacterial strain	Phylum	Family	Phylogenetic cluster	Reference
<i>F. prausnitzii</i> A2-165	Firmicutes	Ruminococcaceae	Clostridium cluster IV	(Duncan 2002)
<i>F. prausnitzii</i> M21/2	Firmicutes	Ruminococcaceae	Clostridium cluster IV	(Louis, Duncan et al. 2004)
<i>F. prausnitzii</i> S3L/3	Firmicutes	Ruminococcaceae	Clostridium cluster IV	(Louis, Duncan et al. 2004)
<i>F. prausnitzii</i> L2-6	Firmicutes	Ruminococcaceae	Clostridium cluster IV	(Duncan 2002)
<i>F. prausnitzii</i> HTF-F	Firmicutes	Ruminococcaceae	Clostridium cluster IV	(Lopez-Siles, Khan et al. 2012)
<i>R. bromii</i> L2-63	Firmicutes	Ruminococcaceae	Clostridium cluster IV	(Barcenilla, Pryde et al. 2000)
<i>E. rectale</i> A1-86	Firmicutes	Eubacteriaceae	Clostridium cluster XIVa	(Barcenilla, Pryde et al. 2000)
<i>E. hallii</i> L2-7	Firmicutes	Eubacteriaceae	Clostridium cluster XIVa	(Duncan, Louis et al. 2004)
<i>B. adolescentis</i> L2-32	Actinobacteria	Bifidobacteriaceae		(Barcenilla, Pryde et al. 2000)
<i>Megamonas</i> sp. Mag1B 95,0% homology* with <i>M. rupellensis</i>	Firmicutes	Veillonellaceae		This work
<i>Clostridium</i> sp. 82B 95,4% homology* with <i>C. hathewayi</i>	Firmicutes	Clostridiaceae	Clostridium cluster XIVa	This work
<i>Clostridium</i> sp. Lac1D 90,5% homology* with <i>C. xylanovorans</i>	Firmicutes	Clostridiaceae	Clostridium cluster XIVa	This work
<i>L. plantarum</i> WCFS1	Firmicutes	Lactobacillaceae		

*based on the sequence homology of 850 nucleotides of the 16S rRNA

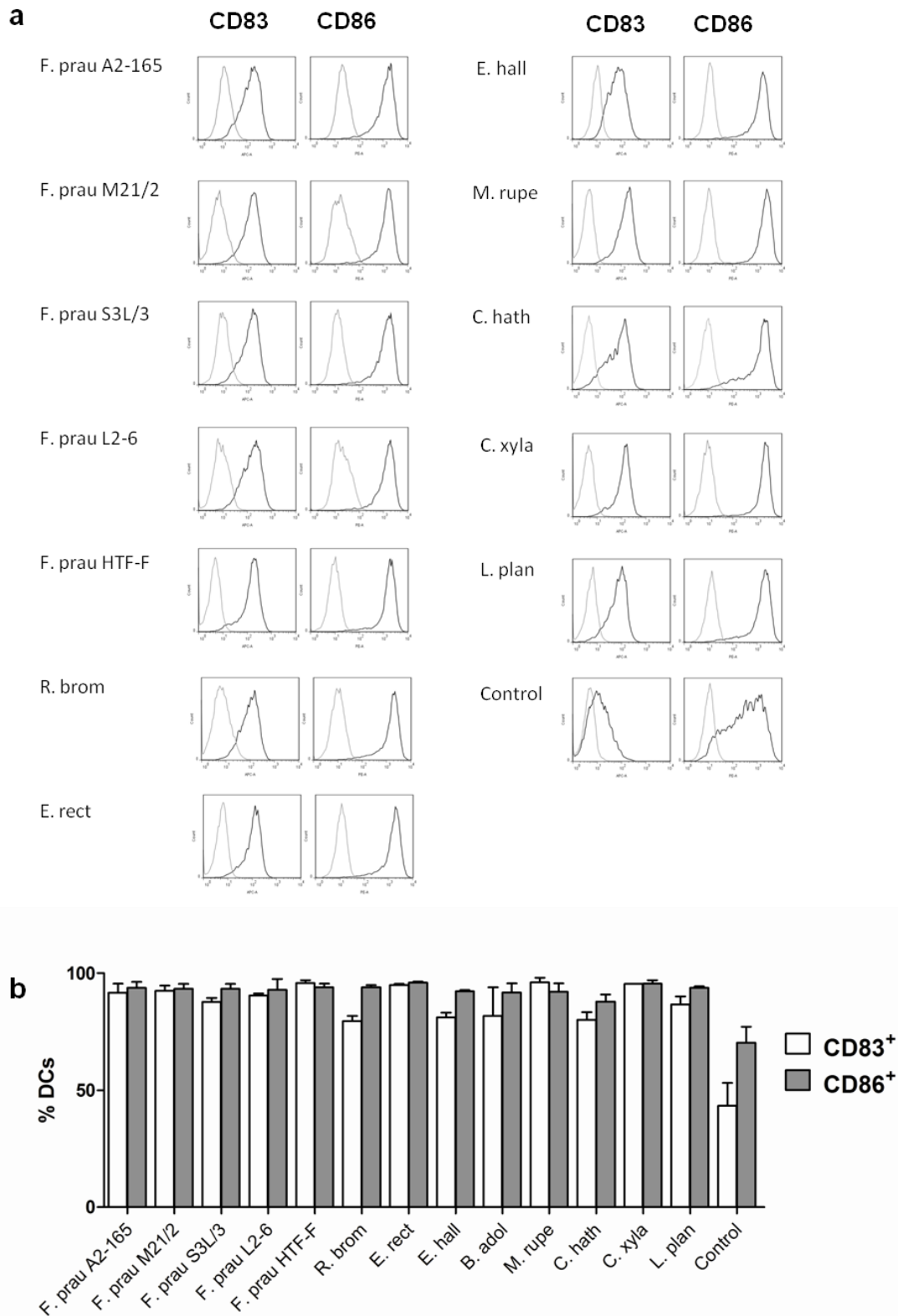


Figure 2: CD83 and CD86 surface expression in DCs after 48 hours of incubation with the bacteria (bacterium: DC ratio 10:1). a) Representative histograms showing the expression of the surface markers CD83 and CD86 on DCs. Grey lines represent the isotype controls and black lines the staining. b) Percentage of CD83⁺ (in white) and CD86⁺ (in grey) DCs from 3 donors. Error bars represent SEM. *F. prau*, *F. prausnitzii*; *R. brom*, *R. bromii* L2-63; *E. rect*, *E. rectal* A1-86; *E. hall*, *E. hallii*; *M. rupe*, *M. rupellensis*; *C. hath*, *C. hathewayi* 82-B; *C. xyla*, *C. xylanovorans* Lac1D; *L. plan*, *L. plantarum* WCFS1.

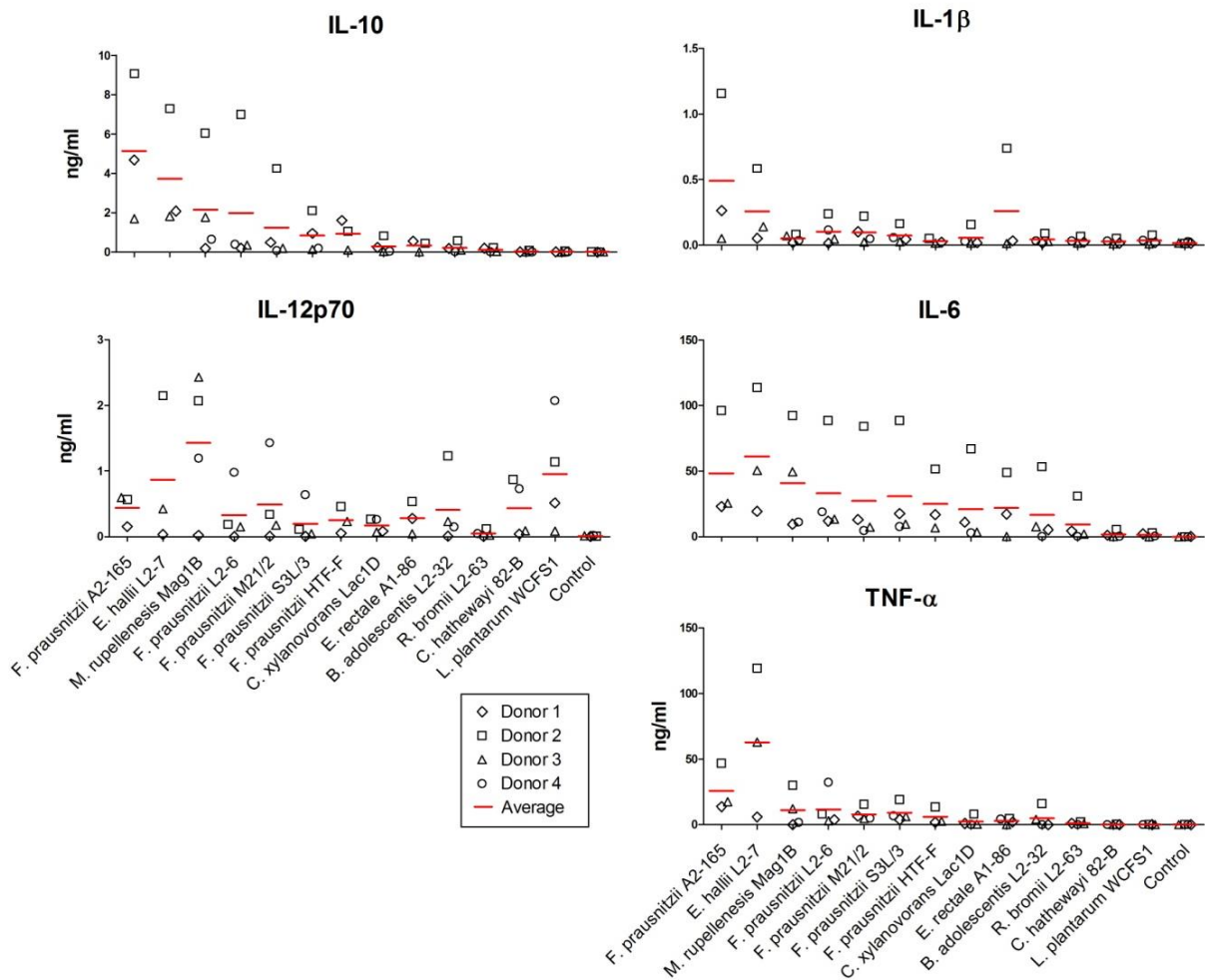


Figure 3: Cytokine secretion by DCs. IL-10, IL-12p70, TNF- α , IL-1 β and IL-6 were measured in the supernatant of DCs from 3 to 4 donors after 48 h of incubation with the bacteria (bacterium to DC ratio 10:1). Each donor is represented with a different symbol and the red lines indicate the average of the donors.

Differential activation of TLRs by commensal bacteria

To investigate the potential relationship between cytokine profiles in DCs and TLR signalling, bacteria were incubated with HEK293 reporter cell lines expressing different human TLRs and a reporter plasmid harbouring the luciferase gene under the control of the NF- κ B promoter. Incubation of each TLR expressing cell line with the respective agonist induced high luciferase activity (Pam2CSK4 is the agonist of TLR2 and TLR2/6, flagellin and LPS are the agonists of TLR5 and TLR4 respectively) compared to cells stimulated with medium alone. HEK293 cells transfected with the reporter plasmid alone did not produce luciferase in response to TLR agonists or bacteria demonstrating that the NF- κ B activation was TLR dependent (data not shown).

The *F. prausnitzii* strains, *R. bromii* L2-63, *E. rectale* A1-86, *E. hallii* L2-7 and *B. adolescentis* L2-32 induced high NF- κ B activation in the TLR2/6 and TLR2 reporter cell lines (Figure 4). Agonists of TLR2 include acylated lipoprotein anchors and lipoteichoic acid present in Gram-positive and Gram-negative bacterial cell envelopes. As previously reported (Erridge, Duncan et al. 2010), the flagellated strain *E. rectale* A1-86 (Duncan and Flint 2008) induced NF- κ B activation via TLR5 signalling. Surprisingly, *E. hallii* L2-7 and *M. rupellensis* Mag1B activated TLR4 signalling although they are considered Gram-positive. *M. rupellensis* Mag1B however, has been described previously as Gram-negative to Gram-variable (Chevrot, Carlotti et al. 2008). *C. hathewayi* 82-B and *C. xylanovorans* Lac1D did not induce NF- κ B activation in any of the TLR assays, suggesting that these strains might express MAMPs that are shielded or not recognized by the TLRs tested. Alternatively, these strains may possess immunomodulatory components that inhibit the activation of the NF- κ B pathway.

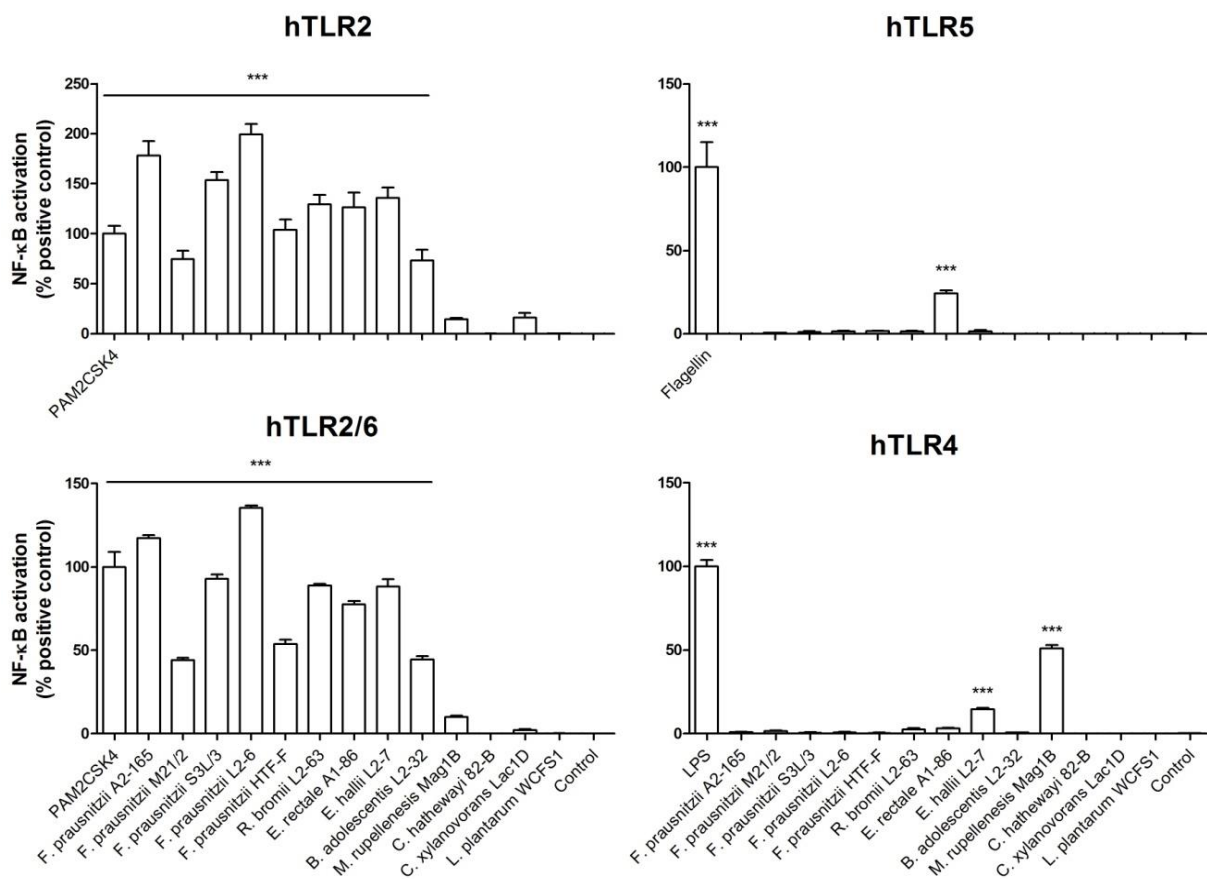


Figure 4: TLR signalling properties of the commensal bacteria. NF- κ B activation was measured using a luminescence reporter in HEK293 cell lines expressing TLR2, TLR2/6, TLR5 and TLR4 after incubation with the bacteria (bacterium: cell ratio 10:1). NF- κ B activation is expressed as percentage of the positive control. Error bars represent SEM, n=6, * indicates p<0.05, ** p<0.01, *** p<0.001 compared to the control.

Principal component analysis

Principal component analysis (PCA) was performed to describe the variance of the cytokine levels (IL-10, IL-12p70, IL-6, IL-1 β and TNF- α) induced by the bacteria.

The PC 1 and PC 2 of the PCA describe 99.9 % of the variance between the cytokine levels induced by the bacteria. The coefficients of PC 1 revealed that the contribution of the different cytokines to the variance was almost equal, the coefficient associated with IL-12p70 was 0.2 while for the other cytokines the coefficient was in average 0.48. The PC 1, PC 2 score plot (Figure 5) revealed that the majority of the bacteria are grouped in the centre of the plot, while *F. prausnitzii* A2-165, *E. hallii* L2-7, *M. rupellensis* Mag1B and *L. plantarum* WCFS1 are clearly separated from the other bacteria and from each other reflecting their capacity to induce distinct cytokine profiles in DCs. Furthermore, PCA revealed that the immune response induced by *F. prausnitzii* A2-165 is very different from the other *F. prausnitzii* strains tested.

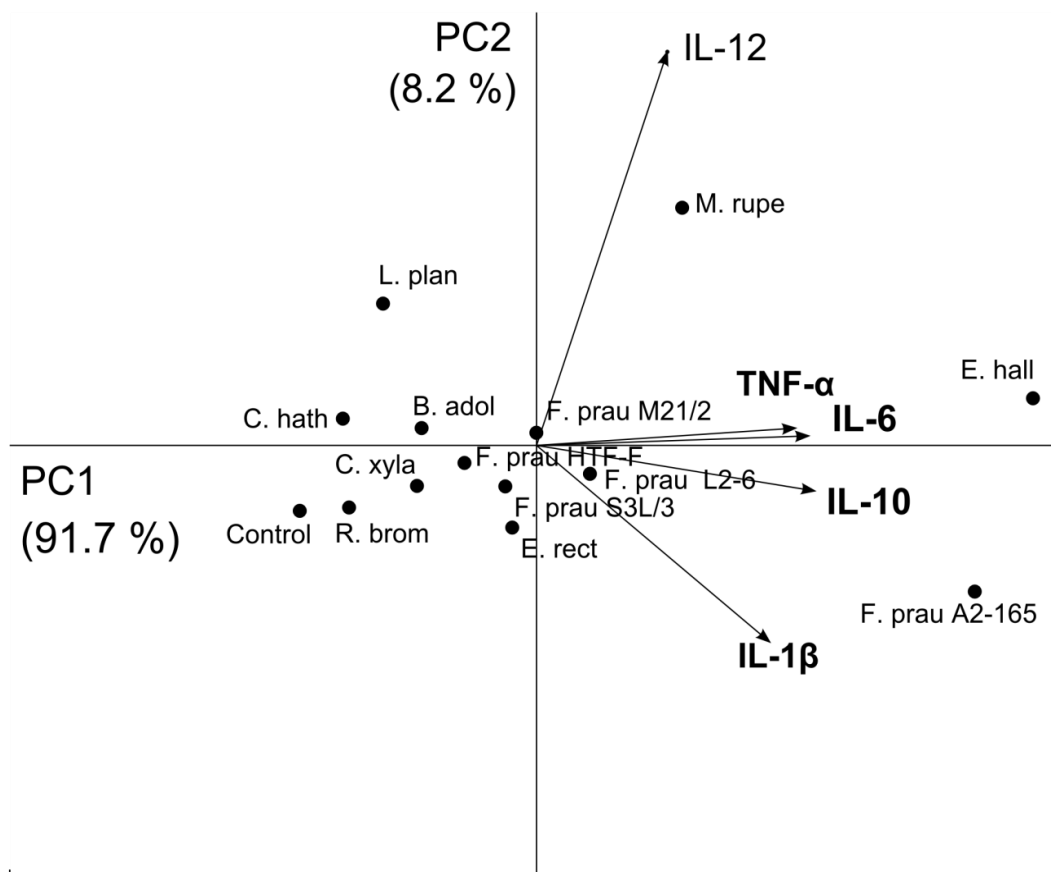


Figure 5: Principal component analysis (PCA) of the cytokine profiles induced by the commensal bacteria in DCs. Each dot represents the average of cytokine levels of a commensal strain in DCs. The variables (level of cytokines) are represented by vectors. The direction and length of a vector indicates how the variable contributes to the two principal components in the plot. (F. prau, *F. prausnitzii*; R. brom, *R. bromii* L2-63; E. rect, *E. rectale* A1-86; E. hall, *E. hallii* L2-7; M. rupe, *M. rupellensis* Mag1B; C. hath, *C. hathewayi* 82-B; C. xyla, *C. xylanovorans* Lac1D; L. plan, *L. plantarum* WCFS1).

Discussion

Reduced counts of *F. prausnitzii* have been correlated with recurrence of colitis in CD patients. *F. prausnitzii* strain A2-165 and its culture supernatant were shown to attenuate colitis in TNBS mouse model. Additionally, incubation of human PBMCs with *F. prausnitzii* A2-165 induced a relatively high IL-10 to IL-12p70 cytokine ratio, suggesting that the bacterium might have an effect on APCs such as DCs (Sokol, Pigneur et al. 2008).

Here we investigated the immune stimulating properties of *F. prausnitzii* and other commensals bacteria on DCs which play important roles in regulating mucosal tolerance and immunity. DCs are key APCs in the intestine, linking innate and adaptive immune responses via the induction of T cells in the mucosal lymphoid tissue. Furthermore, IL-10 produced by APCs in the lamina propria has been reported to enhance the suppressive activity of Foxp3⁺ Tregs in the mucosa (Murai, Turovskaya et al. 2009).

The effects of *F. prausnitzii* on DCs have not yet been described and it is not known whether all *F. prausnitzii* strains behave similarly or if they induce different immune responses in DCs compared to other colonic commensals. To address these questions, we investigated the immunomodulatory properties of five *F. prausnitzii* strains and 8 other abundant intestinal commensal bacteria on human monocyte-derived DCs *in vitro*. The majority of the bacteria tested belong to the Firmicutes phylum, which is characterized by decreased abundance and biodiversity in IBD patients compared to healthy controls (Seksik, Rigottier-Gois et al. 2003; Macfarlane, Blackett et al. 2009). Moreover, *F. prausnitzii*, *R. bromii*, *E. hallii* and *E. rectale* belong to the Clostridium clusters IV and XIVa (Clostridium leptum and coccoides groups, respectively) that have been shown to induce the expansion and activation of Tregs after colonization of germ-free mice (Atarashi, Tanoue et al. 2011).

All the bacteria tested were able to induce efficient maturation and activation of DCs (Figure 2a and b). The highest levels of IL-10 were induced by *F. prausnitzii* A2-165, *E. hallii* L2-7 and *M. rupellensis* Mag1B (Figure 3). *F. prausnitzii* A2-165 was among the weakest inducers of IL-12p70 while *M. rupellensis* Mag1B and *E. hallii* L2-7 were among the strongest inducers (Figure 3). Additionally, these strains induced the largest amounts of TNF- α and IL-6. The *F. prausnitzii* strains, *E. hallii* L2-7 and *E. rectale* A1-86 induced also relatively large amounts of IL-1 β .

PCA revealed that *F. prausnitzii* A2-165, *M. ruppellensis* Mag1B and *E. hallii* L2-7 cause distinct effects on the immune cells when compared to the other strains. These differential effects were due to the high levels of IL-10 induced by *F. prausnitzii* A2-165, the high levels of IL-12p70 and TNF- α induced by *M. ruppellensis* Mag1B and the high levels of IL-10 and IL-12p70 induced by *E. hallii* L2-7. Furthermore, *F. prausnitzii* A2-165 is separated from the other *F. prausnitzii* strains. Strain dependent differences in cytokine profiles have been described previously for *L. plantarum* and linked to genetic factors such as bacteriocin production and altered TLR recognition due to enzymes modifying structures of MAMPs (Meijerink, van Hemert et al. 2010). These results also have implications for the exploitation of *F. prausnitzii* as a therapeutic in IBD as the protective effects described to date have only been described for strain A2-165 which induces larger amounts of IL-10 than the other *F. prausnitzii* strains.

Several studies have correlated *in vitro* cytokine profiles induced by lactobacilli with their anti-inflammatory properties *in vivo*. The *in vivo* protective effects of lactobacilli correlated with high IL-10 and low IL-12p70 induction *in vitro* in PBMCs suggesting that these two cytokines, especially IL-10, were the main factors involved in protection (Foligne, Nutten et al. 2007). In our experiments using DCs, *F. prausnitzii* A2-165 induced high IL-10, low IL-12p70 but also high amounts of the pro-inflammatory TNF- α and IL-6 compared to the other bacteria.

In our experiments, bacteria and DCs were co-incubated in the presence of antibiotic to prevent bacterial overgrowth and medium acidification which would affect DC viability and cytokine secretion. However, *F. prausnitzii* A2-165 culture supernatant and the short chain fatty acid butyrate were tested separately for their capacity to activate and induce cytokines in DCs. Neither *F. prausnitzii* A2-165 culture supernatant nor butyrate induced cytokine secretion or the expression of surface activation and maturation markers in DCs (not shown).

In general, high TLR signalling activity was associated with the ability of commensal bacteria to induce cytokines in the DC assays. The ability of *E. rectale* A1-86 to induce NF- κ B activation through TLR5 is in agreement with a previous study (Erridge, Duncan et al. 2010). TLR4 signalling was induced by *E. hallii* L2-7 and *M. ruppellensis* Mag1B. Phylogenetically, *M. ruppellensis* Mag1B and *E. hallii* L2-7 belong to the Firmicutes lineage. TLR4 signalling induced by the Firmicutes bacteria is ambiguous, as by definition these bacteria are considered Gram-positive. However, due to their unique cell wall composition, upon Gram staining these bacteria appeared to be Gram-negative to Gram-variable (Duncan and Flint 2008, Chevrot 2008). *C. hathewayi* 82-B and *C. xylanovorans* Lac1D induced low or no NF- κ B activation in the

TLR assays and low or undetectable cytokines in DC assay (Figure 3 and 4) despite the fact that they induced activation and maturation of DCs (Figure 2). This suggests that these strains might express MAMPs that are shielded or not recognized by the TLRs tested or that they possess immunomodulatory components that inhibit activation of the NF- κ B pathway. This appears to be a unique property and may be an evolutionary adaptation to avoid immune recognition by the host as proposed for *Bacteroides fragilis* (Weintraub, Zahringer et al. 1989).

Materials and methods

Bacterial strains and culturing conditions

All the commensal bacteria used (Table 1) were isolated from human faeces and identified by 16S rRNA sequencing, *L. plantarum* WCFS1 was isolated from human saliva.

F. prausnitzii strains A2–165, L2-6, M21/2, S3L/3, HTF-F, *R. bromii* L2-63, *E. rectale* A1-86, *E. hallii* L2-7 and *B. adolescentis* L2-32 have been described elsewhere (Barcenilla, Pryde et al. 2000; Duncan 2002; Duncan, Louis et al. 2004; Louis, Duncan et al. 2004; Lopez-Siles, Khan et al. 2012). Strains *C. hathewayi* 82-B, *M. rupellensis* Mag1B and *C. xylanovorans* Lac1D were isolated from freshly voided human faeces as described previously (Lopez-Siles, Khan et al. 2012). Briefly, strains were isolated by plating 1 µl of the faecal material directly on yeast extract, casitone, fatty acid and glucose medium (YCFAG, described in (Lopez-Siles, Khan et al. 2012)). After 12 h to 16 h of incubation at 37°C in an anaerobic tent (80% N₂, 12% CO₂, and 8% H₂), 500 translucent colonies per sample were selected and cultured on fresh plates. After growth, the colonies were presumptively identified based on morphology, further purified and Gram stained. Up to 5 colonies per sample were identified by 16S rRNA gene sequencing. All the commensal bacteria were maintained at 37°C on YCFAG in an anaerobic tent. In all experiments bacteria were grown in YCFAG broth to an optical density at 600nm (A₆₀₀) of 0.8, which corresponds to the late exponential or early stationary growth phase. *L. plantarum* WCFS1 was cultured overnight to stationary phase in deMan, Rogosa Sharpe broth (MRS, Merck, Darmstadt, Germany) at 37°C. Bacteria were harvested by centrifugation, washed in phosphate buffer saline (PBS), resuspended in PBS containing 20% glycerol and stored at -80°C prior to use in the immunoassays. Bacteria were quantified by fluorescent *in situ* hybridization (FISH) or phase contrast microscopy. All buffers and media used for the anaerobic bacteria were deoxygenated by flushing with oxygen free nitrogen for 30 minutes.

Human PBMCs and DCs assays

This study was approved by Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Buffy coats from healthy blood donors were obtained from the Sanquin Blood bank in Nijmegen (The Netherlands). A written informed consent was obtained from each volunteer before sample collection.

PBMCs were isolated from buffy coats of healthy donors using Ficoll Paque Plus density gradient (GE Healthcare, Diegem Belgium) according to the manufacturer's protocol. After centrifugation, the mononuclear cells were collected, washed in IMDM + glutamax (Invitrogen, Breda, The Netherlands) and resuspended in IMDM + glutamax supplemented with 10% foetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO). PBMCs were seeded at 1×10^6 cells/ well in 48-well plates and, after 2 hours, cells were treated with the bacteria (bacterium to cell ratio, 10:1) or were left untreated. After 24 hours, the supernatant was collected for cytokine measurement.

The immune response of DCs to the bacteria was investigated by measuring the expression of surface markers in human monocyte-derived DCs and the cytokines secreted in the supernatant. Monocytes were isolated from PBMCs by positive selection of CD14⁺ cells using CD14-specific antibody coated magnetic microbeads (Miltentyi Biotec, Leiden, The Netherlands). CD14⁺ cells were cultured for 6 days in complete medium in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN) to differentiate into immature monocyte-derived DCs. At day 6, cells were seeded at 10^6 cells/ well in 24-well plates and were treated with the bacteria (bacterium to cell ratio, 10:1). After 48 hours, the supernatant was collected for cytokine measurements.

During the culture period and the stimulation, DCs were cultured in RPMI (Invitrogen) supplemented with 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO) and no bacterial growth was observed. On day 6 and 8, the activation and maturation status of the CD14⁺ cells were assessed by measuring CD83 and CD86 surface expression and the cell viability was measured using annexin V and propidium iodide (PI). Cells were stained with fluorescence conjugated monoclonal antibodies specific for CD83, CD86, their isotype-matched controls and with annexin V and PI (BD Biosciences, Breda, The Netherlands) and analysed on a flow cytometer (FACS Canto II, BD). CD86 and CD83 were expressed at low levels on immature or untreated DCs and were strongly expressed after stimulation. On days 6 and 8 the viability of the cells was between 60–80% (not shown).

TLR signalling assays

TLR assays were performed using human embryonic kidney cells (HEK293) stably expressing human TLR2 and TLR2/6, TLR4 or TLR5 (Invivogen, Toulouse, France) and transfected with a reporter plasmid (pNiFTY, Invivogen) containing the luciferase gene under the control of the

NF- κ B promoter. HEK293 cells expressing the different TLRs and pNiFTY were seeded at 6×10^4 cells/ well in 96-wells plates and incubated with bacteria (bacterium to cell ratio, 10:1), TLR agonists, Pam2CSK4 (Invivogen) for TLR2 and TLR2/6, flagellin (Invivogen) for TLR5 and LPS for TLR4, or medium alone as a control. After 6 hours of incubation, the medium was replaced with Bright glow (Promega), and the luminescence was measured using a Spectramax M5 (Molecular Devices). As a negative control HEK293 cells not expressing TLRs but harbouring pNiFTY were tested in the same conditions and did not show any luciferase activity. Mean values are shown for 6 replicates, error bars represent SEM.

Cytokine analysis

Cytokine concentrations in the DC culture supernatants were determined using Cytokine bead array (BD) and a flow cytometer (FACS Canto II, BD). In human PBMC and DC studies, the limits of detection were the following: for IL-1 β 7.2 pg/ml, for IL-10 3.3 pg/ml, for TNF 3.7 pg/ml, for IL-12p70 1.9 pg/ml and for IFN- γ 1.8 pg/ml.

Statistics and principal component analysis

Differences in NF- κ B activation in the TLR assays were analysed by one way Anova with Dunnett's multiple comparison test using the GraphPad PRISM (GraphPad Software). Principal component analysis (PCA) was used to analyze the variance of the level of cytokines along the different types of bacteria studied. PCA was computed on the standardized data by using the function princomp of the software Matlab.

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Chapter 5

Modulation of dendritic cell-T cell interactions by *Faecalibacterium prausnitzii*

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In preparation

Abstract

Faecalibacterium prausnitzii is one of the most abundant commensal bacteria in healthy humans. *F. prausnitzii* has anti-inflammatory properties both *in vitro* and *in vivo* but the underlying mechanisms are still unknown. The aim of this work was to investigate the modulatory effects of *F. prausnitzii* strain A2-165 on the induction of mucosal T cell responses to the model antigen ovalbumin (OVA) *in vitro* and *in vivo*. The influence of *F. prausnitzii* A2-165 on OVA-specific T cell activation and polarization was studied *in vitro* using naive CD4⁺ T cells and mouse bone marrow-derived dendritic cells (BMDCs) and *in vivo* using a model for nasal tolerance induction to OVA. *F. prausnitzii* A2-165 was compared to another commensal *Clostridium hathewayi* 82-B, which induced very low cytokine secretion in BMDCs despite inducing their activation. Our results demonstrate that *F. prausnitzii* A2-165 has immune-regulatory properties which may be related to its strong capacity to induce IL-10 by BMDCs and T cells. *In vivo*, these effects translated into enhanced OVA-specific T cell proliferation in the nose-draining cervical lymph nodes (CLNs) after combined nasal application of *F. prausnitzii* strain A2-165 and OVA. Strikingly, this was associated with a reduction in the differentiation of IFN- γ secreting T cells in the CLNs. In contrast, *Clostridium hathewayi* 82-B significantly decreased the percentage of dividing OVA-specific T cells in the CLNs and spleen. This may be related to the fact that molecules CD40 and CD86 were expressed at lower levels on BMDCs incubated with *C. hathewayi* 82-B than on BMDCs incubated with *F. prausnitzii* A2-165.

Overall these data demonstrate that *F. prausnitzii* can modulate the OVA-specific mucosal T cell response indirectly by affecting antigen presentation but also directly by acting on T cells leading to reduced differentiation of IFN- γ secreting T cells *in vivo*.

Introduction

In the intestine, the epithelial surface is protected by a secreted mucus layer that spatially compartmentalizes bacteria to the intestinal lumen, however, molecules and metabolites produced by bacteria can diffuse through the mucus and affect epithelial gene expression. Harmless antigens that pass the epithelial barrier can elicit adaptive immune responses in mucosa-associated lymphoid tissue such as Peyer's patches (PPs) or in gut-draining mesenteric lymph nodes (MLNs). At these sites, antigen presenting cells (APCs), such as dendritic cells (DCs), can sample microbes and food proteins and prime mucosal naive T cells driving their activation, clonal expansion and differentiation. In steady-state conditions, regulatory T cell (Treg) conversion in response to presentation of harmless protein antigens occurs with a higher frequency in PPs and MLNs when compared to non-mucosa-draining lymphoid tissue. It has long been known that this preferential tolerance induction at mucosal sites is dependent on microbial sensing (Kiyono, McGhee et al. 1982; Tanaka and Ishikawa 2004). Recent advances in the field have now made it possible to dissect how individual components of the microbiota endow the mucosal immune system with tolerogenic potential. Over the past 5 years, it has become apparent that not all components of the microbiota are equal in terms of their impact on host physiology. It has been shown that particular commensal bacteria can steer mucosal T cell polarization. For example, colonization of germ-free mice with the mouse commensal segmented filamentous bacteria induces an increase in the number of CD4⁺ T cells, in particular T helper 1 cells (Th1), Th17 and Foxp3⁺ Tregs in the small intestine and colon (Gaboriau-Routhiau, Rakotobe et al. 2009). Conversely, colonization of germ-free mice with a cocktail of 46 commensal Clostridia, induces increased numbers of Foxp3⁺ Tregs expressing IL-10 in the colon while mildly inducing Th17 cells and not affecting Th1 cells (Atarashi, Tanoue et al. 2011). Accumulation of colonic IL-10 secreting Foxp3⁺ Tregs is also induced by colonization of germ-free mice with *Bacteroides fragilis* (Round and Mazmanian 2010). The mechanisms by which these commensal bacteria affect mucosal T cells appear diverse. Colonization with Clostridia acts on Foxp3⁺ Tregs through the induction of TGF- β and indoleamine 2,3-dioxygenase (IDO) in epithelial cells (Atarashi, Tanoue et al. 2011) while *B. fragilis* acts on T cells via TLR2 ligation (Round and Mazmanian 2010). This implies that commensal bacteria may act upon mucosal T cells at different stages during activation, differentiation, accumulation in the lamina propria and maintenance in the lamina propria.

Faecalibacterium prausnitzii is one of the most abundant species of human intestinal bacteria (Barcenilla, Pryde et al. 2000; Duncan 2002). This Firmicute has recently raised interest because strain A2-165 attenuates 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced colitis in mice, and induces high IL-10 to IL-12p70 ratio in human peripheral blood mononuclear cells (hPBMCs). Crohn's disease (CD) and ulcerative colitis (UC) are forms of inflammatory bowel disease characterized by a chronic and relapsing inflammation of the gastrointestinal tract. Patients with active CD and UC have lower faecal counts of Firmicutes, in particular lower counts of *F. prausnitzii*, compared with healthy subjects (Sokol, Seksik et al. 2009). Moreover, *F. prausnitzii* along with *Roseburia* spp. are the prominent butyrate producers in the intestine (Pryde, Duncan et al. 2002). Butyrate is a primary energy source for the epithelial cells lining the colon (Yin, Laevsky et al. 2001) and appears to have both anti-inflammatory and cancer chemopreventive activities (Yin, Laevsky et al. 2001; Pryde, Duncan et al. 2002; Place, Noonan et al. 2005). We previously showed, using human monocyte-derived DCs (hDCs), that *F. prausnitzii* strain A2-165 has very distinct immunomodulatory properties compared to other 5 *F. prausnitzii* strains and 8 other abundant commensal strains. This was due to the high levels of IL-10 and low levels of IL-12p70 produced by hDCs incubated with *F. prausnitzii* A2-165 (Chapter 4).

The aim of this work was to investigate the modulatory effects of *F. prausnitzii* A2-165 on the induction of mucosal T cell responses to the model antigen ovalbumin (OVA) *in vitro* and *in vivo*. First, the influence of *F. prausnitzii* A2-165 on OVA-specific T cell activation and polarization was studied *in vitro* using naive CD4⁺ T cells and mouse bone marrow derived DCs (BMDCs). Thereafter, we used a well characterized model for nasal tolerance induction to OVA to determine the effect of *F. prausnitzii* A2-165 on mucosal T cell differentiation *in vivo*. Nasal tolerance is known to have many similarities to oral tolerance induction (Unger, Hauet-Broere et al. 2003; Samsom 2004) and application of bacteria via the nasal route effectively targets responses in the nose-draining cervical lymph nodes (CLNs).

The effects of *F. prausnitzii* A2-165 were compared to *Clostridium hathewayi* 82-B which was selected from a panel of human intestinal commensals on the basis of cytokine production in culture with BMDCs. Strain *Clostridium hathewayi* 82-B was chosen for inclusion in a mouse study because it activated BMDCs but induced very low levels of all cytokines in comparison to *F. prausnitzii* A2-165.

Results

Co-stimulatory molecules expression on mouse BMDCs incubated with commensal bacteria

The immune response of BMDCs incubated with *F. prausnitzii* A2-165 and 5 other human commensal bacterial strains was measured in order to select a strain to compare with *F. prausnitzii* A2-165 in subsequent experiments. The candidate strains were previously tested in culture with hDCs (Chapter 4) and chosen on the following basis: *F. prausnitzii* A2-165 was the highest inducer of IL-10 release by hDCs, *C. hathewayi* 82-B and *C. xylanovorans* Lac1D induced low or undetectable cytokine responses, *Lactobacillus plantarum* WCFS1 was one of the highest inducers of IL-12p70 release and *Eubacterium rectale* A1-86 and *Bifidobacterium adolescentis* L2-32 induced intermediate levels of secreted cytokines compared to the other strains.

Incubation with bacteria did not significantly affect the percentage of CD40 or CD86 positive cells within the BMDCs cultures (Figure 1a). Overall, all the bacterial strains induced increased surface expression of the co-stimulatory molecules CD86 and CD40 by BMDCs compared to the control untreated BMDCs (Figure 1b and c). However, the degree of induction of co-stimulatory molecules expression varied upon bacterial strain as the CD86 and CD40 mean fluorescence intensity (MFI) values induced by *C. hathewayi* 82-B were lower compared to the other strains (Figure 1b and c). In addition, CD86 expression on BMDCs was lower in cultures with *C. xylanovorans* Lac1D when compared to the other strains (Figure 1b). Thus, all strains induced increased expression of BMDC surface molecules known to co-stimulate T cells during antigen presentation.

Mouse BMDCs cytokine responses to commensal bacteria

Culture of BMDCs with *F. prausnitzii* A2-165 induced the largest amount of IL-10 release (mean \pm SEM, 8098 ± 176 pg/ml), followed by *E. rectale* A1-86 (4390 ± 154 pg/ml) and *B. adolescentis* L2-32 (3849 ± 181.6 pg/ml). IL-12p70 release by BMDCs was mostly induced by *F. prausnitzii* A2-165 and *L. plantarum* WCFS1 (136 ± 24 and 161 ± 10 pg/ml respectively, Figure 2). However, for all the strains tested the levels of IL-12p70 were around 10 fold lower than those obtained in similar assays with hDCs (Chapter 4). The amounts of IFN- γ in the culture supernatants of stimulated BMDCs were low (10-15 pg/ml) but high levels of TNF release were induced by virtually all bacterial strains with *L. plantarum* WCFS1 and *F. prausnitzii* A2-165

being the strongest inducers while *C. hathewayi* 82-B and *C. xylanovorans* Lac1D were low to weak inducers. As *C. hathewayi* 82-B was the lowest inducer of all cytokines, it was selected for comparison to *F. prausnitzii* A2-165 in subsequent experiments *in vitro* and *in vivo*.

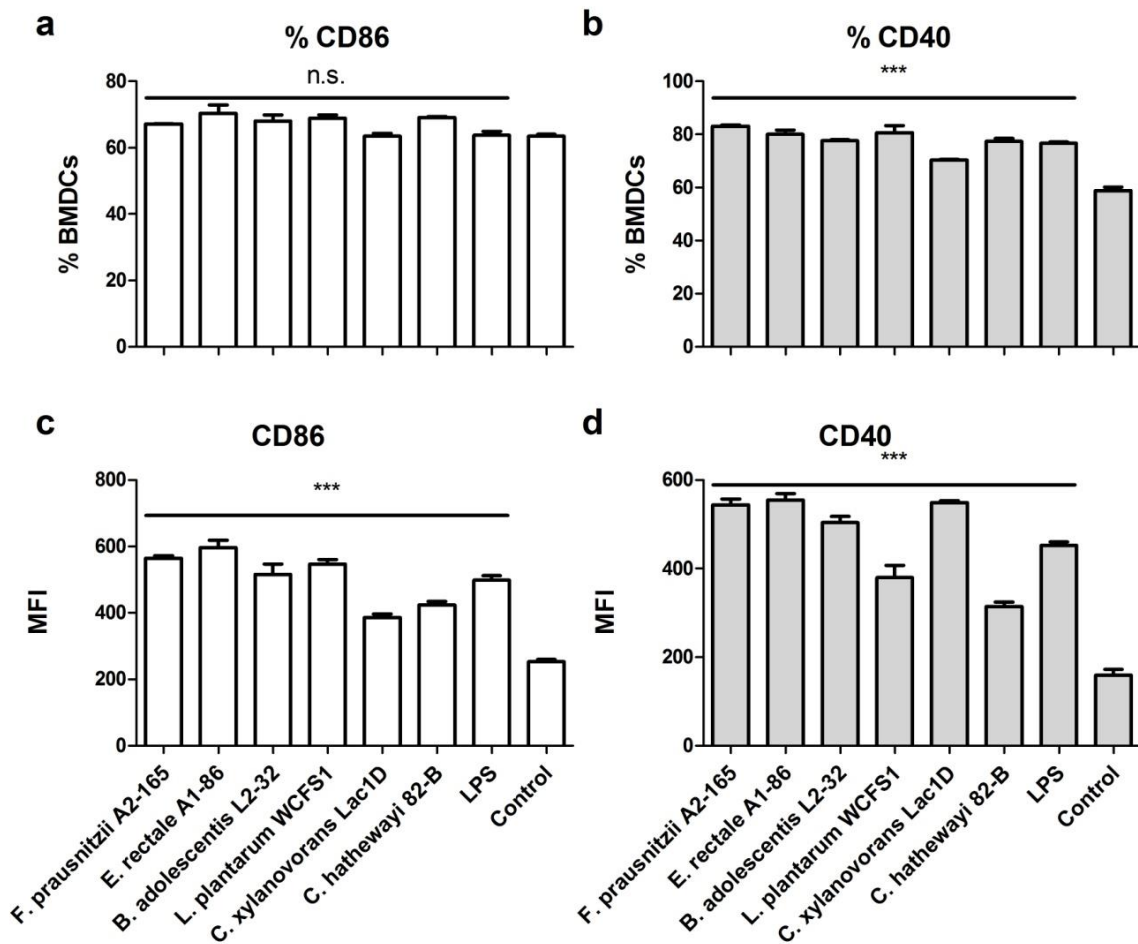


Figure 1: CD86 and CD40 surface expression in BMDCs. Percentage of CD86⁺ (a) or CD40⁺ (b) BMDCs and mean fluorescence intensity (MFI) of CD86 (c) and CD40 (d) after 24 h of incubation with bacteria (bacterium: BMDC, 10:1). Error bars represent SEM, n=3, *** indicates p<0.001, n.s. indicates non significant compared to the control.

Bacteria differentially affect T cell polarization in BMDC-T cell co-cultures

In order to investigate the influence of *F. prausnitzii* A2-165 and *C. hathewayi* 82-B on T cell activation and polarization in the presence of APCs, BMDCs were cultured in the presence or absence of the bacteria or LPS, after 24 h, OVA was added and after other 6 h, naive CFSE labelled OVA-specific CD4⁺ T cells (OVA-specific T cells) were added for additional 72 h.

All conditions of BMDC stimulation increased the number of OVA-specific T cells undergoing proliferation compared to the unstimulated BMDC control. No significant differences in the

percentage of dividing T cells were observed between BMDC stimulation with LPS, *F. prausnitzii* A2-165 or *C. hathewayi* 82-B (Figure 3a and b).

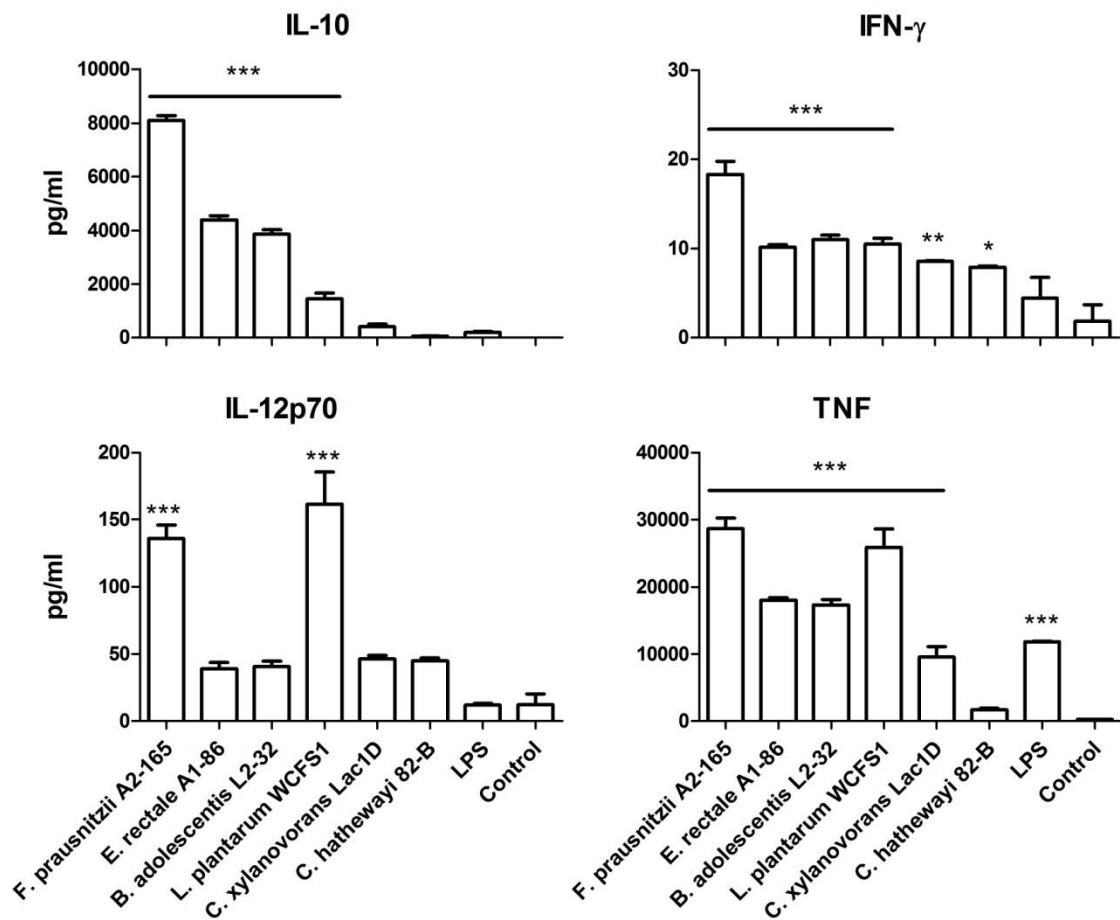


Figure 2: Cytokine secretion by BMDCs. IL-10, IL-12p70, TNF and IFN- γ were measured in the supernatant of BMDCs (5×10^5 BMDCs/well in 24w plate) after 24 h of incubation with bacteria (bacterium: BMDC, 10:1). Error bars represent SEM, n=3, *** indicates $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to the control.

Intracellular staining of OVA-specific T cells revealed that *F. prausnitzii* A2-165 decreased the percentage of total IFN- γ^+ T cells compared to the control and had no effect on IL-17 $^+$ and Foxp3 $^+$ T cells (Figure 4a). In contrast, *C. hathewayi* 82-B induced an increase in the percentage of IFN- γ^+ , IL-17 $^+$ and Foxp3 $^+$ T cells compared to the control (Figure 4a). Despite the bacteria-induced changes in the percentage of IFN- γ^+ and IL-17 $^+$ T cells, the levels of secreted cytokines were not different to the control (Figure 4b). Notably, BMDCs pre-incubated with *F. prausnitzii* A2-165 induced increased IL-10 secretion during OVA-specific T cell proliferation (Figure 4b) compared to the control. As no T cells stained positive for intracellular IL-10, we could not determine which cells produced the IL-10 measured in the

culture supernatants. None of the bacteria altered the levels of secreted IL-2, IL-6 or TNF compared to the control (not shown).

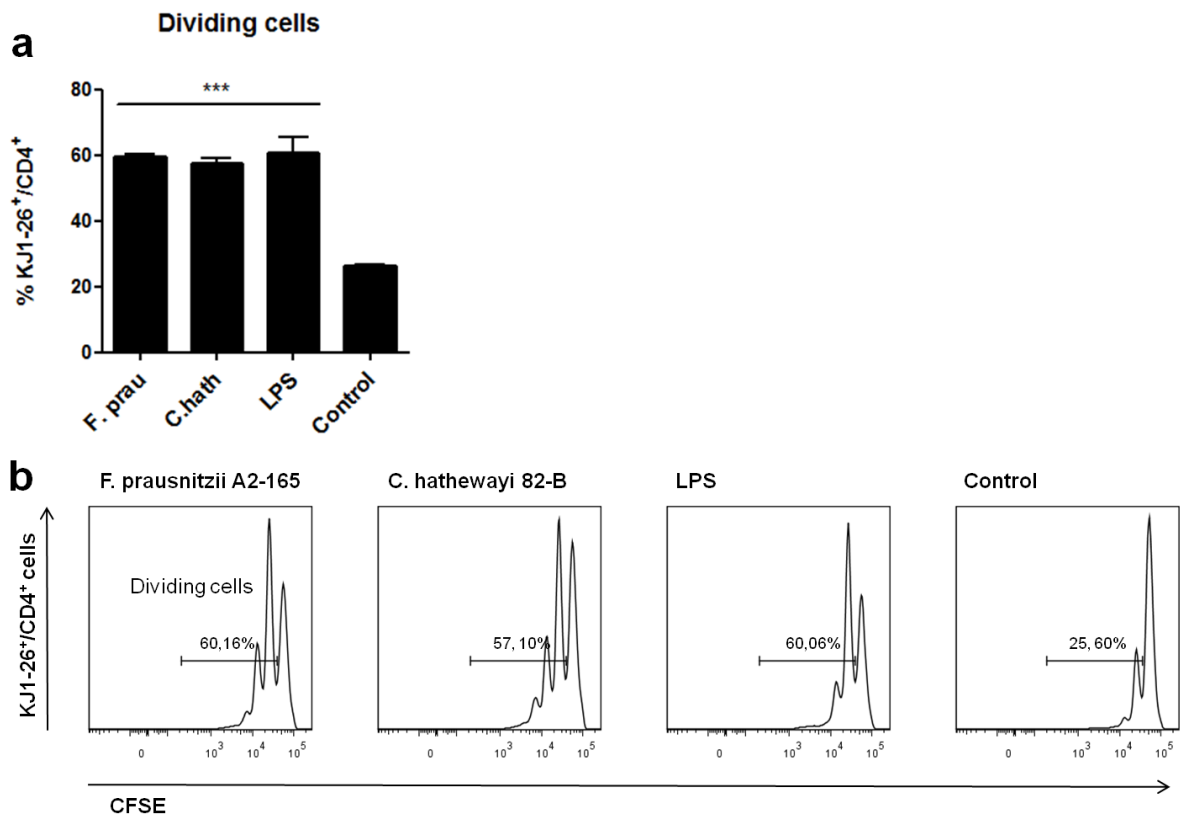


Figure 3: Dividing OVA-specific T cells (KJ1-26⁺/CD4⁺) after incubation with BMDCs pre-stimulated with bacteria. BMDCs were cultured in the presence or absence of the bacteria or LPS and after 24 h, CFSE labelled OVA-specific T cells were added for additional 72 h. a) Percentage of dividing OVA-specific T cells. b) Representative histograms showing divided CFSE labelled OVA T-cells. Error bars represent SEM, n=3, *** indicates p<0.001 compared to the control.

Bacteria influence differentiation of activated T cells in the absence of antigen presenting cells

As *B. fragilis* is proposed to directly modulate mucosal T cells through TLR2 (Round and Mazmanian 2010), we wished to investigate the potential direct effects of *F. prausnitzii* A2-165 and *C. hathewayi* 82-B on T cells in an APC free system. OVA-specific T cells were activated *in vitro* with anti-CD3 ϵ /CD28 mAb in the presence of bacteria and OVA. At 72 h of culture, effects on T cell proliferation, intracellular protein expression and cytokine secretion were measured. As expected, anti-CD3 ϵ /CD28 mAb treatment induced OVA-specific T cell activation which was not influenced by the presence of bacteria (not shown). However, the direct contact with bacteria during T cell receptor triggering modulated the intracellular cytokine secretion and cytokine release compared to the control indicating a direct effect of bacteria on T cell polarization. *F. prausnitzii* A2-165 induced an increase in the percentage of

IL-10 secreting and IFN- γ secreting T cells (Figure 5a) and enhanced IFN- γ , IL-10, IL-6, IL-17 and TNF secretion compared to the control (Figure 5b). Similar but smaller changes were observed in T cells that were activated in the presence of *C. hathewayi* 82-B. It should be noted that although both bacterial strains increased the secretion of IL-17 no changes were observed in the percentage of IL-17⁺ cells (Figure 5a and b). Treatment with either one of the bacteria during cellular activation did not affect the percentage of Foxp3⁺ T cells (not shown).

These results show that the bacterial strains can have a direct effect on T cell differentiation in the absence of APCs. In particular, *F. prausnitzii* A2-165 induces a 5 fold increase in the percentage of IL-10 secreting T cells and a 4 fold increase in IL-10 secretion. The percentage of IFN- γ ⁺ T cells increased 2 fold after incubation with *F. prausnitzii* A2-165 and 1.8 fold after incubation with *C. hathewayi* 82-B.

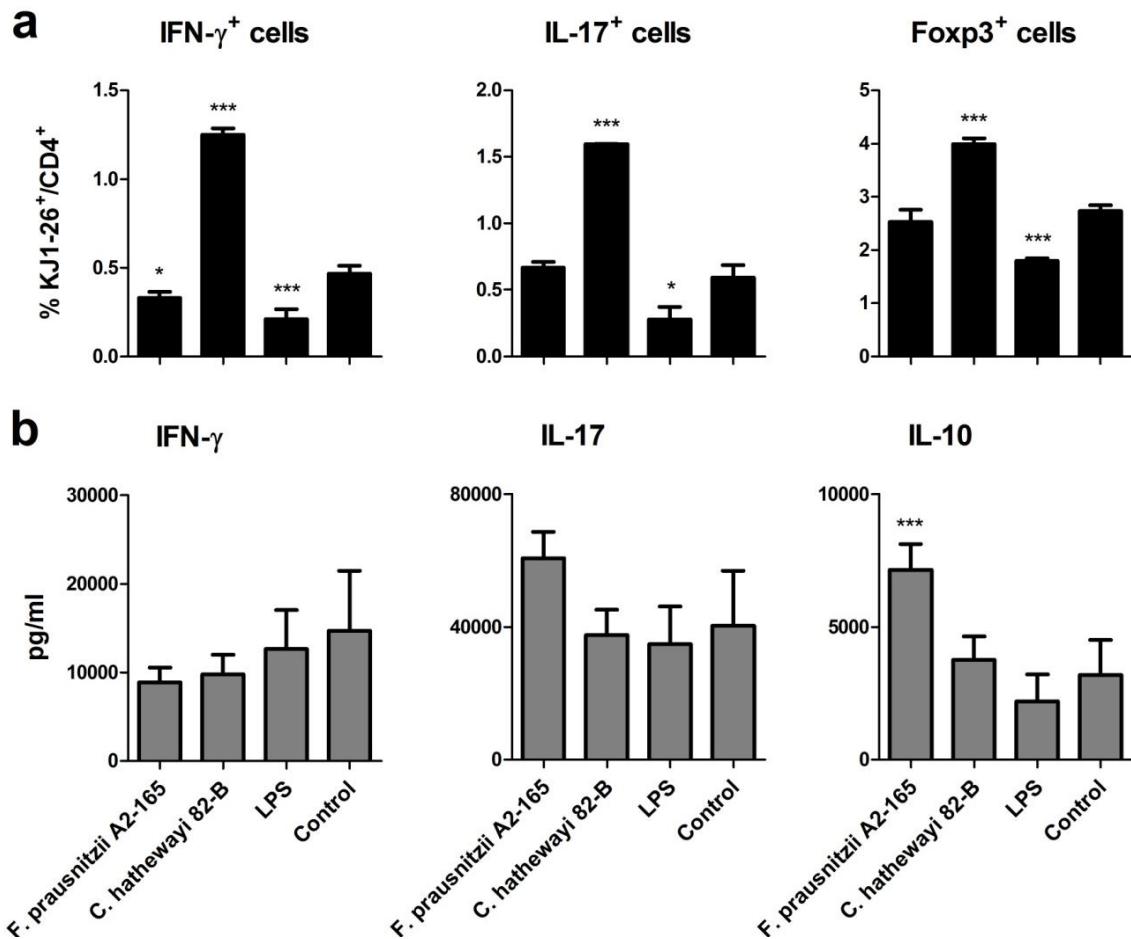


Figure 4: Percentage of IFN- γ ⁺, IL-17⁺, Foxp3⁺ cells (a) and cytokine secretion (b) after incubation of OVA-specific T cells (KJ1-26⁺/CD4⁺) with BMDCs pre-stimulated with bacteria (bacterium: BMDC, 10: 1). BMDCs (5×10^4 BMDCs/well in 96w plate) were loaded with OVA (0.5mg/ml) and cultured in the presence or absence of the bacteria or LPS and after 24 h CFSE labelled OVA-specific CD4⁺ T cells (5×10^5 T cells/well) were added, after 72 h cytokine secretion in the supernatant and intracellular markers were measured. Error bars represent SEM, n=3, *** indicates p<0.001, *p<0.05 compared to the control. Intracellular IL-10 staining was negative for all samples.

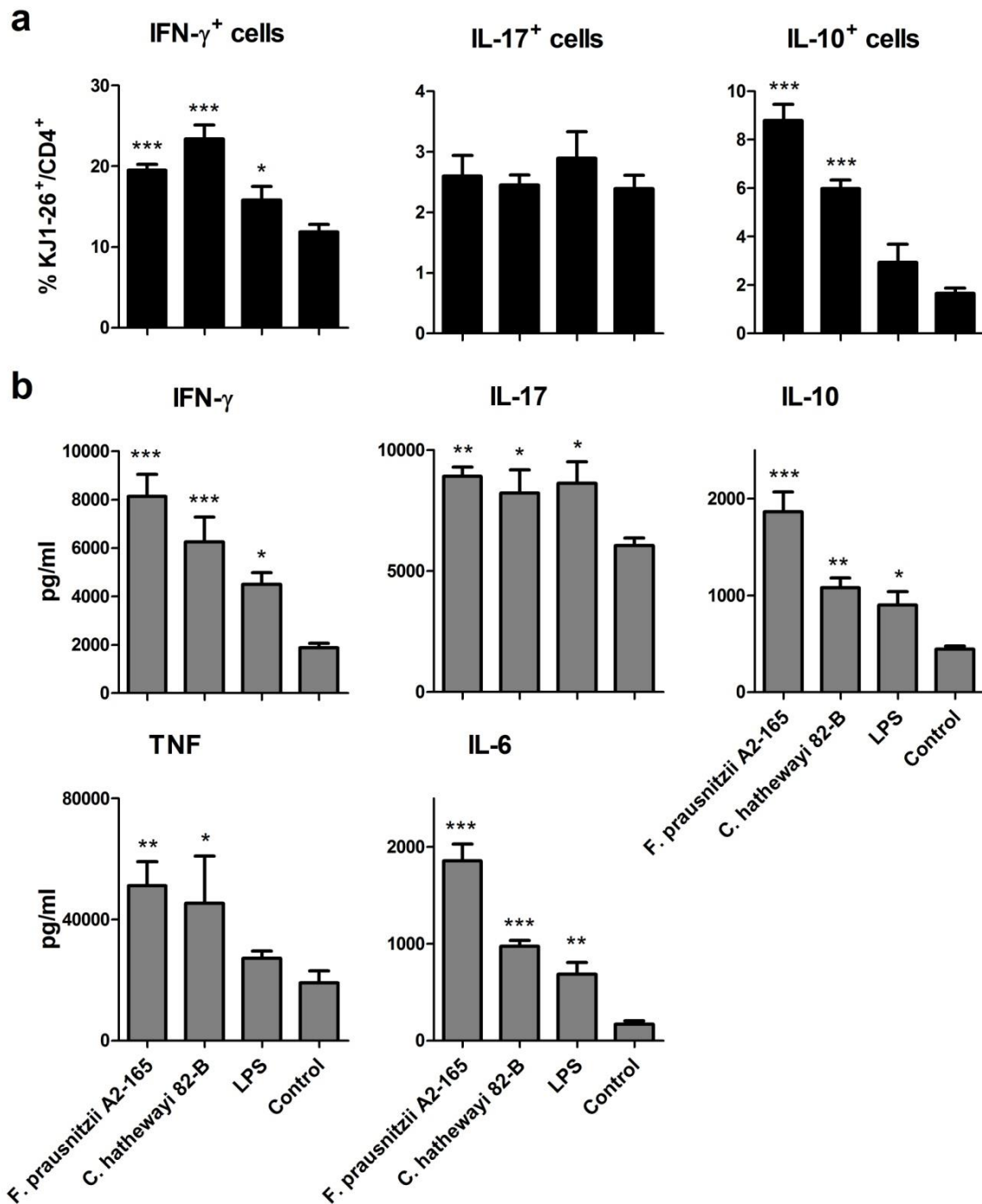


Figure 5: Percentage of IFN- γ^+ , IL-17 $^+$, IL-10 $^+$ (a) and cytokine secretion (b) after incubation of OVA-specific T cells (KJ1-26 $^+$ /CD4 $^+$) with bacteria (bacterium: T cell, 1:1). CFSE labelled OVA-specific T cells (5×10^5 T cells/well) were activated *in vitro* with anti-CD3 ϵ /CD28 mAb and incubated with bacteria, after 72 h cytokine secretion in the supernatant and intracellular markers were measured. Error bars represent SEM, n=3, *** indicates p<0.001, ** p<0.01, *p<0.05 compared to the control.

In vivo CD4⁺ T cell response

To investigate the impact of the strains on the development of mucosal T cell responses *in vivo*, a model for nasal tolerance induction was used (Unger, Hauet-Broere et al. 2003). In short, BALB/c acceptor mice were adoptively transferred with CFSE labelled naive DO11.10 OVA T cell receptor (TCR) transgenic T cells and after 24 h, received an intranasal (i.n.) application of either the bacteria together with OVA or OVA alone. After 72 h, single cell suspensions isolated from cervical lymph nodes (CLNs) and spleens were analysed to assess the proliferation and phenotype of OVA-specific T cells.

In *F. prausnitzii* A2-165 treated mice the percentage of dividing OVA-specific T cells in the CLNs was increased compared to the control mice administered OVA alone. This was found to be significant in a second independent experiment (Supplementary Figure 1). In the spleen of control mice, that received OVA i.n., a population of OVA-specific T cells is found that are in third, fourth or fifth division. In agreement with previous observations no CFSE fluorescence was detected corresponding with T cells in first or second division. Therefore, this population reflects cells that have differentiated in the CLNs and entered the circulation upon exiting the CLNs. Administration of *F. prausnitzii* A2-165 together with OVA significantly increased the number of divided OVA-specific T cells isolated from spleens compared to control mice (Figure 6a and Supplementary Figure 1). Together, these data demonstrate that i.n. administration of *F. prausnitzii* A2-165 enhances the number of OVA-specific T cells that divide in the CLNs. In contrast, *C. hathewayi* 82-B administration significantly decreased the proliferation of OVA-specific T cells isolated from CLNs compared to the control and *F. prausnitzii* A2-165. This effect was also observed in a second independent experiment although it was not significant (Figure 6a and Supplementary Figure 1). In agreement, no increased numbers of divided OVA-specific T cells were detected in the spleen (Figure 6a).

Intranasal administration of either *F. prausnitzii* A2-165 or *C. hathewayi* 82-B together with OVA significantly decreased the number of IFN- γ secreting OVA-specific T cells isolated from the CLNs. In particular, *F. prausnitzii* A2-165 induced an approximately three fold reduction in the percentage of IFN- γ secreting cells (Figure 6b). However, these bacteria did not effect the intracellular expression of Foxp3, IL-17, or IL-10 in OVA-specific T cells isolated from the CLNs (not shown).

In a second independent experiment, performed using the same setup, single cells were recovered from CLNs and spleens of mice at 72h post OVA treatment and characterized after

ex vivo re-stimulation with OVA. Similarly to the first *in vivo* experiment (Figure 6), co-administration of *F. prausnitzii* A2-165 with OVA induced an increased number of dividing OVA-specific T cells in CLN and spleen compared to the control and *C. hathewayi* 82-B group. Again, proliferation of OVA-specific T cells from CLNs was decreased in the mice administered *C. hathewayi* 82-B compared to control although these changes were not statistically significant (Figure 7).

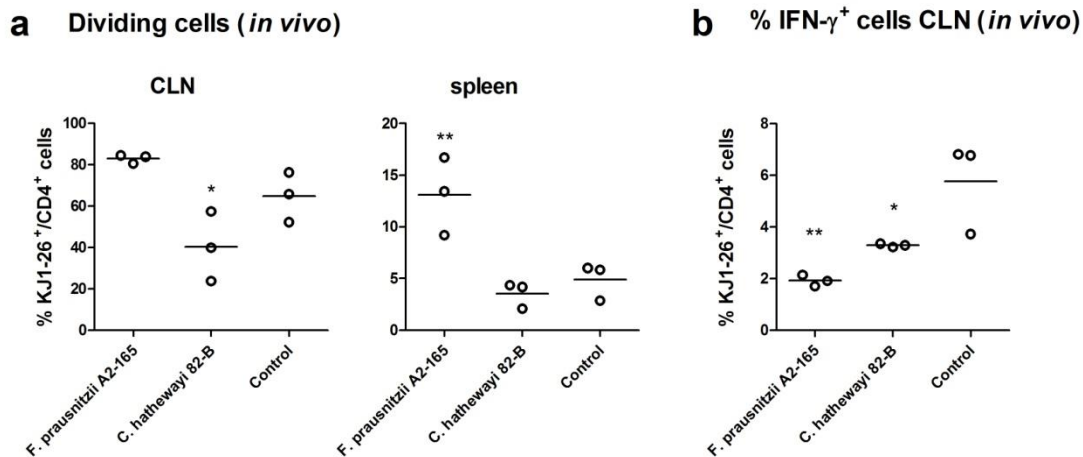


Figure 6: Percentage of dividing and IFN- γ ⁺ OVA-specific T cells *in vivo*. CFSE labelled naive OVA-specific T cells (KJ1-26⁺/CD4⁺) were adoptively transferred in BALB/c mice, after 24 h, mice were administered intranasally with bacteria plus OVA and after additional 72 h, OVA-specific T cells were isolated from cervical lymph nodes (CLNs) and spleens and analysed. a) Percentage of dividing OVA-specific T cells (KJ1-26⁺/CD4⁺) isolated from CLNs or spleens. b) Percentage of IFN- γ ⁺ OVA-specific T cells (KJ1-26⁺/CD4⁺) isolated from CLNs. * indicates p<0.05, ** indicates p<0.01 compared to the control administered OVA alone.

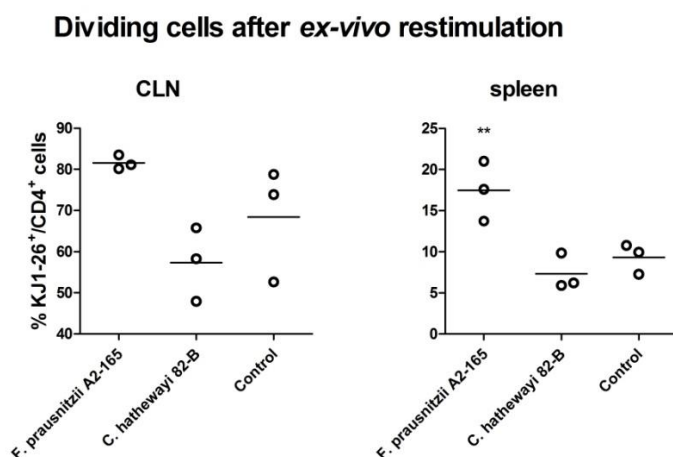


Figure 7: Percentage of dividing OVA-specific T cells after *ex-vivo* re-stimulation. CFSE labelled naive OVA-specific T cells (KJ1-26⁺/CD4⁺) were adoptively transferred in BALB/c mice, after 24 h, mice were administered intranasally with bacteria plus OVA and after additional 72 h, OVA-specific T cells were isolated from nose-draining cervical lymph nodes (CLNs) and spleens and re-stimulated with OVA for additional 24 h and then analysed. ** indicates p<0.01 compared to the control administered OVA alone.

Overall re-stimulation led to low and variable levels of cytokine release. This is likely to be due to the low frequency of OVA-specific T cells within the total CLN cell population. Notably, CLN cells of *C. hathewayi* 82-B treated mice released lower amounts of cytokines; this effect was statistically significant only for IL-10 and MCP-1 (Figure 8).

Cytokine secretion after *ex vivo* restimulation (CLN)

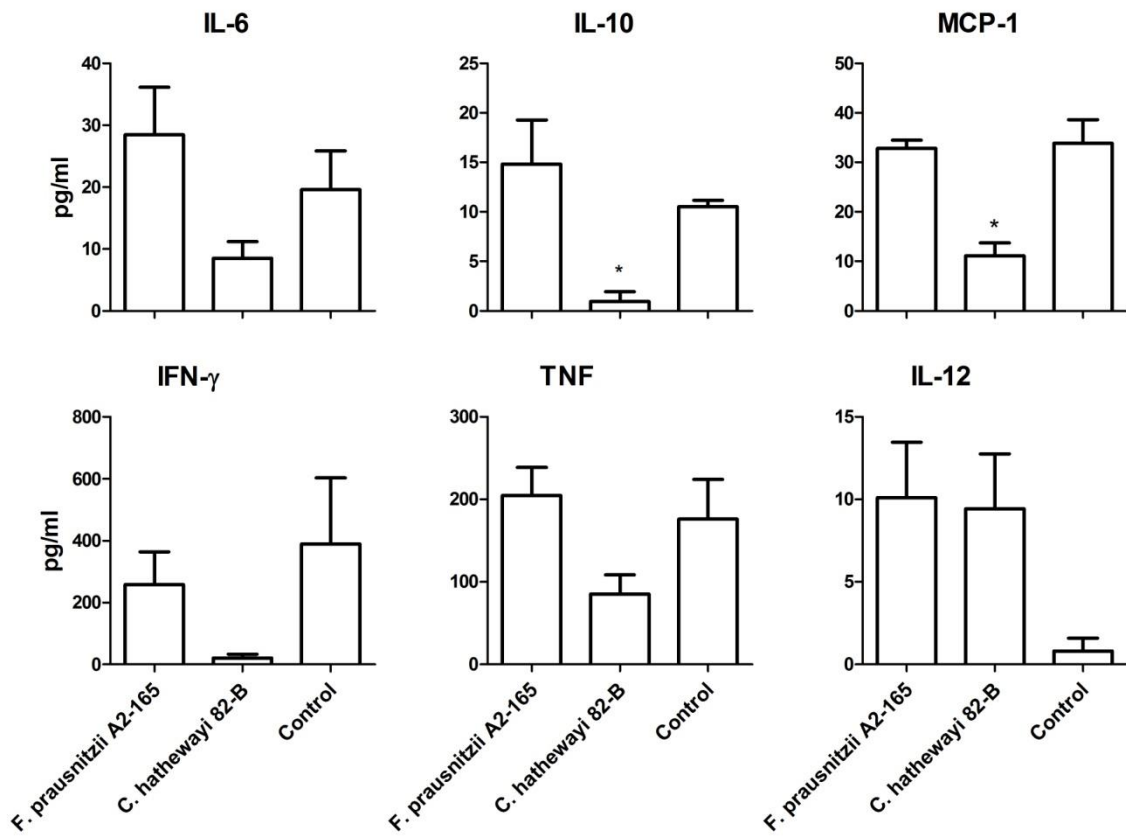


Figure 8: Cytokine secretion by OVA-specific T cells after *ex vivo* re-stimulation. CFSE labelled naive OVA-specific T cells were adoptively transferred in BALB/c mice, after 24 h, mice were administered intranasally with bacteria plus OVA and after additional 72 h, OVA-specific T cells were isolated from CLNs and re-stimulated with OVA for additional 24 h and then analysed. * indicates $p < 0.05$ compared to the control administered OVA alone.

Discussion

F. prausnitzii strain A2-165 is one of the most abundant colonic bacteria in humans and has anti-inflammatory properties *in vitro* and *in vivo*. In particular, *in vitro* it induces high IL-10 to IL-12 ratio in hDCs (Chapter 4) and hPBMCs and *in vivo* it is capable of attenuating TNBS-induced colitis in mice (Sokol, Pigneur et al. 2008). The mechanisms of these protective and anti-inflammatory effects have not yet been unravelled. In this study we show that *F. prausnitzii* strain A2-165 directly modulates murine DCs by enhancing CD86 and CD40 expression and stimulating IL-10, IL-12p70 and TNF release (Figure 1 and 2). As a result, these DCs stimulate increased antigen-specific T cell division in co-culture which is associated with increased IL-10 release (Figure 3 and 4). In addition, *F. prausnitzii* strain A2-165 can directly impact on T cells by stimulating differentiation of IL-10 or IFN- γ producing T cells without affecting the number of proliferating cells (Figure 5). *In vivo*, these effects translate into enhanced OVA-specific T cell proliferation in the nose-draining CLNs after combined nasal application of *F. prausnitzii* strain A2-165 and OVA. Strikingly, this is associated with a reduction in the differentiation of IFN- γ secreting T cells in the CLNs (Figure 6). These data demonstrate that *F. prausnitzii* A2-165 has immunoregulatory properties and indicate that the bacterium has a strong capacity to induce IL-10 by both DCs and T cells.

The effects of *F. prausnitzii* A2-165 were compared with *C. hathewayi* 82-B, a bacterium that had little or no capacity to induce TLR signalling and induced small amounts of cytokines when incubated with hDCs (Chapter 4) or with mouse BMDCs *in vitro*. Upon interaction with BMDCs, *F. prausnitzii* A2-165 induced very large amounts of IL-10 (8098 ± 176 pg/ml) compared to BMDCs incubated with *C. hathewayi* 82-B (63 ± 8 pg/ml) or other commensal bacteria (Figure 2). Similar results were described using hDCs and hPBMCs (Chapter 4) where, among 5 *F. prausnitzii* strains and a total of 13 commensal bacterial strains, *F. prausnitzii* A2-165 was the highest inducer of IL-10. The molecules of *F. prausnitzii* responsible for the high IL-10 induction are not known but might involve the anti-inflammatory factor present in the bacterial culture supernatants (Sokol, Pigneur et al. 2008).

The concomitant enhanced proliferation and high levels of IL-10 secretion as seen in the co-culture of stimulated BMDCs with OVA-specific T cells seems counterintuitive as IL-10 is a suppressive cytokine. In this respect, it should be emphasized that at onset of proliferation T cells may not be sensitive to IL-10 suppression because they down regulate expression of the

IL-10R α chain (Liu, Michalopoulos et al. 1994). *In vivo*, *F. prausnitzii* A2-165 also increased the number of dividing OVA-specific T cells in the CLNs and spleen compared to the control group of mice administered only OVA (Figure 6). This may be due to the uptake of *F. prausnitzii* A2-165 or microbial associated molecular patterns (MAMPs) resulting in increased DCs activation and CD40 expression as also seen in the *in vitro* experiments (Figure 1). These activated DCs may drive enhanced antigen presentation of OVA in the CLNs. Although *F. prausnitzii* A2-165 administration increased the percentage of dividing T cells *in vivo*, it decreased the percentage of IFN- γ ⁺ T cells without affecting the percentages of Foxp3⁺ or IL-17⁺ T cells (Figure 6). This finding suggests that *F. prausnitzii* A2-165 modulated the phenotype of mucosal DCs that migrate from the lamina propria to suppress IFN- γ differentiation in the draining CLNs. Unfortunately; we were not able to detect intracellular IL-10 staining in CLNs cells. IL-10 may suppress differentiation of IFN- γ secreting cells in the CLNs once the cells have proliferated and re-express their IL-10R. Future experiments using *in vivo* blocking with anti-IL-10R antibodies would provide an answer to this hypothesis.

In contrast to the expectation based on the prior *in vitro* data, *C. hathewayi* 82-B had substantial immunomodulatory capacity. BMDCs incubated with this bacterium appeared to be highly effective in stimulating T cells *in vitro*. Indeed, the percentage of T cells staining positive for intracellular markers of different T cell subsets (i.e. IFN- γ , IL-17 and Foxp3) were significantly higher for T cells incubated with BMDCs pre-stimulated with *C. hathewayi* 82-B than BMDCs pre-stimulated with *F. prausnitzii* A2-165, LPS or left unstimulated (Figure 4). In fact, this is surprising as the surface molecules CD40 and CD86 were expressed at lower levels on BMDCs incubated with *C. hathewayi* 82-B than on BMDCs incubated with *F. prausnitzii* A2-165 (Figure 1).

In contrast, *in vivo*, *C. hathewayi* 82-B significantly decreased the percentage of dividing T cells in the CLNs and spleen (Figure 6). The reasons for this are not clear but the bacterium may not release MAMPs and only activate DCs when phagocytosed. Alternatively, *C. hathewayi* 82-B may inhibit the migration of DCs from the mucosa to the draining lymph nodes, blocking the activation and expansion of T cells within the CLNs.

Interestingly, different effects of the bacteria are observed when they are incubated with T cells activated with anti-CD3 ϵ /CD28 mAb compared to T cells incubated with pre-stimulated BMDCs. In both experimental setups the bacteria have the capacity to interact with the T cells although they may be phagocytosed after 24h incubation with DCs. In contrast to the BMDC T

cell co-culture assays, using activated T cells, *F. prausnitzii* A2-165 induced significantly higher percentage of IFN- γ producing T cells and IL-10⁺ T cells and this was observed also after incubation with *C. hathewayi* 82-B (Figure 5). As in the anti-CD3 ϵ /CD28 mAb assay a higher percentage of T cells are activated than in BMDC T cell co-culture assays, it is possible that the co-stimulatory effect provided by the bacterium is better detected.

The culture supernatants of activated T cells incubated with bacteria and LPS contained significantly larger amounts of IFN- γ , IL-17, IL-10 and IL-6 than the supernatants of activated T cells alone (Figure 5). This may be due to the interaction of bacterial MAMPs and LPS with TLR receptors expressed on activated T cells. *In vivo* the direct interaction of commensals and mucosal T cells is likely to be a rare event except in the Peyer's patches. However, in inflammatory bowel disease, damage to the gut barrier is known to increase bacterial translocation into the lamina propria where direct interactions of bacteria with T cells may occur (Waeytens, Ferdinande et al. 2008).

Materials and methods

Mice

Animal experiments were approved by the Animal Experimental Committee of the Erasmus Medical Center. BALB/c mice were obtained from Charles River Laboratories (Maastricht, The Netherlands) and kept in the Erasmus University Medical Centre. DO11.10 transgenic mice on BALB/c background, which have a T cell receptor (TCR) specific for the OVA 323-339 peptide, were bred at the Erasmus University Medical Centre. Mice were kept under specific pathogen-free housing conditions.

Bacterial strains and culturing conditions

F. prausnitzii strains A2-165, *E. rectale* A1-86, *B. adolescentis* L2-32, *C. hathewayi* 82-B, have been described elsewhere (Barcenilla, Pryde et al. 2000; Duncan 2002; Duncan, Louis et al. 2004; Louis, Duncan et al. 2004; Chapter 4) and were maintained at 37°C on yeast extract, casitone, fatty acid and glucose medium (YCFAG, described in (Lopez-Siles, Khan et al. 2012)) under anaerobic conditions. Bacteria were grown in YCFAG broth to an optical density at 600nm (A₆₀₀) of 0.8, which corresponds to the late exponential or early stationary growth phase. *L. plantarum* WCFS1 was cultured overnight to stationary phase in deMan, Rogosa Sharpe broth (MRS, Merck, Darmstadt, Germany) at 37°C. Bacteria were harvested by centrifugation, washed in phosphate buffer saline (PBS), resuspended in PBS containing 20% glycerol and stored at -80°C prior to use in the assays. Bacteria were quantified by Fluorescent *in situ* hybridization (FISH) or phase contrast microscopy. All buffers and media used for the anaerobic bacteria were deoxygenated by flushing with oxygen free nitrogen for 30 minutes.

Mouse BMDCs assays

Bone marrow-cells were isolated by flushing femurs and tibiae of BALB/c mice, and the cell suspension was filtered through 100 µm gauze to obtain single cells. Cells were seeded at 2x10⁶ cells/ petri dish in IMDM (Invitrogen) supplemented with 10% heat inactivated FCS, 100 U sodium penicillin-G (Bio-Whittaker), 2mM GLutamax (Invitrogen), 50mM 2-mercaptoethanol and 20 ng/ml murine rGM-CSF (X63-GM-CSF-producing cell line supernatant (Stockinger, Zal et al. 1996)) and cultured for 7 days to induce differentiation into dendritic cells. On day 7, cells were seeded at 5x10⁵ cells/well in 24-well plates and were stimulated

with bacteria (bacteria to cell ratio 10:1), 1 µg/ml LPS or left unstimulated. After 24 h the supernatant was collected for cytokine measure and cells were stained for surface protein detection.

Purification of DO11.10 T-cells

For the *in vitro* experiments, DO11.10 transgenic T cells were isolated from DO11.10 mice and CFSE labelled, as described previously (Hauet-Broere, Unger et al. 2003; Unger, Hauet-Broere et al. 2003). Briefly, at 8–12 wk of age, DO11.10 transgenic mice were sacrificed, and spleens and lymph nodes (LNs) were collected. Spleens and LNs were filtered through 100 µm gauze to obtain single cells and erythrocytes in the splenocyte suspension were lysed by incubation with lysis buffer (150 mM NH₄Cl, 1 mM NaHCO₃, pH 7.4) for 7 min on ice. Cell suspensions were depleted of B cells, CD8⁺ T cells, and APCs by incubation with a mixture of mAb containing anti-B220 (clone 6B2), CD8, CD11b (MAC-1), MAC-2, F4/80, and anti-MHC-II (m5/114), and subsequent incubation with sheep anti-rat IgG Dynabeads, according to manufacturer's instructions (DynaL Biotech). The CD4⁺ T cell-enriched fraction was labelled with CFSE (Molecular Probes) and the proportion of DO11.10 transgenic CD4⁺ T cells in suspension was determined by flow cytometric analysis of an aliquot of cells using the clonotypic Ab KJ1-26 (anti-OVA transgenic TCR, Caltag) and anti-CD4 (GK1.5, BD).

For the *in vivo* adoptive transfer, DO11.10 T cells were isolated from spleens of DO11.10 rag^{-/-} mice, as described above, but the single cell suspension was not enriched.

In vitro assays with DO11.10 T cells

For the *in vitro* BMDCs-T cells assay, enriched DO11.10 T cells together with OVA were co-cultured with BMDCs stimulated for 24 h with bacteria. BMDCs were cultured as described above and on day 7, they were seeded at 5x10⁴ cells/well in 96-well plates and stimulated with bacteria (bacteria to cell ratio 10:1), 100 ng/ml LPS or left unstimulated. After 24 h, OVA (Calbiochem) was added to a final concentration of 0.5 mg/ml and, after additional 6 h, 5x10⁵ CFSE labelled KJ1-26⁺ CD4⁺ T cells/well were added. After 72 h, cells were treated with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA) and 500 ng/ml Ionomycin to elicit T cell responses; and the intracellular transport of protein was blocked adding monensin (GolgiStop, BD) for the last 4 h of stimulation. Next, the supernatant was collected for cytokine measure

and cells were analyzed by flow cytometry for cell division and stained for surface and intracellular protein detection.

For the *in vitro* T cell assays, enriched DO11.10 T cells were stimulated with hamster anti-mouse CD3 ϵ -chain mAb and hamster anti-mouse CD28 mAb (145-2C11 and 37.51, respectively, BD), bacteria, LPS or left unstimulated. Twenty four-wells plates were pre-coated with 1 μ g/ml anti-CD3 ϵ in PBS and incubated at 37°C. After 3 h non-bound antibody was removed and 5×10^5 CFSE labelled KJ1-26⁺ CD4⁺ T cells/well were seeded together with 1 μ g/ml anti-CD28 and bacteria (bacterium to cell ratio 1:1), 100 ng/ml LPS or left unstimulated (no bacteria). After 72 h, cells were treated with 50 ng/ml PMA and 500 ng/ml Ionomycin to elicit T-cell responses and the intracellular transport of protein was blocked adding monensin (GolgiStop, BD) for the last 4 h of stimulation. Next, the supernatant was collected for cytokine measure and cells were analyzed by flow cytometry for cell division and stained for surface and intracellular protein detection.

Adoptive transfer of DO11.10 T cells and intranasal challenge

For the *in vivo* study, acceptor BALB/c mice received 6×10^6 CFSE labelled KJ1-26⁺ CD4⁺ T cells in 200 μ l saline (NaCl 0.9 %, Brown) by injection in the tail vein. DO11.10 rag^{-/-} T cells were isolated as described above. Twenty four h after the adoptive transfer, mice were administered intranasally with 2×10^8 bacteria in 15 μ l of saline containing 400 μ g of OVA or 15 μ l of saline with OVA as a control. Throughout the *in vivo* experiment, groups of mice that received different treatments were housed separately. After 72 h, mice were sacrificed, nose-draining superficial and deep cervical lymph nodes (CLNs) and spleens were collected and single cell suspensions were analyzed by flow cytometry for cell division and stained for surface or intracellular protein detection or used for *ex vivo* T-cells restimulation.

Ex vivo DO11.10 T-cell restimulation

For *ex vivo* DO11.10 T-cell restimulation, single cells from CLNs (seeded at a density of 3×10^6 cell/well in 48-wells plates) and from spleens (at a density of 10^7 cells/well in 24-wells plates) of BALB/c acceptor mice were restimulated *in vitro* with 0.2 μ g/ml of OVA peptide (OVA 323-339, synthesized and purified by HPLC at the Netherlands Cancer Institute, Amsterdam, the Netherlands). Intracellular transport of protein was blocked adding monensin (GolgiStop, BD) for the last 4.5 h of stimulation. After 24 h, the supernatant was collected for cytokine

measure and cells were analyzed by flow cytometry for cell division and stained for surface and intracellular protein detection.

Cytokine analysis

Cytokines concentrations in the supernatants were determined using Cytokine bead arrays (BD) and a flow cytometer (FACS Canto II, BD). The limits of detection were the following: for IL-6 5 pg/ml, for IL-10 17.5 pg/ml, for IFN- γ 2.5 pg/ml, for TNF 7.3 pg/ml and for IL-12p70 10.7 pg/ml.

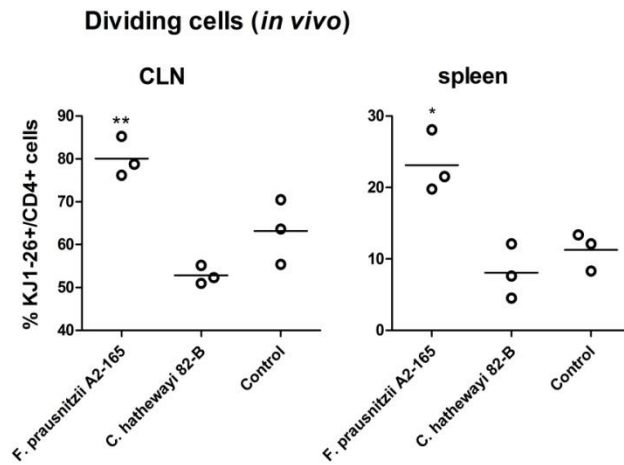
Flow cytometry

BMDCs were stained after stimulation with rat anti-mouse CD40 (clone 3/23, BD) followed by donkey anti-rat PE (Jackson), and rat anti-mouse CD86 (BD) and analysed by flow cytometry (FACS Calibur, BD Bioscience). DO11.10 T cells used *in vitro* and single cells isolated from CLNs and spleens of mice were analysed by flow cytometry to determine their phenotype and division. Cell division was determined based on the decrease of fluorescence intensity of single CFSE peaks. Single cells were stained for surface expression with KJ1-26 (Caltag), anti-CD4 (GK1.5, BD) and for intracellular expression of IL-10 (JES5-16E3), Foxp3 (FJK-16s) IFN- γ (XMG1.2, eBioscience) and IL-17A (TC11-18H10, BD) and their isotype matched control. For flow cytometry analysis, at least 2×10^4 KJ1-26⁺/CD4⁺ cells were used.

Statistics

Differences in cytokine secretion, surface marker expression and percentage of dividing or positive cells were analysed comparing samples treated with the bacteria or LPS with the untreated control. Data were analysed by one way Anova followed by Dunnett's multiple comparison test for the *in vitro* assays or Newman-Keuls multiple comparison test for the *in vivo* assays using the GraphPad PRISM (GraphPad Software).

Supplementary material



Supplementary figure 1: Percentage of dividing OVA-specific T cells *in vivo*. CFSE labelled naive OVA-specific T cells (KJ1-26⁺/CD4⁺) were adoptively transferred in BALB/c mice, after 24 h, mice were administered intranasally with bacteria plus OVA and after additional 72 h, OVA-specific T cells were isolated from nose-draining CLNs and spleens and analysed. * indicates p<0.05, ** indicates p<0.01 compared to the control administered OVA alone.

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Chapter 6

***Faecalibacterium prausnitzii* strain HTF-F and its extracellular polymeric matrix attenuate clinical parameters in DSS-induced colitis**

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Abstract

A decrease in the abundance and biodiversity of intestinal bacteria within the Firmicutes phylum has been associated with inflammatory bowel disease (IBD). In particular, the anti-inflammatory bacterium *Faecalibacterium prausnitzii*, member of the Firmicutes phylum and one of the most abundant species in healthy human colon, is underrepresented in the microbiota of IBD patients. In this study we investigated the capacity of *F. prausnitzii* strain A2-165, the biofilm forming strain HTF-F and the extracellular polymeric matrix (EPM) isolated from strain HTF-F, to suppress inflammation in the mouse dextran sodium sulphate (DSS) colitis model. The two *F. prausnitzii* strains have anti-inflammatory effects in the DSS colitis model. *F. prausnitzii* HTF-F is more effective than A2-165 partly because of the immune-regulating properties of the EPM. The immunomodulatory effects of the EPM are mediated through the TLR2-dependent modulation of IL-12 and IL-10 cytokine production in antigen presenting cells. However, the precise anti-inflammatory mechanism of EPM awaits identification of the active component. Both *F. prausnitzii* HTF-F and the EPM may have a therapeutic use in IBD.

Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is a chronic inflammatory disorder of the gastrointestinal tract characterized by diarrhoea, bloody stools, abdominal pain and weight loss. UC and CD are driven by an aberrant inflammatory T cell response to intestinal microbiota in a genetically susceptible host. Profound changes in the diversity and composition of the microbiota are associated with UC and CD (Seksik, Rigottier-Gois et al. 2003; Macfarlane, Blackett et al. 2009). A decrease in the frequency of the phyla Bacteroidetes and Firmicutes and an increase of Proteobacteria and Actinobacteria has been observed in the faecal microbiota of IBD patients (Frank, St Amand et al. 2007). In particular, *Faecalibacterium prausnitzii*, a member of the Firmicutes phylum and one of the most abundant species in the healthy human colon (Flint, Scott et al. 2012), is underrepresented in the microbiota of IBD patients (Sokol, Seksik et al. 2009). Mucosal-associated counts of *F. prausnitzii* from ileal biopsies are also lower in CD patients with active disease than in patients in remission (Sokol, Pigneur et al. 2008).

F. prausnitzii was reported to be an anti-inflammatory bacterium on account of its capacity to induce high amounts of IL-10 in human peripheral blood mononuclear cells (PBMCs, (Sokol, Pigneur et al. 2008)), and dendritic cells (DCs, Chapter 4). Furthermore, treatment of Caco2 cells with *F. prausnitzii* culture supernatant was reported to reduce IL-1 β -induced NF- κ B activation and secretion of IL-8. This was attributed to an as yet unidentified factor secreted in the medium (Sokol, Pigneur et al. 2008). Additionally, administration of *F. prausnitzii* strain A2-165 and its culture supernatant have been shown to protect against 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice (Sokol, Pigneur et al. 2008). This model is thought to resemble CD because the resulting mucosal inflammation is mediated by a T helper 1 (Th1) response with excessive production of IFN- γ , TNF- α and IL-12.

The aim of this study was to test the capacity of *F. prausnitzii* strains A2-165 and HTF-F to suppress inflammation in the mouse dextran sodium sulphate (DSS) colitis model. Strain *F. prausnitzii* HTF-F undergoes intercellular aggregation and forms a dense biofilm in liquid culture, while strain A2-165 does not. The biofilm might provide some advantage to bacterial colonization *in vivo*. As bacterial exopolysaccharides are common components of biofilm matrices and have been previously shown to have immunomodulatory effects (Mazmanian, Round et al. 2008; Round and Mazmanian 2010; Fanning, Hall et al. 2011), the purified

extracellular polymeric matrix (EPM) of strain HTF-F was also tested for its potential anti-inflammatory effects in the DSS model of colitis.

Results

Phenotypic characteristics of F. prausnitzii strain HTF-F and purification of the extracellular polymeric matrix

Although both *F. prausnitzii* strains HTF-F and A2-165 form colonies with mucoid appearance on solid agar, only strain HTF-F forms mucoid biofilm in liquid culture (Figure 1a). This phenotype is commonly associated with the production of extracellular polysaccharides and intercellular aggregating proteins (Flemming and Wingender 2010). The EPM of strain HTF-F is revealed by Gram staining (Figure 1b) and is observed in transmission electron micrographs as a diffuse and irregular surface layer (Figure 2a, arrow) resembling the capsule polysaccharide (CPS) of *Streptococcus suis* (Figure 2b, (Meijerink, Ferrando et al. 2012)).

The cell bound the EPM produced by strain HTF-F was isolated, concentrated and filtered to remove possible bacterial contaminants. The EPM yield was 1.2 mg/ml from approximately 2.5×10^{11} bacteria. Luciferase-based TLR signalling assays for human TLR2, TLR2/6, TLR4 and TLR5 indicated that microbe-associated molecular patterns (MAMPs) were not present in amounts that would influence activation of immune cells *in vitro* (Figure 3). This was confirmed by the fact that the EPM did not induce activation or cytokine secretion after incubation with hDCs (Figure 4 b).

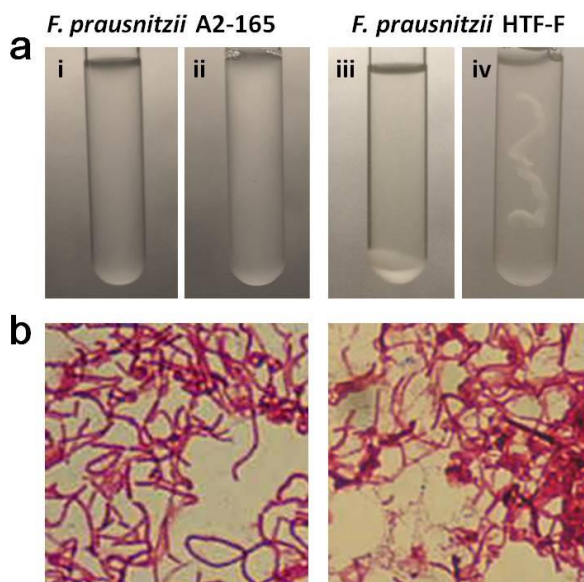


Figure 1: a) Growth and biofilm formation of *F. prausnitzii* strains HTF-F and A2-165 in YCFAG medium under anaerobic conditions. i and ii) *F. prausnitzii* A2-165 before and after shaking respectively; iii and iv) *F. prausnitzii* HTF-F before and after shaking respectively. b) Gram staining of *F. prausnitzii* A2-165 (left panel) and HTF-F (right panel).

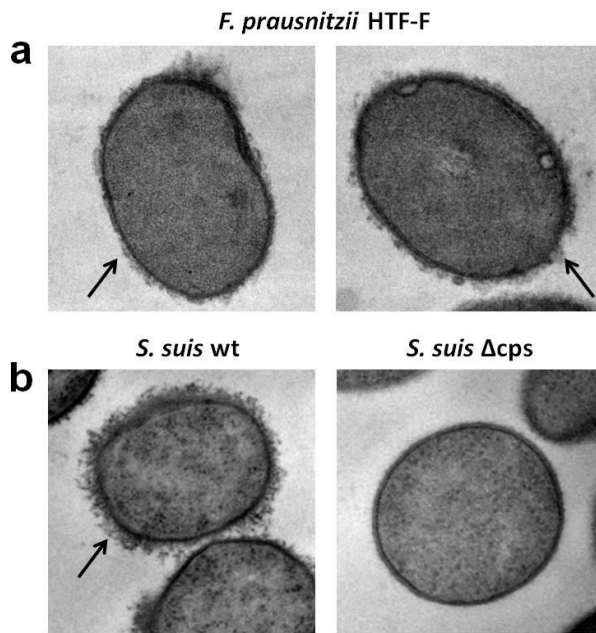


Figure 2: Detection of *F. prausnitzii* HTF-F EPM by transmission electron microscopy. *F. prausnitzii* HTF-F (a) possess a diffuse and irregular surface layer (arrow) which is thinner but similar to the capsule polysaccharide (CPS) of *S. suis* wild type strain (arrow, *S. suis* wt, left panel b) and absent in *S. suis* CPS deletion mutant (*S. suis* Δ cps, right panel b).

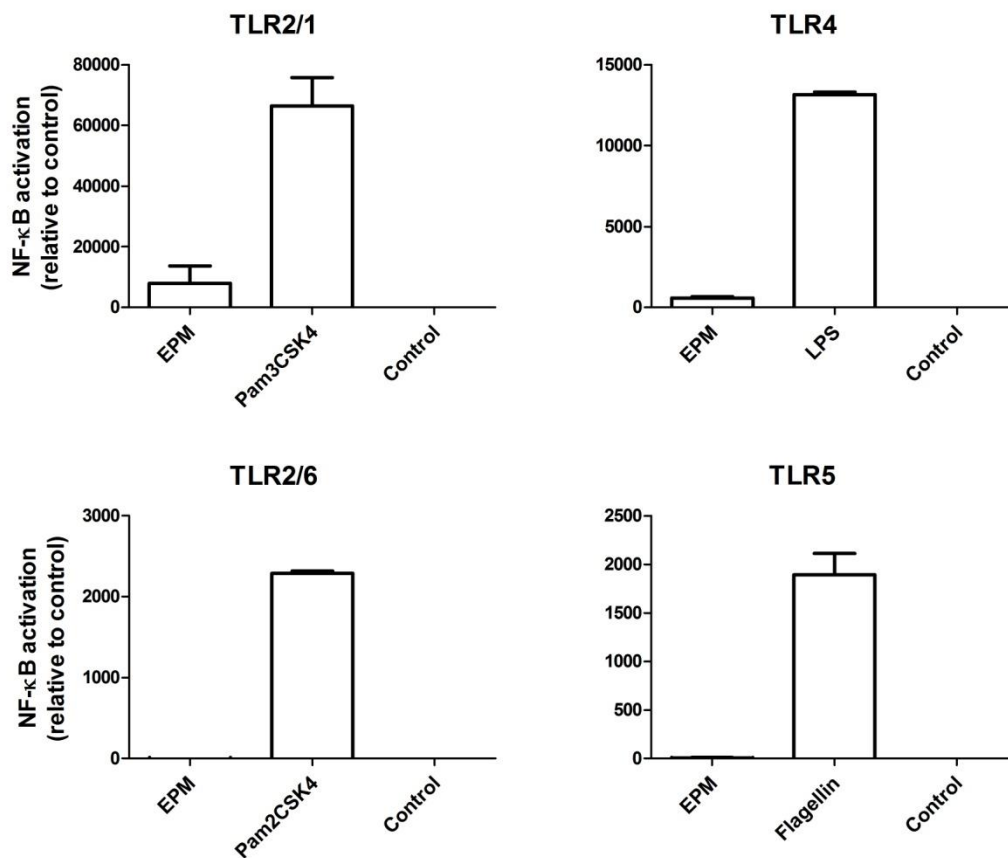


Figure 3: TLR signalling properties of *F. prausnitzii* HTF-F EPM. NF-κB activation was measured using a luminescence reporter in HEK293 cell lines expressing TLR2/1, TLR2/6, TLR5 and TLR4, relative values were calculated deducting the medium control values to the measured values. Error bars represent SEM, n=6.

The EPM of F. prausnitzii HTF-F decreases transcription and production of pro-inflammatory IL-12p70 in L. plantarum-activated hDCs

The immunomodulatory properties of *F. prausnitzii* A2-165, HTF-F and the EPM were tested *in vitro* using human monocyte-derived DCs (hDCs). DCs were chosen because they are one of the most important antigen presenting cells with the capacity to prime naive T cells at mucosal sites and to drive the immune response.

Incubation of hDCs with *F. prausnitzii* strains A2-165 and HTF-F induced large amounts of IL-10 and small amounts of IL-12p70 compared with *L. plantarum* (Figure 4a) or other lactobacilli (not shown). The amount of IL-10 produced after incubation of hDCs with strain HTF-F was lower than with strain A2-165 (Figure 4a). This was in agreement with an earlier comparative study of hDC immune responses to different *F. prausnitzii* strains and other commensals (Chapter 4).

The EPM alone had no effect on the expression of activation or maturation markers and cytokine expression compared to untreated hDCs (Figure 4b) confirming the lack of TLR signalling activity (Figure 3). Therefore, the EPM was combined with *L. plantarum* in hDC cultures to investigate whether it would modulate cytokine production. In combination with *L. plantarum*, the EPM reduced the secretion of pro-inflammatory IL-12p70 but had no effect on the IL-10, IL-1 β or TNF- α elicited by *L. plantarum* (Figure 4a, IL-1 β and TNF- α not shown). This was not due to an effect of the EPM on hDCs maturation and activation as evidenced by the measurement of the co-stimulatory molecules CD83 and CD86 (Figure 4b).

In order to investigate whether the reduced secretion of IL-12p70 was due to transcriptional regulation, quantitative RT-PCR was performed on IL-10, IL-12, IL-1 β and TNF- α mRNA extracted from hDCs at 6 and 20 h after incubation with *L. plantarum*, *L. plantarum* combined with the EPM, EPM alone or unstimulated hDCs. The addition of the EPM had no significant effect on transcription of IL-1 β , TNF- α or IL-10 in hDCs stimulated with *L. plantarum* (not shown) but significantly decreased the transcript levels of IL-12 at 20h by about 2 fold (Figure 5).

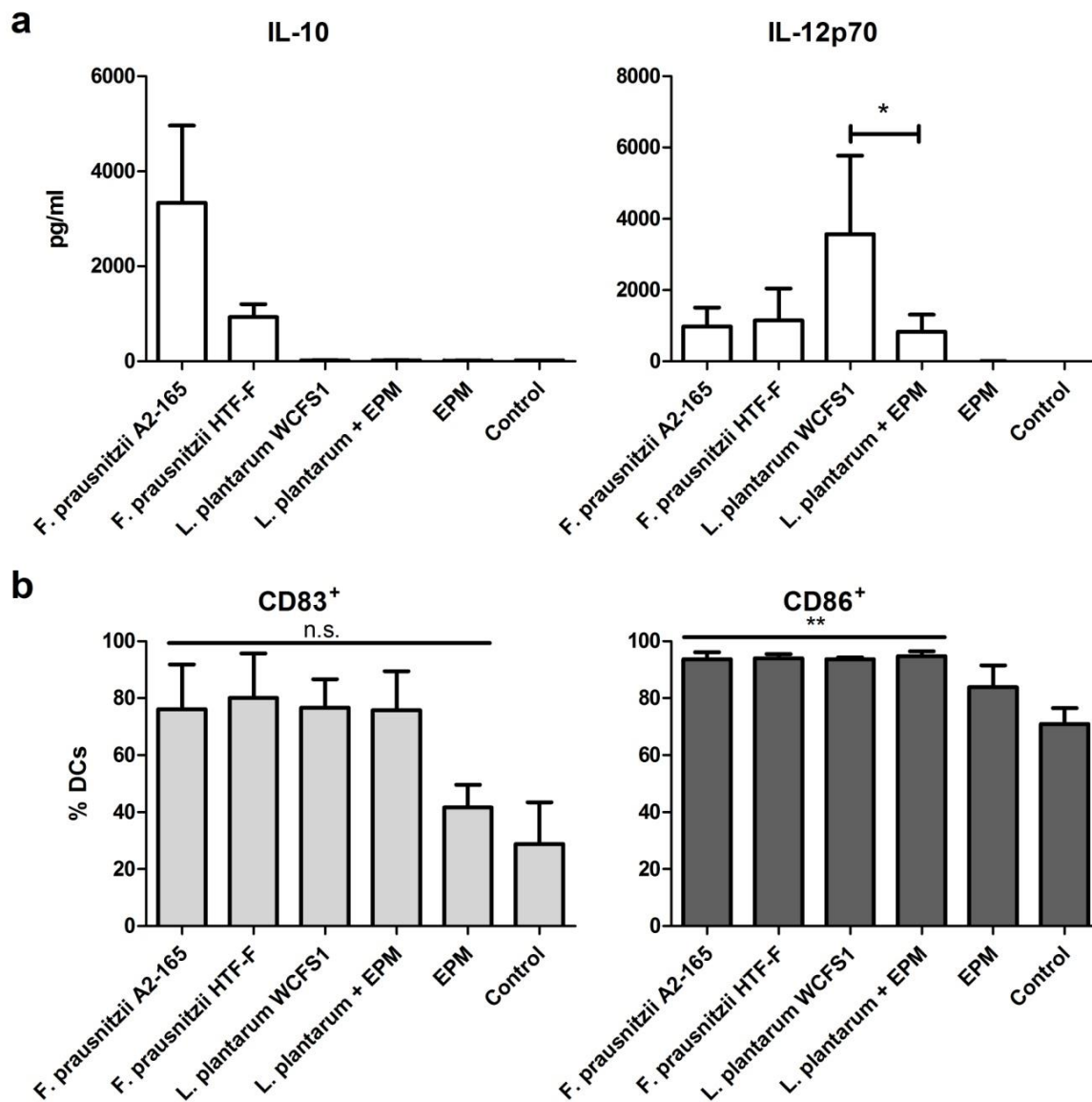


Figure 4: Cytokine secretion and surface marker expression in hDCs after 48 h of incubation with *F. prausnitzii* A2-165 (3 donors), *F. prausnitzii* HTF-F (3 donors), *L. plantarum* (5 donors), *L. plantarum* + EPM (5 donors), EPM (3 donors) or left unstimulated (5 donors). a) IL-10 and IL-12p70 were measured in the supernatant of hDCs. Error bars represent SEM, * indicates $p < 0.05$ compared with *L. plantarum* treated samples. b) Percentage of CD83⁺ (left panel) and CD86⁺ (right panel) hDCs. Error bars represent SEM, ** indicates $p < 0.01$, n.s. indicates non significant compared with the control.

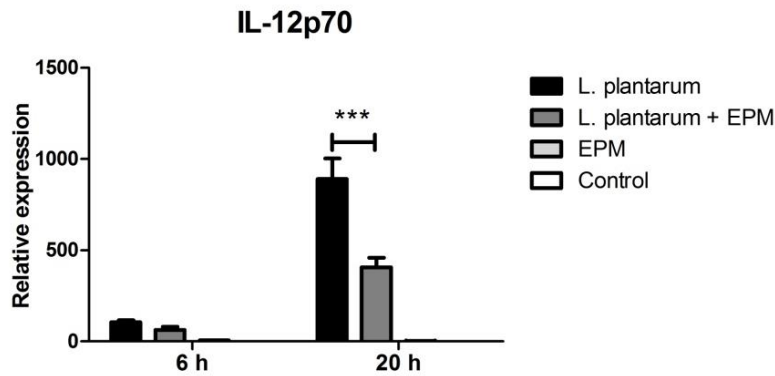


Figure 5: Relative gene expression levels in hDCs determined by quantitative RT-PCR. RNA was extracted from hDCs after 6 and 20 h of incubation with *L. plantarum* (in black), *L. plantarum* + EPM (in dark grey), EPM (in clear grey) or from unstimulated cells (in white) and the expression levels of IL-12p70 gene was calculated relative to the expression levels of the housekeeping gene GAPDH. Error bars represent SEM, n=3, *** indicates p<0.001 compared with *L. plantarum* treated samples.

F. prausnitzii and the EPM of strain HTF-F attenuate clinical symptoms in DSS-colitis

The potential protective effects of *F. prausnitzii* strains A2-165, HTF-F and the EPM were assessed in mice using the DSS-induced colitis model. The bacteria or the EPM were administered to mice intrarectally ten days prior to DSS exposure and continuously administered daily over a period of eight days in which DSS was given in the drinking water to induce colitis. The severity of colitis was evaluated for individual mice in each group by measuring disease activity index (DAI), histological damage score of the colon, body weight and colon length. The DAI and the histological colon damage score after DSS treatment were assessed according to the scale (0–4) of Cooper et al. (Cooper, Murthy et al. 1993).

F. prausnitzii A2-165, HTF-F and EPM administration significantly decreased the DAI compared to colitis control mice which received PBS intrarectally and DSS in the drinking water, although the score was higher than in untreated mice (Figure 6a).

The histological colon damage score was grade 0 in untreated mice (Figure 6b and 7a). *F. prausnitzii* HTF-F administration significantly decreased colon damage score compared to the colitis control mice (grade 1.65 and 3.2 respectively, Figure 6b, 7c and 7b), while *F. prausnitzii* A2-165 and EPM administration did not significantly affect the colon damage score compared to colitis control mice (grade 2.8 and 3.3 respectively, Figure 6b, 7d and 7e).

The colon length was reduced in all DSS treated groups compared to untreated mice but the *F. prausnitzii* HTF-F treated group had significantly longer colons compared with colitis control mice (Figure 6c) indicating reduced severity of colitis.

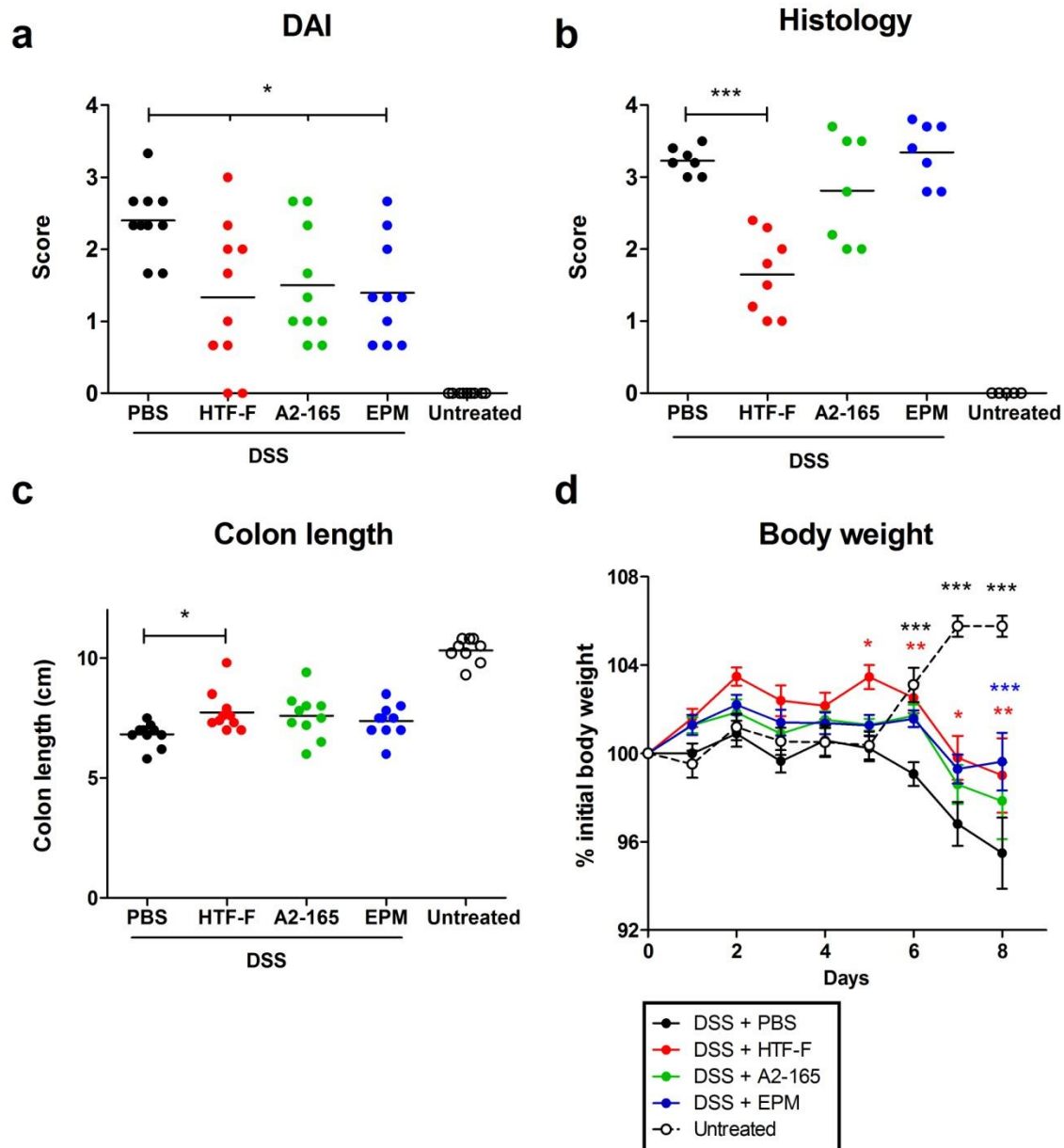


Figure 6: Disease activity index (DAI), colon histological damage score and clinical evaluation of DSS treated mice. Mice were left untreated (in white) or treated with DSS during 8 days and administered intrarectally with PBS (in black), EPM (in blue) or *F. prausnitzii* strains HTF-F (in red) or strain A2-165 (in green). DAI, histological score and colon length (a, b and c respectively) were evaluated at the end of the experiment. Mice body weight (d) was measured throughout the experiment, body weight values are expressed as percentage of the initial value measured at day 0 before DSS administration. Error bars represent SEM, n=10, * indicates p<0.05, ** p<0.01, *** p<0.001 compared with the control colitis mice that received DSS + PBS.

The body weight of mice was measured throughout the period of DSS treatment and compared to the weight before treatment. In untreated mice, the body weight increased by approximately 5 % from day 5 to day 8 (Figure 6d). However, in colitis control mice, the body weight decreased by approximately 5 % from day 5 to day 8. In mice administered *F.*

prausnitzii HTF-F, A2-165 or the EPM, the decrease in body weight was delayed by one day compared to the colitis control mice. In mice administered *F. prausnitzii* HTF-F, A2-165 and the EPM, the decrease in body weight from day 6 to day 8 was between 2 and 4 % and for *F. prausnitzii* HTF-F and the EPM, it was lower than for the colitis control group (Figure 6d).

Taken together these results indicate that *F. prausnitzii* strains A2-165, HTF-F as well as the EPM from strain HTF-F can attenuate the clinical symptoms of DSS-induced colitis.

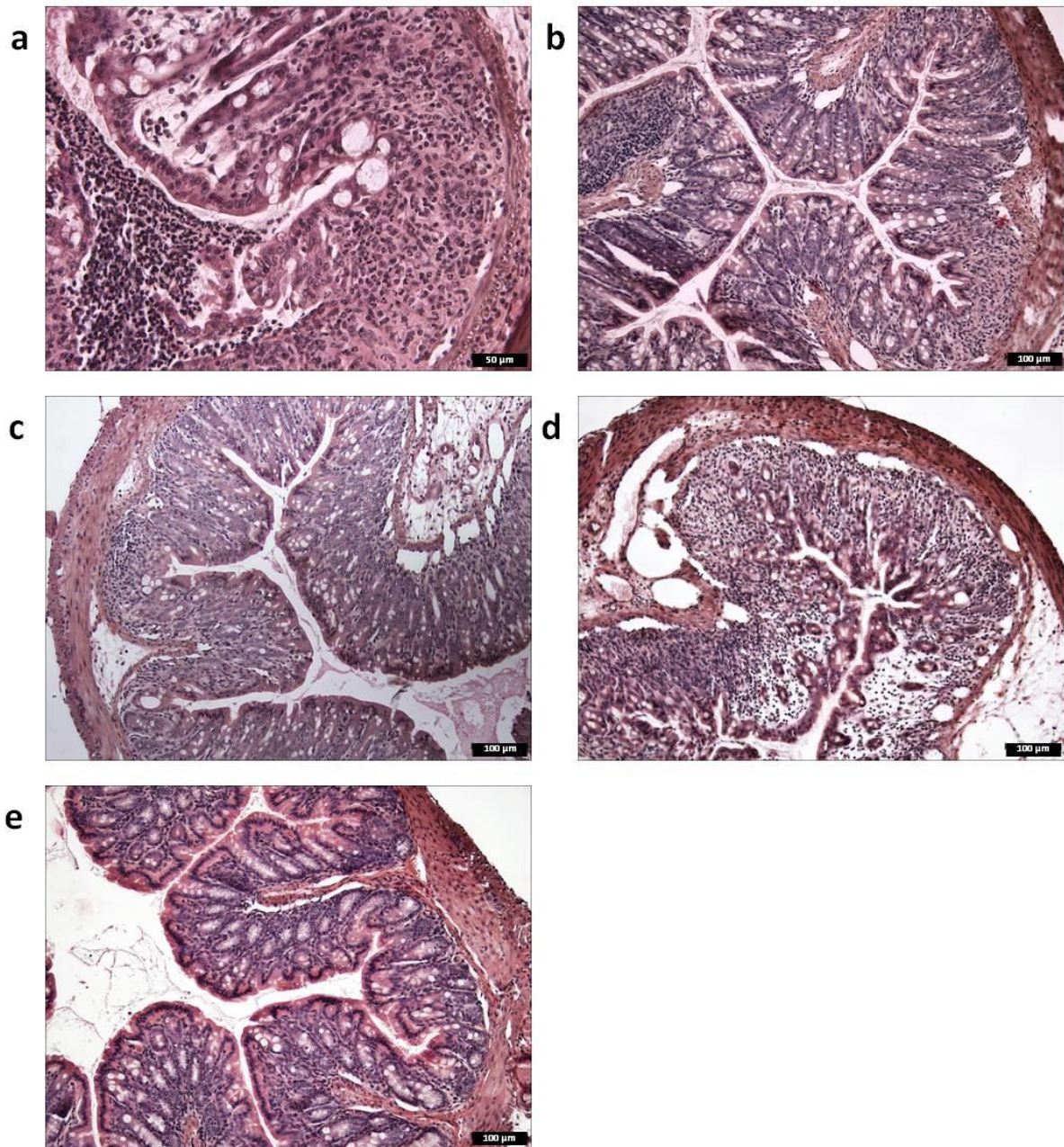


Figure 7: Histological cross-sectional views of colon descensens of untreated or DSS-treated mice: a) colitis control, PBS-DSS-treated mice (damage grade 3-3.5) b) HTF-F-DSS-treated mice (damage grade 1-2.4); c) A2-165-DSS-treated mice, (damage grade 2-3.7); d) EPM-DSS-treated mice (damage grade 2.8-3.8); e) untreated mice (damage grade 0).

Effects of F. prausnitzii and F. prausnitzii EPM on Foxp3 expression in mesenteric lymph nodes and spleen of DSS treated mice

To investigate the potential role of Foxp3⁺ Tregs in the attenuation of DSS-induced colitis, we measured the number of CD4⁺ T cells isolated from mesenteric lymph nodes (MLNs) and spleens that express intracellular Foxp3 by fluorescence-activated cell sorting (FACS). DSS treatment did not significantly affect the levels of Foxp3⁺ CD4⁺ T cells in the MLNs or spleen compared to untreated mice. Administration of *F. prausnitzii* also had no effect on Foxp3⁺ CD4⁺ T cells compared to untreated mice. However, administration of the EPM induced a small but significant increase in Foxp3⁺ CD4⁺ T cells in the MLNs but not in the spleen (Figure 8).

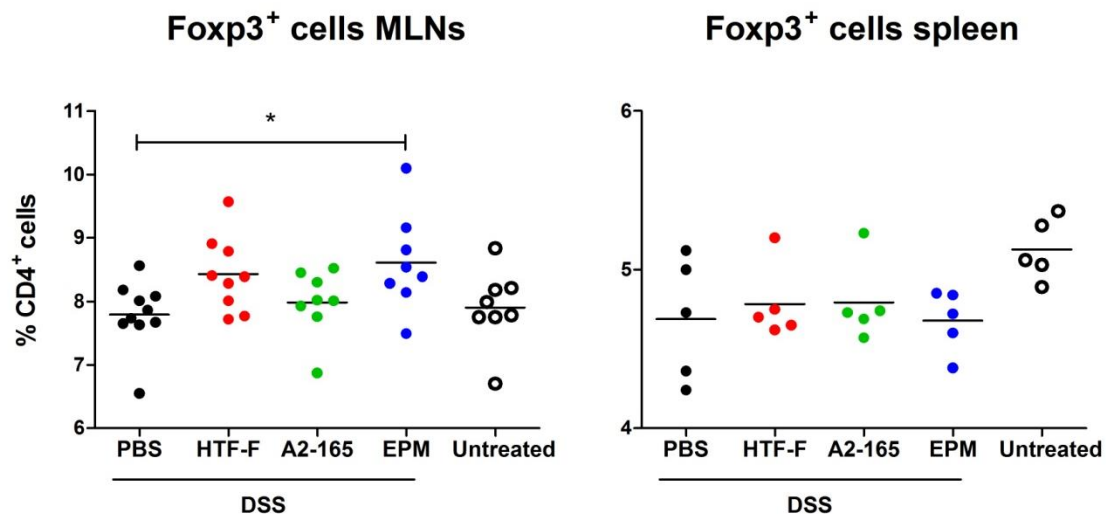


Figure 8: Percentage of Foxp3⁺ CD4⁺ T cells isolated from mesenteric lymph nodes (MLNs, left panel) and spleens (right panel) of mice untreated (in white) or treated with DSS during 8 days and administered intrarectally with PBS (in black), EPM (in blue) or *F. prausnitzii* strains HTF-F or strain A2-165 (HTF-F in red and A2-165 in green, respectively).

The immunomodulatory effects of the EPM are TLR2 dependent

To investigate whether the anti-inflammatory effects of the EPM on IL-12p70 production by DCs contribute to the protective effects observed in DSS-induced colitis, we performed an *in vitro* experiment using mouse bone marrow-derived DCs (BMDCs) stimulated with *L. plantarum* with and without EPM. As found using hDCs, the presence of EPM reduced the secretion of pro-inflammatory IL-12p70 by mouse BMDC stimulated with *L. plantarum*. Strikingly, the EPM also increased the production of IL-10 in BMDC stimulated with *L. plantarum* which was already induced at much higher levels than in hDCs stimulated with *L.*

plantarum. These effects were not due to the induction of cytokine secretion by EPM alone (Figure 9a). Additionally, we tested whether the immunomodulatory effects of the EPM were dependent on TLR2 signalling by including a TLR2 blocking antibody or an irrelevant antibody of the same isotype in the assays. The effects of the EPM on IL-12p70 and IL-10 production by *L. plantarum* stimulated BMDCs were inhibited in the presence of TLR2 blocking antibody but not in the presence of the isotype antibody. As EPM itself did not induce TLR2 signalling (Figure 3) the mechanism leading to reduced transcription of IL-12p70 and increased production of IL-10 was dependent on the TLR2 signalling by *L. plantarum* (Figure 9b).

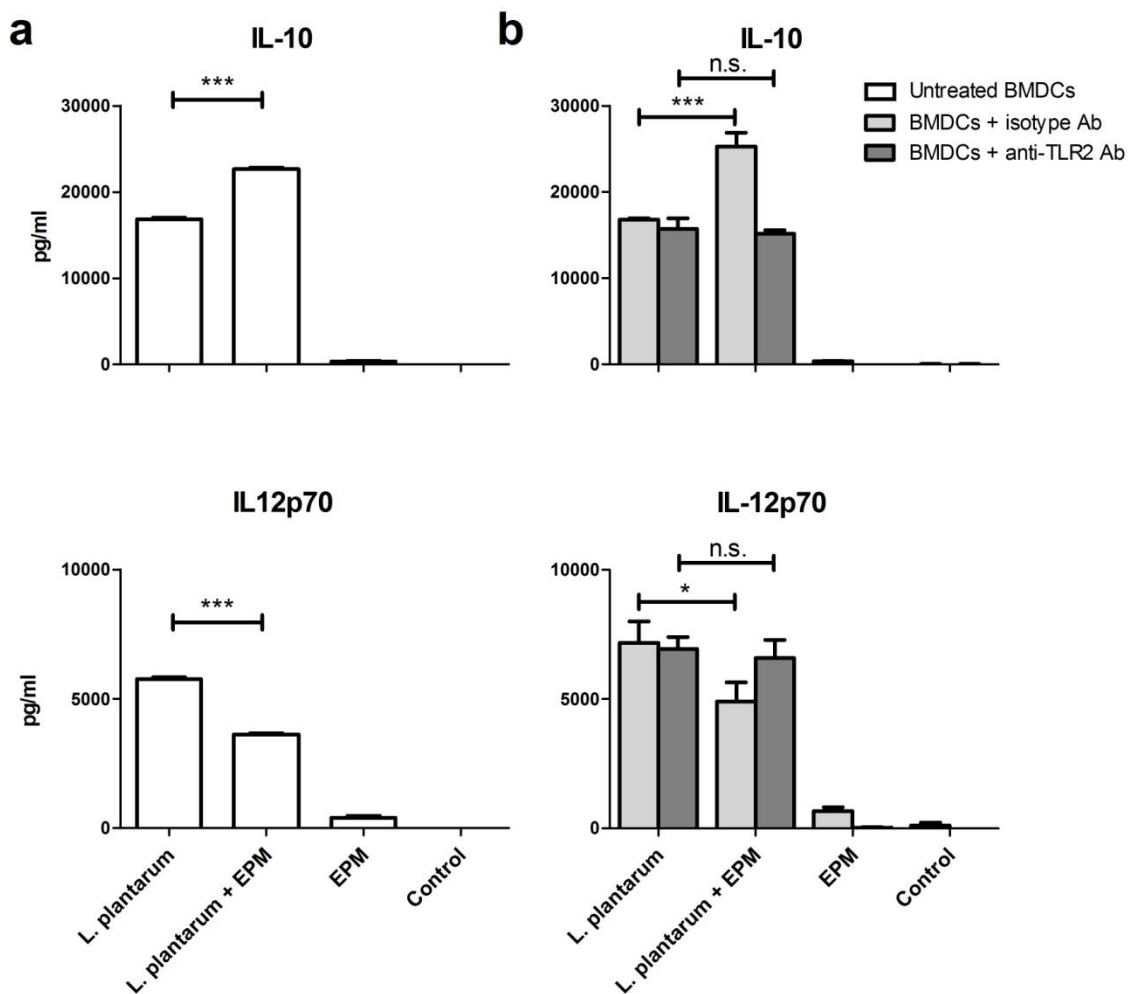


Figure 9: Cytokine secretion in mouse BMDCs. a) IL-10 and IL-12p70 were measured in BMDC supernatants after incubation with *L. plantarum*, *L. plantarum* + EPM, EPM and unstimulated DCs. b) IL-10 and IL-12p70 were measured after incubation of BMDCs with the same samples as in panel (a) except that anti-TLR2 blocking antibody (anti-TLR2 Ab, dark grey bars) or an isotype control (isotype Ab, clear grey bars) were included during the incubation period. Error bars represent SEM (n=3), *** indicates p<0.001, * indicates p<0.01, n.s. non significant compared to *L. plantarum* treated samples.

Discussion

In this study, we demonstrated that *F. prausnitzii* strain A2-165, the biofilm forming strain HTF-F as well as the EPM isolated from strain HTF-F can attenuate the clinical symptoms of DSS-induced colitis. Strain HTF-F had a stronger effect than strain A2-165 or the EPM alone in suppressing inflammation as evidenced by the colon damage score and colon length (Figure 6). The administration of purified EPM alone decreased the DAI indicating that it contributes to the protective effect of strain HTF-F and may be responsible for the stronger protection seen with strain HTF-F compared to A2-165. However, we cannot rule out other possible strain differences, for example in colonization potential, stress resistance or fitness *in vivo* which might contribute to the efficacy of immune suppression.

Previously, *F. prausnitzii* A2-165 and its supernatant were shown to attenuate 2,4,6-trinitrobenzenesulfonic acid (TNBS) colitis in mice by daily intragastric administration prior to and during the induction of colitis (Sokol, Pigneur et al. 2008). In the study of Sokol et al., the colons of mice treated with either *F. prausnitzii* A2-165 or its supernatant had a reduced amount of IL-12p70 and an elevated amount of IL-10 compared with the colitis control group. This is compatible with the relatively high amount of IL-10 induced by *in vitro* culture of *F. prausnitzii* A2-165 with mouse BMDCs, hDCs (Figure 4) and human PBMCs (Sokol, Pigneur et al. 2008). IL-10 is fundamental for the maintenance of homeostasis in the intestine (Geuking, Cahenzli et al. 2011; Veenbergen and Samsom 2012). It is secreted by DCs as well as Foxp3⁺ and Foxp3⁻ T cells in the lamina propria. Secretion of IL-10 by DCs is important for the maintenance of functional Foxp3⁺ Tregs during intestinal inflammation (Murai, Turovskaya et al. 2009). IL-10 also inhibits the production of pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-6 and IL-12. Moreover, IL-10 was shown to play a role in controlling pro-inflammatory responses to translocated microbes by abrogating IL-23 production (Manuzak, Dillon et al. 2012). Nevertheless, differences in IL-10 produced *in vitro* by DCs cultured with *F. prausnitzii* A2-165 and strain HTF-F cannot explain the better protection seen with HTF-F as it induced less IL-10 than A2-165.

Butyrate produced in the colon by *F. prausnitzii* may also contribute to the anti-inflammatory effects observed in experimental colitis model as oral administration of sodium butyrate has been recently shown to attenuate inflammation in experimental UC (Vieira, Leonel et al. 2012). Microbially-produced butyrate is considered important for colonic health, and in the

prevention of colorectal cancer owing to its use as an energy source for epithelial cells and as a modulator of oxidative stress and inflammation (Hamer, Jonkers et al. 2008). Moreover, butyrate enemas have been reported to be effective in the therapy of UC (Hamer, Jonkers et al. 2010).

In vitro assays indicated that the anti-inflammatory mechanism of the EPM was not due to contamination with MAMPs or activation of DCs. However, when the EPM was added together with *L. plantarum* as an inflammatory stimulus to hDCs it decreased the production of IL-12p70 compared to *L. plantarum* alone and had no effect on IL-10, IL-1 β and TNF- α production (Figure 4). A similar effect of the EPM was seen on IL-12p70 production by mouse BMDCs (Figure 9) but, in contrast to hDCs, IL-10 was significantly increased. The effect of EPM on IL-12p70 production occurred at the transcriptional level (Figure 5) suggesting the involvement of the EPM in cell signalling. This mechanism was dependent on TLR2 signalling although the EPM itself did not induce TLR2 signalling in reporter assays, or activate hDCs or mouse BMDCs as in the case of synthetic TLR2 agonists (Figure 9). The mechanism may involve interaction of carbohydrate structures in the EPM with C-type lectin receptors, some of which are known to modulate cytokine production in response to TLR agonists. In the DSS colitis model, administration of EPM but not *F. prausnitzii* increased the number of Foxp3⁺ T cells in the MLNs. Similarly, the extracellular polysaccharide A of *Bacteroides fragilis* (Round and Mazmanian 2010), has also been shown to expand the number of mucosal Foxp3⁺ T cells and confers a TLR2-dependent protection in mouse colitis models (Mazmanian, Round et al. 2008; Round and Mazmanian 2010).

In summary, we demonstrated the anti-inflammatory effect of two different *F. prausnitzii* strains in the mouse DSS colitis model. Furthermore, we showed that *F. prausnitzii* HTF-F which produces a thick biofilm is more anti-inflammatory and this may in part be due to the immune-regulating properties of the EPM. *In vitro* immune assays suggest that the immunomodulatory effects of the EPM are mediated through the TLR2-dependent modulation of IL-12 and IL-10 cytokine production in antigen presenting cells. However, the precise anti-inflammatory mechanism of EPM awaits identification of the active component. Both *F. prausnitzii* HTF-F and the EPM may have a therapeutic use in IBD.

Materials and methods

Animals

BALB/c mice were reared in conventional conditions. Two-month-old females were used for these studies and their body weights were measured before and after each experiment. Animal experiments were approved by the Ethical Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic.

Bacterial strains and culturing conditions

F. prausnitzii strain HTF-F and A2-165 have been described elsewhere (Barcenilla, Pryde et al. 2000; Duncan 2002; Lopez-Siles, Khan et al. 2012) and were maintained at 37°C on yeast extract, casitone, fatty acid and glucose medium (YCFAG, described in (Lopez-Siles, Khan et al. 2012)) under anaerobic conditions. For EPM production, *F. prausnitzii* strains were cultured in YCAG broth, which have the same composition as YCFAG medium but, except acetate, all short chain fatty acids were omitted. *L. plantarum* WCFS1 was cultured overnight until stationary phase in deMan, Rogosa Sharpe broth (MRS, Merck, Darmstadt, Germany) at 37°C. Bacteria were harvested by centrifugation at 4°C, 3300 g for 15 min, washed in phosphate buffer saline (PBS), resuspended in PBS containing 20% glycerol and stored at -80°C prior to use. For the BMDCs assays, *L. plantarum* WCFS1 grown in MRS at 37°C overnight were inactivated with 1% formaldehyde-PBS as described previously (Schabussova, Hufnagl et al. 2012).

Bacteria were quantified by fluorescent *in situ* hybridization (FISH) or phase contrast microscopy. All buffers and media used for the anaerobic bacteria were deoxygenated by flushing with oxygen free nitrogen for 30 minutes.

Isolation and staining of the F. prausnitzii extracellular polymeric matrix

The cell bound EPM was extracted as previously described (Ricciardi, Parente et al. 1998). Briefly, 250 ml of 24 h cultures of *F. prausnitzii* strains HTF-F or A2-165 were recovered by centrifugation at 18400 g for 10 min (4°C). The pre-washed cell pellet was suspended in 8 ml of PBS by vortexing for 5 min allowing the cell bound EPM to dissolve. Planktonic cells were subsequently pelleted by centrifugation at 18400 g for 10 min (4°C). The supernatant was then carefully removed and added to 4 volumes of ice cold absolute ethanol to precipitate the

EPM. After centrifugation at 3300 g for 30 min, the EPM precipitated pellet was washed with 70% ethanol, then lyophilized and stored at -20°C. For further experiments, lyophilized EPM fractions were dissolved in PBS at the desired concentrations. The EPM was shown to be free of bacterial contamination by visual inspection after Gram staining. TLR assays (described in the following section) showed that the EPM was free of contaminating MAMPs.

TLR signalling assays

TLR assays were performed using human embryonic kidney cells (HEK293) stably expressing human TLR2/6, TLR2/1, TLR4 or TLR5 (Invivogen, Toulouse, France) and transfected with a reporter plasmid (pNiFTY, Invivogen) containing the luciferase gene under the control of the NF-κB promoter. HEK293 cells expressing the different TLRs and pNiFTY were incubated with the EPM (1.2 % v/v), the TLR agonists, Pam2CSK4 (Invivogen) for TLR2/6, Pam3CSK4 (Invivogen) for TLR2/1, flagellin (Invivogen) for TLR5 and LPS for TLR4, or medium alone as a control. After 6 hours of incubation, the medium was replaced with Bright glow (Promega), and the luminescence was measured using a Spectramax M5 (Molecular Devices). As a negative control, HEK293 cells not expressing TLRs but harbouring pNiFTY were tested in the same conditions and did not show any luciferase activity. The limits of sensitivity for the TLR reporter cell lines were determined in independent experiments using a dose range for each purified agonist. The limits of detection for these reporter cell lines were determined as follows: 2 ng/ml for Pam2CSK4 (TLR2), 8 ng/ml for flagellin (TLR5) and 50 pg/ml for LPS (TLR4); MAMPs present at concentrations lower than this would not activate immune cells in our *in vitro* assays and, as expected, the EPM did not activate hDCs or mouse BMDCs (Figure 4 and 9).

Human DCs assays

This study was approved by Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Buffy coats from blood donors were obtained from the Sanquin Blood bank in Nijmegen (The Netherlands). A written informed consent was obtained before sample collection.

The immunomodulatory properties of the EPM was investigated by measuring the expression of surface markers and the cytokines secreted in the supernatant after incubation of human monocyte-derived DCs (hDCs) with *F. prausnitzii* A2-165 (hDCs from 3 donors), *F. prausnitzii*

HTF-F (hDCs from 3 donors), *L. plantarum* (hDCs from 5 donors), EPM (hDCs from 3 donors) or *L. plantarum* together with EPM (hDCs from 5 donors). Bacteria were used at a bacterium: DC ratio of 10:1, EPM at 1.2% v/v. Mononuclear cells were isolated from buffy coats of healthy donors using Ficoll Paque Plus density gradient (GE Healthcare, Diegem Belgium) according to the manufacturer's protocol. After centrifugation, mononuclear cells were collected and monocytes were isolated by positive selection of CD14⁺ cells using CD14-specific antibody coated magnetic microbeads (Miltenyi Biotec, Leiden, The Netherlands). CD14⁺ cells were cultured for 6 days in complete medium in the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems, Minneapolis, MN) to differentiate into immature monocyte-derived DCs. At day 6, cells were seeded at 10⁶ cells/ well in 24-well plates and were treated with *L. plantarum* (bacterium: DC, 10:1) in the presence or absence of the EPM isolated from *F. prausnitzii* (1,2% v/v) or left untreated. After 48 hours of co-incubation, the supernatant was collected for cytokine measurements.

During the culture period and the stimulation, DCs were cultured in Rosewell Park Memorial Institute (RPMI) 1640 culture medium (Invitrogen) supplemented with 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St. Louis, MO) and no bacterial growth was observed. On day 6 and 8, the activation and maturation status of the CD14⁺ cells were assessed by measuring CD83 and CD86 surface expression and the cell viability was measured using Annexin V and propidium iodide (PI). Cells were stained with fluorescence conjugated monoclonal antibodies specific for CD83, CD86, their isotype-matched controls and with annexin V and PI (BD Biosciences, Breda, The Netherlands) and analysed on a flow cytometer (FACS Canto II, BD). CD86 and CD83 were expressed at low levels on immature or untreated DCs and were highly expressed after stimulation. On days 6 and 8 the viability of the cells was between 60–80% (not shown).

RNA isolation and real-time qPCR

Total RNA was isolated from hDCs using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer instructions. cDNA synthesis was performed using 500 ng of isolated total RNA and Q-script (Quanta bioscience, Gaithersburg, MD) according to the manufacturer instructions. cDNA was diluted in nuclease-free water to a final volume of 100 µl and stored at -20°C until further use. Primers for IL-10 (forward 5'-GTGATGCCCAAGCTGAGA-3', reverse 5'-CACGGCCTTGCTCTTGT-3'), IL-12p40 (forward 5'-

CTCTGGCAAACCTGACC-3', reverse 5'-GCTTAGAACCTCGCCTCCTT-3'), IL-1 β (forward 5'-GTGGCAATGAGGATGACTTGTTTC-3', reverse 5'-TAGTGGTGGTTCGGAGATTCGTA-3'), TNF- α (forward 5'-CTGCTGCACTTTGGAGTGAT-3', reverse 5'-AGATGATCTGACTGCCTGGG-3'), and the reference genes GAPDH (forward 5'-CTGCACCACCAACTGCTTAG-3', reverse 5'-GTCTTCTGGGTGGCAGTGAT-3') and β -actin (forward 5'-TTGCGTTACACCCTTCTTG-3', reverse 5'-CACCTTCACCGTTCCAGTTT-3') were designed using PRIMER3 software (Rozen and Skaletsky, 2000). Quantitative RT-PCR (qPCR) was performed using the GoTaq qPCR mastermix (Promega), briefly, 5 μ l cDNA (20x dilution), forward and reverse primers (300 nM each) were added to 7 μ l qPCR mastermix and demineralised water was added to a final volume of 14 μ l. The qPCR reaction (2 min 95°C, 40 cycles of 15 s at 95°C, 60 s at 60°C) was carried out on a Rotorgene 6000 real-time cycler (Qiagen). Raw data were analysed using the comparative quantitation method of the Rotor-gene Analysis Software V5.0 and relative gene expression levels were determined as ratio of target gene vs. reference gene and were calculated according to the Δ Ct method described by Pfaffl (Pfaffl 2001) using the following equation: Ratio = $(E_{\text{target}})^{Ct_{\text{target}}(\text{control-sample})} / (E_{\text{reference}})^{Ct_{\text{reference}}(\text{control-sample})}$. Where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value. Dual internal reference genes (GAPDH and β -actin) were incorporated in all qPCR experiments and results were similar following standardization to either gene. For each sample a controls that was not treated with reverse transcriptase was included and no amplification above background levels was observed. Non-template controls were included for each gene in each run and no amplification above background levels was observed. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked at least once by sequencing.

Mouse BMDCs assays

Mouse BMDCs from BALB/c mice were prepared as previously described (Lutz, Kukutsch et al. 1999). Briefly, bone marrow cells isolated from femurs and tibias were seeded at 2×10^5 cells/ml in bacteriological Petri dishes in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 150 μ g/ml gentamycin and 20 ng/ml mouse rGM-CSF (Sigma-Aldrich, USA). Fresh medium was added at day 3 and 6 and BMDC were used on day 8 of culture. Where indicated BMDCs (10^6 cells/ml) were incubated with anti-TLR2 antibody (InvivoGen, USA) or control

isotype antibody IgG2a (eBioscience, USA) at concentration 10 µg/ml for 1 hour 37°C prior to stimulation with *L. plantarum*, EPM or *L. plantarum* together with EPM for 20 h. *L. plantarum* was used at a bacterium: DC ratio of 10:1, EPM at 1.2 % v/v. Culture supernatants of stimulated BMDC were stored at -20°C until use. For cell surface marker analysis, BMDCs were collected after cultivation and pre-incubated with anti-mouse CD16/CD32 (eBioscience, USA) for 5 min on ice prior to staining for 30 min at 4°C with anti-mouse FITC-conjugated CD11c, APC-conjugated MHCII and PE-conjugated CD40, CD80 or CD86 monoclonal antibody (eBioscience, USA). The sample data were acquired on a FACSCalibur flow cytometer (Becton-Dickinson, USA) and analyzed with FlowJo software 7.6.2 (TreeStar, USA).

Cytokine analysis

Cytokines concentrations in the hDC culture supernatants were determined using Cytokine bead arrays (BD) and a flow cytometer (FACS Canto II, BD). In hDCs studies, the limits of detection were the following: for IL-1β 7.2 pg/ml, for IL-10 3.3 pg/ml, for TNF 3.7 pg/ml and for IL-12p70 1.9 pg/ml. Mouse IL-10 was assayed in culture supernatants by enzyme-linked immunosorbent assay (ELISA) using Ready-Set-Go! kit (eBioscience, USA) according to manufacturer's instructions. Levels of IL-12p70 were measured with matched antibody pairs (BD Pharmingen, USA).

Intrarectal administration of bacteria or the EPM and induction of acute ulcerative colitis

The experimental groups of 10 mice and their respective treatments are shown in Table 1. Mice from groups 2, 3, 4 and 5 received 2.5 % DSS (molecular weight 40 kDa; ICN Biomedicals, Ohio, USA) in the drinking water ad libitum for one week. Mice from the untreated control group 1 received only drinking water. Mice from groups 3 and 4 received intrarectally (via tubing) daily doses of 2 to 3 x 10⁹ CFU of *F. prausnitzii* HTF-F and A2-165, respectively in 100 µl PBS for ten days prior the DSS exposure and during the eight days of DSS treatment. Mice from group 5 received intrarectally daily doses of 50 µg the EPM in 100 µl PBS for ten days prior the DSS exposure and during the eight days of DSS treatment. Mice from the colitis control group received intrarectally 100 µl PBS. The following clinical symptoms were measured or assessed: firmness of faeces, rectal prolapses, rectal bleeding and colon length after the sacrifice. The colon descendens was taken for myeloperoxidase assay, isolation of mRNA, histological assessment and for intestinal fragment cultivation.

Table 1: DSS induced colitis experimental groups

Group	Intrarectal treatment	DSS
1	PBS	-
2	PBS	+
3	<i>F. prausnitzii</i> HTF-F	+
4	<i>F. prausnitzii</i> A2-165	+
5	the EPM	+

Disease activity index

Disease activity index (DAI), measured according to Cooper et al. (Cooper, Murthy et al. 1993), is a combined score of weight loss, stool consistency and bleeding divided by 3. Acute clinical symptoms are diarrhoea and/or grossly bloody stools. The scores are explained in Table 2.

Table 2: Scoring of DAI (modified according to Cooper et al. 1993)

Score	Weight loss	Stool consistency*	Occult/gross bleeding
0	None	Normal	Normal
1	1 - 5%	Normal	Normal
2	5 - 10 %	Loose	Hemacult +
3	1 – 20 %	Loose	Blood in colon Starting bleeding from anus
4	> 20 %	Diarrhoea	Gross bleeding

* Normal stools, well formed pellets; loose stools, pasty and semifformed stools which do not stick to the anus; diarrhoea, liquid stools that stick to the anus.

Histological evaluation of colon damage

Colon tissue was fixed in Carnoy's fluid for 30 min, transferred into 96% ethanol and embedded in paraffin. Paraffin-embedded tissue (5 mm) were sectioned and stained with haematoxylin and eosin (H&E) and Alcian Blue and poststained with NuclearFastRed (Vector, Burlingame, CA) for mucin production. Samples were examined using an Olympus BX 40 microscope equipped with an Olympus Camedia DP 70 digital camera, and the images were analysed using Olympus DP-Soft. The degree of damage to the surface epithelium, crypt distortion and mucin production in individual colon segments were evaluated according to Cooper et al. (Cooper, Murthy et al. 1993).

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Chapter 7

General discussion

Oriana Rossi

The human gastrointestinal tract (GI-tract) is colonized by a multitude of microorganisms collectively known as the microbiota. The microbiota includes archaea, fungi, viruses and protozoa but is largely dominated by bacteria. The composition and density of the microbiota vary along the GI-tract and can reach densities of 10^{12} bacteria per gram of luminal content in the colon (Whitman, Coleman et al. 1998) with an estimated total of 10^{14} bacteria in a healthy adult amounting to 1 kg in weight. The intestinal microbiota of healthy adults is dominated by bacteria belonging to the Bacteroidetes, Firmicutes and Actinobacteria phyla (Backhed, Ley et al. 2005; Eckburg, Bik et al. 2005; Ley, Hamady et al. 2008). The relationship between mammals and the microbiota has evolved to be mutualistic and largely symbiotic in nature. In the colon, the microbiota obtains energy from the degradation of plant polysaccharides and other non-digestible carbohydrates increasing the energy harvest for the host. Moreover, the microbiota contributes to homeostasis of tolerance and immunity through interactions of microbial metabolites and other components with the host innate and adaptive immune systems. The coevolution of a mutualistic relationship between the host and the microbiota required the mucosal immune system to develop the ability to defend the body against infections of pathogenic microorganisms while avoiding excessive inflammatory responses to harmless antigens and commensal bacteria. This is achieved by the presence of an effective gut barrier to prevent bacterial contact with the epithelium and a finely tuned network of mechanisms for microbial recognition and tolerance to the microbiota. These mechanisms are described in detail in *Chapter 1*, where it is shown how the host immune system actively controls the microbiota and the microbiota, in turn, actively shapes the host immune response. The contribution of different components of the microbiota to the homeostasis of this mutualistic relationship is still being unravelled. The research described in this thesis aimed at enhancing our understanding of this mutualism between the microbiota, its components and the host. The two broad aims of this thesis were, firstly, to investigate the physical interaction of bacteria with the mucosa and mucosal lymphoid tissue *in vivo* and the microbe-epithelial Toll-like receptor (TLR) signalling *in vitro* and secondly, to investigate the interactions of an abundant colonic commensal, *Faecalibacterium prausnitzii*, with the immune system *in vitro* and *in vivo*.

Host-microbe interactions at the mucosal interface

In *Chapter 3*, using histology techniques we showed that the secreted mucus presents a formidable physical barrier to the direct interaction of bacteria with the epithelial surface. Nevertheless, intact and viable intestinal bacteria were found intracellularly in the Peyer's patches (PPs) and the mesenteric lymph nodes (MLNs, most likely within dendritic cells (DCs)). Intestinal DCs were previously shown to be restricted to the mucosal tissues, PP, and MLNs (Macpherson 2004). Retinoic acid, synthesized by the intestinal epithelium and gut-associated DCs, induces the expression of the specific mucosal homing receptors integrin $\alpha 4\beta 7$ and chemokine receptor CCR9 in T cells residing in the mucosal lymphoid tissue, ensuring their homing to the mucosa. This ensures that immune responses to commensal bacteria are induced locally, without potentially damaging systemic immune responses (Iwata, Hirakiyama et al. 2004). The compartmentalisation of mucosal immunity is one of the mechanisms by which pathological immune responses to the intestinal microbiota are avoided. The preliminary study described in *Chapter 3* focused on PPs and antigen sampling. It is now appreciated that the colon also contains an additional type of lymphoid tissue, the solitary intestinal lymphoid tissues (SILT) which change in response to the luminal microbiota and develop in mature isolated lymphoid follicles (ILFs) (Hamada, Hiroi et al. 2002; Lorenz, Chaplin et al. 2003; Pabst, Herbrand et al. 2006). ILFs can also function as inductive sites for mucosal immune responses and possess a follicle associated epithelium containing M cells as well as B cell follicles, with germinal centers and interfollicular T cells (Lorenz, Chaplin et al. 2003). The conclusion of the preliminary work discussed in *Chapter 3* was that the uptake of bacteria occurs mainly in organized lymphoid structures such as PPs or ILFs because, with the exception of segmented filamentous bacteria (SFB), bacteria were not seen below the mucus and in contact with the intestinal epithelium. However, these observations are based on inspection of microscopy images and do not exclude the possibility that at low frequency, commensal bacteria breach the mucus barrier and contact the epithelium. Furthermore, microbial associated molecular patterns (MAMPs) of commensal organisms could diffuse through the mucus and trigger pattern recognition receptor signalling in epithelial cells. The recognition of bacterial components by innate TLRs on intestinal epithelial cells (IECs) was studied using Caco-2 and HT-29 cell lines in *Chapter 2*. In the intestinal mucosa, several adaptations of TLR signalling have evolved to avoid chronic inflammatory responses to the presence of commensal microbes. In this chapter, we investigated whether polarized IECs

might regulate inflammatory responses by secreting interleukin 8 (IL-8) in a vectorial fashion (i.e. apical versus basolateral) depending on the location of the TLR stimulus. In addition, we hypothesized that CXCR1, the G-protein coupled receptor (GPCR) for IL-8, might control specific functions in polarized IECs depending on its location. The vectorial secretion of IL-8 was studied stimulating the polarized IEC lines Caco-2 and HT-29 with TLR agonists applied from the apical or basolateral side and IL-8 was measured in the two compartments. Both Caco-2 and HT-29 stimulation with TLR agonists induced apical secretion of IL-8 suggesting that, depending on the TLR stimulus, the chemokine could be secreted either from the apical or the basolateral side of the epithelial monolayer. CXCR1 was located on the apical side of Caco-2 cells and of differentiated epithelial cells in human duodenum and colon but not in crypt epithelial cells. Moreover, transcriptome analyses suggested that IL-8 induced GPCR signalling in Caco-2 cells and was involved in the regulation of cell differentiation but not proliferation. Previous studies have reported that IL-8 promotes wound repair in epithelial cells by stimulating cell migration. Luminal IL-8 has been measured in the colon milieu of healthy volunteers (Keshavarzian, Fusunyan et al. 1999). This result, together with the apical location of CXCR1 in human intestinal tissues and the microarray analysis of Caco-2 cells treated with IL-8, support the hypothesis that this chemokine has an autocrine function *in vivo*. Apical IL-8 could be induced *in vivo* by steady-state activation of TLRs by MAMPs of commensal bacteria and have an autocrine role on IECs. The polarized secretion of IL-8, IL-1 and IL-6 has been reported in epithelial cell lines of different tissue origin (Cudicini, Kercret et al. 1997; Holtkamp, Van Rossem et al. 1998; Sonnier, Bailey et al. 2010). A similar autocrine role has been proposed for CCL20, which is also induced by inflammatory pathways in epithelial cells. CCL20 binds to the CCR6 receptor on the apical pole of differentiated epithelial cells (Yang, Ogawa et al. 2005) and induces epithelial cell migration *in vitro* (Vongsa, Zimmerman et al. 2009).

In summary, the findings of *Chapter 2* suggest that IL-8 is secreted apically *in vivo* and that it has a role in epithelial repair. However, this hypothesis remains to be confirmed by *in vivo* studies.

Effects of the commensal F. prausnitzii on mucosal dendritic cells and T cells

Faecalibacterium prausnitzii was selected as a commensal to study in more detail due to its reduced abundance in inflammatory bowel disease (IBD) patients and reported anti-inflammatory effects (Sokol, Pigneur et al. 2008; Sokol, Seksik et al. 2009). The demonstration that *F. prausnitzii* can attenuate TNBS-induced colitis in mice highlights its potential to be exploited as a therapeutic for IBD (Sokol, Pigneur et al. 2008). However, it is necessary to understand the mechanisms involved in the suppression of inflammation and whether all strains of *F. prausnitzii* have similar immunomodulatory effects on the immune system. The latter is especially important considering the fact that the beneficial effects of probiotics in animal models and clinical trials has been shown to be strain-dependent (Rijkers, Bengmark et al. 2010). *F. prausnitzii* is one of the most abundant commensal bacteria in humans and is a strict anaerobe; this bacterium is most abundant in the colon where it metabolizes butyrate as the main end product of fermentation. Being a highly abundant member of the colonic microbiota, *F. prausnitzii* could be sampled by M cells in the follicular epithelium of ILFs and influences primary T cell responses. Additionally, anti-inflammatory components of *F. prausnitzii* could diffuse through the mucus layer and interact with epithelial cells. In this respect, Sokol et al., showed that an unidentified component of *F. prausnitzii* supernatant inhibits IL-8 secretion induced by IL-1 β in Caco-2 cells (Figure 1) (Sokol, Pigneur et al. 2008). Furthermore, colitis results in translocation of intestinal bacteria into the mucosal tissues (Waeytens, Ferdinande et al. 2008) highlighting the relevance of also studying the direct interactions of *F. prausnitzii* with antigen presenting cells and T cells in the lamina propria and the immune response in mouse colitis models.

Chapter 4, 5 and 6 focused on the effects of *F. prausnitzii* on the host immune system, in particular, on DCs and T cells *in vitro* and on mucosal T cells *in vivo*.

In Chapter 4, thirteen human commensal bacteria, including five *F. prausnitzii* strains, were tested for their immunomodulatory properties *in vitro* on human monocyte-derived DCs (hDCs). All commensal strains induced increased surface expression of the co-stimulatory molecules CD83 and CD86 but among these, *F. prausnitzii* A2-165, *Megamonas rupellensis* Mag1B and *Eubacterium hallii* L2-7 elicited distinct patterns of cytokine response in hDCs. These differential immunomodulatory effects were due to the high levels of IL-10 and low levels of IL-12 secretion induced by *F. prausnitzii* A2-165, the high levels of IL-12 and TNF- α induced by *M. rupellensis* Mag1B and the high levels of IL-10 and IL-12 induced by *E. hallii* L2-

7. *Clostridium hathewayi* 82-B and *C. xylanovorans* Lac1D induced low or no NF- κ B activation in the TLR assays and low or undetectable cytokines in hDCs despite the fact that they induced their activation and maturation; suggesting that these strains might express MAMPs that are shielded or not recognized by the TLRs tested or that they possess immunomodulatory components that inhibit activation of the NF- κ B pathway. This appears to be a unique property and may be an evolutionary adaptation to avoid immune recognition by the host as proposed for *Bacteroides fragilis* (Weintraub, Zahringer et al. 1989). Interestingly, *F. prausnitzii* A2-165 had a different immune profile compared to the other *F. prausnitzii* strains tested. The high IL-10 to IL-12 ratio induced by *F. prausnitzii* A2-165 in hDCs, was previously observed in human peripheral blood mononuclear cells (hPBMCs) and was suggested to be linked to the protective effects *in vivo* against 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis (Sokol, Pigneur et al. 2008). In Chapter 5, we showed that *F. prausnitzii* A2-165 stimulation of mouse bone marrow-derived DCs (BMDCs) enhanced CD86 and CD40 expression and induced IL-10 and TNF secretion but, in contrast with what observed in hDCs, it also induced IL-12 secretion. The induction of IL-10 secretion by hDCs in response to *F. prausnitzii* may be linked to its anti-inflammatory properties (Figure 1) because IL-10 is important for the maintenance of functional Foxp3⁺ Tregs during intestinal inflammation (Murai, Turovskaya et al. 2009). IL-10 also inhibits the production of pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-6 and IL-12 and it was shown to enhance the suppressive activity of Foxp3⁺ Tregs in the mucosa (Murai, Turovskaya et al. 2009).

The effects of BMDCs stimulated with *F. prausnitzii* A2-165 on antigen-specific CD4⁺ T cell proliferation as well the direct effect on T cells were studied *in vitro*. BMDCs stimulated with *F. prausnitzii* A2-165 induced increased proliferation of antigen-specific CD4⁺ T cells. When added directly to CD4⁺ T cells, *F. prausnitzii* A2-165 stimulated differentiation of IL-10 or IFN- γ producing T cells without affecting the number of proliferating cells. In these experiments, *C. hathewayi* 82-B was used for comparison. Despite inducing little or no cytokine secretion in hDCs or mouse BMDCs, BMDCs pre-stimulated with *C. hathewayi* 82-B appeared to be highly effective in stimulating T cells *in vitro*. Indeed, the percentage of T cells staining positive for intracellular markers of different T cell subsets (i.e. IFN- γ , IL-17 and Foxp3) were significantly higher for T cells incubated with BMDCs pre-stimulated with *C. hathewayi* 82-B than with BMDCs pre-stimulated with *F. prausnitzii* A2-165.

Commensal bacteria play an essential role in shaping T cells responses, in particular in the induction of Tregs in the colon (Kiyono, McGhee et al. 1982; Tanaka and Ishikawa 2004). Accumulation of colonic IL-10-secreting Foxp3⁺ Tregs is also induced by colonization of germ-free mice with *B. fragilis* (Round and Mazmanian 2010). Additionally, in conventional mice, administration of Bifidobacterium species has been shown to induce IL-10 producing Tregs in the colon (Jeon, Kayama et al. 2012). Thus, commensals can act on mucosal T cells at different stages during their activation, differentiation, accumulation in the LP and maintenance in the LP.

In order to gain insight into how the *in vitro* immune properties of *F. prausnitzii* A2-165 and *C. hathewayi* 82-B would translate to effects on T cell priming *in vivo*, in Chapter 5 we investigated their effects in a model for nasal tolerance induction to the antigen ovalbumin (OVA). Nasal tolerance is known to have many similarities to oral tolerance induction (Unger, Hauet-Broere et al. 2003; Samsom 2004) and application of bacteria via the nasal route effectively targets responses in the nose-draining cervical lymph nodes (CLNs). The *in vitro* immunoregulatory effects of *F. prausnitzii* A2-165, translated *in vivo* into enhanced OVA-specific T cell proliferation in the nose-draining CLNs after intranasal administration of *F. prausnitzii* A2-165 with OVA. Interestingly, this was associated with a reduction of IFN- γ ⁺ T cells in the CLNs. In contrast to *F. prausnitzii* A2-165, *C. hathewayi* 82-B significantly decreased the percentage of dividing T cells in the CLNs and spleen *in vivo*. These data show that the *in vitro* and *in vivo* outcomes can differ. *In vivo* the epithelium and other stromal cells can influence the crosstalk between signals in the lumen and the underlying immune cells. *In vivo* factors produced by enterocytes and other stromal cells such as TGF- β and retinoic acid and thymic stromal lymphopoietin (TSLP) induce tolerogenic DCs expressing CD103 and CCR7 that migrate to the draining lymph nodes and induce Foxp3⁺ Tregs (Iliev, Spadoni et al. 2009). These observations do not explain why intranasal administration of *C. hathewayi* 82-B with OVA inhibits OVA-specific T cell proliferation in the nose-draining CLNs. This might be related to effects of the bacterium on epithelium signalling or *C. hathewayi* 82-B might inhibit the migration of DCs from the mucosa to the draining lymph nodes, blocking the activation and expansion of T cells within the CLNs. Alternatively, the suppression of DC cytokine production induced by *C. hathewayi* 82-B may have a negative influence on their capacity to induce T cell priming in the CLNs. Future experiments are in progress to investigate these hypotheses *in vivo*.

In summary, our data demonstrate that *F. prausnitzii* A2-165 has immunoregulatory properties and indicate that the bacterium has a strong capacity to induce IL-10 by both DCs and T cells. Moreover, *F. prausnitzii* A2-165 administration *in vivo* induced an increase in proliferating T cells and a decrease of IFN- γ producing T cells without modifying the other T cell subsets analyzed (IL-10⁺, IL-17⁺, Foxp3⁺) suggesting that another T cell population might be affected (Figure 1).

Effects of F. prausnitzii on colitis

The protective effects of *F. prausnitzii* strain A2-165, HTF-F and the extracellular polymeric matrix (EPM) produced by strain HTF-F were explored using the dextran sodium sulphate (DSS)-induced colitis model in *Chapter 6*. *F. prausnitzii* HTF-F was selected on the basis of its distinct capacity to undergo intercellular aggregation and form a dense biofilm in liquid culture. Bacterial exopolysaccharides are common components of biofilm matrices and have been previously shown to have immunomodulatory effects (Mazmanian, Round et al. 2008; Round and Mazmanian 2010; Fanning, Hall et al. 2011). For this reason, we also characterised the immune properties of the cell-free EPM from strain HTF-F *in vitro* and compared its protective effects to *F. prausnitzii* strains A2-165 and HTF-F in DSS-induced colitis. Both strains of *F. prausnitzii* attenuated the clinical symptoms of DSS-induced colitis, decreased the disease activity index (DAI) and reduced the loss of body weight. *F. prausnitzii* HTF-F appeared to have a stronger anti-inflammatory effect than strain A2-165 because it also significantly reduced the colon histological damage score and increased colon length.

A decrease in the frequency of Bacteroidetes and Firmicutes and an increase of Proteobacteria and Actinobacteria has been observed in the faecal microbiota of inflammatory bowel disease patients (Frank, St Amand et al. 2007). Patients with active Crohn's disease and ulcerative colitis have lower faecal counts of Firmicutes, in particular lower counts of *F. prausnitzii*, compared with healthy subjects (Sokol, Seksik et al. 2009). Moreover, *F. prausnitzii* A2-165 and its culture supernatant were shown to attenuate TNBS-induced colitis in mice by daily intragastric administration prior to and during the induction of colitis (Sokol, Pigneur et al. 2008). Intraperitoneal injection of *F. prausnitzii* A2-165 also significantly improved survival of mice with TNBS-induced colitis (Sokol, Pigneur et al. 2008).

In the study of Sokol et al., the colons of mice treated with either *F. prausnitzii* or its supernatant had a reduced amount of IL-12 and an elevated amount of IL-10 compared with

the colitis control group which is compatible with the high amount of IL-10 induced by *F. prausnitzii* A2-165 *in vitro* in hPBMCs (Sokol, Pigneur et al. 2008) and our results in human and murine DCs. The TNBS colitis model is thought to resemble Crohn's disease because the resulting mucosal inflammation is mediated by a Th1 response with excessive production of IFN- γ , TNF- α and IL-12. The DSS model mimics the clinical and histological characteristics of UC (Yan, Kolachala et al. 2009); this could explain the differences between our results and that previously reported.

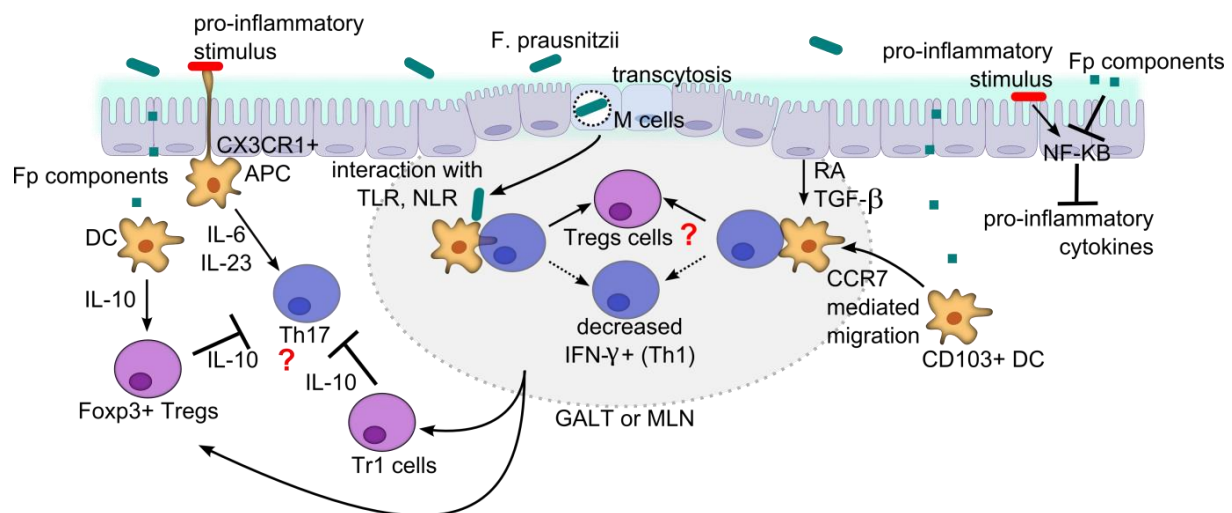


Figure 1: Proposed anti-inflammatory mechanism of *F. prausnitzii*.

The supernatant of *F. prausnitzii* blocks NF- κ B activation induced by a pro-inflammatory stimulus (Sokol, Pigneur et al. 2008). *F. prausnitzii* components might interact with CD103⁺ dendritic cells (DCs) in the lamina propria and stimulate their migration to mesenteric lymph nodes (MLN) or gut associated lymphoid tissue (GALT) and the induction of Tregs. *In vivo*, *F. prausnitzii* stimulates mucosal T cell priming to a model antigen but decreased the IFN- γ ⁺ (Th1) response through an unknown mechanism. This may occur through M cell transcytosis of *F. prausnitzii* in organized lymphoid structures (GALT or MLN) or via the migrating population of CD103⁺ DCs. The capacity of *F. prausnitzii* to induce high amounts of IL-10 in antigen presenting cells, such as DCs, may enhance the suppressive activity of Foxp3⁺ Tregs and block Th17 cells induced by pro-inflammatory stimuli.

The purified cell-bound EPM isolated from *F. prausnitzii* HTF-F contains carbohydrate and protein which are commonly involved in intercellular aggregation of biofilms (Flemming and Wingender 2010). To investigate the contribution of the EPM to the protective effects of *F. prausnitzii* HTF-F, the immunomodulatory properties of the EPM were investigated *in vitro* and *in vivo*. If added together with *L. plantarum* as an inflammatory stimulus to hDCs, the EPM decreased the production of IL-12 compared to *L. plantarum* and had no effect on IL-10, IL-1 β and TNF- α . A similar effect was seen on IL-12 production by mouse BMDCs but, in contrast to

hDCs, the EPM induced an increase in IL-10. Furthermore, the EPM had an anti-inflammatory effect in the DSS-induced colitis model and induced an increase in Foxp3⁺ T cells in MLNs suggesting that it contributes to the protective effects of *F. prausnitzii* HFT-F. Although the EPM itself did not activate DCs or induce TLR2 signalling in reporter cell lines, its effect on cytokine production *in vitro* was dependent on TLR2, suggesting an interaction with this signalling pathway. This might involve interactions via carbohydrate receptors such as C-type lectins some of which are known to modulate cytokine production in response to TLR agonists. Similarly to the EPM, polysaccharide A (PSA) from *B. fragilis* can activate T cell-dependent immune responses and confer protection against TNBS-induced colitis through a TLR2-dependent mechanism (Round and Mazmanian 2010) but in this case a direct interaction of PSA with TLR2 has been proposed.

Major conclusions and future perspectives

In conclusion, this work increased our knowledge on the role of the microbiota and its components on host immunity.

Chapter 2 demonstrated that the chemokine IL-8 can be secreted from the apical side of IECs and have an autocrine role on IECs. Moreover, we hypothesise that IL-8 might be involved in epithelial repair but this hypothesis remains to be confirmed by *in vivo studies*. In *Chapter 3* we showed that the uptake of bacteria occurs mainly in the organized lymphoid structures such as the PPs or ILFs and their presence in the MLN cells suggests an influence on mucosal T cell priming. The results presented in this chapter, are preliminary and one of our future aims will be to confirm the reported lack of mucus over the follicular epithelium of the PPs and to stain for both *L. plantarum* and DCs in tissue section of the ileum, colon and PPs.

In the studies presented in *Chapter 4 to 6*, we demonstrate that the commensal bacterium *F. prausnitzii* has immunomodulatory effects on DCs and on T cells both *in vitro* and *in vivo*. *F. prausnitzii* strain A2-165, which was previously described for its anti-inflammatory properties and for its protective effects (Sokol, Pigneur et al. 2008), induces a unique cytokine profile compared to other commensal bacteria in hDCs. This strain induced large amounts of secreted IL-10 in hDCs and mouse BMDCs and *in vivo*, it reduced the numbers of IFN- γ producing T cells but had no effects on IL-10, IL-17 or Foxp3 expression. The cytokine profiles induced by *F. prausnitzii* were strain dependent and among 5 strains tested; strain A2-165 induced the largest amount of IL-10 and the smallest amount of IL-12. Strain dependent differences in

cytokine profiles have been described previously for *L. plantarum* and linked to genetic factors such as bacteriocin production and altered TLR recognition due to enzymes modifying structures of MAMPs (Meijerink, van Hemert et al. 2010). Gene-trait matching could be used to identify genetic loci correlated with the immune response. At the moment only the genome of two *F. prausnitzii* strains is complete and only one has been annotated. Therefore, microarray-based genome composition studies are already feasible using labelled genomic DNA from different strains. Furthermore, *F. prausnitzii* strain HTF-F which produces a thick biofilm in liquid culture is protective against DSS-induced colitis and this may in part be due to the immune-regulating properties of the EPM. *F. prausnitzii* HTF-F and the EPM are interesting candidates for the treatment of IBD because they were more effective at suppressing inflammation in DSS colitis than the previously described *F. prausnitzii* strain A2-165. Although we could prove that the EPM was not contaminated with MAMPs, its composition and its level of purity are still unknown. We hypothesise that the major component of the EPM is an exopolysaccharide (EPS). EPS production is common among different bacteria and has roles in colonization, protection from bile conditions and low pH. Examples of beneficial surface-polysaccharide from commensal bacteria are the cell surface-associated EPS from *Bifidobacterium breve* which promotes the *in vivo* persistence of the bacterium (Fanning, Hall et al. 2011) and the PSA from *B. fragilis* which increases the numbers of Foxp3⁺ Tregs in MLNs and confers protection from TNBS-induced colitis in mice (Mazmanian, Round et al. 2008; Round and Mazmanian 2010). Efforts are underway to purify the EPS and determine its composition and structure so that future experiments can be performed with purified components. Once the EPS is purified it might be interesting to identify the EPS-encoding genetic loci and to create EPS deletion mutants. Presently, no methods have been described for the genetic manipulation of *F. prausnitzii* but this would open up possibilities for using genetic approaches to confirm the anti-inflammatory and protective role of the EPS.

Apart from the study on *F. prausnitzii*, our work identified other commensal bacterial strains with interesting effects on immune cells *in vitro* and *in vivo*. *C. hathewayi* 82-B for example, induced very low cytokine production in DCs despite inducing their activation *in vitro*. Furthermore, this strain significantly reduced the number of dividing antigen-specific T cells *in vivo*. *C. hathewayi* 82-B might prevent DCs migration from the periphery to the CLNs or it might affect the capacity of DCs to prime naive T cells and will be the subject of future studies.

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Summary

Samenvatting

Summary

The intestinal microbiota plays role in intestinal homeostasis via interactions with the epithelium and innate and adaptive immune mechanisms of the gut thereby profoundly shaping mammalian mucosal immunity and tolerance. However, in some diseases, such as inflammatory bowel disease (IBD), the microbiota may assume a pathologic character accentuating the damage to the host. Despite the recent advances in our understanding of the microbiota structure at different stages of life and the perturbations associated with disease, our understanding of its functional impact and the contribution of individual microbial components on health is still in its infancy. The aim of this thesis was to contribute to our knowledge and understanding of several aspects of the interactions between gut commensal bacteria and host, focusing on one of the most abundant colonic microbes, *F. prausnitzii*, for the detailed mechanistic studies.

In **Chapter 2**, we investigated whether polarized intestinal epithelial cells regulate inflammatory responses to microbes by secreting the chemokine IL-8 in a vectorial fashion depending on the location of the Toll-like receptor (TLR) stimulus. In the Caco-2 model of polarized epithelium, apical stimulation with TLR2 and TLR5 ligands resulted in the apical secretion of IL-8. The CXCR1 receptor for IL-8 was expressed only on the apical membrane of Caco-2 cells and differentiated epithelial cells in the human small intestine and colon suggesting that IL-8 has an autocrine function. Transcriptome analyses revealed that Caco-2 cells respond to stimulation with IL-8, supporting the hypothesis of autocrine signalling via CXCR1. We speculated that this mechanism might be an evolutionary adaptation to the coexistence with microbiota whereby bacterial microbe-associated molecular patterns are utilized to trigger repair mechanisms in epithelial cells.

In **Chapter 3**, mono-associated and conventional mice were used to gain new insights into the interaction of bacteria with the host mucosa and mucosal-associated lymphoid tissue. The vast majority of the bacteria are kept at a distance by the host physical and chemical barriers except for segmented filamentous bacteria which appear to have unique and specialized physical interactions with the epithelium.

Chapter 4, 5 and 6 focused on the immunoregulatory mechanisms of the human commensal *F. prausnitzii*, which has been proposed to have anti-inflammatory properties and is underrepresented in IBD patients with active disease.

In **Chapter 4**, we compared the effects of *F. prausnitzii* and other commensal bacterial strains on human monocyte derived dendritic cells (hDCs). *F. prausnitzii* strain A2-165 induced a very different cytokine profile in hDCs compared with four other *F. prausnitzii* strains and eight other commensal strains, this was due to the high levels of secreted IL-10 and low levels of IL-12.

The effects of *F. prausnitzii* A2-165 and another commensal strain on the induction of mucosal T cell responses to the model antigen ovalbumin (OVA) *in vitro* and *in vivo* were studied in **Chapter 5**. Here we demonstrated that *F. prausnitzii* A2-165 had immunoregulatory properties *in vitro* which may be related to its strong capacity to induce IL-10 by both DCs and T cells. These effects translated *in vivo* into enhanced OVA-specific T cell proliferation in the nose-draining cervical lymph nodes (CLNs) after combined nasal application of *F. prausnitzii* A2-165 and OVA. Strikingly, this was associated with a reduction in the differentiation of IFN- γ secreting T cells in the CLNs. In contrast, a different commensal, *Clostridium hathewayi* 82-B, significantly decreased the percentage of dividing OVA-specific T cells in the CLNs and spleen.

In **Chapter 6**, we investigated the immunomodulatory effects of *F. prausnitzii* strain A2-615, strain HTF-F which forms a biofilm in liquid culture and the extracellular polymeric matrix (EPM) purified from strain HTF-F. The protective capacity of the two strains and the EPM were investigated in the DSS-induced colitis model. Both *F. prausnitzii* strains attenuated the disease activity index, but the biofilm producing strain HTF-F conferred increased protection compared to strain A2-165 and this was partly due to the immunomodulatory properties of the EPM.

Chapter 7 summarizes and discusses the results of the thesis in the context of wider literature on host-microbe interactions and mucosal immunology. Furthermore, the implications of this work on intestinal health and the possible directions for future research are discussed.

Samenvatting

De darm microbiota speelt een rol in het bewaren van evenwicht in de darm via interacties met epitheel en aangeboren en adaptieve immuunsystemen in de darm, tegelijkertijd een aanzienlijke bijdrage leverend aan het tot stand komen van mucosale immuniteit en immunotolerantie in zoogdieren. Echter, in een aantal ziektes waaronder ontstekingsziekten van de darm (IBD) kan de microbiota een ziekteverwekkend karakter aannemen, onder die omstandigheden bijdragend aan schade aan de gastheer. Ondanks de recente doorbraken in ons begrip van de microbiota samenstelling gedurende verschillende levens-stadia en de verstoringen welke gepaard gaan met ziekte staat ons begrip van het functionele belang en de bijdrage van individuele microbiële componenten op gezondheid nog steeds in de kinderschoenen. Het doel van dit proefschrift was: bijdragen aan onze kennis en begrip van een aantal aspecten van de interacties tussen symbiotische bacteriën in de darm en hun gastheer, met speciale aandacht voor één van de meest voorkomende bacteriën van de dikke darm: *Faecalibacterium prausnitzii*, in gedetailleerde mechanistische studies.

In **Hoofdstuk 2** hebben we onderzocht of gepolariseerde darmepitheel cellen een ontstekingsrespons op microbes induceren door het gericht uitscheiden van de chemokine IL-8, afhankelijk van de locatie van een Toll-like receptor (TLR) prikkel. In het Caco-2 model van gepolariseerd epitheel leidde prikkelen vanaf de apicale zijde met TLR2 en TLR5 liganden tot de apicale uitscheiding van IL-8. De IL-8 receptor CXCR1 kwam alleen op het apicale membraan van Caco-2 cellen en gedifferentieerde epitheelcellen in de menselijke dunne en dikke darm tot expressie, suggererend dat IL-8 een autocriene functie zou kunnen hebben. Transcriptoom analyses lieten zien dat Caco-2 cellen reageren op prikkeling met IL-8, wat de hypothese van het bestaan van autocriene signalen via CXCR1 ondersteunde. We speculeerden dat dit mechanisme een evolutionaire aanpassing aan het samenleven met de microbiota zou kunnen zijn, waarbij bacteriële microbe-geassocieerde moleculaire patronen worden benut om weefsel-herstelmechanismen in epitheelcellen te stimuleren.

In **Hoofdstuk 3** werden muizen gekoloniseerd met 1 bacterie-stam samen met standaard gekweekte muizen gebruikt om nieuwe inzichten in het samenspel van bacteriën met de mucosa en het mucosaal-geassocieerde lymfoïde weefsel van hun gastheer te verwerven. De overgrote meerderheid van de bacteriën worden op afstand van hun gastheer gehouden

door fysieke en chemische barrières; dit gold niet voor gesegmenteerde filamenteuze bacteriën die unieke en gespecialiseerde interacties met het epitheel lijken te hebben.

Hoofdstuk 4, 5 en 6 richtten zich op immuun-regulerende processen van de humane commensale bacterie *Faecalibacterium prausnitzii*, welke verondersteld wordt ontstekingsremmende eigenschappen te hebben, en welke in lagere aantallen voorkomt in IBD patiënten met actieve ziekte.

In **Hoofdstuk 4** vergeleken we de effecten van *F. prausnitzii* en andere commensale bacteriestammen op uit monocyten verkregen dendritische cellen (hDCs) van de mens. *F. prausnitzii* stam A2-165 induceerde, als gevolg van hoge niveau's van uitgescheiden IL-10 en lage niveau's van IL-12, een sterk afwijkend cytokine profiel in hDCs in vergelijking met vier andere *F. prausnitzii* stammen en acht andere commensale stammen.

De effecten van *F. prausnitzii* A2-165 en een tweede commensale stam op de inductie van mucosale T cel responsen op het model antigeen ovalbumin (OVA) *in vitro* en *in vivo* werden in **Hoofdstuk 5** bestudeerd. We tonen hier aan dat *F. prausnitzii* A2-165 *in vitro* een aantal immuun-regulerende eigenschappen had welke gerelateerd zouden kunnen zijn aan de sterke capaciteit van deze stam om IL-10 productie door DCs en T cellen te induceren. Deze effecten vertaalden zich *in vivo* in een toegenomen OVA-specifieke T cel vermenigvuldiging in de lymfeknopen uit de nek (CLNs) die de neus draineren, na een gecombineerde toediening van *F. prausnitzii* A2-165 en OVA via de neus. Het was opmerkelijk dat dit gepaard ging met een afname van de differentiatie van gamma-interferon (IFN- γ) uitscheidende T cellen in de CLNs. In contrast hiertoe stond een andere commensale stam, *Clostridium hathewayi* 82-B, welke een significante afname van het percentage delende OVA-specifieke T cellen in de CLNs en milt bewerkstelligde.

In **Hoofdstuk 6** onderzochten we immuun-modulerende effecten van *F. prausnitzii* stam A2-615, van stam HTF-F welke een biofilm vormt in vloeibaar medium, en van de extracellulaire polymeer matrix (EPM) gezuiverd uit stam HTF-F. De beschermende capaciteit van de twee stammen en de EPM werden onderzocht in het DSS-colitis muis model. Beide *F. prausnitzii* stammen beperkten de ziekte-activiteits score, maar de biofilm-producerende stam HTF-F bewerkstelligde een verhoogde bescherming in vergelijking tot stam A2-165 en dit was gedeeltelijk het gevolg van de immuun-modulerende eigenschappen van het EPM.

Hoofdstuk 7 vat de resultaten uit dit proefschrift samen en bediscussieert ze in een brede literatuur context in het licht van gastheer- microbe interacties en mucosale immunologie.

Voorts worden de voortvloeiels van dit onderzoek op gezondheid van de darm, en de mogelijkheden voor verder onderzoek bediscussieerd.

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Oriana

About the author

Curriculum vitae

Oriana Rossi was born the 27th of May 1979 in Torino (Italy). After finishing the high school, she started her study in Industrial Biotechnology in the University of Torino where she graduated in 2005. Her MSc thesis focused on the characterization of the binding sites of the neuropeptide Urotensin II on rat cortical astrocytes. This project was performed in the Laboratory of Molecular and Cellular Neuroendocrinology of the University of Rouen (INSERM, France), in collaboration with the Animal and Human Biology Department of the University of Torino within the European project "Erasmus". After her graduation, she worked in the Laboratory of Lactic Acid Bacteria and Opportunistic Pathogens of INRA of Jouy en Josas (France) on a research project focusing on *Enterococcus faecalis* genes expressed in the mouse gastro-intestinal tract within the Marie Curie project "LabHealth" supported by the European Union. From September 2008 to March 2013, Oriana did the PhD project described in this thesis in the laboratory of Host Microbes Interactomics at Wageningen University within the Marie Curie Initial Training Network "Crosstalk".

List of publications

Rossi, O., Karczewski, J., Stolte, E.H. , Brummer, R.J.M. , van Nieuwenhoven, M.A., Meijerink, M., van Neerven, J.R.J., van Ijzendoorn, S.C.D., van Baarlen, P., Wells, J.M. Vectorial secretion of interleukin-8 mediates autocrine signalling in intestinal epithelial cells via apically located CXCR1. Submitted.

Rossi, O., Khan, M.T., Duncan, S.H., Taverne, N., Harmsen, H.J.M., Flint, H.J., Wells, J.M. Differential cytokine induction and TLR signalling by *Faecalibacterium prausnitzii* and other commensal intestinal bacteria. Submitted.

Rossi, O., Khan, M.T., Schwarzer, M., Hudcovic, T., Srutkova, D., Duncan, S.H., Stolte, E.H., Kozakova, H., Flint, H.J., Samsom, J.N., Harmsen, H.J.M., Wells, J.M. *Faecalibacterium prausnitzii* strain HTF-F and its extracellular polymeric matrix attenuate clinical parameters in DSS-induced colitis. Submitted

Rossi, O., van Baarlen, P., and Wells, J.M. (2011). Host-Recognition of Pathogens and Commensals in the Mammalian Intestine. Current topics in microbiology and immunology.

Wells, J.M., Rossi, O., Meijerink, M., and van Baarlen, P. (2010). Microbes and Health Sackler Colloquium: Epithelial crosstalk at the microbiota-mucosal interface. Proc Natl Acad Sci U S A.

Jarry, M., Diallo, M., Lecointre, C., Desrues, L., Tokay, T., Chatenet, D., Leprince, J., Rossi, O., Vaudry, H., et al. (2010). The vasoactive peptides urotensin II and urotensin II-related peptide regulate astrocyte activity through common and distinct mechanisms: involvement in cell proliferation. Biochem J 428, 113-124.

Overview of completed training activities

Discipline specific activities

Conferences and meetings

- 2nd Microbiota, Probiota and Host Conference, Seeon, Germany, 2009
- Crosstalk Kick-off meeting, Paris, France, 2009
- Yakult Symposium, Amsterdam, The Netherlands, 2009
- Innate immunity session at BCZ 2009, Wageningen, The Netherlands, 2009
- Crosstalk meeting (oral presentation), Aberdeen, United Kingdom, 2009
- Sackler Colloquium on Microbes and Health (poster), New port beach, USA, 2009
- MetaHIT International Conference (poster), Shenzhen, China, 2010
- Crosstalk meeting, Shenzhen, China, 2010
- 2nd TNO Beneficial Microbes Conference (poster), Noordwijkhout, The Netherlands, 2010
- 3rd Joint conference of the DGHM and the VAAM (poster), Hannover, Germany, 2010
- Crosstalk meeting (oral presentation), Debrecen, Hungary, 2010
- Microbes for health Conference (poster), Paris, France, 2010
- 4th Microbiota, Probiota and Host Conference (poster), Seeon, Germany, 2011
- Crosstalk workshop (poster), Lessons from host-pathogens interactions, Milan, Italy, 2011
- Crosstalk meeting (oral presentation), Wageningen, The Netherlands, 2011
- Crosstalk workshop (oral presentation), Host-microbes cross-talk: From animal models to human patients, Oslo, Norway, 2012
- Crosstalk final symposium (poster), Paris, France, 2012

Courses

- Host-microbes interactions workshop, Cagliari, Italy, 2008
- Light in the Intestinal Tract Tunnel Course, Helsinki, Finland, VLAG, 2009
- Advanced visualisation, integration and biological interpretation of ~omics data Course, Wageningen, The Netherlands, 2009
- Confocal light microscopy course, Amsterdam, The Netherlands, 2010
- Advanced Immunology Course , Utrecht, The Netherlands, 2011

General courses

- Crosstalk fall school, Aberdeen, United Kingdom, 2009
- Crosstalk fall school, Debrecen, Ungary, 2010
- Philosophy and Ethics of Food Science and Technology, VLAG, 2011
- Crosstalk fall school, Wageningen, The Netherlands, 2011
- Scientific Writing Course, Wageningen, The Netherlands, 2012

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