Interaction between the gut and its microbiota in inflammatory bowel disease
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CHAPTER 1

General introduction
Chapter 1

INTRODUCTION

The gut has a challenging task to take up fluids, nutrients but not potential pathogens. The gut harbors multiple physical (acid, mucus, tight junctions) and immune-mediated defense mechanisms in order to keep the mucosa free of pathogens. This mucosal immunity is normally in a tolerant state (armed peace) towards the own microbiome. In case of a gastrointestinal infection the immune system is activated causing an inflammation of the gut mucosa that resolves after the pathogens have been removed.

However, in some cases there is an ongoing mucosal inflammation that does not spontaneously resolve. These inflammatory bowel diseases (IBD) are characterized by a variety of symptoms that include abdominal pain, diarrhea that can be hemorrhagic, weight loss and fatigue. Crohn’s disease (CD) and ulcerative colitis (UC) are the major forms of IBD.

In UC, the inflammation is limited to the superficial mucus layer of the colon. UC has an annual incidence of 24.3 per 100,000 persons in Europe. In CD, the inflammation can be present along the whole gastrointestinal tract (from mouth to anus, predominantly the ileocecal region) and is characterized by trans-mural ulcers that can cause fistula. It has an annual incidence of 12.7 per 100,000 persons in the same region.

The incidence of IBD has increased significantly in the recent six decades in the Western industrialized countries and there is growing evidence that the incidence also increases in Eastern Europe and developing countries, such as China and India, that adapt the Western lifestyle.

IBD treatment regimens mainly include anti-inflammatory drugs, such as corticosteroids, and 5-aminosalicates (5-ASA), like mesalazine, and immunosuppressant drugs, such as thiopurines, methotrexate, tacrolimus and cyclosporine. Mesalazine, as an anti-inflammatory drug, is a first-line treatment for mild to moderate cases of UC and is recognized to be an effective drug with less side effects compared to other medication. Use of this medicine reduces oxidative stress in the intestinal tract that will effectively prolong the period of the remission in UC patients.

In the nineties, anti-inflammatory anti-TNFα biologicals, such as infliximab, adalimumab, certolizumab and golimumab, have been prescribed primarily to patients with moderate to severe CD and UC and those not responding to steroids and/or immunosuppressants. Anti-TNFα therapy is effective in up to 60-80% of the patients with CD and 30-40% of the UC patients. Just recently, biologicals that inhibit the migration of leucocytes to the mucosa, such as vedolizumab, have been added to the armamentarium.

As a last resort, removal of the colon can cure UC and a diversion of the fecal stream by ileostomy or colostomy can ameliorate CD. However, CD will always reoccur after restoration of continuity.
Introduction

Alternative treatments, such as fecal microbiota transplantation (FMT) are increasingly considered as a treatment option for IBD\textsuperscript{12}. Recent successful treatment of recurrent *Clostridium difficile* infection with FMT opened a new window for the management of IBD with such an approach. FMT restores beneficial members of the microbiota and might re-establish intestinal homeostasis, which is disturbed in IBD patients. The role of intestinal microbiota in the pathogenesis of IBD will be discussed in more detail in this thesis.

A variety of factors are involved in the pathogenesis of the IBD. These factors include genetic susceptibility genes, dysregulated immune responses of the gut-associated lymphoid tissue (GALT), dysbiosis between the intestinal microbiota and the host cells and environmental factors, like Western life style, diet, higher levels of hygiene and smoking (Figure 1)\textsuperscript{13-15}. Genome-wide association studies have linked IBD with many susceptibility loci. Mutations in these loci may cause a chain of miss-targeted immune responses against intestinal microbiota, both beneficial commensals and potentially pathogenic ones, so called pathosymbionts. The number of IBD susceptibility loci increased to 163 in recent studies, of which 110 are associated with both CD and UC\textsuperscript{16}. Previous epidemiological studies already suggested that Crohn’s disease runs within families. However, accurate heritability measures for relatively rare complex diseases, such as Crohn’s disease, were difficult. Shared environmental factors among family members with IBD confounded the conclusions about the heritability of the disease\textsuperscript{17}. Recent epidemiological studies have shown that there is a 10 to 15-fold increased risk of incidence of IBD in first-degree relatives of IBD patients. The high concordance rate in monozygotic twins of CD (30-36%) compared with UC (15-16%) indicates a stronger genetic contribution in CD compared to UC\textsuperscript{18,19}. Junjie Qin et al. described differences between gut microbial composition of healthy volunteers and IBD patients\textsuperscript{20}. They showed that the microbiota of CD patients differ significantly from those of healthy volunteers and UC patients. Interestingly, the bacterial composition of UC is much closer to the one of healthy volunteers (Figure 2). These findings suggest that changes in the bacterial composition of the gut play a more prominent role in the pathogenesis of CD than for UC and stress the importance of microbiota studies for better understanding of CD.

Several rodent studies have provided evidence for the importance of intestinal microbiota in the development of colitis. Sellon et al. showed that the resident enteric bacteria are necessary for the development of spontaneous colitis and activation of the immune system in IL-10-deficient mice. Germfree interleukin 10 (IL-10)-deficient mice did not develop colitis spontaneously, thus it seems that the normal intestinal bacterial flora causes gut pathology in these mice. In addition, Dianda et al. showed that specific bacteria are responsible for the induction of inflammation in T cell receptor (TCR)-α/β mice gut rather than the general presence of bacteria\textsuperscript{21}. Recent human studies also show a clear relation between the intestinal microbiota and the pathogenesis of CD, mainly with a decrease in
abundance and percentages of specific potentially beneficial bacteria and an increase in those that could be considered as pathosymbionts.

**Figure 1.** Interaction of various factors involved in the pathogenesis of IBD. Factors that contribute to the pathogenesis of IBD include intestinal microbiota and their antigens, which stimulate immune responses and may lead to inflammation or, in contrast, develop specific immune tolerance. Environmental factors, like Western lifestyle, play a role in the pathogenesis for instance by triggering inflammation. Genetic susceptibility increases the vulnerability in some individuals.

**Genetics**

As mentioned above, within the past decade the number of IBD susceptibility loci has gone up to 163. However, IBD is not purely a result of genetic factors, but strongly relates to the interaction between genetics, host immune system and intestinal microbes. Among the genes that are involved in the pathogenesis of CD, **ATG16L1** and **NOD2** are strong susceptibility genes that taught us a lot about how the mucosal immune system respond to bacterial invasion. **NOD2** was the first CD susceptibility gene that was identified and explains up to 20% of the total genetic predisposition of CD. **NOD2** is an intracellular sensor that triggers an immune response after binding to muramyl dipeptide, which is a peptidoglycan mainly present in the cell wall of both gram-positive but also in gram-negative bacteria. Patients homozygous for the **NOD2** risk allele have more bacteria adhered to their mucus layer. **NOD2** has recently been identified as a potent regulator of autophagy. A physical interaction of **NOD2** and Autophagy-related gene 16-like (ATG16L1) appeared to be required for autophagic clearance of intracellular pathogens. The **ATG16L1-T300A** risk variant is associated with a 2.2-fold overall increased risk for CD and is especially associated with ileal CD.
Introduction

Figure 2. The bacterial profile differentiates IBD patients and healthy individuals. Principal component analysis based on the abundance of 155 species with ≥1% genome coverage by the Illumina reads in at least 1 individual of the cohort was carried out with 14 healthy individuals and 25 IBD patients from Spain. Microbial composition of CD patients are negatively correlated with both PC1 and PC2 and are completely separated from the compositions of UC and healthy volunteers while the later ones had an overlap which is positively correlated with both PCs. Taken from Junjie Qin et al, Nature. 2010 Mar: 59-65

Autophagy is a process that allows cells to deal with damaged or malfunctioning organelles and/or proteins that ultimately leads to their degradation inside lysosomes. Over 30 different autophagy-related genes (ATG) genes/proteins play a role in this process. Autophagy is crucial for cell homeostasis and is a cell survival mechanism to face stress conditions, like nutrient deprivation and oxidative stress. In addition, autophagy is required for effective eliminating of internalized pathogens (xenophagy). Xenophagy literally means “eat foreign invaders” and is an infection-triggered process of pathogen targeting to autophagosomes. Invading microorganisms and their related components activate Toll-like receptors that recognize pathogen-associated molecular patterns. This allows autophagosomes to kill micro-organisms and present pathogen components to the innate and adaptive immune systems.

Immune system
The mucus layer of the intestinal tract is exposed to a variety of antigens from microbial, environmental and dietary sources. The mucosa and the submucosal epithelial layer form a
physical barrier against those antigens and at the same time allows the gastrointestinal tract to absorb nutrients. Moreover, this barrier needs to distinguish between harmless and harmful antigens. This discrimination is essential for maintaining tolerance or inducing immune responses towards the harmless and harmful antigens, respectively. This way, adequate mechanisms are controlling inflammation and develop tolerance against specific antigens, including bacterial ones. This allows the intestinal immune system to adjust its response against pathosymbionts that adhere to the mucus layer or invade the intracellular cavity. However, the immune system creates specific tolerance towards harmless or beneficial bacteria in or close to the mucus layer.

CD is considered to be a consequence of an impaired response of the adaptive immune system towards the host microbiota, which is predominantly derived by CD4+ T-cell activation towards microbiota\textsuperscript{33,34}.

A leaky gut is an important phenomenon in the pathogenesis of the CD. This leakage leads to increased presence of immunoglobulins in the gut lumen. These immunoglobulins can bind to specific antigens of the microbiota. A recent study showed that CD patients contain fecal bacteria that bind more IgG than those from healthy individuals after \textit{in vitro} incubation with either autologous (their own) serum or heterologous serum from patients. Furthermore, CD patients have a more intense immune response against specific \textit{E. coli} strains. Experiments with serum samples from different patients indicate that this is a bacterial strain-specific phenomenon rather than disease-specific\textsuperscript{35}.

**Microbiota**

The gastrointestinal tract (GIT) is basically sterile at birth, but its colonization by different bacterial strains begins during birth. This will develop to be the largest reservoir of bacteria in the human body that reaches numbers up to $10^{11}$ or $10^{12}$ cells/g of luminal contents in the colon\textsuperscript{36}. In comparison, the bacterial numbers in the stomach and duodenum are between 0-10$^3$ per ml of content due to the short retention time, exposure to the gastric acid with bacteriocidal activity in the stomach and bile salts in the duodenum. Bacterial numbers per ml of jejunum content is less than 10$^4$ and for the ileum is between 10$^5$-10$^8$ per ml.

\textit{Firmicutes} and \textit{Bacteroidetes} are the main bacterial phyla making up to 90% of the intestinal microbiota populations. Other bacterial divisions present in lower numbers are \textit{Actinobacteria}, \textit{Proteobacteria}, \textit{Verrucomicrobia} and \textit{Cyanobacteria}\textsuperscript{37-40}. Notwithstanding this limited variation in divisions, the human intestinal microbiota profile could be considered as an individual-specific property. This specificity is a result of the wide variability in strains and species belonging to those bacterial phyla\textsuperscript{41}. This vast amount of microbes in the colon provides important beneficial aspects for the host. These include (i) digestion of substrates that are indigestible by the host due to
Introduction

enzymatic limitations of human digestive system, (ii) providing nutrients for intestinal tract cells, (iii) stimulating and disciplining the host immune system to tolerate the beneficial bacteria and (iv) suppressing the colonization of the GIT by pathogenic microorganisms and pathosymbionts42.

Several studies have shown anti-inflammatory effects of specific members of human microbiota. Those so-called symbionts, such as faecalibacteria, bifidobacteria and lactobacililli, possibly protect the gut epithelial layer from inflammation. These protective mechanisms might include the stimulation of the anti-inflammatory IL-10,43,44 or down-regulation of inflammatory cytokines such as IL-8 and TNFα45.

*Faecalibacterium praunsnitzii*

*F. praunsnitzii* is a non-motile Gram-positive member of the Firmicutes phylum. Initially, it was named *Fusobacterium praunsnitzii* due to its negative Gram stain and morphology, however, phylogenetic studies showed that these bacteria are members of the *Clostridium* cluster IV (the *Clostridium leptum* group) rather than the *Fusobacteriaceae*46,47. *C. leptum* is the second largest bacterial group in human feces after the *Clostridium coccoides* group. *F. praunsnitzii*, as a major member of *C. leptum* group, represents generally 5% of the total bacterial counts in human fecal samples, but this number can go up to 25% in some cases, being one of the most abundant bacterial species in the human GIT48,49. Previously, Lopez-Siles *et al.* categorized human *F. praunsnitzii* in two phylogroups, namely 1 and 2 50. This was based on rRNA sequencing and physiological tests. It is not yet known if these phylogroups have the same anti-inflammatory properties and/or the same in vivo physiological features, such as SCFA production50.

*F. praunsnitzii* is an extremely oxygen sensitive member of the gut microbiota and is proposed to have anti-inflammatory properties. Importantly, it appears to be underrepresented in CD, specifically in the ileal type of the disease35,44,51. Previously it has been shown that abundance of *F. praunsnitzii* is reduced in ileal CD biopsies together with a concomitant increase in numbers of *E. coli*, specifically the pathogenic Adherent Invasive *E. coli* (AIEC) (Figure 3)51. Moreover, reduced numbers of *F. praunsnitzii* have been associated with a higher risk of earlier relapse in CD patients that underwent an operation52. This increased risk of recurrence is also correlated with the higher presence of pathosymbionts, such as fusobacteria and *E. coli*53.

*F. praunsnitzii* is one of the major butyrate producers in the human intestinal tract22,54. Intestinal microbiota metabolize dietary particles, mainly fibers, to produce short chain fatty acids (SCFAs) that include butyrate, propionate, acetate, lactate and formate, which may provide anti-inflammatory effects on intestinal epithelial layer and can be used by colonocytes as energy source. These SCFA producers are represented by *Lachnospiraceae* and *Roseburia* and their numbers are decreased in CD alongside *F. praunsnitzii*55.
Figure 3: Twin studies reveal specific imbalances in the mucosa-associated microbiota of patients with ileal Crohn’s disease. Quantitative real-time PCR for F. prausnitzii 16S rRNA gene (A) and adherent invasive E. coli uidA (B) in mucosal DNA from ileum, ascending colon, transverse colon, descending colon and rectum (displayed from left to right) from individuals with predominantly colonic Crohn’s disease (CCD), healthy controls (HC), and with predominantly ileal Crohn’s disease (ICD). Interestingly, F. prausnitzii is absent in most ICD patients, while these patients have highest percentages of E. coli while the same counterbalance is not applicable for CCD patients and healthy volunteers. Taken from Willing et al Inflammatory Bowel Diseases 2008 NOV 653-660

Butyrate

Butyrate is an important energy source for colonocytes\textsuperscript{56,57}. Beside a role in intestinal physiology, butyrate contains anti-inflammatory properties as well. A recent study has shown that SCFAs, including butyrate, promote the expansion of colonic regulatory T-cells, thus playing a role in the development of the intestinal immune system\textsuperscript{58}. Sokol et al. described the anti-inflammatory properties of F. prausnitzii, its secreted products in the medium supernatant and its main metabolite butyrate for the treatment of chemically-induced inflammation of the mouse intestinal tract\textsuperscript{52}. It appeared that butyrate inhibited the invasion of potentially pathogenic bacteria (pathosymbionts) in the intestinal epithelial layer. Moreover, it suppressed the development of malignancies\textsuperscript{56}. However, the anti-inflammatory properties of F. prausnitzii are most likely not limited to butyrate
Production alone\textsuperscript{52}. In the same study, \textit{F. prausnitzii} cell extracts and the supernatants from the growth medium provide more protection to mice suffering from chemically-induced intestinal inflammation (Figure 4). This shows the competency of \textit{F. prausnitzii} to produce several anti-inflammatory compounds. However, due to its extreme oxygen sensitivity, the possible anti-inflammatory properties of living \textit{F. prausnitzii} on gut mucosa remains elusive.

\textbf{Figure 4} Protective effects of Intraperitoneal administration of \textit{F. prausnitzii}, its supernatant, butyrate and Dexamethasone on mice after 2,4,6-trinitrobenzenesulphonic acid (TNBS) challenge. Survival rate of mice with intestinal inflammation induced by TNBS after 20 days. Figure shows that the supernatant of \textit{F. prausnitzii} culture gives 100% survival rate compared to the 50% survival rate resulted from \textit{F. prausnitzii} cells. In comparison, Dexamethasone and butyrate give lower survival rates. Taken from \textit{PNAS} 2008 Oct 16731-16736

\textbf{\textit{F. prausnitzii} in the gut microbiome.}

Given the fact that \textit{F. prausnitzii} is not the only butyrate producer in the human gut, its anti-inflammatory effects should be investigated in conjunction with other butyrate producers such as \textit{Roseburia} species, which are also dominantly present in the intestinal tract. Therefore, in addition to the bacterium-host interactions, bacterium-bacterium interaction may influence the host’s health state as well. For example, the cross-talk relation between \textit{F. prausnitzii}, as an acetate consumer and butyrate producer, and other dominant commensal gut bacteria, such as \textit{Bacteroides thetaiotaomicron} as an acetate producer, has been described recently by Wrzosek \textit{et al}. These two commensal and metabolically complementary bacteria are capable of enhancing the intestinal-epithelial layer’s integrity by modulating goblet cell differentiation and mucin glycosylation \textsuperscript{59}. Therefore, \textit{F. prausnitzii}’s anti-inflammatory properties should be studied in the microbial community, preferably a co-culture setting with living eukaryotic cells, applying an aerobic-anaerobic interface, as in the gut.
Figure 5 The host selects mucosal and luminal associations of co-evolved gut microorganisms. A novel concept hypothesizes the microbiome selection by the host using the mucus layer, innate and adaptive immune systems. The mucus layer consists of two layers, the outer one that is less firm and allows certain types of bacteria to penetrate, while the layer attached to the epithelial cells is denser in immune-related compounds and has a higher oxygen level from blood circulation. These immune system-related molecules include antimicrobial peptides (AMPs) and antibodies such as IgA. Specific adhesion capabilities of some bacterial strains, in addition to the mucin-degrading capacities, lower oxygen sensitivity and AMP resistance generates a unique mucosa-associated microbial profile. Several bacterial strains able to colonize the mucus layer (Mucosal bacteria) are particularly important as they train the immune system as symbiotic bacteria. Outgrowth of specific bacterial strains with this ability like AIEC cause inflammation. The interaction between bacteria and epithelial cells could be due to: 1) a direct cell contact between bacterial strains and intestinal epithelial cells and 2) indirect interaction through the diffusion of microorganism-associated molecular patterns (MAMPs), which is the way that luminal bacteria interact with the host. The outer layer of mucus layer, which contains diluted immune compounds and a lower amount of oxygen, might be a suitable reservoir niche for microbiota to reside in a less harsh environment. This reservoir might act as an inoculum to restore disturbed microbiota after disease conditions, such as chronic antibiotic therapy, diarrhea and colonization of mucus layer by pathosymbionts. Adopted from Van den Abbeele et al. FEMS Microbiology Reviews 2011 Jul: 681-704

The eco-physiology of F. prausnitzii
Despite the fact that F. prausnitzii is extremely oxygen-sensitive, it has the unique ability to deploy riboflavin and thiols as mediators for extracellular electron transfer (EET) to oxygen\textsuperscript{60}. In the presence of the oxygen, free thiols of cysteine or glutathione can be
oxidized to disulfide bonds and act as the electron acceptor, while flavins act as redox mediators for faecalibacteria. This phenomenon, is not applicable for other anaerobic gut bacteria or bacteria like *Pseudomonas* and *E. coli*, which are capable of producing riboflavin, but cannot use it for EET. This unique feature of *F. prausnitzii* may explain why it is able to colonize the oxic-anoxic phase close to the intestinal mucus layer, where oxygen is penetrating from the blood circulation (Figure 5)\(^61-63\).

**Phylogroups of *F. prausnitzii***

As mentioned previously, there are two different phylogroups of *F. prausnitzii* in the human intestinal tract of which cultured representatives are characterized. However, the differences between these two phylogroups regarding their localization in the GIT and the possible differences between their ability to colonize the oxic-anoxic phase of the feces are not demonstrated yet. There are still crucial questions about *F. prausnitzii* physiology to be answered. For instance, it is unclear whether *F. prausnitzii* can deploy riboflavin for shuttling electrons *in vivo*, as well as whether this electron shuttling has an effect on epithelial cells. Moreover, it is important to determine whether dietary riboflavin supplementation affects the abundance of *F. prausnitzii* in the gut. Other topics to be studied are the possible differences in the effect of two different phylogroups of *F. prausnitzii* on the intestinal epithelial layer and overall intestinal homeostasis. Furthermore, the effect of riboflavin on overall balance of microbiome *in vitro* and *in vivo* remains elusive so far.

**SCOPE OF THE THESIS**

In Chapter 2, we studied the localization of the two major phylogroups of *F. prausnitzii* in fecal samples of healthy volunteers and we show differences between the two phylogroups in their ability to grow in the oxic-anoxic zone of the intestinal tract. In addition, the ability of *F. prausnitzii*, as well as *Clostridium* group XIVa and *Roseburia*, to utilize specific food particles were analyzed.

In Chapter 3, based on the described ability of *F. prausnitzii* to deploy riboflavin for electron transfer, here the effect of daily supplementation of 100 mg of riboflavin on the abundance of *F. prausnitzii* in fecal samples of healthy volunteers was studied. Moreover, the abundance of some other major members of microbiota, specifically *Clostridium* group XIVa and *Roseburia*, as major butyrate producers was studied. CD is associated with a decrease in the abundance of beneficial *F. prausnitzii* and an increase of hostile in the *E. coli*, therefore, the possible effect of riboflavin supplementation on *E. coli* abundance and the balance between the two species was studied as well.

Different studies have been performed to investigate the anti-inflammatory properties of *F. prausnitzii* *in vivo* and *in vitro*. However the extreme oxygen sensitivity of *F. prausnitzii* is a
strong obstacle in analyzing those effects on human cells that require sufficient oxygen. Furthermore, the possible effects of host cells, specifically GIT cells, on \textit{F. prausnitzii} is fully unexplored. In \textbf{Chapter 4}, we developed a novel and simple method to study the interaction between Caco-2 cells (human intestinal epithelial cell line) in co-culture with \textit{F. prausnitzii} and analyzed the effect on both cell types, focusing potential anti-inflammatory and anti-stress effects of Caco-2 cells and growth stimulating effects of Caco-2 cells on \textit{F. prausnitzii}.

Up to now, 163 loci have been identified to play role in the pathogenesis of the IBD and an important one is the \textit{ATG16L1} gene, where the \textit{ATG16L1-T300A} is the CD risk allele and the \textit{ATG16L1-T300} is the protective allele. The possible differences between these two genotypes in the way they handle invading bacteria is unknown. Moreover, the interrelationship between the \textit{ATG16L1} genotype and the composition of mucosal microbiota has not been studied before. In \textbf{Chapter 5}, we studied the microbial composition of ileal biopsies from inflamed and non-inflamed regions of CD patients homozygous for either the \textit{ATG16L-T300} protective allele or the \textit{ATG16L-T300A} risk allele. Moreover, we determined the intracellular survival rate of adherent-invasive \textit{E. coli} (AIEC), an important bacterium in the pathogenesis of CD, in primary monocytes isolated from healthy volunteers homozygous for either \textit{ATG16L1} protective allele or risk allele.

In \textbf{Chapter 6}, we analyzed whether the humoral immune response in IBD patients is directed towards a selective group of intestinal microbiota. Specifically, we identified the bacteria to which a IgG response is directed in fecal samples from CD and UC patients.

In \textbf{Chapter 7}, we summarize our results and speculate on future perspectives of our findings.

\textbf{REFERENCES:}

Introduction

Chapter 1

Introduction


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Chapter 1