CHAPTER 1

GENERAL INTRODUCTION

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INTRODUCTION

The small and large intestines are primarily responsible for food processing and the absorption of fluids and nutrients. It does so in symbiosis with a large number and great variety of commensal bacteria that assist in digestion and outnumber the human cells 10-fold. Apart from being a digestive and absorptive organ, the gut is also an unique organ where a refined balance is maintained between food antigens, the microflora, environmental factors and immune responses that allow symbiosis with commensal bacteria (tolerance), but also effectively eliminates pathogens. The intestinal mucosa is covered by a single cell layer epithelium that has a very high turnover rate, being completely renewed every 4-6 days throughout human lifetime. To maximize absorptive efficiency, the intestinal mucosa is extensively folded consisting of villi and crypts generating a surface of 300-400 m². To cope with the highly complex and dynamic microbial load and diversity and to maintain a mutually beneficial symbiosis, the mucosal immune system is under continuous alert to restrict penetration and invasion of deleterious luminal contents. The single layer of epithelial cells is the primary barrier to prevent excessive entry of antigens into the circulation and limits inflammation to a subclinical immune response. This so called tolerance is characterized by a defined, but fragile balance between several regulatory mechanisms that closely interact with each other. A disbalance in the signaling platforms results in disruption of the epithelial barrier, allowing luminal pathogens to pass the epithelium, triggering the immune system and leading to intestinal inflammation and mucosal injury. Genetic, experimental and clinical research in the last decade has significantly deepened our understanding of the mechanisms that lead to loss of tolerance towards normal gut flora and lead to chronic and/or relapsing intestinal inflammation in patients with Inflammatory Bowel Disease (IBD).

INFLAMMATORY BOWEL DISEASE

IBD is the common denominator for two distinct pathological entities, Crohn’s disease (CD) and ulcerative colitis (UC). Both disorders are characterized by mucosal ulcers caused by a chronic inflammation of the gastrointestinal tract and both disease share several clinical features, including abdominal pain, bloody diarrhea, vomiting, nausea, weight loss, fever and fatigue. Despite these similarities, they can be classified as two distinct pathologies by their clinical characteristics and etiology. Crohn’s disease can occur anywhere in the digestive tract, from the oral cavity to the anus, but it typically involves the terminal ileum (Figure 1).
It is associated with transmural and often granulomatous inflammation in a discontinuous pattern, causing deep ulcers, abscesses, strictures and fistulas (Figure 2). Ulcerative colitis is characterized by inflammation of the mucosa in the colon, typically starting in the rectum and extending in a continuous manner upwards to one or several colonic segments (Figure 1 and 2). In addition to the gastrointestinal symptoms, there may be extra intestinal manifestations, including primary sclerosing cholangitis, arthritis, erythema nodosum, pyoderma gangrenosum, uveitis, episcleritis, pleuritis, myocarditis and ankylosing spondylitis. IBD is typically diagnosed in late childhood or early adulthood, peaking in the second and third decades of life. Approximately 10-20% of IBD patients are diagnosed during early childhood, which is then correlated with a more severe disease phenotype. Currently, there is hardly any curative therapy for IBD and the patient's best medical therapy is managing the disease to induce and maintain
remission after an acute episode and relieve the symptoms of diarrhea, abdominal pain and rectal bleeding. CD is initially treated with corticosteroids, followed by immunosuppressive therapy with thiopurines (azathioprine, 6-mercaptopurine) or methotrexate. When this is ineffective, CD is treated with antibodies against TNF-α, which neutralize TNF-α and prevents it from binding to the TNF-α receptor. In addition, these antibodies can bind to membrane-bound TNF-α, thereby inducing apoptosis in lamina propria T-lymphocytes, which suppresses the inflammatory response. Moreover, these anti-TNF-α antibodies induce regulatory macrophages that inhibit T-lymphocyte proliferation. Anti-TNF-α therapy has shown efficacy to induce and maintain remission in CD and to a lesser extent in UC. In the majority of CD patients, the disease eventually progresses from a mild inflammation to a more complicated phenotype with strictures, abscesses or fistulas, which require surgical intervention. However, CD will re-occur after surgery as soon as continuity is restored, therefore drug therapy remains necessary. Mesalazine is an important drug to induce remission in UC. If patients do not respond to mesalazine, corticosteroid therapy is started. Corticosteroids are effective in inducing remission in most patients, but is not suitable as a maintenance drug because of its side effects. Thiopurines can be added to decrease steroid use, maintain remission and prevent relapses. In contrast to CD, UC is limited to the colon and can therefore be cured by complete resection of the colon.

**Epidemiology**

Epidemiological studies have shown that IBD is predominantly a disease of the industrialized Western world, as in North America, Western Europe, Australia and Scandinavian countries (Figure 3). In these areas, incidence rates range from 0.7-9.8 cases per 100,000 people per year for CD and from 1.5-20.3 cases per 100,000 people per year for UC. The prevalence for UC is approximately 235 per 100,000 and for CD 200 per 100,000, respectively. In the Netherlands, prevalence of IBD is estimated at 90,000 and every year approximately 1,000 new CD patients and 1,500 new UC patients are diagnosed. The prevalence and incidence are much lower in underdeveloped countries, such as South America, southeast Asia and Africa, with the exception of South Africa (Figure 3). A strong north-south geographical gradient in prevalence exists, although this seems to become less pronounced in recent years, as the incidence rises rapidly in southern regions, especially in Brazil and China. The reason for this increase is unclear, but is likely related to environmental and
life style changes associated with development. This includes industrialization, better sanitation and hygiene and improved access to specialized health care\textsuperscript{17,21}. It is unlikely that the rapid changes in IBD incidence and global distribution are explainable by variations in the genetic background of affected individuals, suggesting that the external environmental is the main trigger of the disease. Racial (e.g. African Americans, Asians, Hispanics, Caucasians) and/or ethnical (e.g. Jewish vs. non-Jewish) differences in disease prevalence are most evident shown by studies in multicultural societies and studies on migrated populations\textsuperscript{22,23}. In a study performed in Singapore, for instance, the overall prevalence of UC was 6 per 100,000 in Chinese and Malaysian migrants, while the prevalence for Indian migrants was

\begin{figure}
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\includegraphics[width=\textwidth]{fig3}
\caption{Global map of Crohn's disease incidence (top) and ulcerative colitis incidence (bottom). Adapted from Ng S.C., 2013, Gut\textsuperscript{17}.}
\end{figure}
significantly higher (16.2 per 100,000)\textsuperscript{24,25}. Together with the association of IBD with other immune-mediated disorders, most notably psoriasis and ankylosing spondylitis, this may provide insight into possible etiological factors and supports the involvement of the genetic background as well as the external environment. The strongest evidence for the contribution of both genetic and environmental factors is reflected in familial aggregation and twin concordance studies. The concordance of disease is higher in monozygotic twins than in dizygotic twins, especially for CD (50\% vs. 4\% for CD and 18\% vs. 6\% for UC, respectively) and the increased risk for IBD in siblings of IBD patients support the heritability of IBD\textsuperscript{26-28}. Since it is clear that genetic contribution is not solely responsible for the risk for disease and IBD is not heritable in a Mendelian fashion, it is evident that non-genetic factors (e.g. environmental triggers) affect the development of IBD. Most likely, the environmental factors interact with genetic factors\textsuperscript{29-32}, resulting in varying susceptibility to disease and variable phenotypic presentations.

ENVIRONMENTAL FACTORS

Given the complex nature of IBD, the etiology has not yet been fully defined and may never become fully understood. Several environmental and life style factors have been associated with an increased risk for IBD, including smoking, diet, (antibiotic) drugs, appendectomy, stress, climate and (personal) hygiene. At present, a general consensus exist that IBD develops from an unfavorable interaction between the individual’s environment and its specific genetic make-up that together impair the proper host response to the gut microbiome. However, the effect of individual genetic variants discovered so far, as well as the cumulative effect of those, remain relatively small and are thought to account for only a minor proportion of disease risk, e.g. 13.6\% for CD and 7.5\% for UC\textsuperscript{29,33-36}. As depicted in Figure 4, odd ratios for the identified IBD susceptibility loci are low and range from 0.67 to 1.45. It is likely that additional genetic risk alleles for IBD will be identified in the near future, by analyzing larger cohorts and cohorts of other ethnic backgrounds. However, such new IBD susceptibility genes will probably have even smaller risk effects than the variants identified so far and thus will hardly add to the cumulative genetic risk for IBD. This implies that the “external environment” harbors the strongest risk factors for IBD, but remain ill-defined (Table 1). Important progress in this area can only be made when such life style and environmental factors are documented in detail in large patient cohorts. Large-scale epidemiological studies have attempted to identify causative environmental factors and/or provide potential mechanisms by
which these factors cause disease. Table 1 shows the current status of the association of commonly studied environmental factors, such as smoking, dietary components, hygiene and medication, with IBD. Notably, the odd ratios of the individual factors range from 0.24 to 7.80 and are higher than those found for genetic factors (Figure 4). However, it remains to be established whether such factors alone can cause IBD or that this only occurs in a genetically susceptible individual. It is therefore important to study the mutual interaction of genetic and environmental factors in the initiation and disease progression in IBD.

**Smoking**

An intriguing environmental/life style factor that is associated with IBD is cigarette smoking\(^2\). Most remarkably, smoking appears to have opposite effects on CD and UC, being detrimental for CD and protective in UC\(^{41-43}\). In an European IBD cohort it was found that only 10-15% of UC patients are current smokers compared to 25-40% of matched control subjects\(^{44-46}\). Moreover, smoking has dose-dependent positive effects on UC disease course and severity, exemplified by a reduced need for oral steroids, hospitalizations and colectomy in smokers compared to nonsmokers\(^{38,45,47-49}\). In contrast, the relative number of smokers is significant higher among patients with CD compared to matched controls\(^{37,38,50,51}\). Moreover, CD patients were more likely to
have prenatal smoke exposure or passive smoke exposure during childhood than controls\textsuperscript{50}. Smoking may also influence the CD phenotype, as a higher prevalence of ileal disease and a reduced colonic involvement in smokers has been reported\textsuperscript{50,52}. In addition, CD patients who smoke develop more extensive fistulae and strictures, indicative of a more severe disease phenotype\textsuperscript{50}.

The mechanisms underlying the differential effect of smoking in CD and UC are obscure. Tobacco smoke contains an estimated number of 5,000 different compounds, including nicotine, free radicals, acrolein and carbon monoxide. Smoking affects both systemic and mucosal immunity, altering a wide range of innate and adaptive immune functions\textsuperscript{66,53,54}. For instance, smoking alters T-cell populations and their differentiation into regulatory T-cells\textsuperscript{55-57}, modulates apoptosis (Regeling A et al. submitted) and cellular stress responses (e.g. heat shock proteins)\textsuperscript{58-62}. Further,
it significantly decreases serum and mucosal immunoglobulins levels (IgA) and pro-and anti-inflammatory cytokines, especially IL-1β and IL-846,63. Smoking also targets macrophage function as it alters their antigen presenting capacity and impairs the ability to kill internalized bacteria64,65. Furthermore, it enhances small bowel permeability and colonic mucus production66-68. Taken together, it remains to be determined how such general effects may ultimately be beneficial for one type of IBD (UC) and detrimental for the other (CD).

MICROBIOTA
The colon is inhabited by a 1,000 or more different bacterial species belonging to four main phyla, e.g. Bacteroidetes, Firmicutes and to a lesser degree Proteobacteria and Actinobacteria. The intestinal microbiome can be considered as an interface between the external environment and the host. The normal gut microbiota contribute to host metabolism and development of the immune system and protects the host from pathogenic bacterial colonization. A complex network of interactions is therefore necessary to generate either an immune response for clearance of pathogens or a response of tolerance for dietary compounds and commensal microbes. In healthy conditions, the gut microbiome exists in a state of symbiotic tolerance with its host (eubiosis) and remains relatively stable over time. However, environmental factors such as geography, economic living conditions, age, diet and lifestyle (e.g. smoking) have been shown to influence the composition of the intestinal microbiome69-71. Obviously, use of antibiotics may drastically change the microbiome composition as well30,72,73. Dysbiosis reported in IBD is likely to be a consequence of such changes in environment, resulting in an imbalance in the microbe-host relationship, which leads to mucosal barrier dysfunction and inflammatory conditions74-76. Recent years have brought new insights in alterations in the microbiome due to host-related and environmental factors. The gut microbiome in IBD patients is less diverse74,77-79, having reduced numbers of beneficial/protective bacteria (e.g. Bacteroides, Firmicutes, Lactobacilli)80,81 and enhanced numbers of pathogens (e.g. adherent invasive Escherichia coli (AIEC), Mycobacterium avium paratuberculosis sspp (MAP), Salmonella) compared to healthy controls36,82. Especially Faecalibacterium prausnitzii (F. prausnitzii) has been shown to be less abundant in the intestinal microbiota of IBD patients78,81. F. prausnitzii is a prominent butyrate-producing bacterium in the gut and is known for its anti-inflammatory properties. Butyrate serves as a major source of energy for colonic epithelial cells83 and acts as an inhibitor of pro-inflammatory cytokine production in the intestinal mucosa via suppression of NF-κB signaling84.
Moreover, butyrate reinforces the mucosal barrier by promoting the production of mucin and antimicrobial peptides, as well as by strengthening the epithelial barrier integrity through directly increasing the expression of tight junction proteins. Decreased butyrate levels could therefore promote intestinal inflammation and it is considered to be of possible therapeutic value in the treatment of IBD. Detrimental changes in intestinal microbiota, which may be caused by environmental factors, could therefore lead to alterations in gut barrier and immunity and create a microenvironment that stimulates pathogenic colonization. Alternatively, pathogenic bacteria may overactivate the immune response, which in turn affects the normal gut flora. For example, AIEC are abundantly present in CD patients, especially those with ileal disease. AIEC have type I pili and flagellae that bind CEACAM6, which is a characteristic intestinal epithelial surface protein upregulated both by the bacterium itself and by host pro-inflammatory cytokines. This upregulation leads to increased numbers of these bacteria in the lamina propria and in macrophages, in which they survive and replicate in the harsh environment of the phagolysosome. This provokes a strong pro-inflammatory immune response leading to intestinal inflammation and permeability.

The risk for IBD is not simply adding up disease-associated factors (e.g. susceptibility genes, microbiome, environmental factors, etc.), but instead, these causative factors interact closely and modify each other. For example, environmental factors cause (epigenetic) modifications, which affect signaling pathways of the immune system that are modulated by genetic variables, leading to an inadequate response to maintain intestinal homeostasis. It is therefore tempting to speculate that the host genome influences the microbiome and thereby affect inappropriate pathogenic colonization of the gut. IBD susceptibility genes that play a role in the immune system may increase the vulnerability or response to a pathogen as opposed to promoting an inaccurate response to gut commensals.

**Autophagy in IBD**

Autophagy (literally: “self-eating” in Greek) was originally described as a process of cellular self-digestion that is essential for maintaining cellular homeostasis, particularly under nutritional limitations. It is involved in recycling and degradation of cytosolic contents and damaged organelles, primarily aimed at promoting cell survival. Nowadays, it is also known to play a central role in the immune response by clearing intracellular microbes and, thus, providing resistance against infection (e.g. host response). The autophagy program begins with the formation of a membrane,
termed phagophore, which can develop from membrane components from various intracellular sources, including endoplasmic reticulum (ER), mitochondria and the plasma membrane. This process is followed by elongation and closure of the phagophore to form the autophagosome and is finalized by fusion with the lysosome, where the content is degraded. During this process, portions of the cytoplasm (micro-autophagy), protein aggregates (chaperone-mediated autophagy), complete organelles, for example mitochondria (mitophagy) or bacteria (xenophagy) are engulfed by the autophagosome for subsequent fusion with lysosomes. The degradation of proteins and organelles by the lysosomal hydrolytic enzymes provides the cell with the nutrients that are required to adapt to conditions of stress, such as starvation. Each stage of this process requires tight regulation to control autophagosome assembly and includes the sequential activation of the Beclin1/class III phosphatidylinositol 3-kinase (PI3K) complex and two ubiquitin-like protein complexes. The two ubiquitin-like systems involved in autophagy generate the ATG5/12/16 complex and promote the lipidation of microtubule-associated structures.

Figure 5. Schematic diagram of the autophagy pathway. During autophagy, cytoplasmic constituents are enclosed in an isolation membrane that is elongated mainly through the action of two ubiquitin-like conjugation systems into a double-membrane autophagosome. Autophagosomes fuse with lysosomes to form autolysosomes where the vesicle content is broken down along with the autophagosome inner membrane.
protein 1 light-chain 3 (LC3). ATG12 is first conjugated to ATG7, subsequently transferred to ATG10 and then conjugated to ATG5. The ATG5–12 complex is further stabilized by ATG16L1 and localizes to the outer membrane of the growing phagophore. This complex stimulates lipidation of LC3-I to LC3-II mediated by ATG4, ATG7 and ATG3 (Figure 5). LC3-II is a component of the phagophore membrane and is the biochemical marker for autophagosome assembly used in most studies. The essential role of autophagy in life is evident from the observation that the absence of single components of the autophagy machinery, such as ATG16L1, in mice results in death within the first day of life\textsuperscript{100,101}.

Advances in genetic testing and high-throughput technologies have paved way for genome-wide association studies (GWAS), which identified multiple single nucleotide polymorphisms (SNPs) associated with IBD. At present, the number of IBD-associated gene loci is 163, of which 110 are associated with both diseases, 30 are CD-specific and 23 are UC-specific\textsuperscript{36,102}. The strongest associations with IBD are found in genes that are involved in anti-bacterial defence systems, e.g. recognition and internalization (e.g. \textit{NOD2}) and processing and/or elimination (e.g. \textit{ATG16L1}, \textit{IRGM}). In the pre-GWAS area, nucleotide-binding oligomerization domain-containing protein 2 (NOD2) was the first gene to be identified of which specific variants increase the risk for CD\textsuperscript{103}. It encodes an intracellular pattern recognition receptor that recognizes the bacterial cell wall components peptidoglycan (PGN) and muramyl dipeptide (MDP) of Gram-negative and Gram-positive bacteria\textsuperscript{104-106}. NOD2 detects invading pathogens and plays a central role in the production of cytokines and anti-microbial peptides via NF-κB and MAP kinases\textsuperscript{107,108}. Patients carrying the \textit{NOD2} mutations exhibit an impaired immune response to microbial infections and bacterial ligands\textsuperscript{75,109}. As a result, \textit{NOD2} deficiency in mice leads to exaggerated intestinal inflammation\textsuperscript{110,111}.

One of the strongest genetic associations for CD identified by GWAS is the autophagy-related 16 like 1 (\textit{ATG16L1}) gene\textsuperscript{36,112}, which is an essential component of autophagy. The CD-associated G allele (marked by rs2241880; A=protective, G=risk) leads to an amino acid substitution at position 300 from Threonine to Alanine (T300A). The \textit{ATG16L1-T300A} risk variant is the dominant allele in the European population, having a frequency of 0.55. It confers only a two-fold increase in CD susceptibility, which indicates that gene-environmental interactions are probably required for disease initiation and/or progression\textsuperscript{112-114}.

Intestinal macrophages, monocytes, dendritic cells and granulocytes continuously sample bacteria from the gut lumen through phagocytosis to maintain tolerance
towards commensal bacteria and initiate immune responses towards pathogens. Autophagy plays a central role in the intracellular clearance of phagocytosed bacterial (xenophagy), thus, these processes are tightly linked. In response to MDP, NOD2 physically interacts with ATG16L1 at the plasma membrane, which is a crucial step in the autophagy-dependent antibacterial pathway. CD-associated mutations in NOD2 prevent complex formation and impair the effective processing of intracellular pathogens, which may alter the microbial composition in the intestine and promote survival of intracellular bacteria in the underlying tissues, leading to chronic uncontrolled intestinal inflammation. Defects in autophagy genes promote intestinal inflammation and the association of these autophagy genes with CD strongly supports the hypothesis that the inability in eliminating intracellular pathogens combined with abnormal innate immune responses contributes to the pathogenesis of this disease.

Since it was established as a CD susceptibility gene in 2008, numerous in vitro and in vivo studies have been performed to define the functional role of ATG16L1 in autophagy and its contribution to IBD. Cadwell et al. reported that an ATG16L1 hypomorphic mouse (ATG16L1(HM)) strain that expresses only 1% of the normal level of ATG16L1 show Paneth cell granule abnormalities, which are also observed in the ileal epithelium of patients with CD that are homozygous for the ATG16L1-T300A risk variant. Notably, the ATG16L1(HM) mice do not develop spontaneous colitis nor do they display enhanced susceptibility to dextran sodium sulphate (DSS)-induced colitis compared with wildtype mice. However, when the mice were infected with persistent murine norovirus (MNV) prior to the onset of DSS-induced colitis, ATG16L1(HM) mice, but not wildtype mice, showed typical pathology of human Crohn’s disease. These findings demonstrate a virus-plus-susceptibility gene interaction and support the important role of environmental factors to initiate and/or maintain CD. Additionally, autophagy is important for the clearance of AIEC and ATG16L1 deficiency enhances intracellular AIEC survival and replication, leading to an increased production of pro-inflammatory cytokines (e.g. TNF-α). This implies that ATG16L1 plays a significant role in antibacterial activity towards this organism and protection against intestinal inflammation. Taken together, these studies show that autophagy plays an important role in human inflammatory disorders by direct elimination of intracellular bacteria. As autophagy and IBD are both controlled by genetic and environmental factors, it is tempting to speculate that modulation of autophagy may hold both the origin as well as the potential therapeutic targets for IBD.
CHAPTER 1

In summary, impaired processing of gut microbes, either commensal or pathogenic, by the innate immune system is assumed to play a key role in initiating inflammation in IBD, followed by an exaggerated adaptive immune response leading to chronic inflammation of the intestinal tract. It is now of particular interest to define how the interaction between the genetic and environmental factors leads to chronic intestinal inflammations and what molecular/cellular mechanisms are involved.

OUTLINE OF THESIS

Accumulating evidence suggests that an imbalance between commensal gut microbes and host innate immune responses underlie the initiation and disease course of IBD. Factors from the environment and/or life style are likely to alter gut microbiome and initiate the disease in genetically prone individuals. The aim of this thesis is to understand how environmental factors (cigarette smoking, intestinal flora) interact with genetic susceptibility and lead to chronic intestinal inflammation. Knowledge about the interaction between the life style and genetic background is crucial to optimize personalized health care for patients with IBD.

In chapter 2, we review the importance of innate immune responses in controlling intestinal flora (with data available up to 2010). We provide an overview of the contribution of several gene products associated with Crohn’s disease on the functionality of the innate immune system, in particular autophagy. More recent data that is relevant for this topic is included in the introductory chapter.

In chapter 3, we analyzed the smoke-induced kinase signaling pathways and studied their putative role in regulation of apoptosis in intestinal epithelial cells and immune cells in order to explain why cigarette smoking aggravates CD and ameliorates UC.

In chapter 4, we extended these studies by identifying genes of which transcription is induced by cigarette smoke and that may have a protective function in UC.

In chapter 5, we determined the effect of cigarette smoke on uptake (phagocytosis) and intracellular degradation (autophagy) of CD-associated pathogens by monocytes from genetically prone individuals (homozygous for the $ATG16L1$-$T300A$ CD risk allele).

In chapter 6, we further investigated the impact of the CD-associated $ATG16L1$-$T300A$ risk variant on the microbial composition of the ileal mucosa and whether this is affected by mucosal inflammation.

In chapter 7, we summarize the results presented in this thesis and discuss their implications for improving future therapies.
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