Inflammatory Intestinal Disorders

Genetic background and consequences of gene-defects on immune activation
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Inflammatory Intestinal Disorders

Genetic background and consequences of gene-defects on immune activation

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<tbody>
<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>CARD</td>
<td>CAspase Recruitment Domain</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster of Differentiation 4</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's Disease</td>
</tr>
<tr>
<td>CeD</td>
<td>Celiacs Disease</td>
</tr>
<tr>
<td>Chr</td>
<td>Chromosome</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DLG5</td>
<td>Disk Large Homologue 5</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran Sulfate Sodium</td>
</tr>
<tr>
<td>EBI3</td>
<td>Epstein-Barr-virus-Induce gene 3</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>Gαi2</td>
<td>G alpha inhibitory 2</td>
</tr>
<tr>
<td>GI tract</td>
<td>GastroIntestinal tract</td>
</tr>
<tr>
<td>GITR</td>
<td>Glucocorticoid-Induced TNF receptor related protein</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen complex</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IC</td>
<td>Indeterminate Colitis</td>
</tr>
<tr>
<td>ICE</td>
<td>IL-1β Converting Enzyme</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>IKBKG</td>
<td>Inhibitor of Kappa light polypeptide gene enhancer in B-cells, Kinase Gamma</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor Kappa-B Kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>IL-1 Receptor Antagonist</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon-Stimulated Responsive Element</td>
</tr>
<tr>
<td>JAM</td>
<td>Junction Adhesion Molecule</td>
</tr>
<tr>
<td>JNK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>LOD</td>
<td>Logarithm of the Odds</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRS</td>
<td>Likelihood Ratio Statistics</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-Rich Repeat</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipoteicoic Acid</td>
</tr>
<tr>
<td>LT-β</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>MAGUK</td>
<td>Membrane Associated Gyanylate Kinase</td>
</tr>
<tr>
<td>MAP</td>
<td>Mycobacterium avium subspecies paratuberculosis</td>
</tr>
<tr>
<td>MD-2</td>
<td>Myeloid Differentiation-2</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl Dipeptide</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multi Drug Resistance</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MKK</td>
<td>Map Kinase Kinase</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation factor 88</td>
</tr>
<tr>
<td>MYO9B</td>
<td>Myosin IXB</td>
</tr>
<tr>
<td>MZ</td>
<td>Marginal Zone</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-Kappa-B Essential Modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor of kappa light polypeptide gene enhancer in B-cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding Oligomerization Domain</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-Steroid Anti Inflammatory Drugs</td>
</tr>
<tr>
<td>OCTN</td>
<td>Carnitine/Organic Cation Transporter</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>pANCA</td>
<td>anti-neutrophil cytoplasmic antibody</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononucleated Cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>PT</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative Trait Locus</td>
</tr>
<tr>
<td>RAC-1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RIP-2</td>
<td>Receptor-Interacting Protein-2</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>T2</td>
<td>Transitional type 2</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TDT</td>
<td>Transmission Disequilibrium Test</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIRAP</td>
<td>Toll/IL-1R domain-containing Adaptor Protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNBS</td>
<td>Tri-Nitro-Benzene Sulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>Toll-IL-1R domain-containing adaptor inducing IFN-β-related adaptor molecule</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN-beta</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>VA</td>
<td>Villous Atrophy</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
Chapter 1

1 Definition of Disease

Inflammatory Bowel Disease (IBD) is the common denominator for at least two distinct pathological entities, Crohn’s disease (CD) and ulcerative colitis (UC). Both disorders are characterized by chronic inflammation of the gastrointestinal tract and share several clinical characteristics, including abdominal pain, diarrhea, occult blood loss, fever and fatigue. Despite these similarities, they are regarded as two different diseases with their own characteristics and etiology. This can be inferred from differences in the development, localization, histological and immunological features, and complications of the disease. Therefore, both diseases will be described as two distinct pathologies. First a summary of the general clinical presentation of the diseases and their epidemiology will be given, followed by a general discussion of the environmental factors implicated in the etiology of IBD. Finally, a description of the immunological processes involved will be given, followed by a discussion of the genetic factors that are implicated in the susceptibility to and pathogenesis of intestinal inflammation.

1.1 Crohn’s Disease

Crohn’s disease was coined after the description by Burill B. Crohn, Leon Ginsberg and Gordon D. Oppenheimer in 1932 as ‘a disease of the terminal ileum, affecting mainly young adults, characterized by a sub acute or chronic necrotizing and cicatrizi ng inflammation’ \(^1\). Although Crohn gave his name to the disease, it was already described in detail some 20 years before, by T. Dalziel, as chronic interstitial enteritis \(^2\).

When patients with this disease first report to their physician, most of them suffer from abdominal pain, diarrhea, weight loss, and fatigue. Disease can manifest anywhere in the gastrointestinal (GI) tract, with predilection sites in the terminal ileum, the colon and the perianal region. Endoscopically, the tissue appears swollen, with a narrowed lumen of the bowel. Histologically, the inflammation extends throughout the entire bowel wall, affecting the mucosa and the underlying muscular and serosal layers of the intestine. The inflammation is characterized most typically by non-caseating granulomas, and so called ‘skip lesions’ in which affected parts of the intestine are segmented by healthy patches, which may result in a cobblestone aspect of the mucosa.

In a subgroup of patients, the upper gastrointestinal tract is affected. The typical non-caseating granulomas can be found in any area of the upper GI tract, from the esophagus to the duodenal bulb. Some patients even have disease manifestations in the oral cavity, and may present with a swollen upper lip or mucocutaneous lesions. In addition to the gastrointestinal symptoms, there can be extra intestinal manifestations, including inflammation of the joints, eyes, skin, and liver.

Usually, the first onset of CD is during adolescence or in early adulthood, affecting mainly people between 15 and 35 years of age. A small second incidence peak occurs in individuals in their 6\(^{th}\) or 7\(^{th}\) decade of life.

So far, there is no curative treatment for CD. Conventional treatment of the disease involves anti-inflammatory and immunosuppressive medication including aminosilicates and corticosteroids, sometimes combined with antibiotics. More severely affected patients are also being treated with immunomodulatory agents including azathioprine/6-mercaptopurine, and methotrexate. More recently,
Introduction
cytokine modulating agents, in particular anti-tumor necrosis factor (TNF)-α based treatments, have 
been added to the therapeutic arsenal (discussed in more detail in section 3.2.1.1). Although these 
therapies may effectively suppress the disease activity in the majority of patients, a substantial 
number of patients will experience flare-ups of the disease. In the more severely affected patients, 
complications like bowel obstruction, stenoses and fistulas may occur, and resection of the affected 
area may be necessary. Patients with longstanding CD have increased risk for carcinomas in the 
affected areas.

1.2 Ulcerative Colitis
Ulcerative colitis was first described as early as 1859 by Wilks as ‘the morbid appearance of the 
intestine of miss Banks’. Although publications dating as far back as the 18th century describe UC-
like illnesses, Wilks’ description led to the recognition of UC as a separate, non-infectious pathology. 
The main symptom of UC is diarrhea, which is generally bloody and may be associated with crampy 
abdominal pain. Loss of appetite and subsequent weight loss are common, as is fatigue. In cases of 
severe bleeding, anemia may also occur. The symptoms of ulcerative colitis do tend to come and go, 
with fairly long periods between flare-ups, in which patients may experience no distress at all. 
Contrary to CD, UC only involves the colon. Typically, the inflamed area starts at the level of the 
rectum. In the mildest form of UC, only the distal part of the large intestine is involved, referred to as 
proctitis. In more severe cases it can extend to the left side of the colon, or extend throughout the 
total colon as a pancolitis. The inflammation seen in UC is continuous, with no interspacing healthy 
tissue. It involves only the mucosal layer of the bowel wall, and is histologically characterized by large 
infiltrates of lymphocytes, plasma cells, and polymorphonuclear granulocytes, accompanied by 
depletion of Goblet cells and crypt hyperplasia. Endoscopic examination of the colon shows a tender 
colon, which is easily damaged, swollen and hyperemic with superficial ulcerations, which can be 
deep in the more severely affected patients. Furthermore, pseudopolyps are commonly found.
Similar to CD, UC is usually diagnosed in young adults, but can occur at any age. There is a second 
incidence peak in individuals between 50 and 60 years old, and in this group, incidence is slightly 
higher in men than in women. 
Treatment is aimed at controlling the inflammatory reaction and restoring losses of fluid, salts, and 
blood. Treatment of UC involves anti-inflammatory and immunosuppressive drugs including 
aminosilicates and corticosteroids. Treatment with azathioprine/6-mercaptopurine and cyclosporine is 
used in the more severely affected patients. If there is no improvement, surgery may become 
necessary. Since UC is restricted to the colon, complete resection of the colon will be curative, 
although disease may manifest in the rectal stump (proctitis), or in the case of ileoanal anastomosis in 
the newly formed ileoanal pouch (‘pouchitis’). 
Complications of the disease include perforation of the colon. This may be preceded by severe 
inflammation of the entire colon with accompanying weakening and ballooning of the intestinal wall, in 
which case the dilated colon becomes at risk of rupturing. Similar to Crohn’s disease, patients with UC 
have an increased risk of developing colon cancer.
1.3 Indeterminate colitis

In approximately ten to fifteen percent of patients with colitis, no definitive diagnosis of either ulcerative colitis or Crohn’s disease can be made by colonoscopy or histological examination. This type of IBD is called indeterminate colitis, after introduction of the term in 1978 by Ashley B. Price. Most cases of indeterminate colitis are characterized by fulminant colitis, a condition in which the classic features of ulcerative colitis or Crohn’s disease may be obscured by severe ulceration with early superficial fissuring ulceration, transmural lymphoid aggregates, and relative rectal sparing. Currently, it is not clear whether these patients represent a distinct entity within IBD. The ability to discern if IC is in fact a predecessor for CD or UC is specifically important in patients that need to undergo surgery in which a pouch needs to be formed, since the risk of developing pouchitis is three to four times higher in patients that prove to have CD.

2 Epidemiology

It can be stated that inflammatory bowel disease is predominantly a disease of the industrialized, Western world, especially of Northern America and Europe. Incidence rates are typically expressed as new cases per 100,000 people per year, and can be as high as 20.2 for CD and 25 for UC. Roughly, this leads to an estimated 3.2 million people that are affected by either CD or UC worldwide. Incidence rates are much lower in Asian and African countries, and even lower in Latin American countries, although here should be added that there is limited data available for these countries.

Table 1 Incidence and Prevalence rates of Crohn’s disease and Ulcerative Colitis throughout the world

<table>
<thead>
<tr>
<th>Area</th>
<th>Incidence</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD</td>
<td>UC</td>
</tr>
<tr>
<td>Europe³</td>
<td>8</td>
<td>15-24,5</td>
</tr>
<tr>
<td>Scandinavian ⁸-¹⁵</td>
<td>3,6-5,8</td>
<td>9,2-24,5</td>
</tr>
<tr>
<td>United Kingdom ¹⁶-¹⁸</td>
<td>5,6-9,8</td>
<td>13,9</td>
</tr>
<tr>
<td>Northern Europe (Netherlands, N.W France, Germany) ¹⁹, ²⁰</td>
<td>4,9-6,9</td>
<td>3,2-10,0</td>
</tr>
<tr>
<td>Southern/Central Europe (Croatia, Italy, Spain, Greece) ²¹-²⁷</td>
<td>0,7-3,4</td>
<td>1,5-9,6</td>
</tr>
<tr>
<td>North America⁷, ²⁸-³³</td>
<td>3,6-20,2</td>
<td>2,3-19,5</td>
</tr>
<tr>
<td>Asia³⁴-³⁸</td>
<td>0,5-4,2</td>
<td>1,2-6,0</td>
</tr>
<tr>
<td>Africa³⁹</td>
<td>0,3-2,6</td>
<td>0,6-5,0</td>
</tr>
<tr>
<td>Latin America⁴⁰</td>
<td>0-0,03</td>
<td>1,2-2,2</td>
</tr>
</tbody>
</table>

Incidence rates are given in number of new cases per year, per 100,000 people. Prevalence rates are given in number of known cases at a certain time point, per 100,000 people. N/D Not determined.

The highest incidence rates are found in the Scandinavian countries and the north of the North American continent, with a strong north-south geographical gradient in prevalence. This geographical gradient seems to get less pronounced over years, but is still clearly present. One could plead that this is due to mixing of genetic backgrounds between the populations of these areas, but another possibility could be that the people in the south of Europe are changing their daily habits, in such a way that they resemble more those in the north. In this respect it is noteworthy that Ashkenazi Jews...
living in Eastern Europe have a high incidence of Crohn's disease, however, the incidence rates are significantly lower in the group of Ashkenazims that has moved to Israel. This implies that there must be other than genetic factors that influence CD susceptibility.

3 Etiology
The exact etiology of IBD remains to be elucidated. So far, general consensus has come to the recognition that IBD is an ongoing and unwanted perpetuating activation of the immune system directed towards the normally non-pathogenic intestinal flora in a genetically susceptible host. The exact mechanism by which these factors come together and result in IBD however, largely remains to be determined.

In the following sections, the different etiological factors and how they fit in our current understanding of the etiology of IBD will be discussed. We will start with 'the outside world' (the environmental factors), then move on to the 'outside world inside the host' (the bacteriological factors), and finally the host factors themselves, i.e., the immunological and genetic factors.

As mentioned above, the different forms of IBD (CD, UC and IC) are illnesses that are related, but despite the fact that they share many characteristics, they also exhibit distinct features. Therefore, the etiology of these diseases could conceivably share some mechanisms, but may also differ significantly for others. For all the factors discussed below, I will first focus on the general impact on IBD, and then, when applicable, discuss how these factors are implicated in the different forms of IBD.

3.1 Environmental and exogenous factors
Virtually every environmental factor one can think of has been implied as an etiological factor for IBD. These include climate, dietary factors, (personal) hygiene and life-style habits like smoking.

The diet has been studied as an obvious environmental factor in the etiology of IBD. However, although some IBD patients find that they do not tolerate certain food-substances, and benefit from excluding these substances from their diet, there are no convincing data that any food substances would be causative for IBD, nor that certain diets would influence the cause of disease. One exception to this thesis may however be a high fat intake. Excessive intake of fat results in high levels of deoxycholate, a substance that is associated with increased risk for IBD. In addition, it was recently shown that mice fed deoxycholate develop colitis, providing some circumstantial evidence that indeed in some cases, dietary changes may be advisable.

Another environmental factor that, in epidemiological studies, has been implicated as a risk factor for IBD, in particular CD, is the use of oral contraceptives. The use of non-steroid anti-inflammatory drugs (NSAIDs) has been shown in some studies to aggravate the disease activity of IBD. The strongest exogenous risk factor for IBD known so far is smoking, although it has opposite effects on CD and UC. The risk for developing CD is twice as high in smokers vs. non-smokers. Also, the risk of relapses and need for operations is twice as high in smokers. On the contrary, the risk for developing UC is approximately 2.2 times lower in smokers as compared to non-smokers, and smoking has been associated with a positive effect on disease severity. The highest incidence of UC is found in former smokers. Why smoking exerts these effects on IBD, especially the contradicting
effects in CD versus UC, remains to be elucidated. Studies assessing the different substances in cigarette smoke have pointed towards nicotine as the substance that dampens the immune response in UC, but not in CD, however the results are inconclusive and the possibility that other substances may also contribute to the effects cannot be ruled out\textsuperscript{55-57}. In conclusion, while many environmental factors have been implicated to be of some influence on IBD pathogenesis and disease course, there are currently not enough data available to deduce generally applicable conclusions from these findings.

### 3.1.1 Microbiological factors

When thinking of the pathogenesis of IBD, one has to keep in mind that the gut lumen belongs to the milieu extérieur, which is in direct contact with the outside world. In fact, it is a highly specialized environment, that houses approximately 100 trillion (\(10^{14}\)) bacteria, 10 times more than the number of cells that make up the human body. Several of these bacteria are necessary for the breakdown of certain food substances that cannot be processed by the human digestive system, as well as for the production of certain essential amino acids and vitamins. In addition, the presence of non-pathogenic bacteria prohibits colonization with pathogenic bacteria to which the GI-tract is frequently exposed. In this respect microbiological entities are obvious candidates as causative agents in any inflammatory disease like IBD. Although the commensal intestinal flora is typically harmless, and in fact essential for a functional digestion, the symbiosis with the host is a delicate balance. An ever-growing amount of data is pointing towards this normal enteric flora as one of the key players in IBD development\textsuperscript{58}. Clinical observations of beneficial effects of antibiotics in the treatment of CD, and to a lesser extent UC, have been appreciated for years. In addition, it has been demonstrated in IBD patients that the numbers and concentrations of bacterial populations that make up the biofilms covering the epithelium is much higher than in healthy subjects\textsuperscript{59}, and loss of immune tolerance against the autologous enteric aerobic and anaerobic flora has been reported\textsuperscript{60}. More recently, the use of probiotics, primarily lactic acid bacteria, in the treatment of IBD has shown some clinical effectiveness. It is believed that probiotics beneficially affect the host by not only modulating gut microbial balance and induction of tolerance towards the non-pathogenic flora\textsuperscript{61}, but also by their ability to modulate gut permeability\textsuperscript{62, 63}. The epithelial barrier is eminently important in protecting the host from invasion of pathogenic organisms. This concept is further discussed in section 3.2.2.2. Finally, as will be more extensively discussed in Chapter 2, it has been amply shown that in virtually all animal models for IBD known so far, colitis does not develop when the animals are kept in a germ-free environment. This implies that, since these animals lack an intestinal flora, the commensal bacteria are needed to develop colitis.

### 3.1.2 Infectious agents

Several micro-organisms, including \textit{Listeria monocytogenes}, \textit{Escherichia coli (E. coli)}, \textit{Cytomegalovirus}, and \textit{Saccharomyces cerevisiae}, have been proposed in the etiology of IBD\textsuperscript{64-67}. Whether they truly do have a role in the pathogenesis of IBD remains a topic of debate, since most organisms have only been found in individual studies and have not been confirmed by others. An exception may be \textit{Mycobacterium
Introduction

There are quite some striking similarities between CD and intestinal tuberculosis in man, caused by *M. tuberculosis*. Additionally, in cattle, the closely related *Mycobacterium avium* subspecies paratuberculosis (MAP) has been shown to cause Johne’s disease, a chronic granulomatous ileitis in ruminants. These observations have led to the theory that a zoonosis could be involved in the development of CD. Indeed, MAP has been detected more frequently in CD patients than in controls by polymerase chain reaction (PCR) techniques, microscopy, and by detecting antibodies to MAP in serum, although not all studies confirm these data. Also, anti-mycobacterial treatments have proven inconclusive and disappointing. Therefore, despite the fact that there seems to be a relationship of MAP and CD, so far it remains to be determined whether MAP is really a causative agent or whether its increased prevalence in Crohn’s disease is an epiphenomenon. This concept of a pathogenic microorganism causing IBD is further corroborated by the demonstration of the ability of *E. coli* to colonize and cause inflammation and higher prevalence of adhesive *E. coli* strains in CD patients.

More recently, the role of viruses, in particular the measles virus (rubeola), as causative agents have received a fair amount of attention. It was shown in some studies that early childhood infection with the virus, or infection of the mother during pregnancy, increased the risk of developing CD. The finding of paramyxovirus-like particles in endothelial granulomas of CD patients led to the suggestion that CD is a chronic vasculitis caused by the persistence of the measles virus in the mucosa. Also, vaccination with live virus was implicated as an increased risk of developing both CD and UC. However, data on this topic so far are scarce and contradictory. In this respect it is interesting to note that the progressive decline of measles virus infection in the last decades coincides with a concomitant rise of CD during the same period.

Summarizing, it remains very difficult to either imply or rule out the role of either pathogenic or commensal micro-organisms in the etiology of IBD, especially in CD, in which MAP stays a topic of debate. So far, there is no conclusive data to prove whether the occurrence of certain microorganisms is either causative for IBD in general or in specific sub-forms of IBD, or alternatively, an epiphenomenon.

### 3.2 Host factors

So far, the discussion has focused on potential external factors that can influence the pathogenesis of IBD. Although all these factors may have their own contribution to the disease, probably the most important factor that determines whether or not IBD develops in a person, is the type and strength of response of the host to the ‘outside’ world. In essence, the immune system has a pre-set manner of responding to many different stimuli. The strength and type of this response is not only dependent on the type of stimulus, but is at least in part also determined by the genetic make-up of the host. Here, first a brief overview of the immunological aspects of IBD will be given. In subsequent sections the different approaches in finding the genes involved in IBD will be discussed followed by an overview of the genes that are currently known to be relevant for IBD susceptibility.
3.2.1 Mucosal Immunology

As already alluded to in the previous sections, CD and UC are two distinct pathologies that share several clinical characteristics, but differ substantially upon closer examination. In recent years it has become apparent that the underlying immune responses are fundamentally different in these diseases. First, a brief description of these processes in general will be provided, followed by an overview of the diseases-specific immunological phenomena.

Maybe the most striking property of the intestinal immune system is its ability to discern between the myriad of antigens present in the gut lumen. Against the potentially dangerous antigens from pathogenic organisms a sound and strong immune response is mounted, whereas under normal conditions, there is no response against harmless food antigens or the commensal flora. This process of not mounting an unwanted response against these ‘safe’ antigens that could be potentially harmful for the host, is referred to as tolerance. This process has been found to involve several different mechanisms, including anergy and deletion as well as active suppression through specialized T-cells, known as regulatory cells. An in depth description of all the processes involved is beyond the scope of this overview; for reviews, see 83, 84.

As in all immune responses the mucosal immune response in the gastrointestinal tract consists of two main pillars, the innate and adaptive immune response.

The innate immune system is comprised of cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. It is able to promptly respond to pathogens, but it does not provide long-lasting or protective immunity. Recognition of pathogens relies on the recognition of well preserved patterns, so called pathogen associated molecular patterns (PAMPs), through pattern recognition receptors (PRRs) 85. An example of an important family of these PRRs is the Toll-like Receptor (TLR) family 86, 87. There are currently 11 different mammalian TLRs known, all with their own ligand specificity and cellular expression (Table 2). These receptors are found on many effector immune cells, including macrophages, dendritic cells (DCs), and B cells. One of their modes of action is by triggering immune cells to produce chemoattractant substances to attract more effector immune cells to come to the site of infection. In addition, they also function on non-immune cells to secrete anti-microbial substances, including defensins and angiogenins produced by Paneth cells 88-90 (see also sections 3.4.2.2.a and b). The broad spectrum of ligands that is recognized by these receptors, and their widespread expression illustrates that the innate immune system provides a broad first line of defense that also involves non-immune cells, but is rather non-specific. The innate immune response is quick and general, but it does not provide long-lasting immunity, or memory to specific antigens. However, activation of the innate immunity may induce antigen presenting cells (APCs) to stimulate T cells to trigger an adaptive response, and in this way it is involved in inducing antigen specific immune responses and long-lasting memory.

The adaptive immune response is mediated by T cells, which can be divided in either cytotoxic T cells, characterized by the expression of CD8 on the cell surface, or T helper cells, characterized by the expression of CD4. T helper cells can be further divided based on their cytokine secretion patterns. In T\(\text{H}1\) type responses, APCs like DCs or macrophages express high levels of the cytokine interleukin (IL)-12 after antigen recognition. IL-12 is one of the hallmark cytokines in T\(\text{H}1\) responses and mainly functions in the stimulation of T\(\text{H}1\) lymphocytes and natural killer (NK) cells 91. These cells in turn produce the second
Introduction

Cytokines play a crucial role in the regulation of immune responses. In T helper cell (Th) responses, two main profiles are distinguished: Th1 and Th2 responses. Th1 responses are characterized by the production of interferon-gamma (IFN-γ), which in turn stimulates the production of interleukin-12 (IL-12). This positive feedback loop promotes Th1 differentiation, which is important for cell-mediated immunity against intracellular pathogens. Th1 cells activate macrophages, NK cells, and antigen-specific cytotoxic T lymphocytes, and they are also involved in inducing opsonizing antibodies by B cells. Th1 cytokines, including IFN-γ, IL-12, TNF-α, IL-6, and IL-18, are produced by cells like DCs and macrophages.

Th2 cells, on the other hand, produce cytokines like IL-4, IL-5, and IL-13, which inhibit Th1 responses. Th2 responses are crucial for the induction of humoral immunity, promoting B-cell proliferation and antibody production. These responses are mediated by cytokines such as IL-4 and IL-10, which promote Th2 differentiation and suppress Th1 responses.

During the last decade, a third subset of T cells has been identified, known as regulatory T cells (Tregs). Originally described as CD4+CD25+ T cells, Tregs express several inhibitory receptors (CTLA-4, TGF-β, GITR) and are typically found in situations that require immune suppression. Tregs are activated by TCR stimulation with self-antigens and are important in controlling the balance between Th1 and Th2 responses. They can suppress effector T cells by direct contact or by producing cytokines such as IL-10 and TGF-β.

In murine models of inflammatory bowel disease (IBD), Tregs have been shown to have a protective role. In human IBD, studies have identified differences in Treg expression and function compared to healthy subjects. For example, patients with IBD have been found to have decreased numbers of CD8+ Tregs, which may contribute to the persistence of immune responses. Patients with IBD have also been shown to have defects in their ability to generate Tregs in response to oral tolerance induction, even after immunization with keyhole limpet hemocyanin (KLH). These findings have led to further studies to understand the role of Tregs in the pathogenesis of IBD.
Table 2  Summary of known human Toll-Like Receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
<th>Origin of Ligand</th>
<th>Adapter molecules</th>
<th>Cell type</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipoproteins</td>
<td>Bacteria and mycobacteria 112, <em>Neisseria meningitidis</em> 113</td>
<td>MyD88/TIRAP</td>
<td>Myofibroblasts 114</td>
<td>Cell surface</td>
</tr>
<tr>
<td>TLR2</td>
<td>Lipoprotein/lipopeptides</td>
<td>Various pathogens 118, Gram-positive bacteria 119,120, Glycoinositolphospholipids</td>
<td>MyD88/TIRAP</td>
<td>Myofibroblasts 114, Circulating PMNs 115, 116, 117</td>
<td>Cell surface</td>
</tr>
<tr>
<td></td>
<td>Peptidoglycan</td>
<td>Gram-negative bacteria 120, Staphylococcus epidermidis 122, Trypanosoma cruzi 123</td>
<td></td>
<td>Lamina propria macrophages (low) 130</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipoteichoic acid</td>
<td>Mycobacteria 121, Treponema pallidum 124, Neisseria 125</td>
<td>MyD88/TIRAP</td>
<td>Myofibroblasts 114</td>
<td></td>
</tr>
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<td></td>
<td>Liparabinomannan</td>
<td>Gram-negative bacteria 121, Leptospira interrogans 126</td>
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<td></td>
<td>Phenol-soluble modulin</td>
<td>Gram-positive bacteria 120, Porphyromonas gingivalis 127, Fungi 128</td>
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<td></td>
<td>Glycoconjugates</td>
<td>MyD88</td>
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<td>Glycolipids</td>
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<td></td>
<td>Peptidoglycan</td>
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<td>Porins</td>
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<td>Atypical lipopolysaccharide</td>
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<td>Heat-shock protein 70*</td>
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<tr>
<td>TLR3</td>
<td>Double stranded RNA</td>
<td>Viruses 132</td>
<td>TRIF</td>
<td>DCs 133</td>
<td>Cell surface</td>
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<td></td>
<td>poly I:C</td>
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<td></td>
<td>Myofibroblasts 114</td>
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<tr>
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<td>Gram-negative bacteria 134, Respiratory syncytial virus 136, Mouse mammary-tumour virus 137, Chlamydia pneumoniae 138,139</td>
<td>MyD88/TIRAP/TRIF/TRAM</td>
<td>Lamina propria macrophages (low) 130</td>
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<tr>
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<td>Taxol</td>
<td></td>
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<td>DCs 133</td>
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<td>Envelope protein</td>
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<td>Myofibroblasts 114</td>
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<tr>
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<td>Heat-shock protein 60*</td>
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<td></td>
<td>Polysaccharide fragments of heparan sulphate*</td>
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<td></td>
<td>Fibrinogen*</td>
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<td>Flagellin</td>
<td>Bacteria 146</td>
<td>MyD88</td>
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<td></td>
<td>Endothelial cells 147</td>
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<td>Circulating PMNs 115, 131</td>
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</tr>
<tr>
<td></td>
<td>Lipoteichoic acid</td>
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<td>MyD88</td>
<td>Myofibroblasts 114</td>
<td>Intracellular</td>
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<tr>
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<td>Imidazoquinoline</td>
<td></td>
<td></td>
<td>Circulating PMNs 131</td>
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<td>Loxoribine</td>
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<tr>
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<td>TLR9</td>
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<td>Circulating PMNs 131</td>
<td>Cell surface</td>
</tr>
</tbody>
</table>

TLR, Toll Like Receptor; MyD88, myeloid differentiation factor 88; TIRAP, Toll/IL-1R domain-containing adaptor protein (also called MAL); TRIF, Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN-beta; TRAM, Toll/IL-1R domain-containing adaptor inducing IFN-beta-related adaptor molecule. *It is possible that these ligand preparations, particularly those of endogenous origin, were contaminated with lipopolysaccharide and/or other potent microbial components, so more-precise analysis is required to conclude that TLRs recognize these endogenous ligands. Adapted from S. Akira and K. Takeda and MT. Abreu, M. Fukata, and M. Arditi.
3.2.1.1 Immunological aspects of IBD

As briefly described above, the adaptive immune system can grossly be divided in Th1 type responses on the one hand, and Th2 type responses on the other. Immunologically, CD is found to be a Th1 mediated disease, in which the inflamed area extends throughout the entire bowel wall, including the muscular and serosal layers of the intestine. Typical for Th1 type responses is the high expression of IL-12 by APCs after antigen recognition, and indeed, high levels of IL-12 are produced by macrophages of CD patients, and increased amounts of activated T cells are found in affected areas in CD patients, where they produce high levels of IFN-γ and TNF-α, and decreased amount of IL-4. Also, high expression levels of the IL-12β receptors and the typical Th1 transcription factor T-bet signal transducer and activator of transcription (STAT) 4 are found, all indicative of IL-12 signaling. The most convincing evidence for the role of IL-12 has come from the observation that antibodies to IL-12 could both prevent and cure disease in an animal model of colitis, and similarly, a clinical trial in CD involving anti-IL-12 antibodies has proven its clinical applicability. However, part of these effects may be attributable to the more recently identified cytokine IL-23, which is structurally related to IL-12 as it shares the p40 subunit, but has a p19 subunit instead of p35. Similar to IL-12, it has the ability to activate Th1-cells, but appears to function also more downstream in the Th1 response, sustaining the immune response rather than starting it, and inducing Th1 memory cells more effectively than IL-12. In addition, IL-23 activates a subset of T cells characterized by IL-17 production, the so called Th17 T-cell subset, that has been found to mediate chronic inflammation and autoimmune disease in animal models. In a mouse model of mucosal inflammation (the IL-10-deficient mouse model for IBD) it was shown that inflammation was more dependent on IL-23 than IL-12, and administration of IL-23 could exacerbate colitis in these mice. Administration of anti-IL-23p19 antibodies suppressed intestinal inflammation, and in two other murine models of IBD it was found that genetically inactivating the p19 subunit inhibited production of several other pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, and IL-17, specifically at mucosal sites, rather than influencing the systemic responses. Elevated levels of IL-23 production by lamina propria APCs are also found in human CD, and administration of anti-IL-12p40 antibodies, specific for both IL-12 and IL-23 down-regulated IFN-γ, TNF-α, IL-6 and IL-17 secretion. All this data combined, it appears now that IL-23 might have an even more important, or at least more mucosal specific function, in mucosal inflammation than IL-12.

Yet another cytokine from this IL-12 family that is now being recognized to possibly play a part in the etiology of IBD is IL-27. IL-27 consists of Epstein-Barr-virus-induce gene 3 (EBI3), which is related to IL-12p40, and IL-27p28, a protein with similarities to IL-12-p35, and secreted by APCs. On the one hand IL-27 has been shown to have pro-inflammatory Th1-activating properties, similar to IL-12. It induces rapid clonal expansion of naive CD4+ T cells and triggers IFN-γ secretion, and in fact sensitizes T cells for the Th1-cell polarizing effects of IL-12, thereby directly inhibiting production of IL-4 by CD4+ T cells. Also, IL-27 can induce mast cells and monocytes to produce IL-1 and TNF-α. On the other hand IL-27 has been shown to have anti-inflammatory effects, negatively regulating mast cell and macrophage function. Mice deficient for a subunit of the IL-27 receptor, WSX-1 display elevated levels of IL-4, IL-6, TNF-α and IFN-γ, also pointing towards immunosuppressive properties of IL-27. In investigating the role and expression of IL-27 in IBD, several groups have...
identified an elevated expression of IL-27 in CD \textsuperscript{178-180}, but not in UC, in concordance with the possible T\textsubscript{h}1 role of IL-27. Although there is increasing evidence for the involvement of IL-27 in IBD, the exact contribution and mechanisms still need to be found.

Although the above combinations of the related proteins IL-12p40, EBI3 and IL-12p35, IL-23p19, IL-27p28 are all somehow implicated in CD, and are not found to be upregulated in UC, another novel cytokine that is formed by a combination of EBI3 and IL-12p35 \textsuperscript{181} is found to be upregulated in UC \textsuperscript{178, 182}. This cytokine is thought to have IL-12 antagonizing functions, thus inhibiting T\textsubscript{h}1 responses \textsuperscript{183}. The precise properties and role of this novel cytokine in IBD however are still under investigation (Figure 1).

That exactly this cytokine is found in UC, and not CD, emphasizes the fact that CD and UC are two immunological distinct pathologies. Unlike CD, UC is characterized by contiguous superficial inflammation of the colonic lamina propria, and the immunological background of UC is of a more T\textsubscript{h}2 type response. T\textsubscript{h}2 cells are more than T\textsubscript{h}1 cells equipped to promote B cell activation and antibody production.

**Figure 1** Overview of IL-12/23 family members and their receptors

- IL-12 is a covalently linked heterodimer composed of a light chain (IL-12p35) and a heavy chain (IL-12p40). The IL-12 receptor comprises IL-12R\textsubscript{β}1 and IL-12R\textsubscript{β}2, both of which have homology to gp130. The IL-12p40 component of IL-12 can also dimerize with IL-23p19 to form IL-23. The receptor for this heterodimer is formed by the association of IL-12R\textsubscript{β}1 and IL-23R. The next member of this family of cytokines is IL-27, which is composed of EBI3 (Epstein–Barr-virus-induced molecule 3) and IL-27p28. IL-27 binds a receptor composed of gp130 and WSX1 (named after the WSXWS protein motif that is found in the carboxyl terminus of many type I cytokine receptors; also known as TCCR). The last member of this family is a cytokine composed of EBI3 and IL-12p35. Currently, it has not been assigned to a receptor, and its function remains elusive, but appears to antagonize the functioning of IL-12.
This is reflected in the production of various autoantibodies, like anti-neutrophil cytoplasmic (pANCA) and anti-tropomyosin antibodies that are found in many UC patients \(^{184, 185}\). This autoantibody production however, does not seem to play a central role in UC pathogenesis, but rather seems to reflect different disease subgroups within UC. The type of subclasses of antibodies produced are characteristic for T\(_h2\) type responses, as the antibodies found in UC are mostly of the IgG1 and IgG4 subclass \(^{186}\). Furthermore, although there are also elevated levels of TNF-\(\alpha\), the key cytokines in this condition are IL-13 and IL-4 and IL-5 \(^{159, 187, 188}\).

Of special interest here is IL-13. IL-13 is a T\(_h2\) type cytokine that enhances the expression of CD23/Fc\(\varepsilon\)RII and class II major histocompatibility complex (MHC) antigens on monocytes/macrophages as well as on B cells; it stimulates B cell proliferation and it induces IgE and IgG4 class switching and production. Furthermore, it inhibits the production of proinflammatory and T\(_h1\) type cytokines including IL-1\(\alpha\), IL-6, and IL-12 by monocytes. Moreover, it synergizes with IL-2 in regulating IFN-\(\gamma\) synthesis in large granular lymphocytes \(^{189-193}\). In a mouse model for intestinal inflammation, in which colitis is induced by intrarectal administration of oxazolone it was shown that this cytokine in particular is the driving force behind the intestinal inflammation seen in these animals. NK T cells and/or IL-13 were shown to be directly cytotoxic to the epithelial cells, providing an explanation for the lesions observed in UC \(^{194}\). Even more, IL-13 is typically highly expressed in affected colonic tissue in UC \(^{187}\).

Together, these results emphasize the important role that various cytokines play in the induction and maintenance of the different forms of inflammatory bowel disease, and illustrate how the inflammations seen in CD and UC have clearly distinct cytokine profiles. This does not only reflect putative different etiologies but has also implications for the development and use of cytokine-based immunotherapy.

### 3.2.1.2 Immunotherapy

Clearly, there is an important role for T\(_h1\) and T\(_h2\) cytokines in CD and UC, respectively. Recently, cytokines are being recognized as promising therapeutic targets. The best-known example of cytokine modulating therapy in IBD is directed towards TNF-\(\alpha\). This typical pro-inflammatory cytokine is present in large amounts in CD patients, and is also found in elevated levels in UC patients \(^{159}\). The main local effects of TNF-\(\alpha\) comprise the induction of the classical signs of inflammation, heat, swelling, redness and pain. TNF-\(\alpha\) induces other pro-inflammatory cytokines including IL-1 and IL-6 and enhances leukocyte migration by inducing expression of adhesion molecules by endothelial cells and leukocytes \(^{195}\). As said, TNF-\(\alpha\) is found in elevated levels in the lamina propria of IBD patients, and has even been found in stools of pediatric CD patients \(^{196}\), making this cytokine an interesting target for therapy based on biologicals. Indeed, during the past two decades, anti-TNF-\(\alpha\) therapy has become one of the most effective and widespread used treatments for Crohn’s disease.

Currently, there are two types of anti-TNF-\(\alpha\) therapy, the one consisting of monoclonal antibodies directed against soluble TNF-\(\alpha\), and the other being a small recombinant human fusion protein consisting of the extracellular portion of the TNF receptor and IgG. After treatment, patients show a downmodulation of the activation normally attained by soluble TNF-\(\alpha\), reflected by a significant reduction in numbers of neutrophils.
and activated T cells in the lamina propria \(^{197}\). It had been shown that treatment with monoclonal antibodies induces lysis of inflammatory cells carrying membrane-bound TNF-\(\alpha\), and apoptosis in lamina propria T cells and PBMCs by activation of caspase-8, -9, and -3 \(^{198-201}\). The mechanism through which anti-TNF-\(\alpha\) treatment attains its effects, is not purely through neutralizing soluble TNF-\(\alpha\) however. This can be deducted from the differential effect of the two types of anti-TNF-\(\alpha\) treatment, since the effects of the fusion protein, which basically can only bind free soluble TNF-\(\alpha\), are less outspoken than those seen after monoclonal antibody treatment, hence, neutralizing soluble TNF-\(\alpha\) cannot be the only mode of action of the therapies \(^{202, 203}\).

At any rate, as with other anti-cytokine therapies such as anti-IL-12, or anti-IL-23 therapy, systemic administration of antibodies is a quite costly form of treatment, and can have some (severe) safety problems, mainly concerning immunogenicity directed towards the administered antibodies, leading to infusion reactions, loss of response, and serum sickness–like delayed infusion reactions. Also, more opportunistic infections are seen, mainly in patients concomitantly treated with immunosuppression \(^{204, 205}\).

### 3.2.2 Genetic aspects of IBD

There are multiple lines of evidence that suggest a genetic contribution to the pathogenesis of IBD. These include epidemiologic data on racial and ethnic differences in disease prevalence, familial aggregation and twin studies as well as the association of IBD with recognized genetic syndromes, including psoriasis, eczema, and ankylosing spondylitis. The most striking support for the contribution of both genetics and environmental factors in the pathogenesis of IBD is provided by twin concordance studies, especially for CD. Should a disease be purely genetically determined, then the concordance between monozygotic twins should be close to 100%, whereas the concordance rates for dizygotic twins should not be different from that for all siblings. However, in IBD the concordance rates for monozygotic twins are significantly less than 100%, (42% to 58% for CD, 6% to 17% for UC \(^{206, 207}\)) indicating that there is a reduced penetrance for the IBD genotype, and that this is most likely due to non-genetic factors, such as environmental triggers. From these studies it has become evident that IBD is not inherited in a purely Mendelian fashion, but rather has a complex genetic etiology, with an intricate interaction with environmental factors (for reviews, see \(^{208-210}\)). In addition, based on these findings it is questionable whether IBD represents a spectrum of a variety of diseases with entirely different etiologies rather than the two currently known clinical disease entities (UC and CD). Due to this complexity, identifying the genes involved in IBD has proven to be difficult and time consuming, and so far, only a few genes have been irrefutably implicated in the etiology of IBD.

Identifying genetic defects is only the first step in unraveling the genetic background of the pathogenic processes involved in IBD. Although our knowledge about mucosal immunology, genetics, and regulation of gene expression is growing rapidly, there are still quite a few questions about how the immunological balance in the GI tract is genetically orchestrated in the normal situation, let alone in the diseased situation. Therefore, the second step in understanding the genetic component of IBD etiology, determining the role of the found genetic defects, has proven to be very difficult.

Here, I will first give an overview of the different approaches in finding IBD susceptibility genes, and then give an overview of the genes that are now thought to be involved in IBD, and how these could affect the immune system of IBD patients.
3.2.2.1 Approaches to identify genes involved in IBD

Nowadays, quite a few different complementary approaches are being applied to identify genes involved in IBD susceptibility, both in humans and in animal models of IBD. I will first describe the types of studies that are performed in the human population, and then continue with the possibilities in animal models.

At first, candidate gene studies have been performed, based on the suspicion of a role of the gene product in IBD, and involving genes associated with immune regulation or a role in epithelial barrier function. When polymorphisms are found, the frequency of these polymorphisms can then be determined in both affected and unaffected individuals. When the frequency is significantly different in the affected group, this could indicate a causative relation between the gene and the disease susceptibility or etiology.

Later, with the development of more powerful methods of genomic screening, genetic linkage studies were used to genome-wide scan and type genetic microsatellite markers in families containing more than one affected member. If the actual disease-susceptibility gene is located close enough to the marker tested, it is less likely to be separated during meiosis due to crossing-over, and will therefore be co-inherited. This principle of cosegregation, allows for the identification of chromosomal regions shared in affected individuals in excess of statistical expectation. Testing for many markers spread throughout the genome, broad genomic regions can be identified that are shared more often by affected pairs of relatives (e.g., affected sibling pairs). This way, the areas that are involved in IBD, the susceptibility loci, can be mapped on the genome. Thus far, six major susceptibility loci (IBD1-6), have been identified

<table>
<thead>
<tr>
<th>IBD locus designation</th>
<th>Chromosomal Location</th>
<th>Study</th>
<th>Diagnosis</th>
<th>Candidate genes within or near locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBD1 16q12</td>
<td>Hugot</td>
<td>CD</td>
<td>NOD2</td>
<td></td>
</tr>
<tr>
<td>IBD2 12q13</td>
<td>Satsangi</td>
<td>UC</td>
<td>VDR, IFN-g</td>
<td></td>
</tr>
<tr>
<td>IBD3 6p13</td>
<td>Hampe</td>
<td>CD, UC</td>
<td>MHC I and II, TNF-a</td>
<td></td>
</tr>
<tr>
<td>IBD4 14q11</td>
<td>Ma, Duerr</td>
<td>CD</td>
<td>TCR alpha/beta complex, IL-3, IL-4, IL-5, IL-13,</td>
<td></td>
</tr>
<tr>
<td>IBD5 5q31–33</td>
<td>Rioux</td>
<td>CD</td>
<td>CSF-2, OCTN, CD14</td>
<td></td>
</tr>
<tr>
<td>IBD6 19p13</td>
<td>Rioux</td>
<td>CD, UC</td>
<td>ICAM-1, C3, TXA2R, LTB4H</td>
<td></td>
</tr>
<tr>
<td>Other 1p36</td>
<td>Cho</td>
<td>CD, UC</td>
<td>TNF-R family, CASP</td>
<td></td>
</tr>
<tr>
<td>Other 7q</td>
<td>Satsangi</td>
<td>CD, UC</td>
<td>MUC-3</td>
<td></td>
</tr>
<tr>
<td>Other 3p</td>
<td>Satsangi</td>
<td>CD, UC</td>
<td>HGFR, EGFR, GNA2</td>
<td></td>
</tr>
<tr>
<td>Other 10q23</td>
<td>Hampe</td>
<td>CD</td>
<td>DLG5</td>
<td></td>
</tr>
<tr>
<td>Other 4q</td>
<td>Vermeire</td>
<td>UC, CD</td>
<td>NFKB1</td>
<td></td>
</tr>
</tbody>
</table>

Adapted and updated from Bonen and Cho 2003
NOD2, nucleotide binding oligomerization domain 2; VDR, vitamin D receptor; IFN-g, interferon-gamma; TCR, T-cell receptor; IL, interleukin; CSF, cerebrospinal fluid; ICAM, intercellular adhesion molecule 1; TXA2R, thromboxane A2 receptor; LTBH4H, leukotriene B4 hydroxylase; TNF-R, tumor necrosis factor-receptor; CASP, caspase; MUC3, mucin 3; HGFR, hepatocyte growth factor; EGFR, epidermal growth factor; GNA2, inhibitory guanine nucleotide-binding protein; DLG5, discs, large homolog 5; NFKB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
Yet another possible approach to identify genes that can be applied in the search for IBD susceptibility genes includes the use of microarray analysis in which differences in mRNA expression can be compared between patients and healthy controls, leading to the identification of genes that are differentially expressed in IBD patients (for a extensive description of the technique, see review \cite{223}). Differences in expression can indicate a difference in regulation of a certain gene, or gene family, or a pathway involved which could help pinpointing genes that are involved in the susceptibility \cite{224,225}. Especially when this technique is combined with a classical genome screen, it can be a valuable tool in identifying possible candidate genes \cite{226}.

Whenever a certain gene becomes suspect of being a susceptibility gene, either because it colocalizes to a linkage region, or because its product has been implicated in disease etiology because of its function or differences in mRNA expression levels, it can be sequenced to look for polymorphisms, and the classical candidate gene approach can then be applied to see if there truly is an association of this gene with the disease susceptibility.

Recently, a new technique related to the mRNA expression microarray technique has been applied in the search for IBD susceptibility genes \cite{227}. In the so-called ‘genome-wide association study’, a microarray chip is used to simultaneously genotype over 300,000 known single nucleotide polymorphisms per sample, spread throughout the genome. The advantage of this technique over classical candidate gene approaches is that these studies do not have an \textit{a priori} assumption about where the risk variants may reside, but have the disadvantage that they are limited in their coverage of rare genetic variants, that might also have a contribution to the genetic architecture of the disease.

It has proven very difficult to identify susceptibility genes in IBD. Besides the technical difficulties of some methods, the main setbacks are the immense genetic variability within the human population, and the possibility that IBD in fact reflects a group of illnesses, instead of just two pathologies, with similar symptoms, but with possibly very different etiologies and genetic backgrounds. Also, many genes that have been implicated so far, have proven to have a very low penetrance. All this combined makes that very large sample sizes are needed to actually prove a relation with the disease susceptibility and a gene, and makes this process a very difficult and time consuming task.

Another approach in finding IBD susceptibility genes can be sought in the use of animal models. Here, I will discuss the advantages and disadvantages of using animal models over human studies.

In contrast to the human population where a large genetic heterogeneity exists, every mouse of the same inbred strain is in essence genetically identical. Interestingly, between strains there are many genetic differences, and these are responsible for differences in susceptibility to colitis, whether this colitis is induced by administering a chemical substance, or by genetically modifying the model. After generating F2 mice from a susceptible and a resistant strain these can be screened for colitis susceptibility and severity. From these crosses, the parts of the genome that cosegregate with disease susceptibility can be determined (Figure 2). With this straightforward technique, regions that cosegregate with the susceptibility or severity of the disease can be mapped on the mouse genome. These regions are called quantitative trait loci (QTLs), and typically span an area of a chromosome containing several genes. A number of studies have been performed like this, and from them, several QTLs have emerged (Table 4).

Both the human and mouse QTLs can contain many genes. In the ideal situation, the QTL is fairly small, and only contains a few genes, with one of them being the obvious candidate gene because of its function. This
gene can be sequenced to see if it contains polymorphisms that could explain why this particular gene is involved in disease susceptibility. Usually, the areas spanned by the QTLs are quite large, and sequencing an entire QTL is practically not feasible. In this situation, searching for genes in mouse models has a great advantage over human studies, because of the possibility to generate congenic mouse strains. In a congenic strain, mice from one particular strain have a small part of their genome replaced with that same part of genome from another strain. The QTL from the susceptible strain can be transferred to the resistant strain. If this results in the resistant strain now becoming susceptible, this is proof of principle that the QTL in fact does harbor a susceptibility gene. The QTL can now be made smaller by consecutive crossing of mice, until it is small enough to be directly sequenced to search for functional polymorphisms between the strains, and identify the true susceptibility gene (Figure 2). The different approaches, with their advantages and disadvantages, are summarized in Table 5.

3.2.2.2 Genes implicated in IBD susceptibility

Taken together, many genes have been implicated in the etiology of IBD, all with different levels of significance. Interestingly, these genes are involved in a wide array of functions ranging from involvement in the barrier function of the gut epithelium, receptors involved in the recognition of bacteria, to specific cytokines that activate T lymphocytes. An overview of some of these genes, in particular those that have proven their role in the etiology and/or are of relevance for the research described in this thesis are given in the following sections.

3.2.2.2.a NOD2

In 2001, the groups of Hugot  and Ogura  described 3 different mutations in the NOD2 gene, that are associated with a highly increased susceptibility to CD. This gene maps to the locus on chromosome 16 previously identified as a susceptibility locus for IBD in a genome-wide scan. Individuals homozygous for these mutations, either as the consequence of the same mutation on each chromosome, or because of the combination of 2 different mutated alleles (so-called compound heterozygosity) leads to a 20-40 fold higher chance of developing CD, whereas the risk of developing the disease was found to be approximately four times higher in individuals that are heterozygous . So far, this is the only gene found that is so strongly associated with CD.

NOD2 is a receptor for a PAMP, called muramyl dipeptide (MDP). MDP is a dipeptide that is part of peptidoglycans, which are part of the bacterial wall of both Gram-positive and Gram-negative bacteria. NOD2 has been shown to display NF-κB activating properties, after binding of its ligand MDP . NOD2 is primarily expressed in monocytic cells, dendritic cells and granulocytes.
Figure 2 Principle of genome-wide linkage analysis and generating congenics
**Table 4  Quantitative Trait Loci found in animal models for Inflammatory Bowel Diseases**

<table>
<thead>
<tr>
<th>Study</th>
<th>QTL designation</th>
<th>Chromosoma location</th>
<th>Human orthologous region</th>
<th>Candidate genes within or near QTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mahler '99</td>
<td>Dssc1</td>
<td>Chr 5, 72 cM</td>
<td>C57Bl/6J</td>
<td>Selpl, Pdgfa</td>
</tr>
<tr>
<td>3.5% DSS colitis, induced in C3H/HeJx C57BL/6J outcrosses</td>
<td>Dssc2</td>
<td>Chr 2, 47 cM</td>
<td>C57Bl/6J, C3H/HeJ</td>
<td>Itgα4, M1a, M1b, Fabp1, Egf, Nfkb1, Gbp1</td>
</tr>
<tr>
<td>Farmer'01</td>
<td>Cds1</td>
<td>3</td>
<td>C3H/HeJ</td>
<td>2q</td>
</tr>
<tr>
<td>IL-10 KO in C3H/HeJx C57BL/6J</td>
<td>Cds2</td>
<td>1</td>
<td>C3H/HeJ</td>
<td>Casp8, Cds2, Cfl4, Icos, Thbs1, B2m, M11, Pcn</td>
</tr>
<tr>
<td></td>
<td>Cds3</td>
<td>2</td>
<td>C3H/HeJ</td>
<td>15q 2q</td>
</tr>
<tr>
<td></td>
<td>Cds4</td>
<td>8</td>
<td>C57Bl/6J</td>
<td>8p</td>
</tr>
<tr>
<td></td>
<td>Cds5</td>
<td>17</td>
<td>C57Bl/6J</td>
<td>6p</td>
</tr>
<tr>
<td></td>
<td>Cds6</td>
<td>18</td>
<td>C57Bl/6J</td>
<td>18q or 5q</td>
</tr>
<tr>
<td></td>
<td>Cds7,8</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cds 9</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bourn '02</td>
<td>Tnbs1</td>
<td>94-48 cM</td>
<td>SJL/J</td>
<td>I10rα, I1α, Claudin-18</td>
</tr>
<tr>
<td>Kozaiwa '03</td>
<td>Tnbs2</td>
<td>9</td>
<td>SJL/J</td>
<td>I12p40, I0</td>
</tr>
<tr>
<td>Borm 05</td>
<td>Dbq1</td>
<td>9</td>
<td>SAMP1/YitFc</td>
<td>SAMP1/YitFc, Apc, Mad2, Mad</td>
</tr>
<tr>
<td>Sugawara '05</td>
<td>Gpdc1</td>
<td>3</td>
<td>C3H/HeN</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gpdc2</td>
<td>1 47 cM</td>
<td>C3H/HeJ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gpdc3</td>
<td>1 42 cM</td>
<td>C3H/HeJ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ibdq2</td>
<td>6</td>
<td>SAMP1/YitFc</td>
<td>Ppary</td>
</tr>
</tbody>
</table>

Selpl, P-selectin ligand; Pdgfa, platelet-derived growth factor A; Itgα4, α-4 integrin; Il, interleukin; Fabp1, fatty acid-binding protein; Egf, epithelial growth factor; Nfkb1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105); Gbp1, guanylate binding protein 1; Casp8, caspase 8; Cds2, cluster of differentiation 28, (costimulatory T cell protein); Cfl4, cytoytic T lymphocyte-associated protein 4; Icos, inducible costimulator; Thbs1, thrombospondin 1; B2m, beta-2-microglobulin; Pcn, proliferating cell nuclear antigen; Defb, defensin B; Defcr, defensin-related cryptdin peptide; Fgf1, the fibroblast growth factor receptor 1; H2-Ea, histocompatibility 2, class II antigen E alpha; C4/Slp, component of complement/sex-limited protein; Tnfa tumor necrosis factor-alpha; Apc, adenomasosis polyposis coli; Mad2, MAD (mamthers against decapentaplegic) homolog 2 (Madr2) encoding SMAD2; Mad4, MAD homolog 4 (Madr4) encoding SMAD4; Cd14, cluster of differentiation 14, lipopolysaccharide co-receptor; Pla2g2a, secretory phospholipase A2 group IIa; Ppary, Peroxisome Proliferator-Activated Receptor-gamma.

**Figure 2  Principle of genome-wide linkage analysis and generating congenics**

a. Two mouse strains with different susceptibility for colitis are crossed to generate a F1 population that is heterozygous over their entire genome. The F2 generation resulting from brother-sister mating of the F1 generation can display all possible different distributions of the parental genome, and they can develop all different grades of colitis. Because of these differences in genetic makeup and disease severity, it is possible to identify loci that are responsible for the susceptibility for colitis with linkage analysis. If at a certain point in the genome, there are no genes that are involved in the disease susceptibility, one will see a normal Mendelian ratio of parental alleles in both the susceptible and resistant groups of mice, i.e., 1:2:1. If there is a gene in the vicinity of a marker, they will cosegregate together, and the ratio of parental genome in both groups will change. In the susceptible group, more mice will be homozygous for the susceptible allele and fewer for the resistant allele, and the other way around for the resistant group, more mice will be homozygous for the resistant allele and less for the susceptible allele.

b. Once a QTL is identified, congenic mouse strains can be generated. In a congenic strain, mice from one particular strain have a small part of their genome replaced with that same part of genome from another strain. By cross-breeding the two strains, a F2 generation similar to that of the initial genome screen can be generated. The mice are genotyped, and mice that are homozygous for the desired region are used for backcrossing, generating mice that are partly homozygous for the original strain, and partly heterozygous. These F3 are intercrossed to generate mice that can be partly homozygous for the donor strain, and after genotyping, mice homozygous for the desired locus can be selected for further crossing. This procedure can be repeated until the congenic region is small enough to be sequenced, to search for polymorphisms that can explain the strain differences in susceptibility.
Table 5  Summary of advantages and disadvantages of different approaches to find susceptibility genes

<table>
<thead>
<tr>
<th>Approach</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Studies in humans</td>
<td>All mice within one strain are genetically identical</td>
<td>Genetical heterogeneity of population</td>
</tr>
<tr>
<td></td>
<td>Environmental factors are controllable</td>
<td>Heterogeneity of diseases</td>
</tr>
<tr>
<td></td>
<td>Relatively easy access to large sample numbers</td>
<td>Disturbing influence of environment factors</td>
</tr>
<tr>
<td></td>
<td>Possibility to genetically engineer models to study relevance of found genes</td>
<td>Limited availability of sufficient patients, however, due to above 3 reasons,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>large sample size is needed to attain statistical significance</td>
</tr>
<tr>
<td>Studies in murine models</td>
<td>Technically relatively simple and straightforward</td>
<td>No models available truly representing all features of either CD or UC</td>
</tr>
<tr>
<td>Candidate gene approach</td>
<td>No a priori assumptions</td>
<td>A priori assumption needed, no investigation of ‘unexpected’ genes</td>
</tr>
<tr>
<td>Genomic screening</td>
<td>No a priori assumptions</td>
<td>Very large sample sizes needed</td>
</tr>
<tr>
<td>Microarray analysis</td>
<td>Functional differences can be found</td>
<td>Read-out is genetic region, not a gene</td>
</tr>
<tr>
<td>Genome-wide association studies</td>
<td>No a priori assumptions</td>
<td>Not all genetic variation lead to difference in mRNA expression, and expression</td>
</tr>
<tr>
<td></td>
<td>Direct read-out is gene</td>
<td>difference does not necessarily be due to variation in the same gene</td>
</tr>
</tbody>
</table>

and to a lesser extent in intestinal epithelial cells, especially Paneth cells \(^{238,239}\). It is also expressed at low levels in T cells, in which expression can be upregulated after TNF-α or lipopolysaccharide (LPS) administration \(^{237,240}\).

NOD2 is a member of the family of the nucleotide-binding oligomerization domain-leucine-rich repeat (NOD-LRR) protein family, which comprises of proteins that all consist of C-terminal ligand recognition domain(s), central NOD domain(s), and N-terminal protein-protein interaction domain(s) \(^{241}\). The NOD2 protein is composed of two N-terminal caspase recruitment domains (CARD), a NOD domain, and C-terminal LRRs \(^{236}\) (Figure 3). Members of the NOD-LRR protein family are known to be involved in regulation of apoptosis \(^{242}\) and NF-κB activation \(^{243}\).

The three different mutations in the NOD2 gene that are responsible for the susceptibility for CD are found in, or close to the LRR region of NOD2. Two mutations lead to a amino acid substitution, (Arg702Trp and Gly908Arg) \(^{212}\), and the third (3020insC) leads to a frameshift mutation that results in a truncated protein, lacking the last 33 amino acids \(^{213}\) (Figure 3). The LRR is essentially important in bacterial recognition and the specific mutations found in CD have been shown to lead to a reduced capacity to induce NF-κB activation \(^{213,234}\). CD however, is characterized by an elevated NF-κB activation. So how can these contradictory findings be explained and why do mutations in NOD2 lead to CD?
NOD2 has a tripartite structure, with two caspase recruitment domains (CARD), a central nucleotide binding and oligomerization domain (NOD), and a C-terminal set of ten leucine-rich repeats (LRRs). Depicted by arrows are the three major mutations that are associated with CD.

There are currently several theories as to how these mutations are involved in disease pathogenesis. Grossly, they can be divided into two groups. The first theory is based on the proposition that CD develops as the consequence of a loss of functionality of the NOD2 protein whereas the other theory assumes that these mutations lead to a hyperactivated state of the mucosal immune system. The different possible routes how NOD2 mutations can lead to CD are discussed below.

**Defective epithelial defense**

In normal intestinal tissue, with functional NOD2, Paneth cells produce sufficient amounts of antibacterial substances, called α-defensins. Normally, the production of these antimicrobial peptides is sufficient to keep the commensal bacteria in check. Paneth cells from individuals that are defective for NOD2 however, produce less α-defensins. In mice, these antimicrobial peptides are called cryptdins, and mice that are NOD2 deficient produce less of these cryptdins, and are more susceptible to bacterial infection. Although there are many other bacterial sensing receptors in the intestine, it appears that without NOD2 function, there is a lack of sufficient antimicrobial peptides. This could lead to overgrowth of the commensal bacterial population, resulting in the colonization of the intestine by pathogenic bacteria and ultimately resulting in CD. Thus, the defects seen in epithelial defense would argue for the loss of function hypothesis.

**Altered Interleukin-1β processing**

IL-1β is a pro-inflammatory cytokine, which is primarily produced by activated macrophages and monocytes. It functions in the generation of systemic and local responses to infection, injury and immunological challenges by generating fever, activating lymphocytes and promoting infusion of leukocytes into the sites of injury or infection (for review see). IL-1β does not have a signal peptide, therefore, it cannot be excreted via the classical secretory pathway (i.e. via the endoplasmic reticulum to the Golgi). Instead, it is translated in the cytosol as an inactive precursor molecule, proIL-1β, that must be proteolytically processed to generate the active mature IL-1β. The enzyme involved in this conversion of proIL-1β to the active mature form of IL-1β is the IL-1β converting enzyme (ICE), also called caspase-1.
NOD2 can have multiple roles in the production and excretion of IL-1β. Firstly, NOD2 can activate the serine/threonine kinase receptor-interacting protein (RIP)2/RICK/CARDIAK pathway, leading to NF-κB activation and subsequent transcription of IL-1β. Secondly, NOD2 can interact directly with ICE, thereby promoting conversion of the proIL-1β to functional IL-1β.

Thus, NOD2 appears to be an activator of IL-1β production and excretion. Indeed, it has been shown in studies using human peripheral blood mononucleated cells (PBMCs) that NOD2 deficiency leads to a decreased IL-1β excretion in homozygous NOD2 deficient patients. This effect appears to be mainly due to a defect in the processing of proIL-1β, since the transcription of mRNA in NOD2 homozygous deficient PBMCs was shown to be upregulated after MDP stimulation, comparable to controls, but the excretion of functional IL-1β was diminished. These results would, like the defects in epithelial defense, argue for the loss-of-function hypothesis.

On the other hand, in a study using knock in mice with NOD2 deficiencies, the opposite was found, with an enhanced IL-1β secretion in mice homozygous for the disease related mutations. In this study, it was shown that only the mutant form of NOD2 could activate the IL-1β converting enzyme ICE, thus arguing for a gain of function mutation.

These contradictory results can be explained by the differences in experimental design in human and mouse models, but can also relate to the different cell types studied. In addition, the process of generating genetically modified mice could theoretically have induced another mutation that could be responsible for the observations. Also, the differential effects could be due to the fact that some defects are related to a knock-out versus a knock-in design. By the use of a knock-in design altering the LRR region of the gene, this leaves the capability of the NOD regions to oligo-dimerize with other NOD/NLR family member molecules. When the entire NOD2 is knocked-out, this process is completely ablated. Thus, in the first (knock-in murine model) functional ability to suppress through interaction of other NOD/NLR may be retained, and in fact gain of functionality of CARD-CARD interactions, resulting in an activation of the IL-1β pathway. In the NOD2 knock-out model, this will not occur. Also, the in vitro studies with human cells might not fully represent the in vivo situation, and one should be careful in translating these findings to general statements on pathogenic mechanisms. Finally, there is also the possibility that NOD2 does not function the same in humans and mice.

Altered interactions with Toll-like Receptor signaling

As mentioned above, NOD2 is an intracellular receptor for PAMPs. However, the most well known large family of PAMP receptors is probably the TLR family. These receptors recognize a wide variety of conserved microbial structures, and are thus essential in pathogen recognition by the innate immune system. Binding of the appropriate ligand to a TLR triggers the host cells to elicit an immune response mediated through NF-κB dependent activation of inflammatory gene transcription (Figure 4).

Thus, both TLR signaling and NOD2 signaling lead to NF-κB activation. Theoretically, simultaneous stimulation through TLRs and NOD2 should lead to an enhanced NF-κB activation, and loss of NOD2 signaling would result in a diminished NF-κB response. Indeed, in human PBMCs, it has been shown that
NOD2 activation acts synergistically with TLR activation, leading to a stronger immune response, and this potentiating effect is lost in cells from CD patients with mutations in \textit{NOD2}\textsuperscript{234, 254-261}. However, recently it has been implicated by Watanabe \textit{et al.}\textsuperscript{262} that NOD2 activation leads to an inhibition of specifically TLR2 signaling in mice. In the absence of functional NOD2, these mice produce substantially larger amounts of specifically IL-12 after stimulation with the TLR2 ligand peptidoglycan (PGN)\textsuperscript{263}. As stated above, MDP is a derivative of PGN\textsuperscript{263}, so that both receptors intrinsically recognize the same bacterial substance, which could provide an explanation as to why NOD2 interacts with specifically this TLR. \textit{Nod2}\textsuperscript{-/-} mice adoptively transferred with OVA specific CD4\textsuperscript{+} T cells display a profoundly elevated IL-12 production after infection with an OVA expressing \textit{E.Coli} strain, and the colitis seen in these mice can be abrogated by anti-IL-12p40 monoclonal antibodies, or introducing a TLR2 deficiency in these mice\textsuperscript{264}. Extrapolating these findings to the human situation, it can be envisaged that when there is a lack of efficient dampening of the TLR2 response, followed by a subsequently high production of IL-12, this

**Figure 4** \textit{Toll-like receptor signaling}

Depicted are the various signaling pathways by which the Toll-like receptors (TLRs) lead to gene activation. TLR 1, 2, 4, 5, and 6 are located on the cellular plasma membrane, whereas TLR 3, 7, and 9 are situated intracellularly. IKK: Inhibitor of nuclear factor kappa-B kinase; MD-2: Myeloid Differentiation-2; MyD88: myeloid differentiation factor 88; NEMO: NF-Kappa-B Essential Modulator; NF-kB: Nuclear factor kappa B Nuclear Factor of kappa light polypeptide gene; NOD: Nucleotide-binding Oligomerization Domain; RIP-2: Receptor-Interacting Protein-2; STAT: Signal Transducer and Activator of Transcription; TRAP: Toll/IL-1R domain-containing adaptor protein; TLR: Toll Like Receptor; TRAF: TNF receptor-associated factor; TRAM: Toll-IL-1R domain-containing adaptor inducing IFN-beta-related adaptor molecule; TRIF: Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN-beta.
could conceivably lead to an unwanted strong immune response. This argues for the loss of function hypothesis, whether it is the loss of efficient TLR2 dampening, leading to a higher IL-12 production, or the lack of enhancing other TLR responses. As with the contradictory results seen in IL-1β processing, these discrepancies between human and mouse studies can be explained by the differences in experimental design in human and mouse models, the design of the genetic manipulations in the murine models, the different cell types studied, the differences in type of mutations, and the possibility that in vitro studies do not fully reflect the in vivo situation, or that NOD2 does not function the same in humans and mice.

Since it is now well established that NOD2 signaling leads to NF-κB activation, it might seem contradictory that after abrogation of fully functional NOD2 signaling, the NF-κB activation is in fact found to be elevated in CD patients. One explanation could be derived from the data from Watanabe et al. \(^{262}\), that NOD2 does not only have an activating effect, but more importantly, functions as a negative regulator of another immune activating process. Thus, cells lacking this dampening effect get hyperactivated, and show a heightened NF-κB activation. Another explanation could be that because of the NOD2 deficiency, the immune response is somewhat belated or less strong, giving pathogens an opportunity to infiltrate. Due to other signaling processes than through NOD2, the inflammation and the subsequent immune response are stronger, and thus higher levels of NF-κB activation are seen.

The above-described theories on how mutations in NOD2 could be affecting the immune response in intestinal inflammation are summarized in Figure 5.

### 3.2.2.2.b Genes involved in epithelial homeostasis

In keeping intestinal homeostasis, the barrier function of the bowel wall is clearly of great importance. The permeability of the epithelial lining is normally tightly regulated, controlling what substances and cells are allowed to cross this barrier.

Defects in epithelial barrier function have been found in both CD and UC \(^{265-269}\) and particularly in patients with CD, an increased intestinal permeability has been postulated as an early predisposing factor for the pathogenesis \(^{270}\). Here, I will discuss the genes that could have an effect on the intestinal homeostasis and that have so far been implicated in IBD susceptibility.

**Epithelial permeability**

**Claudins**

The tight junctions are the main regulators of the epithelial permeability. They are the most apically located intercellular connections and are complexes of proteins that are linked to the actin cytoskeleton. The transmembrane proteins of the tight junctions include occludin \(^{271, 272}\), junction adhesion molecule (JAM) \(^{273}\), and members of the claudin-family \(^{274}\).

Recently, a significant amount of attention is drawn towards the claudin-family. These proteins are the most important proteins in the tight junction complexes contributing most to barrier function \(^{275, 276}\), and alteration of claudin expression has been shown to directly influence the strength and functioning of the tight junctions \(^{277}\). Indeed, accumulating data is provided that claudin expression is altered in CD.
For example, the expression of the pore-forming claudin-2 was found to be increased in CD, and expression of the sealing claudins, claudin-3, -5 and –8, was reduced and redistributed. In our lab, we have recently gained additional evidence for the role of these proteins in an animal model of intestinal inflammation. In a search for genetic risk factors we performed a genome-wide screen in the trinitrobenzene sulfonic acid (TNBS) colitis model. In this screen, we identified a susceptibility locus on chromosome 9. In a subsequent microarray analysis in which we determined the expression of genes on this locus in diseased versus healthy mice we found that Claudin-18 maps to this locus and was significantly upregulated in inflamed colons. These findings point to a genetic difference between colitis susceptible and resistant mice in the upregulation of this tight junction molecule. The relevance for human disease is under current investigation.

Myosin IXB

*Myosin IXB* (*MYO9B*) is a gene that was first found to be of influence in the susceptibility to celiac disease, a disorder of the small bowel that is caused by an intolerance to gluten. Recently, this gene has also been implicated in IBD susceptibility, with a slightly stronger association found in UC.

**Figure 5** Schematic view of how NOD2 can be involved in intestinal inflammation

Depicted are various routes in which NOD2 signaling is involved and through which it could be involved in inflammatory processes leading to Crohn’s disease. For explanation of these routes, see text. MDP: muramyl dipeptide; NOD2: nucleotide binding and oligomerization domain 2; TLR: Toll-like receptor; PAMP: Pathogen Associated Molecular Pattern.
At present it is not known how the genetic variants that are found influence the cellular mechanisms involving MYO9B. MYO9B is expressed most abundantly in cells from the myeloid lineage, but, amongst others, also in intestinal and epithelial cell lines. MYO9B is a member of the myosin superfamily. The gene encodes a single headed motor protein, belonging to the class of IX myosin molecules. These are unique compared to other classes, because they contain a Rho-GTPase-activating domain within their tails. Rho-family GTPases are involved in remodeling of the cytoskeleton and tight junction assembly, both of which result in enhanced epithelial paracellular permeability. Alterations in MYO9B could therefore, through altered Rho-dependent signaling, lead to a perturbation of the epithelial barrier function. Also, MYO9B is also shown to be involved in the control of bacterial invasion of epithelial cells, and activated Rho proteins are found to be involved in healing of human intestinal epithelial wounds.

Further studies are necessary to determine how MYO9B variants influence the susceptibility to IBD, but the fact that this gene is found to be involved in both forms of IBD and celiacs disease, with typical different areas of involvement, points to the notion that certain genes can predispose to a shared general mechanisms underlying intestinal inflammatory disorders. Depending on the combination with other host specific genetic factors, this mechanism can then result in different disease outcome.

**Disc Large Homologue-5**

Disk large homologue 5 (DLG5) is recently implicated as a novel gene involved in IBD etiology. DLG5 is a member of the MAGUKs (Membrane Associated Gyanylate Kinase) family which mediates intracellular signaling, and is involved in maintaining epithelial integrity. A diminished functioning of DLG5 caused by variations in its gene could cause a less strong epithelial barrier, adding to the pathogenesis of IBD. The association found with these variations in the initial study however, was not always confirmed by other studies, rendering DLG5 involvement a topic of debate. Although the association with IBD is not always found, the risk variants of DLG5 do seem to be involved in the phenotypic expression of the disease, especially in perianal and penetrating CD, and being refractory to corticosteroid treatment.

Although DLG5 does not seem to be associated with IBD in many studies, when combined with NOD2 status, the frequency of the risk variations in DLG5 is higher in patients with NOD2 deficiencies. Also, recently it has been shown that the association with DLG5 variation and IBD is mainly found in the male gender. This gender dependence may explain the divergent association findings related to DLG5 and CD, as the gender composition of both the CD and the control cohort influences whether the risk variant is associated with IBD.

**IL-13 and Epithelial Growth Factor**

Next to differences in tight junctions, the increased permeability of the epithelium can also be caused by increased apoptosis of epithelial cells. Especially in UC, in which the number of apoptotic epithelial cells has been reported to be as high as 28% in fulminant disease, apoptosis of the epithelium could be a major factor increasing gut permeability.

As said before, IL-13 is a cytokine that is typically highly expressed in affected colonic tissue in UC. Furthermore, it was recently shown that IL-13 impairs epithelial barrier function by increasing...
epithelial apoptosis, increasing the expression of the pore-forming tight junction protein claudin-2, and decreasing rate of new cell formation in vitro as well as in vivo in UC patients. These functional properties of IL-13, combined with the notion that the gene for this cytokine resides in the IBD5 locus, indicates that alterations in this gene could contribute to the etiology of UC.

Epithelial growth factor (EGF) and EGF receptor are genes that have been implicated in the etiology of IBD both by their location in human and murine susceptibility loci and by their obvious role in maintaining gut integrity. Lack of sufficient properly functional EGF, or its receptor could conceivably aggravate this effect, by not providing enough stimulus for the epithelium to regenerate, as seen in mice lacking the EGF receptor which show an increased susceptibility to acute dextran sulfate-induced colitis. Although to date, no functional polymorphisms in the above genes have been found that could indicate a direct causative relation for these genes in the etiology of IBD, therapy directed against IL-13 or enhancing EGF could prove to be beneficial for some UC patients. Enhancing of EGF functioning has already been shown to be helpful in some UC patients. Although depleting IL-13 producing NK T cells has proven to block inflammation in a murine model for colitis, so far, no clinical data are available for anti-IL-13 treatment in UC patients. Still, anti-IL-13 treatment has proven to be effective in asthma treatment, which is also a Th2 mediated disease with many immunological similarities to UC.

**Bacteriocidal functions of the epithelium**

**Defensins**

In addition to providing a solid boundary for pathogens, the cells of the epithelium also secrete antibacterial substances. The main cell type involved in this is the Paneth cell, a specialized epithelial cell located mainly in the crypts of the small intestine, in close proximity to epithelial stem cells. Among the antibacterial substances are lysozyme, an antimicrobial enzyme that dissolves the cell walls of many bacteria, type II phospholipase A2, an enzyme specialized in the lysis of bacterial phospholipids, angiogenins, that exhibit microbicidal activity against both enteric and systemic bacterial and fungal pathogens, and α-defensins and β-defensins. Many of the genes encoding these substances reside in mouse QTLs (see also Table 4), like defensin B (Defb), defensin-related cryptdin peptide (Defcr), and secretory phospholipase A2 group IIA (Pla2g2a). Recently, it has been shown in mice that NOD2 deficiency results in a reduced production of cryptdins, the mouse orthologue of human α-defensins. These mice show a profound susceptibility to bacterial infection via the oral route, but not through intravenous or peritoneal delivery, indicating the importance of the cryptdins, especially in mucosal immunity. Also, in normal human intestinal tissue, with functional NOD2, Paneth cells produce a sufficient amount of α-defensins, which keep the commensal bacteria in check, but Paneth cells from individuals that are defective for NOD2, produce less α-defensins. Also, in humans, the copy number of β-defensins is highly polymorphic within the healthy population, and recently, it has been shown that people with 3 copies or less have a significantly higher risk of developing colonic CD. Thus, defective defensins functioning could lead to an overgrowth of the commensal-bacterial population, resulting in an uncontrolled inflammation.
Solute Carrier Family 22A4/22A5 gene cluster

Within the IBD5 locus on chromosome 5q two related genes of the solute carrier family, SLC22A4 and SLC22A5 are positioned that code for the carnitine/organic cation transporters OCTN1 and OCTN2, respectively. These cation transporters are involved in maintaining a physiological cation concentration in the intestine. Recently two novel functional single nucleotide polymorphisms (SNPs) have been reported to be associated with CD. The first, Leu503Phe in SLC22A4 leads to decreased activity of OCTN1, and the second, a G->C transversion in the promoter of SLC22A5, leads to a decreased expression of OCTN2 in the intestine\(^\text{305}\). These genes were also found to be implicated in the susceptibility to UC\(^\text{306, 307}\). Not all studies on these genes were able to replicate the associations found. Some studies show an association with perianal disease\(^\text{292}\), where others report a predominant association with ileal involvement\(^\text{308}\) or fibrostenosing disease behavior\(^\text{309}\).

Although there is genetic data available pointing towards SLC22A4 and SLC22A5 as susceptibility genes for IBD, at this time, there are no confirmatory studies demonstrating the altered expression and activity in primary human cells stratified on the IBD5 locus, or studies in murine models that give more insight in the functioning of these genes in IBD pathogenesis. These studies are warranted to further establish the presence or absence of involvement of these genes, and to provide a physiological explanation of the genetic association.

Multi Drug Resistance 1

Within a region of suggestive linkage on chromosome 7 lays the gene Multi Drug Resistance 1 (MDR1). The MDR1 protein is a member of the ATP-binding cassette of membrane transporters, and functions as a drug efflux pump P-glycoprotein 170 (Pgp-170) and is expressed on the surface of lymphocytes and intestinal epithelial cells. It has been found that IBD patients with high expression of MDR1 are more refractory to corticosteroid therapy, providing a possible mechanism for involvement of this gene\(^\text{310, 311}\). However, mice in which the Mdr1 gene has been deleted spontaneously develop colitis\(^\text{312}\).

Clearly, alterations in the expression of this gene appear to be very important in IBD susceptibility. Indeed, an Ala893Ser/Thr polymorphism was found in MDR1, and this appeared to be involved in especially UC susceptibility\(^\text{313, 314}\) and corticosteroid refractory CD\(^\text{315}\), although the association with CD was not generally confirmed\(^\text{316}\).

3.2.2.2.c Genes involved in mucosal immunity

Antigen recognition

In order to have a proper mucosal immune response, the immune system needs to be able to detect when a potentially dangerous pathogen is trying to invade the host. After the recognition of a pathogen by a receptor, the signal needs to be transported to the nucleus of the cell to elicit the proper immune response. Many genes that are involved in this process have been implicated in the etiology of IBD, and some of the most prominent ones will be discussed here.
Introduction

TLR4, CD14 and MD-2

Probably one of the most well known Gram-negative bacterial endotoxic products is LPS. LPS is recognized by TLR4, in close physical combination with myeloid differentiation-2 (MD-2) \(^{317}\), and this recognition is greatly enhanced by either membrane bound or soluble CD14 \(^{318-320}\). Because of their obvious function in innate pathogen recognition, the genes involved in LPS recognition are interesting candidate genes in IBD pathogenesis. Even more, CD14 lays within the \(IDB5\) locus on chromosome 5q, and mice deficient for TLR4 are extremely susceptible for colitis \(^{321, 322}\). Quite some studies have been performed using the candidate gene approach for CD14 and TLR4. Indeed, an Asp299Gly variant within the \(TLR4\) gene has been found that is associated with LPS hyporesponsiveness \(^{323}\). This variant has been associated with CD and UC \(^{324}\) reproducibly, or at least a trend towards association was found in most studies \(^{210}\).

A promoter polymorphism (-159C/T) in CD14 has been implicated in IBD in a number of studies \(^{325, 326}\), especially after stratification for \(NOD2\) mutations \(^{327}\), however this association was not generally reproduced \(^{328}\). A possible explanation for this could be that the populations studies were stratified differently, or that the CD14 polymorphisms is in fact in linkage with other yet to be discovered disease-causing variant(s), or with other genes, like \(SLC22A4/5\), that reside relatively close to CD14.

TLR5

Recently, the bacterial substance flagellin has been identified as a dominant antigen in CD \(^{329}\). Flagellin is recognized by TLR5. Carriage of a relatively common polymorphisms that causes a premature stopcodon in the \(TLR5\) gene has been found to induce decreased serum anti-flagellin antibody levels in controls, and this appears to protect from developing CD in Ashkenazi Jews \(^{330, 331}\).

The Human Leukocyte Antigen gene complex

One of the most consistently replicated IBD linkage regions is the \(IBD3\) locus on chromosome 6 \(^{332}\). Within this locus lays the MHC region, in man called Human Leukocyte Antigen complex (HLA). The MHC region is divided into three subgroups called MHC class I, MHC class II, and MHC class III. The main function of MHC class I and II molecules is to recognize antigens and present them to T cells. Class I molecules are expressed on almost every nucleated cell and present peptides that are derived from cytosolic proteins that have been endogenously processed to CD8\(^+\) T cells. Class II molecules on the other hand, present peptides that are derived from exogenous proteins, that have been endocytosed and digested in lysosomes, to CD4\(^+\) T cells and are expressed only on specialized professional APCs, like DCs and macrophages, and on activated T cells and B cells. The MHC class III region harbors genes that encode for other immune components, such as complement components (e.g., C2, C4, factor B) and cytokines (e.g. TNF-\(\alpha\)).

Within the complete MHC region over 200 different MHC genes are found, that are all highly polymorphic \(^{333}\). Certainly, there must reside susceptibility alleles in this genomic region, however, the immense genetic complexity makes it extremely challenging to find them. Because of the diversity, extremely large sample groups are needed to definitely show linkage with disease susceptibility. A meta-analysis combining results from 29 studies showed significant association of HLA-DR2, HLA-
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DR9 and HLA-DR*0103 for UC, and a protective association for HLA-DR4 with UC. In CD, HLA-DR7, HLA-DRB3*0301 and HA-DQ4 were found to be associated with disease susceptibility, whereas HLA-DR2 and HLA-DR3 were found to be protective.

Signal transduction

Next to the ability to recognize pathogens, one of the most important processes in regulating the immune response is the signal transduction from the receptor downstream to the nucleus, to activate transcription of either pro- or anti-inflammatory genes. Typically, a receptor activates a cascade of kinases that subsequently activate each other by processes like phosphorylation or ubiquitination, ultimately resulting in the release of transcription factors. Normally, when in rest, these transcription factors are kept in the cytosol, but upon activation they migrate to the nucleus, where they can activate gene transcription. Evidently, because of the importance of a fine tuned, well regulated signaling in immune responses, any gene involved in this cascade is a likely candidate gene in a disease that is characterized by an imbalanced immune activation, as seen in IBD.

Nuclear Factor κB

The nuclear factor (NF)-κB family is a group of critical transcription factors involved in a variety of regulatory processes, including innate and adaptive immune responses, cell growth, apoptosis, and tissue differentiation. One of the NF-κB family members, the gene coding for the NF-κB subunit p50, NFKB1 is found on chromosome 4q24. This region was originally implicated in UC susceptibility in North American populations with UC, especially in Ashkenazi Jews, and appeared to epistatically interact with the IBD1 region, and this was later confirmed in European and Canadian studies. In addition, in two separate spontaneous colitis models, the GnaI2−/− mouse model, and the IL10−/− mouse model, a locus on chromosome 3 was found to significantly contribute to colitis susceptibility and severity (called Gpdc1 and Cdcs1 respectively). This region is the mouse orthologue for the human 4q region and harbors mouse Nfkb1. Interestingly, an elevated susceptibility to bacterial infections and intestinal inflammation is demonstrated in Nfkb1 deficient mice but on the other hand, mice with enhanced NF-κB p50 expression show enhanced inducible NF-κB activation, chronic inflammations and elevated susceptibility to bacterial infections. These findings are further corroborated by studies in human IBD, where elevated levels of NF-κB activation are found in the intestinal mucosa of both CD and UC patients. These observations designate NFKB1 to be an interesting candidate gene for IBD.

Indeed, a promoter polymorphism, conferring a ATTG deletion 94 bp upstream of the transcription start of the NFKB1 gene was found to be associated with UC susceptibility. These results were confirmed in our study in an Amsterdam based IBD patient group, but other studies failed to reproduce the association.

Nevertheless, it is clear that any alteration in NF-κB signaling can have profound effects on the immune state of the host. Should the promoter polymorphism in NFKB1 be a true susceptibility allele, the exact mechanism underlying the NFKB1 related disease susceptibility would remain difficult to explain. One can think of several explanations. To exert its effect, p50 binds to p65 to form biologically active
heterodimers, which translocate from the cytoplasm of the cell to the nucleus. Here, they bind to NF-κB binding sites in promoter sequences of inflammatory proteins, including IL-12, TNF-α and IFN-γ and regulate their transcription. Alternatively, p50 is able to form homodimers which block transcription by binding to NF-κB sites in the nucleus. The polymorphisms in the promoter region leads to a less strong activation of NF-κB1 transcription, which seems contraintuitive to the fact that especially in UC, high levels of NF-κB activation are seen. However, if the diminished NFKB1 transcription would lead to less available p50, fewer inhibitory p50/p50 homodimers can be formed. Fewer blocking homodimers could then lead to a stronger activation of transcription of inflammatory genes, leading to the strong NF-κB induced immune response, as seen in UC. Another potential explanation is that low levels of p50 lead to a poor innate immune response. Bacteria that would have normally been handled by this first line of defense are then able to provide for ongoing antigenic signals and subsequently cause an ongoing inflammation.

The importance of the NF-κB signaling route in intestinal inflammation is also shown by the effects of IKKβ deficiency on the gut epithelium. This Inhibitor of nuclear factor Kappa-B Kinase β (IKKβ) is essentially important for the activation of NF-κB. Lack of proper IKKβ functioning in itself does not lead to increased epithelium apoptosis and gut permeability, but after challenge with intestinal ischemia-reperfusion, radiation therapy, or toxin A derived from Clostridium difficile, these animals are far more susceptible to colitis.

Clearly, any change in the delicate balance of NF-κB signaling can lead to an alteration in immune response which can be associated with enhanced susceptibility to colitis, however larger studies with well defined patient groups are warranted to discern whether or not the NFKB1 promoter polymorphism is a true susceptibility allele.

Cytokines and cytokine receptors

Because of their important role in the immune response, in initiating, regulating, and perpetuating it, the genes that code for cytokines and their receptors are an obvious group of candidate genes for IBD.

Tumor Necrosis Factor locus

The gene coding for TNF-α is one of the genes residing in the MHC class III region on chromosome 6, within the IBD3 susceptibility locus. In close proximity of TNFA, lays the gene formerly known as TNFB, now named lymphotoxin-α (LTA). Both genes are interesting candidate genes, not only because of their location, but especially because of their function as some of the most potent pro-inflammatory cytokines. Even more, elevated levels of serum TNF-α have previously been demonstrated in IBD patients, and anti-TNF-α therapies are effective for patients with active CD and UC (See also section 3.2.1.2).

Many studies have been performed looking for polymorphisms in both genes. Especially for TNFA, several polymorphisms have been found, that are associated with enhanced susceptibility to, or severity of a variety of infectious diseases like mucocutaneous Leishmaniasis and death from cerebral malaria, neoplastic disorders like colorectal cancer, and autoimmune like disorders, such as asthma.
These polymorphisms have been found to be associated with IBD in several studies and appear to occur in only five conserved combinations, or haplotypes (TNF-C, -E, -H, -I and –P). Some studies have found that they seem to be more involved in the severity of disease than susceptibility itself.

The *Interleukin 1* cluster

A cluster of cytokines that has been implicated in the susceptibility to IBD is the IL-1 cluster. This cluster comprises of three genes, \(IL1A\), \(IL1B\) and IL-1 receptor antagonist (\(IL1RA\)). IL-1\(\alpha\) and IL-1\(\beta\) are both pro-inflammatory cytokines, mainly expressed in cells of the myeloid lineage and epithelial cells, that have many pro-inflammatory properties, as described before. IL-1RA on the other hand is a substance that is closely related to IL-1\(\alpha\) and IL-1\(\beta\), and binds to the IL-1 receptor but does not trigger it. Thus, IL-1RA can function as a buffer, keeping the IL-1 receptor occupied, preventing binding of the activating ligands IL-1\(\alpha\) and IL-1\(\beta\), thus limiting the activation. An imbalance in the expression of IL-1RA and IL-1 has been shown to influence the susceptibility and clinical course of several inflammatory diseases, like rheumatoid arthritis and inflammatory myopathies, and intravenous administration of IL-1RA has shown to be promising in many of them.

In IBD as well, many studies have been performed showing that an imbalance in the IL-1 system is found in IBD patients. Indeed, polymorphisms in the IL-1RA and IL-1B genes have been found to be involved in the susceptibility to IBD, and even more the severity of UC. However, as with many of the above discussed genes, the results found were not always reproduced when different populations were studied.

*Interleukin-12*

Another cytokine that would be an interesting candidate gene, especially in Crohn’s disease, is IL-12, being a key cytokine for the induction of T\(_h\)1 immune responses and elevated IL-12 levels being one of the hallmark features of the disease. Polymorphisms causative of an elevated IL-12 production in CD would provide a plausible explanation for the disease susceptibility.

Biologically functional IL-12 is a heterodimer formed by an IL-12\(\alpha\) chain (IL-12p35) and IL-12\(\beta\) chain (IL-12p40) encoded by \(IL12A\) and \(IL12B\) respectively. The latter subunit, IL-12p40, was shown to be linked to susceptibility to chemically induced colitis in mice. A logical step would be to try to identify polymorphisms in the human form of the gene. Indeed, there are functional polymorphisms found in human \(IL12B\), that were shown to be associated with susceptibility to several autoimmune diseases, or with high secretion levels of IL-12. However so far, no association of \(IL12A\) or \(IL12B\) polymorphisms with CD, or IBD in general has been proven.

*Interleukin-23 receptor*

Very recently, a highly significant association between Crohn’s disease and the Interleukin-23 receptor (\(IL23R\)) gene on chromosome 1p31, which encodes a subunit of the receptor for IL-23 was found using a genome-wide association study. The uncommon coding variants of the gene found in this study confer protection against CD. These finding were further substantiated in a study in pediatric IBD patients, showing that especially in non-Jewish CD patients with ileal disease, the rare
IL-23R allele is protective for the development of IBD\textsuperscript{382}. At this point, very few data is available on the genetic involvement of IL-23, however, combining the two above studies with previous studies on the proinflammatory role of IL-23 in CD, point out the importance of the IL-23 signaling pathway in CD etiology, and make this pathway a very promising therapeutic target in IBD.

Many genes have been investigated in IBD susceptibility. So far only \textit{NOD2}, based on its strong association, is irrefutably connected to CD. The inconsistent findings with the other above described genes and several other genes that were not discussed here may reflect underpowered cohorts to dissect out the effects of for instance differences in tobacco use, disease behavior, location, and duration. False-positive, or negative results can also occur when the frequencies of the various alleles vary in different racial and ethnic groups, for example the difference of frequency of the \textit{NOD2} polymorphisms in Caucasian and Asian populations, or when the studied groups are not recruited from the same genetic homogeneous population. Furthermore, differences in phenotype definitions can trouble the genetic view.

\textbf{Concluding remarks}

Although Crohn’s disease and ulcerative colitis are the best defined pathologies within IBD it is clear that IBD is in fact a plethora of diseases, all characterized by chronic inflammation of the gastrointestinal tract, but with differences in the development, localization, histological and immunological features, as well as complications of the disease. Complicating factors in defining possible subtypes within either CD or UC can be found in the immense diversity in the human population, and differences in environmental factors, life style, and gut flora. Also, the extent to which these factors have an effect on the susceptibility and development of the diseases remains largely elusive, and is most probably not the same for all the sub-pathologies within IBD. In addition, the underlying genetic defects can be different for the different subtypes of IBD. Although in the past decade much progress is made in our knowledge about the genetic background of IBD, the larger part of the puzzle still remains to be solved. Meta-analyses combining the results of many studies and studies with sufficiently large cohorts of strictly defined patients are warranted to dissect the complex polygenic background of IBD.

Understanding this background, and most probably being able to divide IBD patients in genetically distinct subgroups will be of great aid in understanding the physiology and immunology of the disease and will ultimately lead to the development of specific treatments for all subgroups of the diseases.
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Chapter 2

Animal models of Inflammatory Bowel Diseases

Michelle E.A. Borm and Gerd Bouma

Abstract
Crohn’s disease and ulcerative colitis are chronic idiopathic inflammatory disorders of the gastrointestinal tract. While the exact etiology of these inflammatory bowel diseases remains elusive, both immunological and genetic determinants have been identified that are involved in disease pathogenesis. Murine models of mucosal inflammation have provided a detailed insight in the immunological processes underlying chronic mucosal inflammation. Here, we highlight some recent findings that have contributed to our understanding of human IBD.
Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) are two related but distinct chronic inflammatory disorders of the gastrointestinal tract, commonly denoted as inflammatory bowel diseases (IBD). The exact causes remain elusive, but thus far, IBD is thought to be the result of an inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora in a genetically susceptible host \(^1\). This paradigm has emerged, to a great extent, from studies in animal models of mucosal inflammation. The large variety of animal models that has been identified so far is consonant with the view that many types of imbalance in the gastrointestinal immune system can lead to mucosal inflammation, and by extension, that human IBD is likely to be a common denominator for a group of clinically related disorders with multiple etiologies and distinct clinical characteristics. Even more, there is now convincing evidence that the immunological abnormalities underlying both forms of IBD are entirely different (Box 1).

In Europe and North America, there are an estimated 3.6 million IBD patients. Disease incidence appears to be lower in developing countries; however, as societies become more “Westernized”, with attendant changes in life-style, diet and other environmental exposures, the incidence rates rise, and approximate those of the industrialized areas \(^2\).

Thus, while environmental factors play an important role in disease pathogenesis, there is substantial epidemiological evidence that genetic factors have a decisive role in the susceptibility to IBD as well. The most convincing epidemiological evidence for this thesis comes from twin concordance studies, particularly in CD. These epidemiologic observations are supported by genetic linkage studies in which families containing more than one affected member are analyzed using genome-wide genotyping technology. These studies identified several chromosomal regions harboring IBD susceptibility genes \(^3\). The most prominent and first discovered region is located on chromosome 16 (denoted \(IBD1\)) and is involved in susceptibility to CD but not UC \(^4\). More recently, it was found that polymorphisms in the \(NOD2\) gene (NM_022162) are responsible for the linkage with this region (see further discussion below) \(^5,6\). Importantly, these mutations are found only in a small proportion of CD patients. Therefore, other, as yet unknown gene-defects must exist that are responsible for disease susceptibility in the remainder group of patients.

The broad variety of factors involved in the etiology, the genetic heterogeneity of the human population and complex gene-gene and gene-environment interactions significantly complicate further unraveling the causes of IBD. Therefore, much of our current understanding of IBD comes from the systematic study of well-defined animal models. The scope of the current review will not be to provide a detailed overview of all models, but rather to summarize some general concepts and to highlight some recent advances in our understanding of IBD, that have emerged from these models, with a special emphasis on recent advances in the field of (murine) genetics.

\textit{In vitro models}

The gastrointestinal tract involves many different cell types that are not only involved in digestion and nutrient absorption, but also provide an essential physical and immunological barrier to the milieu exterier. Deficiencies involving any of these cells could lead to an exaggerated mucosal...
inflammation. It follows that such a complex system can not be easily mimicked in vitro. However, by studying just one cell type at a time, specific aspects of the cellular mechanism involving IBD can be explored. In addition, in vitro models for the gut wall function can be safely used in toxicity and efficacy tests for novel drug therapies.

Box 1 Characteristics of human inflammatory bowel diseases

- **General definition**
  Inflammatory Bowel Disease (IBD) is a chronic relapsing idiopathic inflammation of the gastrointestinal tract. The two main clinical forms of IBD are Crohn’s disease (CD) and ulcerative colitis (UC).

- **Epidemiology**
  Disease prevalence is highest in industrialized countries for both CD and UC, ranging from 10-200 cases per 100,000 individuals in North-America and Europe.

- **Areas of involvement**
  - CD: Most commonly the terminal ileum, cecum, peri-anal area and colon, but any part of the gastrointestinal tract can be affected. Characterized by segments of normal bowel between affected regions, known as ‘skip lesions’.
  - UC: Involves the rectum and extends proximally in a continuous fashion, and always remains restricted to the colon. Is sometimes restricted to the rectum as ‘ulcerative proctitis’.

- **Histology and characterization of inflammation type**
  - CD: A transmural inflammation (affecting all layers of the bowel wall), dense infiltration of lymphocytes and macrophages; granulomas in up to 60% of the patients; fissuring ulceration and submucosal fibrosis.
    The inflammation is characterized by a Th1 type response, with high levels of IL-12, IFN-γ and TNF-α.
  - UC: An inflammation affecting the superficial (mucosal) layers of the bowel wall, infiltration of lymphocytes and granulocytes and loss of Goblet cells, accompanied by ulcerations and crypt abscesses.
    The inflammation is characterized by a more Th2 type response, with high levels of IL-5 and IL-13 (but not IL-4), and high levels of autoantibodies, such as anti-neutrophil cytoplasmic antibodies (pANCA), indicative of B-cell activation.

- **Clinical features and complications**
  Both CD and UC involve diarrhea and pain, and can show extra-intestinal inflammatory manifestations in joints, eyes, skin, mouth and liver. Longstanding IBD leads to an increased risk for colon carcinoma, in particular in UC.
  - CD: narrowing of the gut lumen can lead to strictures and bowel obstruction, abcess formation, and fistulization to skin and internal organs.
  - UC: severe diarrhea, blood loss and progressive loss of peristaltic function leading to rigid colonic tube, in severe cases, this can lead to ‘toxic megacolon’ and perforations.
Primary cultures

Primary cultures can be obtained from colorectal tissue specimen or biopsies derived from endoscopy. These cultures have the advantage of containing the same combination of cell populations present in the intestine, and thus represent the *in vivo* situation, however, they are difficult to maintain and generally survive for only a few days.

Cell lines

Several cell lines resembling the gut epithelium, mostly derived from carcinomas, are commercially available (Table 1). The cell line used most often in IBD research is the CaCo2 cell line, which is derived from colorectal carcinoma cells and resembles the columnar epithelium of the small bowel. Also, cell lines derived from human fetal cells, like the H4 cell line derived from human fetal enterocytes have been used. Epithelial cell lines can be applied for pharmacokinetic and toxicity studies of drug therapies. A drawback for using them is the loss of tissue characteristics with the passaging of the cells, and the lack of interaction with other cell types involved in mucosal immunity.

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<td><strong>Pros</strong></td>
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<tr>
<td>Relatively cheap</td>
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<td>Reduced complexity of the system</td>
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<tr>
<td>Incorporate multiple cell types, histologies and processes</td>
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<td>Similar complexity to human system</td>
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<td><a href="http://www.atcc.org">www.atcc.org</a></td>
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In vivo models

During the course of the last fifty years, an amazingly large number of different models of mucosal inflammation has been described all showing some of the characteristics of either CD or UC. A recent excellent and detailed overview of the various animal models of IBD has been provided by Strober and colleagues. Several species have been studied, including mice, rats, rabbits, guinea-pigs and tamarins. Here we will give a brief overview of the different categories of models, with some examples in each category.

1. Induced models

The models studied in greatest detail so far involve induction of colitis by administration of an exogenous colitogenic substance. In general, these models require the co-administration of a substance that temporarily disrupts the mucosal integrity, allowing the colitogenic components to gain access to the mucosal immune system (for examples, Box 2).

In one of the early models of IBD, rabbits were sensitized with ovalbumin. Then, the colonic epithelial barrier was disrupted by rectal administration of formalin, followed by re-administration of the antigen, leading to a transient inflammation with many similarities to UC (the ‘Auer procedure’). An important conclusion from these and subsequent studies was that the perpetuating inflammation in IBD results from a break in ‘tolerance’ to antigens in the normal mucosal microflora. The presence of this nonpathogenic luminal flora is a prerequisite for developing inflammation, since in virtually all models known today, there is no inflammation when animals are kept under germfree conditions. Indeed, early studies in guinea-pigs demonstrated that the inducing agent carrageenan did not provoke ulcerations in germfree animals, while restoration of animals with Bacteroides vulgatus resulted in the development of cecal ulcerations upon carrageenan feeding.

In addition, disease may ameliorate when animals are treated with antibiotics. For example, HLA-B27 transgenic rats develop a spontaneous gut, joint and skin inflammation when housed under conventional conditions. If these animals are kept under germfree conditions however, no intestinal inflammation is seen. Treatment of these animals with antibiotics significantly reduces the severity of the inflammation. While there is evidence that these findings can be extrapolated to human IBD, it must be noted that despite exhaustive research, to date no specific pathogenic microbes have been associated with susceptibility to human IBD.

2. Defective T-cell mediated colitis

Generally, encounters with mucosal antigens do not result in inflammatory responses, but instead in negative regulatory T cell responses, induction of anergy or deletion of antigen-specific T cells, a phenomenon known as “oral tolerance”. This phenomenon was already noticed in the late sixties by Halpern et al., who showed that feeding rats E.coli before immunizing them with dead or live E.coli in Freund’s adjuvant could prevent the development of colitis. Recently, several different regulatory cells, involved in dampening immune responses to harmless antigens, have been identified. It follows that defects in development or function of these regulatory cells may lead to mucosal inflammation. Indeed, a surprising number of murine models of IBD can be traced to a regulatory T
cell defect (Box 2). A notable example is mucosal inflammation that is induced in severe combined immunodeficient (SCID) mice by the adoptive transfer of naïve (CD45RB\(^{hi}\)) T cells lacking regulatory cells, which is prevented by the co-transfer of mature (CD45RB\(^{lo}\)) cells containing regulatory T cell subpopulations\(^{40}\). So far it has been very difficult to trace abnormalities in regulatory T cell function in human IBD. Thus, further work will be necessary to assess the meaning and validity of this finding.

**Box 2 Examples of animal models in differently induced colitis models for IBD**

a. Induction of colitis by administration of an exogenous colitogenic substances:
   - Trinitrobenzene sulfonic acid (TNBS): mouse, rat, rabbit, guinea-pig\(^{26, 29}\),
   - Dextran sulfate sodium (DSS): mouse, rat, guinea-pig\(^{30-32}\),
   - Oxazolone: mouse, rat\(^{33, 34}\)

b. Induction of colitis caused by defective T-cell mediated regulation:
   - Adoptive transfer model in severe combined immunodeficient (SCID) mice\(^{16}\),
   - IL-10 deficient mice\(^{35}\),
   - Transforming growth factor-β (TGF-β) deficient mice\(^{36}\),
   - Tgε26 mice (defects in thymic development of regulatory cells)\(^{37}\)

c. Induction of colitis by introduction of a gene-defect or a transgene\(^1\):
   - Gi2α\(^{-}\) mice\(^{38}\),
   - IL-10\(^{-}\) mice\(^{35}\),
   - TCR-α chain deficient mice\(^{27}\),
   - STAT4 transgenic mice\(^{28}\)

3. **Spontaneous models**

A third category involves spontaneous models of mucosal inflammation. This category can be divided into two related subcategories; animals that spontaneously develop mucosal inflammation, and models in which a gene-defect or a transgene causes an inappropriate mucosal immune response.

3a. **Spontaneous models**:

The most notable example of this kind are cotton-top tamarins, who develop a colitis similar to UC\(^{20}\). Since these animals are relatively closely related to humans, they are theoretically excellent models for efficacy testing of novel drug therapies\(^{41}\). The ethical and practical drawbacks are however apparent.

Another model is the recently recognized SAMP1/YitFc mouse strain that develops a chronic intestinal inflammation localized in the terminal ileum. The resulting ileitis bears a remarkable resemblance to human Crohn's disease with discontinuous, transmural inflammatory lesions in the terminal ileum\(^{21}\).  

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*Animal Models of Inflammatory Bowel Diseases*
3b. Spontaneous colitis in genetically modified animals:

With the introduction of large scale genetic engineering during the early nineties of the last century, it became apparent that inactivating or overexpressing entirely different genes frequently leads to mucosal inflammation (Box 2). This category now represents the largest group of models of mucosal inflammation. An important conclusion relevant to human IBD is that entirely different genetic defects can lead to mucosal inflammation and a more or less similar clinical picture. In other words, it is very unlikely that there is only one gene or a limited group of genes involved in IBD susceptibility.

Recent novel insights in the etiology of IBD derived from animal models;

The Nod2⁻/⁻ mouse

As alluded to above, mutations in the NOD2 gene were identified in 2001 as the first genetic risk factor for CD. Until very recently, the mechanism by which this leads to mucosal inflammation remained enigmatic. NOD2 is an intracellular receptor for muramyl dipeptide (MDP), a part of bacterial peptidoglycan (PGN), and is expressed in a variety of cell types, including monocytes and monocyte-derived cells. MDP recognition by NOD2 leads to NF-κB activation, which leads to production of pro-inflammatory cytokines. The polymorphisms in the NOD2 gene lead to abrogated NOD2-signaling and, as one would think, should lead to diminished NF-κB activation. This is contrary to the inflammatory response seen in CD patients, which is associated with a notably elevated NF-κB activation.

These seemingly contradicting findings were recently partially clarified. Watanabe et al. stimulated splenocytes from Nod2⁻/⁻ mice with ligands for different types of Toll Like Receptors (TLR’s). They found that splenocytes from Nod2⁻/⁻ mice showed an increased NF-κB activation and consequently IL-12 mediated Tʜ₁ responses following stimulation with PGN. This effect was specific for PGN, and did not apply to other TLR ligands. PGN can bind to both TLR2 and, through its MDP component, to NOD2. This indicates that under normal conditions, TLR2 activation by PGN is inhibited by NOD2. Thus, simultaneous signaling through NOD2 leads to a dampening of the NF-κB activating effect of TLR2 signaling. The exact mechanism by which NOD2 signaling inhibits the TLR2-mediated NF-κB activation and Tʜ₁ response and why this leads to gut-specific inflammation remains enigmatic.

Genetic Analysis of IBD using Murine Models

Identifying NOD2 as a susceptibility gene for CD is a major scientific breakthrough. However, mutations in this gene are found in only a subgroup of CD patients. Identification of the remainder set of susceptibility genes may be problematic since it seems likely that their contribution to the disease will be more limited than that of NOD2.

Linkage strategies in humans usually encounter significant difficulties in the final stages of gene identification. Some of these difficulties can be overcome by identification of genetic factors underlying experimental models of mucosal inflammation, followed by a candidate gene approach in humans testing the relevance of the identified mouse gene to human IBD (Table 2). This approach has been successfully applied to several complex immune-mediated diseases, but has only recently received attention in IBD research. In an initial study of this kind, genetic factors underlying susceptibility to dextran sulphate-
induced colitis (DSS colitis) were examined. The approach taken here, and in other genetic studies, was to cross susceptible mice (in this case C3H/HeJ mice) with relatively resistant mice (C57BL/6J mice) and then correlate co-segregation of microsatellite polymorphisms associated with each strain with susceptibility to disease in the F2 progeny. In this study, a number of susceptibility loci (called quantitative trait loci, QTL) were identified indicating the complexity of the genetic factors operating in DSS colitis. So far, the nature of the underlying genes remains unknown.

Other models of inflammation that were analyzed in this fashion are two gene-knockout models; colitis in IL-10 deficient mice, and in G-protein Gia2 deficient mice. The IL-10−/− F2 progeny was analyzed as indicated above and yielded a remarkably strong association with a region on chromosome 3, called Cdcs1. Surprisingly, the exact same region was identified in the Gia2 deficient model. This indicates that even a severe primary genetic defect (either IL-10 or Gia2) may depend on the presence of a secondary factor (encoded by one or more genes within the locus on chromosome 3). It is likely that the latter gene(s) is of major importance in maintaining the balance in the mucosal immune response, and that this balance is disrupted when a key homeostatic gene is inactivated. Exactly what gene(s) is responsible for the disease susceptibility associated with this QTL remains to be established.

Yet another model that has been studied and one that has so far yielded the most information, is the TNBS colitis model. This colitis in SJL/J mice is a Th1 type inflammation and is completely prevented or reversed by treatment with anti-IL-12. In the genetic study performed, comparing susceptible SJL/J mice and resistant C57BL/6 mice, two QTLs were identified. Interestingly, the first QTL, on chromosome 9 has also been found in a preliminary study of the SAMP1/YitFc mice described previously. The occurrence of this locus in two very different models, one of which spontaneously mimics a form of human IBD, suggests that this locus bears further examination.

The second QTL, on chromosome 11, is particularly of interest because it is syntenic to human chromosome 5q33-34 that has been linked to human Crohn’s disease, and is associated with susceptibility to other Th1-mediated disease models, i.e., experimental allergic encephalitis (Eae6) and experimental insulin-dependent diabetes (ltdm18). Peak linkage in this QTL was found near the gene encoding the IL-12 p40 subunit, (Il12b, NM_008352). This gene makes a logic candidate gene, since CD is characterized by elevated IL-12 production, and anti-IL-12 therapy proved very effective, both in mice and in humans. Indeed, we found that Il12b is polymorphic between SJL/J and C57BL/6. Taken together, these data strongly suggest that the high IL-12 responses and susceptibility to TNBS colitis are related, and the polymorphism in Il12b is the most likely genetic abnormality causing TNBS colitis. Whether a similar polymorphism exists in human CD remains to be seen.
**Table 2 Advantages of genetic research in animal models and humans**

<table>
<thead>
<tr>
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<th>Animal models</th>
<th>Human</th>
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<tbody>
<tr>
<td>Genetic composition</td>
<td>+ Inbred population, greatly reduced complexity</td>
<td>- Outbred population</td>
</tr>
<tr>
<td>Environment</td>
<td>+ Constant control of environment and diet, the same for all animals</td>
<td>- Multiple factors in environment, diet and life-style, differing from patient to patient</td>
</tr>
<tr>
<td>Systematical analysis</td>
<td>+ Possibility to generate genetically engineered models varying in only one specific gene, without variations in environmental factors</td>
<td>- Lack of possibility to look at one factor at a time</td>
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<tr>
<td>Numbers</td>
<td>+ Relatively simple generation of large sample sizes</td>
<td>- Limited numbers of patient available for research</td>
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<tr>
<td>Genetical engineering</td>
<td>+ Offers possibility to analyse role of specific gene, making use of congenic, transgenic or knock-out mice</td>
<td>- Legally and ethically not possible</td>
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Summary of advantages of genetic research in animal models compared to human studies

**Concluding remarks**

The study on the causes of IBD, both in animal models as well as in humans, have revealed important new insights in the immunological processes and genetic abnormalities leading to mucosal inflammation. These findings have already, and will further lead to new therapeutic approaches aimed at interfering with these processes. The most notable example includes different strategies to block key inflammatory cytokines such as TNF-α, which has already proven to be a highly effective treatment in CD, achieving positive effects in some 60% of patients. Similar effects have recently been shown for anti-IL-12 therapy in CD, and the list of therapeutic targets is rapidly growing.

A next step will be to tailor therapeutic regimens to the individual patients, i.e., based on correcting the immunological abnormality or genetic defect operative in a particular patient. The feasibility of this possibility is quite obvious with respect to the NOD2 mutation since in this case the lesion is most likely to involve an hematopoietic cell that can be substituted with normal genes.
Glossary

**Twin concordance studies:** a twin pair is concordant when both members exhibit the disease. When concordance is significantly higher in monozygotic (identical) twins, than in dizygotic (non-identical) twins or siblings, this is an indication that a genetic component is involved in disease susceptibility. This was found particularly in CD.

**Polymorphism:** a gene (or genetic region) is polymorph when in a given population, more alleles coexist of the same gene.

**Linkage:** a gene, or genetic region is in ‘linkage disequilibrium’ with a disease if a specific allele of this gene is found in a patient population more often than would be expected by chance.

**Linkage disequilibrium:** the preferential association of a particular allele, for example, a mutant allele for a disease with a specific allele at a nearby locus more frequently than expected by change.

**Transgene (transgenic):** an artificially introduced gene of foreign DNA (a human gene introduced in a mouse).

**Congenic:** an artificially replaced gene or genetic region from another strain, e.g. a mouse of strain A which is homozygous for the distal part of chromosome 2 of strain B.

**Toll-like Receptors (TLR):** A family of receptors that recognize conserved products that are unique to microorganisms, such as lipopolysaccharide. TLR-mediated events signal to the host that a microbial pathogen is present.

References


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Chapter 3

A Major Quantitative Trait Locus on Mouse Chromosome 3 Is Involved in Disease Susceptibility in Different Colitis Models

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Abstract

Background & Aims: Mice with a disrupted gene for the G-protein alpha inhibitory 2 chain (Gnai2\textsuperscript{−/−}) develop a spontaneous colitis resembling human inflammatory bowel disease (IBD). Disease expression differs markedly between inbred strains of mice, indicating genetic control of disease susceptibility. We performed a genome-wide screen to localize the chromosomal regions regulating disease expression. Methods: 284 F2 mice derived from resistant C57BL/6J Gnai2\textsuperscript{−/−} mice and susceptible C3H/HeN Gnai2\textsuperscript{−/−} mice were analyzed in a genome-wide screen for colitis susceptibility and severity. Results: A highly significant locus on chromosome 3 (Gpdc1) contributed to colitis susceptibility and severity (Likelihood ratio statistics (LRS) = 32.4; LOD score = 7, \( P < 1.0 \times 10^{-5} \)). The peak linkage of this locus at 62 cM co-localizes exactly with a previously identified locus controlling colitis susceptibility in IL-10 deficient mice. In addition, evidence for linkage with a locus on chromosome 1 (Gpdc2; LRS = 19.7; LOD = 4.3) was found, and the two loci interacted epistatically (combined LRS = 68.2). A third locus (Gpdc3) was found on chromosome 9 and this locus interacted epistatically with a locus on chromosome 7 which by itself did not have an effect on the trait. Conclusion: The identification of a major locus on chromosome 3 that controls susceptibility to spontaneous colitis in two different gene-knockout models indicates that this locus harbors a gene(s) that plays a key role in maintaining mucosal homeostasis. Identification of this gene(s) may contribute to the further understanding of the mechanisms underlying human IBD.
Introduction

While the exact etiology of the two major forms of inflammatory bowel disease, Crohn’s disease (CD) and ulcerative colitis (UC) is still unclear, there is a growing agreement that they occur as the consequence of a genetically determined dysregulated immune response to one or more antigens from the mucosal microflora.

That this abnormality has strong genetic underpinnings has long been recognized and in recent years a number of IBD susceptibility loci have been established by linkage studies. Moreover, the susceptibility conferred by one of these loci, (IBD1) has been shown to be due to mutations in the NOD2 (CARD15) gene, which when present on both chromosomes, causes CD in 8-17% of Caucasian CD patients. It is now known that NOD2 is an intracellular sensor of a component of bacterial peptidoglycan (muramyl dipeptide) that regulates NF-κB activation; nevertheless, it is not yet known how this abnormality leads to CD and/or a dysregulated immune response. In addition, the fact that NOD2 mutations occurs in only a minority of CD patients underscores the fact that it accounts for only small part of the genetic factors underlying CD as a whole and suggests that the mucosal immune dysregulation causing this disease can have many causes.

An important step forward in the understanding of IBD has come from the identification of animal models of mucosal inflammation that more or less resemble human IBD. One of the key findings to emerge from the study of these models is that susceptibility to colitis in most, if not all, of the experimental models is influenced by the strain of the mouse in which the model is expressed. These strain differences in disease susceptibility offer the opportunity to identify genes that are involved in determining susceptibility/resistance to mucosal inflammation and as such may contribute to the identification of the genes involved in human IBD.

One mouse model that displays such strain differences in susceptibility is the spontaneous colitis model occurring in mice deficient in the G-protein alpha inhibitory chain (Gαi2). Such mice most likely develop mucosal inflammation because signaling through Gαi2 normally inhibits IL-12 production and thus in the absence of this molecule, IL-12 production and the Th1 response is greatly exaggerated. The strain-specific variation in inflammation observed in this model is exemplified by the fact that the C3H/HeN strain is highly susceptible to disease, whereas the C57BL/6J strain maintained under identical circumstances, is highly resistant. As implied above, this introduces the possibility that a genetic analysis of these strains of Gαi2−/− mice might lead to the identification of modifying genes critical to the expression of colitis in these mice.

On this basis, we performed such a genetic analysis of Gαi2−/− mice using genome-wide microsatellite methodology to identify genetic susceptibility loci in Gαi2−/− (C3H/HeNxC57BL/6J) F2 generation intercrosses. This genetic screening procedure allowed identification of a highly significant susceptibility locus on chromosome (Chr.) 3 as well as additional loci on Chr. 1, 9 and the X-chromosome. The importance of the Chr. 3 locus is emphasized by the fact that this locus also is likely to be involved in the strain variation in disease susceptibility found in IL-10 KO mice.
Materials and Methods

Mice

Specific pathogen-free, 5–6-week-old C3H/HeN mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Gαi2 knock-out mice on the C57BL/6J background were bred from homozygous C57BL/6J breeding pairs as described before \(^{10,12}\), and were originally a kind gift of Baylor University School of Medicine. The Gαi2 deficiency was backcrossed from the C57BL/6J background onto the C3H/HeN strain for >6 generations. Since Gαi2 deficiency causes severe inflammation in C3H/HeN mice, causing early death and loss of desire to breed, these mice were maintained in the heterozygous state (Gαi2\(^{+/−}\)). (C57BL/6JxC3H/HeN) Gαi2\(^{−/−}\) F1 hybrids were derived from the reciprocal mating of C57BL/6J Gαi2\(^{−/−}\) mice with C3H/HeN Gαi2\(^{+/−}\) mice, and subsequent genotypic selection for the Gαi2\(^{−/−}\) deficiency. These mice were used to generate a first group of 147 F2 mice by brother-sister mating of F1 Gαi2\(^{−/−}\) mice and this group of mice was analyzed using a genome-wide screen. Meanwhile, an additional group of 137 F2 mice was generated making a total of 284 F2 mice (146 female and 138 male).

Mice were maintained in the National Institute of Allergy and Infectious Diseases animal holding facilities and all breeding pairs were set up randomly. Animal use adhered to National Institutes of Health Laboratory Animal Care Guidelines.

Clinical Evaluation and Definition of Disease

Susceptibility to and severity of colitis in F2 mice was determined by weekly monitoring of clinical signs of colitis, diarrhea and weight loss. Mice were sacrificed when they showed profound weight loss and/or signs of colitis. Mice that showed no signs of disease and no weight loss at the age of 5 months were considered resistant to colitis and sacrificed as well. Blood was drawn from all mice for serological studies and colons were removed for histopathological analysis.

Histologic Assessment of Tissues

Colonic tissue specimens were obtained from all euthanized mice, and fixed in 10% buffered formalin phosphate (Sigma-Aldrich, Zwijndrecht, The Netherlands). The specimens were embedded in paraffin, cut into sections, and stained with H&E. The degree of inflammation on microscopic horizontal sections of the entire colon was graded semiquantitatively from 0 to 4 as described previously \(^{13}\), in short: 0, no evidence of inflammation; 1, low level of lymphocyte infiltration with infiltration seen in 10% high power fields and no structural changes observed; 2, moderate lymphocyte infiltration with infiltration seen in 10%–25% high-power fields, crypt elongation, bowel wall thickening that does not extend beyond the mucosal layer, and no evidence of ulceration; 3, high level of lymphocyte infiltration with infiltration seen in 25%–50% high-power fields, high vascular density, and thickening of the bowel wall that extends beyond the mucosal layer; 4, marked degree of lymphocyte infiltration with infiltration seen in 50% high-power fields, high vascular density, crypt elongation with distortion, and transmural bowel wall thickening with ulceration.
**Definition of Groups**

To define a semi-quantitative and gradual division of the disease expression in F2 mice, we created a total score of disease severity, combining the percentage of weight loss, the age of sacrifice and the histological score by assigning a score from zero to four for each grade of the corresponding trait (Table 1). If mice were found dead (due to rapid disease progression), an extra point was added to the total score. Theoretically, this would result in a maximum score of 13. Animals were considered resistant when they had a total disease score of 3 or less, implicating they displayed no or only minimal clinical or histological signs of disease upon sacrifice aged 20 weeks or older, whereas mice with a total score of 4 or higher were considered susceptible. We had previously established that mice that had not developed any signs of disease by the age of 20 weeks, would not do so if they were followed for a longer period of time (up to 1 year; data not shown). Therefore, mice that did not develop any signs of disease were sacrificed at the age of 20 weeks.

In part of the analysis, the phenotypic extremes were analyzed. These included on the one hand most resistant mice with a disease score of 0 to 2, and on the other hand most susceptible mice with a score of 8 or higher.

**Table 1. Definition of Disease Severity in GnaI2 Deficient F2 Mice**

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<th>Histology</th>
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<td>0</td>
<td>0</td>
<td>0 – 4</td>
<td>26+</td>
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<tr>
<td>1</td>
<td>1</td>
<td>5 – 9</td>
<td>20 – 25</td>
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<tr>
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<td>10 – 19</td>
<td>15 – 19</td>
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<tr>
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<td>3</td>
<td>20 – 29</td>
<td>10 – 14</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>30+</td>
<td>0 - 9</td>
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Total score of colitis was determined by the sum of the score for each trait (histology, weight loss, age of death) and adding an extra point if an animal was found dead.

**Genotyping**

Spleens were taken from all mice and frozen at -70°C. DNA was isolated using a commercial kit as described by the manufacturer (Gentra Systems, Biozym, Landgraaf, The Netherlands). Primers for microsatellite markers that differ at least 6 base pairs in size between susceptible C3H/HeN mice and resistant C57BL/6J mice were selected from online available databases (CIDR; [http://www.cidr.jhmi.edu/mouse/mouse.html](http://www.cidr.jhmi.edu/mouse/mouse.html)) and The Jackson Laboratory (http://www.informatics.jax.org/) and purchased from Invitrogen Life Technologies (Breda, The Netherlands). A total of 134 microsatellite markers equally dispersed along the mouse autosomes and the X-chromosome were selected for genotypic analysis. Using these markers, a genetic map was generated with an average locus distances of 12 cM, with the largest distance (28 cM) on proximal
Chapter 3

Chr. 2 between markers D2Mit1 and D2Mit242. The markers used in this study will be published online (http://www.immunogenetics.nl).

To evaluate which markers cosegregate with disease phenotype, a first group of 147 mice were selected for a genome-wide screen: 56 resistant F2 mice (30 females and 26 males) with a disease score of 3 or less and 91 F2 susceptible mice (47 females and 44 males) with a disease score of 4 or higher. From this group, 76 mice were selected with the most distinct phenotypes (47 resistant, 29 with severe colitis). Genetic regions that showed suggestive or significant linkage, either from the group with the extreme phenotypes or the total first group, were then further investigated by genotyping the remainder of the eligible mice.

Polymerase chain reaction (PCR) amplification was performed in GeneAmp PCR System 9600 and GeneAmp PCR System 9700 (both PE Applied Biosystems, Nieuwekerk aan de IJssel, The Netherlands) in 96-well plates in 10µL volumes using a protocol provided by the manufacturer (Research Genetics, Invitrogen). Thirty cycles of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 60 seconds followed by an extension period at 72°C for 7 minutes were generally used, although some primers required a slightly different annealing temperature for optimum amplification. After PCR amplification, PCR products were run on 4% agarose gels (in a mixture of 2% ultra pure agarose (NuSieve® GTG® Agarose; Cambrex Bio Science Rockland, Rockland ME, USA), and 2% low melting (Invitrogen)) and visualized by ethidium bromide (Invitrogen) staining.

Statistical Analysis

The MapManager software package was used to construct linkage maps with microsatellite markers that experimentally showed polymorphism between the C3H/HeN and C57BL/6J strain (http://mapmgr.roswellpark.org/mmQTX.html). Likelihood ratio statistics (LRS) values for colitis severity were calculated with MapManager QTXb18 version 0.27 in populations of females and males, females alone and males alone. Logarithm of the odds (LOD) scores were determined by LRS/2ln10. Empirical thresholds for suggestive and significant linkage were determined by permutation analysis using the MapManager software package with 1000 permutations. Gene-gene interactions between the different genetic loci were identified using the same statistical program.

For nonparametric linkage analysis of resistant and susceptible mice, χ² analysis was performed using GraphPad InStat version 3.05 for Windows 95 (GraphPad Software, San Diego, CA; http://www.graphpad.com). A χ² test statistic for each marker locus was derived using 2x3 contingency tables to test for linkage.
Results

Distribution of colitis susceptibility in parental strains and their offspring

Parental strains

We generated homozygous C3H/HeN Gnai2-deficient mice by crossing C57BL/6J Gnai2-deficient mice with C3H/HeN mice, backcrossing F1 mice carrying the gene defect to C3H/HeN mice for >6 generations and then intercrossing to obtain homozygous mice. Such homozygous Gnai2 deficient (Gnai2-/-) C3H/HeN mice, when reared under specific-pathogen-free conditions, were highly susceptible to colitis (Fig. 1).

Figure 1. Distribution of Colitis Scores in Different Groups of Mice

As previously described, colitis in C3H/HeN Gnai2-/- mice is characterized by severe diarrhea and weight loss, frequently accompanied by rectal prolapse and dilation of the colon. In general, only the distal half of the colon is involved, with a sharp demarcation between the involved and non-involved areas. Microscopically, massive infiltrations of lymphocytic cells are seen, accompanied by depletion of goblet cells, crypt elongation, high vascular density and transmural bowel wall thickening with ulceration (Fig. 2).

C3H/HeN Gnai2-/- mice display a greatly reduced survival rate and in general breed poorly, probably because most mice develop disease before the reproductive age. Colitis develops in these mice as early as week 6 and even before full onset of gastrointestinal disease, the animals exhibit decreased body weight as compared to their wildtype littermates (data not shown). Therefore, for breeding purposes, the C3H/HeN mice were maintained in a heterozygous state for the Gnai2 deficiency (Gnai2+/-). Based on genotyping the offspring of Gnai2+/- x Gnai2+/- mice, the frequency of homozygous Gnai2 deficient mice was markedly below the expected 25%, indicating intrauterine death in a significant number of Gnai2-/- mice (results not shown). Interestingly, heterozygous Gnai2 C3H/HeN deficient mice did not develop any signs of disease, indicating that colitis only occurs if no functional Gnai2 is present (Fig. 1).

Whereas C3H/HeN Gnai2-/- mice are highly susceptible to colitis, C57BL/6J Gnai2-/- mice are highly resistant and disease did not occur in these mice even when the latter were followed for as long as 6 months (Fig. 1), and in addition, upon necropsy, colons of these mice were normal, both macroscopically and microscopically (Figure 2c, d).
Figure 2 Microscopic Views of Normal and Inflamed Colons
Involvement of mouse chr. 3 in colitis susceptibility

F1 generation
The F1 generation was generated by crossing C3H/HeN Gnaiz/+ mice with C57BL/6J Gnaiz/- mice. As expected, 50% of the offspring was homozygous for the Gnaiz deficiency, and these mice were used for further analysis and breeding purposes. The Gnaiz F1 offspring was relatively resistant to colitis. Thus, in general, these animals did not develop weight loss, diarrhea or macroscopic signs of colitis, although occasionally some histological abnormalities were found (Fig. 2e).

F2 generation
A total of 284 F2 mice (138 males; 146 females) was generated by interbreeding the Gnaiz F1 generation. As can be seen from Figures 2 and 3, the F2 population displayed a broad spectrum of disease, varying from highly resistant with no clinical and histological signs of illness, to highly susceptible, with severe colitis. The severity of colitis was determined using a semi-quantitative classification as described in detail in Materials and Methods. However, as can also be seen from Figure 3, the trait followed an essentially bimodal distribution. Thus, in part of the analysis, the trait was analyzed as a qualitative trait: mice with a disease score of 3 or less (n = 145; 79 males, 66 females) were considered resistant to colitis since they did not exhibit any clinical signs of disease, and, in addition, displayed no or only minimal weight loss and histological abnormalities (Table 1). In contrast, a total of 139 mice (59 males, 80 females) displayed clinical signs of disease accompanied by significant weight loss and histological disease (i.e., a disease score of 4 or higher) and were considered susceptible. Overall, females were somewhat more susceptible to colitis than males. This was reflected in the mean colitis score, which was higher in females than in males (4.9 vs. 4.2; P < 0.05). Occasionally, histological signs indicative of colon carcinoma were found in F2 mice. This occurred exclusively in mice with severe inflammation. However, there was no clear correlation between occurrence of carcinoma and the duration of the disease, since, in some instances, neoplastic lesions were found in mice as young as 9 weeks.

Figure 3 Distribution of Colitis in Gnaiz Deficient F2 Mice

Distribution of colitis in F2 mice. Dotted line indicates cut-off for resistant/susceptible mice. The colitis severity follows a bimodal distribution. Also, females are more severely affected as male mice.

Figure 2. Microscopic Views of Normal and Diseased Colon
Representative H&E stained longitudinal sections (x100) of colons of: a: Wild-type C3H/HeN; b: GNAI2-/- C3H/HeN; c: Wild-type C57BL/6J; d: GNAI2-/- C57BL/6J; e: GNAI2-/- C3H/HeNxC57BL/6J F1; f-j: GNAI2-/- F2 mice, with increasing histological scores
Strategies for Analysis and Distribution of Traits

In order to identify the genetic regions determining the differences in susceptibility between susceptible C3H/HeN and resistant C57BL/6J mice, we genotyped the initial group of 147 F2 mice of our cohort with a panel of 134 microsatellite markers equally dispersed among the genome. We first analyzed disease in the F2 population as a qualitative (binary) trait, i.e. susceptible versus resistant mice. Among the 147 mice, 56 (30 females; 26 males) were relatively resistant, with a disease score of 3 or less, whereas 91 (47 females; 44 males) were susceptible (with a disease score of 4 or higher). Contrasting genotype and allele frequencies of these groups led to the identification of 4 chromosomal regions that exhibited an association suggestive for linkage ($P < 1.6 \times 10^{-3}$ or stronger) \(^{14}\). These loci reside on Chr.s. 1, 3, 13 and the X-chromosome.

Subsequently, the subgroup of mice with the most extreme phenotypes were analyzed. This analysis has the advantage that the phenotypes of these groups are clearly distinct and non-overlapping, and the disadvantage of lower statistical power due to the lower sample number. Contrasting mice with a disease score of 2 or less with mice with a disease score of 8 or higher resulted in essentially the same associations. In addition, this analysis allowed identification of an association with a locus on Chr. 9, which was not found in the entire group of mice.

Finally, disease was considered as a quantitative trait in which the genes contribute to the trait in a continuous fashion rather than in a discrete way. This was accomplished by a total disease score which takes into account histological score, weight loss and age of sacrifice (Table 1). Due to the semi-quantitative nature of our phenotypes we established an empirical genome-wide significance level for our dataset by performing a permutation analysis. The observed genotypes of the F2 animals were kept constant while the phenotypes were randomly shuffled over the genotype data, thereby efficiently disrupting any genotype–phenotype correlation. Linkage analysis was performed and the strongest association (expressed as the likelihood ratio statistics-LRS) in each experiment was recorded. This procedure was repeated 1000 times and through this method the LRS threshold for 95% significance was calculated to be 16.5. Suggestive linkage was calculated to be found above a threshold LRS value of 10.0.

All chromosomes with regions identified with the above strategies that showed at least suggestive linkage were genotyped in the entire cohort of 284 F2 mice, to confirm or discard the linkage.

Identification of major susceptibility locus on chromosome 3

Analysis of the entire F2 population as a qualitative trait confirmed the presence of a region on the distal part of Chr. 3 that is strongly associated with colitis susceptibility. As can be seen from Table 2, the strongest association was found with microsatellite markers D3Mit316 at 59 cM ($\chi^2 = 29.7$; $P < 1.0 \times 10^{-5}$) and D3Mit348 at 61 cM, ($\chi^2 = 29.6$; $P < 1.0 \times 10^{-5}$). Analysis of phenotypic extremes led to a similarly significant association with these markers ($\chi^2 = 32$; $P < 1.0 \times 10^{-5}$, data not shown). When mice were subdivided according to sex, the same significant associations were found in both males and females.
Table 2. Distribution of Genotypes Surrounding the Locus on Chromosome 3 Regulating Susceptibility to Colitis in Gna12 Deficient F2 Mice

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<th>Position (cM)</th>
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<th>Susceptible (n)</th>
<th>P value</th>
<th>( \chi^2 )</th>
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Microsatellite analysis of chromosome 3. Numbers of different genotypes were compared between susceptible and resistant mice in a contingency table using the \( \chi^2 \) test for independence. Markers at peak association are indicated in Bold. cM, centi Morgan; n, number of mice; BB indicates homozygous for the C57BL/6J strain; BC indicates heterozygous; CC indicates homozygous for the C3H/HeN strain.

We then analyzed the F2 population as a quantitative trait. As shown in Figure 5, this analysis confirmed the highly significant association with this region. The strongest association was again with the chromosomal region surrounding D3Mit348 at 61 cM. A peak LRS of 32.4, corresponding to a LOD score of 7 was found for this locus. The strength of the association markedly exceeded the threshold for significant linkage (LRS = 16.5) that was determined empirically by permutation analysis. We have provisionally designated this QTL Gpdc1, for G protein deficiency-induced colitis.

To study the mode of inheritance of this locus, we stratified mice according to their genotype at position D3Mit348, and determined the severity of colitis in these groups. As can be seen from Figure 4, F2 mice that were homozygous for the C75BL/6 allele at this position had a mean (± SEM) colitis score of 4.01 (± 0.31) and this was not significantly different from mice heterozygous for the C57BL/6J allele at this position, who had a mean score of 4.16 (± 0.27). This was in sharp contrast with the colitis score in mice
homozygous for the C3H/HeN allele at this position 6.40 (± 0.32; \( P < 0.001 \)). These findings are consistent with the view that two copies of the C3H allele at this position are required to develop colitis, and help to explain the resistance in the F1 population of mice.

Figure 4. Quantitative Trait Analysis of Chromosomes 1, 3, and 9

On Chr. 1, after stratification by \textit{Gpdc1}, a broad peak is found, spanning a region of approximately 30 cM, from 39 cM to 70 cM (\textit{Gpdc2}). The peak has a LRS of 19.7. On Chr. 3, a very broad region shows linkage to colitis susceptibility, with the 95% confidence interval of 22 cM around the peak marker D3Mit348, which had a LRS of 32.4 (\textit{Gpdc1}). After stratification by \textit{Gpdc1}, on Chr. 9 a peak at 42 cM with marker D9Mit123 just reaches the significance cut-off LRS of 16.5 (\textit{Gpdc3}). All susceptibility loci are C3H/HeN-derived

**Genetic linkage analysis: other QTLs**

Since the association with the region on Chr. 3 was remarkably strong, this locus might theoretically overshadow associations with other genetic loci that otherwise might have an important effect on the trait. We therefore performed sequential regression analyses in which we stratified for marker D3Mit348. This analysis continued to show evidence suggestive of linkage to colitis susceptibility on Chr. 1, 9, and X identified in the initial screen. (Table 3), but did not reveal additional loci. The locus on Chr. 13 that was previously mentioned to be a locus of suggestive linkage in the first group did not retain any association in the total group of mice, and was thus discarded as being a locus of interest.

**Chromosome 1**

A locus on Chr. 1, denoted by D1Mit215 at 47 cM showed suggestive linkage to disease susceptibility in the first group of 147 mice. As can be seen in Table 4, analysis of the entire group of 284 mice as a
Involvement of mouse chr. 3 in colitis susceptibility

Qualitative trait confirmed this finding, but did not markedly increase the strength of the association ($P = 1.5 \times 10^{-3}; \chi^2 = 13.0$).

QTL analysis essentially confirmed the qualitative trait data, revealing a broad peak of approximately 30 cM surrounding the marker D1Mit308 (Fig. 4), with a maximum LRS of 19.7 (17.8 without stratification for D3Mit348) which corresponds with a LOD score of 4.3. According to the results obtained in the permutation test, this LRS value exceeds the threshold for significance in a genome-wide screen, and we have therefore provisionally designated this locus \textit{Gpdc2}.

Similar to \textit{Gpdc1}, only mice that were homozygous for the C3H/HeN allele showed a significant rise in colitis score, as can be seen in Figure 5. Mice that were homozygous for the C57BL/6J allele and mice that were heterozygous at this locus had a mean (± SEM) colitis score of 4.34 (± 0.27) respectively, whereas mice that were homozygous for the C3H/HeN allele had a mean score of 5.77 (± 0.33; $P < 0.01$), indicating that this locus also acts in a recessive manner.

Chromosome 9

An association suggestive of linkage with Chr. 9 was found in the phenotypic extremes in the first group of mice. Although analysis of the entire group of mice as a qualitative trait did not confirm this finding, a significant deviation from the normal genotype distribution was found in the phenotypic extremes of the entire group ($P = 8.0 \times 10^{-4}$). QTL analysis of the entire group of mice revealed suggestive linkage with marker D9Mit123 at 42 cM, with a LRS of 14.2. After controlling for D3Mit348, the LRS increased to 16.5, and thus reached borderline genome-wide significance (Fig. 4; Table 3). We have provisionally named this C3H/HeN-derived locus \textit{Gpdc3}.

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<th>Locus</th>
<th>LRS</th>
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Likelihood Ratio Statistics (LRS) were calculated using marker regression analysis after control for the major QTL on Chr. 3 (right columns) or without control for this QTL (left columns). Suggestive Linkage: LRS > 10.0 (italic); Significant Linkage: LRS > 16.5 (bold). All susceptibility loci are C3H/HeN-derived.
### Table 4. Distribution of Genotypes Surrounding the Locus on Chromosome 1 Regulating Susceptibility to Colitis in Gnaq2 Deficient F2 Mice

<table>
<thead>
<tr>
<th>Marker</th>
<th>Position (cM)</th>
<th>Genotype</th>
<th>Resistant (n)</th>
<th>Susceptible (n)</th>
<th>P value</th>
<th>$\chi^2$</th>
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Microsatellite analysis of chromosome 1. Numbers of different genotypes were compared between the susceptible and resistant mice in a contingency table using the $\chi^2$ test for independence. Markers at peak association are indicated in Bold. cM, centi Morgan; n, number of mice; BB indicates homozygous for the C57BL/6J strain; BC indicates heterozygous; CC indicates homozygous for the C3H/HeN strain.

**X-Chromosome**

Since male mice are hemizygous for the X-chromosome, we performed nonparametric analysis for the allele rather than the genotype frequencies in the qualitative trait analysis (Table 5). This resulted in a $P$ value suggestive of linkage ($P = 3.0 \times 10^{-3}$) in the total group of mice for marker DXMit223 located distally (at 73.3 cM) on the X-chromosome. Separate analysis of male and female mice revealed a stronger association of this locus in female mice than in male mice, although the association in female mice was weaker than in the group as a whole, probably because of the lower statistical power arising from the smaller size of the female subgroup. After stratification for D3Mit348, QTL analysis again revealed a statistical association suggestive of linkage for the total group of mice, with a LRS of 14.1 (Table 3). Again, this susceptibility locus was derived from the C3H/HeN strain.
**Involvement of mouse chr. 3 in colitis susceptibility**

**Figure 5. Distribution of Colitis Score for Different Genotypes at D3Mit348 and D1Mit308 in Gnia2 Deficient F2 Mice**

A significant difference between the C57BL/6J homozygous and C3H/HeN homozygous groups of mice for markers D3Mit348 and D1Mit308 was found. BB indicates homozygous for the C57BL/6J strain; BC indicates heterozygous; CC indicates homozygous for the C3H/HeN strain.

**Epistatic and additive interactions with the identified locus on chromosome 3**

Separately, the QTLs on Chr. 1 and 3, and to a lesser extent the loci on Chr. 9 and X contributed significant main effects to both the qualitative and quantitative disease traits. The MapManager software package was then used to assess epistatic and additive interactions between *Gpdc1* and other chromosomal regions. Only mice that are genotyped for their complete genome are informative for identification of interactions between chromosomal regions; therefore, in this analysis, we used the data from the initially screened 147 mice.

When marker alleles for *Gpdc1* were paired with those of *Gpdc2*, a strong interaction was observed between the two loci. Thus, the combined LRS for both loci was higher than the sum of the LRS of both QTLs individually (Table 6; combined LRS = 68.2). In addition, a C3H/HeN derived locus on Chr. 7 which did not have an effect on the trait individually (LRS = 4.4), showed significant epistatic effects when paired with *Gpdc3* (Table 6).

**Table 5. Allele Frequencies on the X-Chromosome in Gnia2 Deficient F2 Mice**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Position (cM)</th>
<th>Allele</th>
<th>Resistant (n)</th>
<th>Susceptible (n)</th>
<th>P value</th>
<th>( \chi^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXMit68</td>
<td>17.2</td>
<td>B</td>
<td>84 (43)</td>
<td>99 (77)</td>
<td>0.06 (0.27)</td>
<td>3.5 (1.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>90 (59)</td>
<td>154 (121)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DXMit64</td>
<td>45</td>
<td>B</td>
<td>82 (43)</td>
<td>100 (66)</td>
<td>0.1 (0.2)</td>
<td>2.4 (1.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>92 (59)</td>
<td>153 (122)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DXMit223</td>
<td>73.3</td>
<td>B</td>
<td>91 (49)</td>
<td>88 (55)</td>
<td>3.0 \times 10^4</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>83 (53)</td>
<td>165 (133)</td>
<td>(1.4 \times 10^3)</td>
<td>(10.1)</td>
</tr>
</tbody>
</table>

Microsatellite analysis of the X-chromosome. Allele frequencies were compared between susceptible and resistant mice in a contingency table using the \( \chi^2 \) test for independence. Markers at peak association are indicated in bold. Numbers between brackets represent female mice. cM, centi Morgan; n, number of alleles; B indicates C57BL/6J alleles; C indicates C3H/HeN alleles.
Table 6. Summary of Epistatic Effects

<table>
<thead>
<tr>
<th>Locus A</th>
<th>Position (cM)</th>
<th>Individual Effect (LRS)</th>
<th>Locus B</th>
<th>Position (cM)</th>
<th>Individual Effect (LRS)</th>
<th>Combined Effect (LRS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr. 3</td>
<td>62.1</td>
<td>32.4</td>
<td>Chr. 1</td>
<td>61.2</td>
<td>17.8</td>
<td>68.2</td>
</tr>
<tr>
<td>Gpdc1</td>
<td></td>
<td></td>
<td>Gpdc2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chr. 7</td>
<td>41</td>
<td>4.4</td>
<td>Chr. 9</td>
<td>42</td>
<td>14.2</td>
<td>37.8</td>
</tr>
<tr>
<td>(D7Mit350)</td>
<td></td>
<td></td>
<td>Gpdc3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Interactions between individual markers were calculated using the MapManager QTX software package. The interacting region of Gpdc1 conferred D3Mit316 and D3Mit348, and Gpdc2 conferred D1Mit308 and D1Mit215. Only the highly significant results are tabulated ($P < 1.0 \times 10^{-5}$). cM, centi Morgan. All susceptibility regions were C3H/HeN-derived.

Distribution of C3H/HeN and C57BL/6J Alleles in the F2 Study Population

The GnaI2 deficiency of C57BL/6J mice was transferred to C3H/HeN mice by serial backcrossing for six generations (see Methods); this plus the fact that parental C3H/HeN GnaI2<sup>-/-</sup> mice exhibited a reduced survival rate introduced the possibility that the C3H/HeN GnaI2 KO mice carry some excess C57BL/6J DNA that skewed the allele distribution in the F2 generation and thus decreased the number of fully informative mice in this generation.

To determine if this was in fact the case, we first tested the genome-wide allele distribution by comparing the found allele distribution with the expected distribution in a 2x2 contingency table. The genome-wide distribution of C57BL/6J:C3H/HeN alleles did not differ from the expected 1:1 distribution. We then examined the allele distribution in individual chromosomes. For chromosomes 2 and 6, B6 alleles were slightly more expressed, whereas for chromosomes 4, 8, 14 and X C3H alleles were slightly more expressed ($p \leq 0.05$); however the over-expression of C57BL/6J alleles was strongly significant only on chromosome six ($p = 0.0006$) (data not shown). Thus, the possibility exists that Chr. 6 could harbor a susceptibility region that was inaccessible to discovery in this study.

Discussion

During the last decade, numerous animal models have been identified that have provided key insights into the mechanisms underlying both experimental mucosal inflammation and human inflammatory bowel disease<sup>6</sup>. One of the most important of these insights is that the development of colitis in mice with a severe primary immunological defect (such IL-10 deficiency<sup>11</sup>) or in mice with colitis induced by a haptenating agent (such as trinitrobenzene sulphonic acid (TNBS)-colitis<sup>15</sup>) manifest varying levels of colitis depending on their genetic background. This indicates that even a severe (genetically determined) primary defect may depend on the presence of genetically determined secondary factors to produce disease. This is borne out in the present study of colitis development in GnaI2<sup>-/-</sup> mice wherein we show that disease expression is dependent on a secondary factor(s) encoded by one or more genes within susceptibility loci on chromosome 3 and other chromosomes.

A second important insight derived from the animal models is that despite the fact that the cause of inflammation differ widely among the various models, they are almost always due to either an exaggerated $T_h1$ response characterized by high expression of IL-12 and IFN$\gamma$, or, less frequently, by a $T_h2$ response mediated by IL-4/IL-13 and that both types of responses are dependent on cell stimulation...
by antigens in the mucosal microflora. In the Gnai2<sup>-/-</sup> mice studied here the inflammation falls into the Th1 category in that the colitis is characterized by a marked increase in Th1-type cytokine production. In this case the Th1 orientation is best explained by the studies of He et al., who have shown that treatment of normal mice with pertussis toxin (PT), a substance that inhibits Gi protein-signaling, enhanced the capacity of splenocytes to produce IL-12 in response to both microbial and non-microbial stimuli. Thus, the genetically-determined absence of Gi signaling in these mice could result in a resetting of the Th1/Th2 “immunostat” in a way that leads to an excess Th1 in response to microbial antigens in the gut. This formulation is consonant with our previous study that provided strong circumstantial evidence that in the TNBS-colitis model, a strain-specific dysregulation in IL-12 secretion is present which results in over-production of IL-12 in response to TLR4 stimulation. In addition, it is consonant with the Th1 colitides occurring in both IL-10 deficient mice and Stat3 deficient mice (the latter characterized by an IL-10 signaling abnormality) although in these cases Th1 overproduction is likely due to deficient down-regulation of the mucosal Th1 responses elicited by mucosal microflora. If we now combine the concept of a reset immunostat with the fact noted above that in most cases models of colitis exhibit marked strain specificity and thus evidence for the existence of secondary genetic factors, we can say that the latter is in some manner directly or indirectly influencing the increase Th1 response to induce disease. Alternatively, it is creating conditions under which an augmented Th1 response leads to colitis.

As a first step to identify the genetic factors operating in Gnai2<sup>-/-</sup> mice, we performed a genome-wide linkage analysis for colitis susceptibility. We analyzed F2 intercross mice derived form segregating crosses between relatively resistant C57BL/6J and susceptible C3H/HeN mice. This allowed the identification of several chromosomal loci that are involved in colitis susceptibility and/or severity. The locus that most strongly associated with colitis susceptibility, provisionally named Gpdc1, resides on the distal end of mouse Chr. 3 and represents the major determinant of colitis susceptibility in this intercross population. Remarkably, this locus was previously found to control colitis susceptibility in a different model of spontaneous colitis, the IL-10 deficient mouse model. By using virtually the same mouse stocks (the C57BL/6J strain and in this case the C3H/HeJBir strain (which carries the lps mutation in the Toll-like receptor 4 (TLR4), and therefore lacks the capacity to respond to lipopolysaccharide (LPS)), rather than the C3H/HeN strain, Farmer and coworkers identified a major colitogenic locus (designated Cdcs1) at precisely the same position on Chr. 3 as Gpdc1. This finding strongly suggest that this region on Chr. 3 harbors a gene that has a strong modulating effect on the exaggerated Th1 responses seen in both models, which is independent of the primary genetic abnormality that gave rise to these responses. Thus, identification of this gene may provide new fundamental insights in the regulation of pathological Th1 mucosal immune responses.

Whether the modifying gene on Chr. 3 described here and previously in IL-10 KO mice is acting in relation to two very different primary abnormalities, operative in each type of mice, or a single abnormality operative in both type of mice, is an open question. The first possibility is supported by the findings of He et al., already alluded to above which locates the defect in the capacity of dendritic cells in Gnai2<sup>-/-</sup> mice to produce IL-12 and not in the regulation of a normal capacity to produce IL-12, as in IL-10 KO mice. Thus, these data suggest that Gnai2<sup>-/-</sup> mice and IL-10 KO mice have two basically
different immunoregulatory abnormalities. The second possibility is favored by data recently reported by Dalwadi et al. who showed that Gnaiz\(^{-/-}\) mice are characterized by a reduction in marginal zone (MZ), transitional type 2 (T2) and B-1a splenic B cells (that have the capacity to produce IL-10) as well as decreased in vitro (LPS-induced) B cell IL-10 responses\(^ {20}\). Thus, these data suggest that Gnaiz\(^{-/-}\) mice also have an IL-10 defect and that the modifying gene can be affecting the IL-10 production defect in both mouse strains. In view of these somewhat disparate findings it is too soon to decide on whether the modifying gene on Chr. 3 is modifying a single abnormality or two separate abnormalities.

As previously discussed by Farmer et al., there are several genes present in the identified locus on Chr. 3 that would make attractive candidate secondary disease genes\(^ {11}\). One of these is the gene encoding the NF-κB p105 transcription factor (Nfkb1). NF-κB designates a group of critical transcription factors controlling various promoters of pro-inflammatory cytokines, cell-surface receptors, transcription factors, and adhesion molecules\(^ {21}\). Thus, any mutation affecting the Nfkb1 gene would clearly have major implications for a great variety of immunological and/or inflammatory processes. The fact that IL-12 is a cytokine that is under transcriptional regulation of NF-κB makes this gene even more of interest, since in the Gnaiz\(^{-/-}\) model, the IL-12 expression is clearly altered/elevated\(^ {12}\). Indirect evidence that this gene might be important in IBD comes from a recent study by Karban et al., who found an elevated frequency for the \(-94delATTG\) allele in the promoter region of Nfkb1 in UC patients\(^ {22}\), a finding since confirmed by studies of our own IBD patient group (data not shown). Nevertheless, we did not find a polymorphism in the coding region of murine Nfkb1 so that if this gene is a disease gene, the abnormality would have to consist of a subtle change in the way it is activated and thus in the way it functions under different circumstances. This multifaceted question is currently under investigation.

The gene encoding epidermal growth factor (the Egf gene) is a second candidate gene of interest present in the Chr.3 locus. Since EGF induces epithelial cell proliferation polymorphisms or mutations in the Egf gene could conceivably affect the repair mechanisms of the mucosa and thus the susceptibility to mucosal injury due to inflammatory processes. Indeed, treatment of UC patients with EGF has proven useful in some cases\(^ {23}\). Yet a third candidate in the Chr. 3 locus is the gene encoding guanylate binding protein-1 (GBP1), a member of a family of proteins induced by IFN-γ during macrophage induction that shares the ability to bind to guanine nucleotides\(^ {24}\). GBP1 is a GTPase that converts GTP to GMP. GBP expression is abundant in IFN-exposed cells, but little is known about its function\(^ {25}\). Interestingly, type I and II IFNs are capable of inducing the synthesis of GBP1 in some strains of mice, including the colitis susceptible C3H/HeN strain, whereas cells of other strains, including C57BL/6J, fail to synthesize GBP1 in response to both types of IFNs and therefore could comprise an important candidate gene\(^ {26}\).

While the association of disease in Gnaiz\(^{-/-}\) mice with Chr. 3 was quite strong, this locus explained only part of the phenotypic variance. Thus, it is not surprising that we identified other disease susceptibility loci as well. One such locus (designated Gpdc2) was found on Chr. 1 at 47 cM and provided strong evidence of interaction with Gpdc1. That is to say that the strength of the association of the two loci taken together was markedly higher than the sum of association of the individual loci. This indicates that the two loci may act in a combined fashion in determining disease susceptibility. Interestingly, evidence for the presence of a disease gene on Chr. 1 associated with the gene on Chr. 3 (Cdcs 1) was also
found in IL-10-deficient mice \(^1\). It should be noted however that in this case, peak linkage was more proximal and it thus cannot be excluded that the two identified Chr. 1 regions represent different loci. A third susceptibility locus, \(Gpdc3\), was identified on Chr. 9 with peak linkage at 42 cM. At this junction it is important to note that \(Gnai2\) also resides on Chr. 9. However this gene is located at 59 cM, clearly separated from the region of peak linkage, strongly diminishing the possibility that this locus is caused by artificial interference in the knock-out backcross protocol. Furthermore, D9Mit355, located at 53 cM, right in between \(Gpdc3\) and \(Gnai2\), is polymorphic for C57BL/6J, C3H/HeN and 129/SvJ, the strain on which the \(Gnai2\) knock-out model was originally created \(^10\). Using this marker, no residual 129/SvJ DNA was found at this position, adding further evidence that \(Gpdc3\) is a true QTL (data not shown), and that it is unlikely that this QTL is caused by artificial interference with the \(Gnai2\) congenic segment. Although \(Gpdc3\) had only borderline significance for linkage with disease, it is of interest because it's location corresponds to a locus we previously identified in the TNBS-colitis mouse model, \(Tnbs1\) \(^15\). In addition, a similar locus (designated \(Ibdq1\)) has recently been found by Kozaiwa et al., in SAMP1/YitFc mice who spontaneously develop an ileitis similar to that in human Crohn's disease \(^27\). Finally, in the DSS model of induced colitis, evidence for the involvement of Chr. 9 has also been obtained \(^28\). As has been outlined in the study by Kozaiwa, precise localization of genes controlling complex traits using F2 and first backcross animals is generally not possible because of the limited number of informative recombinations produced in cohorts of manageable size and the substantial variability of phenotypic expression in mice with random segregations of other disease-associated alleles. However, it is at least striking that several models have disclosed linkage in overlapping regions on mid-Chr. 9, which might indicate that this region does in fact harbor a susceptibility gene that mediates overall susceptibility to intestinal inflammation, and is not caused by artificial interference or expression differences induced by the mutation.

Studies in congenic mouse stocks will be able to shed light on the question as to whether the same gene(s) are involved in the different colitis models. That genome-wide analysis of experimental models has the power to eventually identify disease causing gene(s) was recently demonstrated by McIntire and colleagues \(^29\). In their study, they used congenic stocks for a genetic region on mouse chromosome 11 initially identified in a genome-wide screen and were able to identify a gene involving asthma susceptibility using positional cloning.

In conclusion, we have identified a major susceptibility locus on mouse Chr. 3 that exerts effect in conjunction with a locus on Chr. 1. The identification of similar if not identical loci in different models of spontaneous mucosal inflammation indicates that these regions harbor genes that play an important role in maintaining overall mucosal homeostasis. Identification of these genes, using the approach described above involving congenic mice, will provide detailed understanding of the factors that facilitate the development of colitis in individuals prone to mount increased T\(_h\)1 responses. This will thus be relevant to the further understanding of human IBD.

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References

17. Mahler M, Leiter EH. Genetic and environmental context determines the course of colitis developing in IL-10-deficient mice. Inflamm Bowel Dis 2002;8:347-55.
Chapter 4

Sequencing *Nfkb1* for polymorphism detection

Addendum to Chapter 3
Introduction

In our studies to locate quantitative trait loci (QTLs) involved in colitis susceptibility in a Gna12 deficient mouse model, we compared the genome of F2 mice derived from the resistant C57BL6/J strain and the susceptible C3H/HeJ mouse strain. We found a major C3H/HeJ derived QTL on distal chromosome 3. Within this locus lay a number of interesting candidate genes, amongst which \textit{Nfkb1} and \textit{Egf}.

Mice with altered NF-\kappa B signaling have proven to have a higher susceptibility to opportunistic infections and show an inflammatory phenotype \textsuperscript{1}. Furthermore, it is known from human studies that both ulcerative colitis (UC) and Crohn’s disease (CD) patients show elevated levels of NF-\kappa B activation \textsuperscript{2,3}. Also, linkage was found previously with chromosome 4q \textsuperscript{4}, were the human orthologue for \textit{Nfkb1} resides. This chromosomal region appeared to epistatically interact with the \textit{IBD1} region on 16p where the first gene identified as a true susceptibility gene for CD, \textit{NOD2} is located \textsuperscript{5, 6}. This linkage of chromosome 4q with IBD susceptibility was confirmed by several studies \textsuperscript{7, 8}, and found to be mainly associated with UC.
NF-κB signaling route

NF-κB stands for Nuclear Factor of kappa light polypeptide gene enhancer in B-cells. The NF-κB family comprises of a group of critical transcription factors, involved in enhancing gene expression of genes involved in a variety of regulatory processes, including innate and adaptive immune responses, cell growth, apoptosis, and tissue differentiation \(^9, 10\). The NF-κB signaling route and its family members are highly conserved throughout the animal kingdom. Here I will focus on the murine forms. Amongst the family members are p50/105 (encoded by \(Nfkb1\)), p52/p100 (encoded by \(Nfkb2\)), p65 or RelA, RelB and c-Rel (encoded by \(Rela\), \(Relb\) and \(Rel\) respectively). In short, they exert their effects by forming complexes with each other, both homo-dimers and heterodimers, resulting in a multitude of possible complexes, all with specific target genes and differential effects in activating their specific genes transcription.

Activation and Regulation

In resting state, the NF-κB complexes are kept in the cytosol by their sequestering proteins, the IκB protein family, and upon activation they are released and translocate to the nucleus, where they exert their effect on gene expression. Recently, activation of the NF-κB signaling pathways has been categorized into the canonical (classical) pathway and the noncanonical (alternative) pathway \(^11\) (Figure 1). The former is characterized by nuclear translocation of complexes containing p50/105, the latter by the formation and translocation of complexes containing p52/p100. The canonical pathway typically involves activation through cell surface receptors like Toll-like Receptors (TLRs) or Tumor Necrosis Factor-Receptor (TNF-R).
Figure 1 Canonical (classical) and non-canonical (alternative) NF-κB pathway

The canonical pathway is characterized by nuclear translocation of complexes containing p50/105. It typically involves activation through cell surface receptors like Toll Like Receptors (TLRs) or Tumor Necrosis Factor-Receptor (TNF-R). Adapter proteins (e.g. TNF receptor-associated factors (TRAFs) and Receptor-Interacting Protein (RIP)) are recruited, that in turn recruit inhibitor of nuclear factor kappa-B kinase (IKK) complexes containing NF-Kappa-B Essential Modulator (NEMO). This complex induces degradation of the sequestering protein IκB, releasing the NF-κB complex that can then migrate to the nucleus. The canonical pathway has an auto-regulatory mechanism, in that NF-κB activates expression of the IκB gene, leading to resequestering of the NF-κB complex.

The noncanonical pathway can be activated by other receptors, (e.g., Lymphotoxin-β (LT-β), B-cell activating factor (BAFF), and CD40). In the noncanonical pathway, receptor binding leads to activation of the NF-κB-inducing kinase (NIK), which phosphorylates and activates an IKKα complex, which in turn phosphorylates the IκB domain of p100, leading to its partial proteolysis and liberation of the p52/RelB complex, that can in turn migrate to the nucleus.

Adapter proteins (e.g. TNF receptor-associated factors (TRAFs) and Receptor-Interacting Protein (RIP)) are recruited, that in turn recruit inhibitor of nuclear factor kappa-B kinase (IKK) complexes containing NF-Kappa-B Essential Modulator (NEMO). Activation of this complex leads to degradation of the sequestering protein IκB, and finally results in translocation of the NF-κB complex to the nucleus. The canonical pathway has an auto-regulatory mechanism, in that NF-κB activates expression of the IκB gene, leading to resequestering of
the NF-κB complex. The noncanonical pathway can be activated by certain receptors, (e.g., Lymphotoxin-β (LT-β), B-cell activating factor (BAFF), CD40). In the noncanonical pathway, receptor binding leads to activation of the NF-κB-inducing kinase (NIK), which phosphorylates and activates an IκKα complex, without NEMO, which in turn phosphorylates two serine residues adjacent to the ankyrin repeat C-terminal IκB domain of p100, leading to its partial proteolysis and liberation of the p52/RelB complex (Figure 1) 12.

NF-κB p50 mediated gene transcription can be regulated in several ways (Figure 1).

To exert its effect, p50 binds to p65 to form a biologically active heterodimer, which can translocate from the cytoplasm of the cell to the nucleus. Here, it binds to a NF-κB binding site in promoter sequences of inflammatory genes, to enhance gene expression. In resting state, the p50/p65 complex is kept in the cytosol by IκB. Activation of kinases that ubiquitinate IκB (IKKs) leads to degradation of these IκBs, resulting in the release of the NF-κB complex. Alternatively, next to binding of ‘regular’ IκBs to NF-κB complexes, dimer formation with an active protein (e.g. p65) and p105 precursor proteins can take place. The IκBγ part of the p105 keeps the complex inactive and in the cytoplasm. Removal of the inhibitory IκBγ C-terminal half of the precursor leads to activation of the NF-κB complex.

Next to sequestering potentially active complexes in the cytosol by IκB, NF-κB mediated gene transcription can be inhibited by blocking the NF-κB binding sites in the promoter region of the genes. The subunit p50 is able to form homodimers, that can also translocate and bind to the NF-κB binding sites, but do not have the gene transcription enhancing properties. This way, p50 can function as its own inhibitor, by preventing the binding of activating complexes 13.

*Nfkb1* gene structure and functioning

As alluded to earlier, *Nfkb1* encodes for NF-κB p50/105 (Figure 2). It is a large gene, residing on the distal part of mouse chromosome 3, coding for 971 amino acids (aa’s) encoded by 24 exons (GeneID: 18033). The protein product of *Nfkb1* is synthesized as an inactive cytoplasmic precursor protein, p105, which upon processing generates the active subunit p50 of the Rel/NF-κB complex. This p50 protein is formed from the N-terminal side of the precursor. From the C-terminal side of the p105 precursor, an IκBγ protein can be formed. This IκBγ protein is a sequestering protein, involved in retaining the NF-κB complex in the cytosol, thus inhibiting NF-κB activation. Both gene products will be discussed here.

*p50*

The p50 part of the protein harbors a Rel homology domain (RHD), which mediates DNA-binding, dimerization and association with the IκB family of inhibitory proteins. The C-terminal domain within the RHD has an immunoglobulin-like fold that binds to DNA. This RHD is shared with the other members of the Rel/NF-κB transcription factor family members. The C-terminal end of p50 harbors a nuclear localization signal (NLS). This sequence does not seem to influence the dimerization and DNA-binding properties, but is involved in binding IκB inhibitors. The IκB proteins bind preferably to the RHD of their specific subunit (e.g. IκBα to p65/RelA, IκBγ to p50) and thus block the NLS of their specific subunit, keeping the complex sequestered in the cytoplasm.
As mentioned above, next to p50, Nfkβ1 also encodes a p70 IκB protein, IκBγ, which is identical to the C-terminal 607 amino acids (aa’s) of p105. IκBγ contains 7 ankyrin–like repeated sequences, that are responsible for association with the RHD of the NF-κB subunits. At the C-terminal side, the IκBγ protein contains a death domain and destruction box. The death domain is necessary for cytoplasmic retention of NF-κB, but is by itself not sufficient for retention in the cytoplasm. It does not participate in IκBγ binding to NF-κB.

Alternative RNA splicing generates two IκBγ isoforms with properties different from those of p70. One 63-kDa isoform, termed IκBγ1 lacks 59 amino acids C-terminal to ankyrin repeat 7, but has a novel 35-aa C terminus, encoded by an alternative reading frame. The second, a 55-kDa isoform, IκBγ2, lacks the 190 C-terminal aa’s of p70IκBγ. In contrast to p70IκBγ, which is a cytoplasmic protein, IκBγ1 is found in both the cytoplasm and nucleus, whereas IκBγ2 is predominantly nuclear. Both IκBγ1 and IκBγ2 are specific for p50 and have different affinities for this subunit, suggesting that alternative RNA splicing may be used to generate IκBγ isoforms that respond differently to intracellular signals.

Taken together, NF-κB mediated activation of gene transcription shows very complex regulation mechanism. Any change in gene transcription or gene function of any of the family members can have significant consequences for the regulation of the immune status of the host. In summary, there is substantial evidence for altered NF-κB activation in both human IBD and in animal models of colitis. In addition, at least one gene of this pathway, Nfkβ1, maps to linkage regions in both animal models as well as in human IBD. Taking this into consideration, we decided to compare the sequences of the Nfkβ1 gene from the two mouse strains described in the previous chapter to see if we could identify polymorphisms in the coding region of the gene that could help to explain the differences in susceptibility between the two strains.
Sequencing Nfkb1 for polymorphism detection

Materials and Methods
For sequencing the transcribed part of Nfkb1 (aa’s 1 until 3601), mRNA was isolated from colons from both C57BL/6J and C3H/HeN mice, using Trizol (Invitrogen, Breda, The Netherlands), according to manufacturers recommendations. cDNA products were generated from the mRNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, St.Leon-Rot, Germany). For sequencing the 5’UTR promoter region, genomic DNA was isolated from spleens from both C57BL/6J and C3H/HeN mice, using a commercial kit as described by the manufacturer (Biozym, Landgraaf, The Netherlands). Overlapping fragments were amplified by PCR using Nfkb1 specific primers designed on the basis of the published genomic DNA sequences (accession NM 008689.1 GI: 6679043).
Amplified DNA and cDNA fragments were purified by gel purification (QIAEX® II Gel Extraction Kit, QIAGEN, Westburg, The Netherlands) cloned into a plasmid vector for sequencing (TOPO TA Cloning® Kit for Sequencing, version H, Invitrogen). Plasmids with the gene fragments were isolated (QIAprep® Spin Miniprep Kit, QIAGEN) and commercially sequenced by BaseClear (Leiden, The Netherlands). Sequences of amplified fragments were compared with each other and with the published Nfkb1 DNA sequences.

Results and Discussion
We found 5 single nucleotide polymorphisms (SNPs) of which one lays in the ‘3-UTR of the gene (Table 1 and Figure 3). These polymorphisms were found by aligning the sequences of the two strains to each other, and comparing them to the published sequences from GenBank.
The first SNP, at position 903 after the translation start, was also found by Bleich et al. (AY 521463, GI: 41350656), and was C57BL6/J derived. All the other polymorphisms found in this study were C3H/HeN derived. We did not find the other (C57BL6/J derived) SNP found by Bleich (at position 1119, C->T transition). All the SNPs identified in the coding region of the gene are silent mutations, i.e. mutations that do not cause a change in amino acid composition of the protein.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Single nucleotide polymorphisms between C3H/HeN and C57BL6/J mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>C3H/HeN</td>
</tr>
<tr>
<td>903</td>
<td>G</td>
</tr>
<tr>
<td>1860</td>
<td>C</td>
</tr>
<tr>
<td>2028</td>
<td>A</td>
</tr>
<tr>
<td>2796</td>
<td>A</td>
</tr>
<tr>
<td>3255 (UTR)</td>
<td>T</td>
</tr>
</tbody>
</table>

Locations given are numbers of basepairs from the transcription start. Amino Acids are; T = Threonine, K = Lysine, Q = Glutamine, V= Valine, N/A not applicable.
Figure 3 Polymorphisms between C3H/HeN and C57BL6/J strains in Nfkb1

Single nucleotide polymorphisms are indicated in **bold**. Top sequences are C3H/HeN derived, bottom sequences are C57BL6/J derived. The nucleotides that differed from the published sequences for that same strain are underlined.

The fact that we did not identify any functional polymorphisms in Nfkb1 does not necessarily mean that this gene is not involved in the difference in susceptibility to colitis as seen in C57BL6/J and C3H/HeN mice. We only sequenced the cDNA, so the translated part of the gene, omitting all the 24 introns. Also, it is still not exactly known what part of the sequence before the known transcribed part comprises the promoter of this gene. Mutations in both the promoter, the introns, and the 3’UTR could have a profound effect on the expression of the gene, or cause so far unknown splice variants to occur. Ongoing studies in congenic mouse strains in which the QTL found on chromosome 3 is transferred from the susceptible strain to the resistant strain and vice versa will be helpful to further discern whether Nfkb1 is involved in colitis susceptibility in the Gna12<sup>−/−</sup> model.
Sequencing Nfkb1 for polymorphism detection

References


Chapter 5

A NFKB1 promoter polymorphism is involved in susceptibility to ulcerative colitis

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³ Department of Internal Medicine Slotervaart Ziekenhuis Amsterdam, The Netherlands

Summary

Nuclear factor κB (NF-κB) designates a group of critical transcription factors involved in a variety of immunologic and/or inflammatory processes. Conceivably, genes involved in the NF-κB pathway make interesting candidate genes for chronic inflammatory disorders, including the Inflammatory Bowel Diseases (IBD), Crohn’s disease (CD), and Ulcerative colitis (UC). In two mouse models of colitis strong linkage has been observed with a locus on chromosome 3 that harbors the \( Nfkb1 \) gene. In addition, a polymorphism in the promoter region of the human \( NFKB1 \) gene was found to be associated with susceptibility to UC. In this study, we searched to confirm this previously found association in IBD in a different population.

Allele and genotype frequencies of the –94ins/delATTG polymorphism were determined in 266 unrelated Dutch Caucasian IBD patients (127 UC, 139 CD), and 155 matched healthy controls. The allele frequency of the deletion was significantly higher in UC patients (\( P=0.019 \)), but not in CD patients, compared to healthy controls, and the UC patients homozygous for the –94 ATTG deletion had a younger age of onset.

Our findings confirm the previously found association between this polymorphism and susceptibility to UC in an independent study population and adds further evidence for the role of this gene in disease susceptibility.
Introduction

Crohn’s disease (CD) and ulcerative colitis (UC) are two related but distinct chronic inflammatory disorders of the gastrointestinal tract, commonly denoted as inflammatory bowel diseases (IBD)\(^1\). The exact cause remains elusive, but thus far, IBD is thought to be the result of an inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora in a genetically susceptible host\(^2\).

Epidemiological linkage studies indicate that genetic factors play a key role in the etiology of IBD\(^3\),\(^4\). Indeed, mutations in NOD2 have been identified in a subgroup of CD patients. However, IBD is clearly not inherited as a simple Mendelian trait but rather has a complex genetic basis with multiple genes involved. This is further substantiated by various linkage studies in which a large number of chromosomal regions have been identified that are linked to disease susceptibility. One of these is found on chromosome 4q24 and harbors, among others, a member of the nuclear factor \(\kappa\B (NF-\kappa\B)\) family, \(NFKB1\). This gene is an attractive candidate gene since its gene product is involved in regulating a large variety of inflammatory responses. \(NFKB1\) encodes two isoforms, the cytoplasmic non DNA-binding p105, and the 50 kDa DNA-binding p50\(^5\). As stated above, NF-\(\kappa\B\) p50/105 is a member of the NF-\(\kappa\B\) family, a group of critical transcription factors involved in a variety of regulatory processes, including innate and adaptive immune responses, cell growth, apoptosis, and tissue differentiation\(^6\),\(^7\). To exert its effect, p50 binds to p65 to form biologically active heterodimers which translocate from the cytoplasm of the cell to the nucleus. Here, they bind to NF-\(\kappa\B\) binding sites in promoter sequences of inflammatory proteins, including IL-12, TNF-\(\alpha\) and IFN-\(\gamma\) and regulate their transcription. Alternatively, p50 is able to form homodimers which block transcription by binding to NF-\(\kappa\B\) sites in the nucleus\(^8\). Interestingly, mice with enhanced p50 expression show enhanced inducible NF-\(\kappa\B\) activation, chronic inflammations and elevated susceptibility to bacterial infections\(^9\).

Additionally, \(NFKB1\) has been identified as a strong candidate gene in two different mouse models for colitis (the \(IL-10\) deficient\(^10\) and \(Gnai2\) deficient\(^11\) mouse models), since in both models strong linkage is observed with the chromosomal region in which the mouse homologue for \(NFKB1\) resides. These findings are further corroborated by studies in human IBD. Thus, elevated levels of NF-\(\kappa\B\) activation are found in the intestinal mucosa of both CD and UC patients\(^12\),\(^13\). In addition, in North American populations with UC, especially in Ashkenazim Jews, linkage was found previously with chromosome 4q\(^14\). This chromosomal region appeared to epistatically interact with the \(IBD1\) region on 16p where the first gene identified as a true susceptibility gene for CD, \(NOD2\) resides\(^15\),\(^16\). The linkage of chromosome 4q with IBD susceptibility was confirmed by studies in Europe, combining patients from England, Germany and Holland\(^17\),\(^18\), and found to be mainly associated with UC.

Recently, Karban et al. described a promoter polymorphism 94 bp upstream of the transcription start of the \(NFKB1\) gene. This polymorphism consists of an ATTG-deletion, at a putative NF-\(\kappa\B\) binding site. The authors found an elevated frequency of the deletion in UC patients in a non-Jewish North American population. Also, oligonucleotides containing the deletion showed strongly diminished binding to nuclear proteins isolated from normal human colonic tissue and cell lines but not to nuclear proteins isolated from normal human ileal tissue\(^19\). This functional polymorphism potentially leads to altered expression of NFKB1 and altered NF-\(\kappa\B\) activation patterns, which, at least in part, could
induce elevated IBD susceptibility. In this study we describe the frequency of the previously described promoter polymorphism in our Dutch Caucasian patient population. We found an elevated frequency of the ATTG-deletion in UC patients, but not in CD patients.

Materials and Methods

Patients

DNA was isolated previously from whole blood using standard isolation procedures. Patients were recruited from the department of Gastroenterology of the Vrije Universiteit Medical Center, Amsterdam. Informed consent was obtained from all participants after approval of the VUmc Medical Ethical Committee. For UC, 127 patients were available, 63 females, 64 males, and for CD 139 patients were available, 102 females, 37 males. The mean age at first diagnosis of IBD was 31.9 +/- 12.3 years for UC and 27.0 +/- 12.0 years for CD.

Severity of disease was defined by means of the localization/extent of inflamed area for UC, e.g. involvement of only the rectum (proctitis), the rectum and the sigmoid or descending colon (left-sided colitis), or the entire colon (pancolitis). Severity of disease in CD patients was defined using the Vienna classification, using codes to indicate age of onset, behavior and localization of the disease. Vienna-A, age at diagnosis, A1= <40 years, A2= >40 years, Vienna-B disease behavior, B1 = inflammatory, B2 = structuring, B3 = perforating, Vienna-L, localization, L1 = terminal ileum, L2 = colon, L3 = ileo-colonic, L4 = upper GI-tract.

Genotyping the −94delATTG human Nfkb1 promoter polymorphism

A restriction enzyme digestion assay was used to genotype a −94ins/delATTG polymorphism as described before 19. In short, DNA samples were used to amplify a 289 bp PCR fragment from genomic DNA (Forward primer TTT AAT CTG TGA AGA GAT GTG AAT G, reverse primer CTC TGG CTT CCT AGC AGG G [Invitrogen Life Technologies, Breda, The Netherlands]). Products were digested with the enzyme PflM1 (New England Biolabs, Beverly, MA, USA) and analyzed on a 4% agarose gel (in a mixture of 2% ultra pure agarose [NuSieve GTG Agarose; Cambrex Bio Science Rockland, Rockland, ME, USA] and 2% low melting agarose [Invitrogen]).

Statistical analysis

For case-control test for genetic association, a $\chi^2$ test for independence statistic was derived using 2x3 contingency tables. Odds ratios with 95% confidence intervals were calculated using Fisher’s Exact Test and the approximation of Woolf. One-way ANOVA was used to compare mean age of onset for patients from different genotypes. For all analyses, GraphPad InStat version 3.05 for Windows 95 was used (GraphPad Software, San Diego, CA; http://www.graphpad.com). All groups were checked for Hardy-Weinberg equilibrium.
Results
We genotyped our 266 Dutch Caucasian IBD patients (127 UC, 139 CD), and matched healthy controls (n = 155) for the –94 ATTG deletion promoter polymorphism (Table 1). All populations were in Hardy-Weinberg equilibrium.
When comparing UC patients with the controls, we found that allele and genotype frequencies of the –94 ATTG deletion were significantly higher in UC patients (p=0.019). No differences in allele or genotype frequencies were found between CD patients and healthy controls (p = 0.39 respectively; Table 1).
Comparing the UC population with the control population revealed a significant risk for developing UC in the subjects homozygous for the –94 ATTG deletion (25% versus 16%; odds ratio = 2.51; 95% confidence interval = 1.28-4.92; p = 0.011) The heterozygote genotype revealed an elevated, but not quite significant risk (48% versus 43%, odds ratio = 1.71; 95% confidence interval = 1.00-2.07; p = 0.058) Comparing the CD population to the controls did not reveal any elevated risk (data not shown).
Subsequently, we investigated whether this polymorphism could be linked to a particular clinical phenotype. First, patients were stratified according to gender. In UC patients, the association with this polymorphism was particularly strong in men, and not in women. Stratification according to gender did not reveal any significant sex differences for CD (Table 1).

Table 1 Genotypes of patients and healthy controls and -94 delATTG allele frequency

<table>
<thead>
<tr>
<th>Genotype (n)</th>
<th>Genotype -94 delATTG</th>
<th>-94 delATTG allele frequency</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>del/del</td>
<td>del/ins</td>
<td>ins/ins</td>
</tr>
<tr>
<td>UC female (63)</td>
<td>11 (17%)</td>
<td>37 (59%)</td>
<td>15 (24%)</td>
</tr>
<tr>
<td>UC male (64)</td>
<td>21 (33%)</td>
<td>24 (37%)</td>
<td>19 (30%)</td>
</tr>
<tr>
<td>UC (127)</td>
<td>32 (25%)</td>
<td>61 (48%)</td>
<td>34 (27%)</td>
</tr>
<tr>
<td>CD female (102)</td>
<td>12 (12%)</td>
<td>52 (51%)</td>
<td>38 (37%)</td>
</tr>
<tr>
<td>CD male (37)</td>
<td>4 (11%)</td>
<td>18 (49%)</td>
<td>15 (40%)</td>
</tr>
<tr>
<td>CD (139)</td>
<td>16 (12%)</td>
<td>70 (50%)</td>
<td>53 (38%)</td>
</tr>
<tr>
<td>Total IBD (266)</td>
<td>48 (18%)</td>
<td>131 (49%)</td>
<td>87 (33%)</td>
</tr>
<tr>
<td>Controls female (80)</td>
<td>13 (16%)</td>
<td>36 (45%)</td>
<td>31 (39%)</td>
</tr>
<tr>
<td>Controls male (75)</td>
<td>11 (15%)</td>
<td>31 (41%)</td>
<td>33 (44%)</td>
</tr>
<tr>
<td>Controls (155)</td>
<td>24 (16%)</td>
<td>67 (43%)</td>
<td>64 (41%)</td>
</tr>
</tbody>
</table>

del/del represents individuals homozygous for the –94 delATTG allele, del/ins represents heterozygous individuals and ins/ins represents individuals homozygous for the wild-type allele. P values are patient groups compared to controls, either as a whole group, or gender matched. N/A not applicable

The mean age of onset was significantly lower in patients homozygous for the –94 delATTG allele (27 years vs. 33 or 34 years, p=0.03, Table 2), indicating that homozygosity for this allele could be a risk factor for early onset of the disease. Stratifying UC patients according to localization of disease led to very small populations, too small to provide valid evidence for relevant observations for all groups (Table 2). Neither did we find an association of the polymorphism with the severity of disease, as defined by the need of colectomy or cyclosporin (CSA) treatment (data not shown).
Table 2 Genotypes of UC patients

<table>
<thead>
<tr>
<th>Phenotype (n)</th>
<th>Genotype</th>
<th>-94 delATTG allele frequency</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>del/del del/ins ins/ins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proctitis (10)</td>
<td>4 (40%) 4 (40%) 2 (20%)</td>
<td>0.6</td>
<td>0.11</td>
</tr>
<tr>
<td>Left sided (54)</td>
<td>13 (24.1%) 26 (48.1%) 15 (27.8%)</td>
<td>0.48</td>
<td>0.15</td>
</tr>
<tr>
<td>Pan colitis (63)</td>
<td>15 (23.8%) 31 (49.2%) 17 (27.0%)</td>
<td>0.48</td>
<td>0.035</td>
</tr>
<tr>
<td>Controls (155)</td>
<td>24 (16%) 67 (43%) 64 (41%)</td>
<td>0.37</td>
<td>N/A</td>
</tr>
<tr>
<td>Age of onset</td>
<td>27 33 34</td>
<td>N/A</td>
<td>0.03</td>
</tr>
</tbody>
</table>

del/del represents individuals homozygous for the –94 delATTG allele, del/ins represents heterozygous individuals and ins/ins represents individuals homozygous for the wild-type allele. P values are patient groups compared to controls. N/A not applicable.

Next, we subdivided the CD patient group according to the Vienna classification. Although the –94 delATTG allele frequency appeared to be higher in patients with perforations (Vienna B3, data not shown), subdividing the CD patients led to very small populations and these groups were too small to provide valid observations.

Discussion

The NFKB1 gene encodes for two isoforms, the cytoplasmic non DNA-binding 105 kDa p105 protein, and the 50 kDa DNA-binding p50 protein, and is one of the most prominent members of the NF-κB family. The NF-κB family consists of transcription factors involved in a variety of immune responses, and it has been implicated in many inflammatory pathologies, such as asthma, arthritis, atherosclerosis, and IBD. There is linkage evidence that the region where NFKB1 resides, 4q24, is involved in IBD susceptibility.

The region of the mouse genome where Nfkb1 is located has been implicated in two separate spontaneous colitis models, in which F2 backcrosses derived from resistant C57BL/6J Gna12-/- and susceptible C3H/HeN Gna12-/- mice, or C57BL/6J Il-10-/- and susceptible C3H/HeJ IL-10-/- mice, were analyzed in a genome-wide screen for colitis susceptibility and severity, identifying a highly significant locus on chromosome 3 contributed to colitis susceptibility and severity (called Gpdc1 and Cdcs1 respectively). In addition mouse Nfkb1 is involved in chronic inflammations and an elevated susceptibility to bacterial infections and intestinal inflammation is demonstrated in Nfkb1 deficient mice. These observations designate NFKB1 to be an interesting candidate gene for IBD.

In this study we describe the frequency of the previously described –94 ATTG-deletion polymorphism of the Nfkb1 promoter in a Dutch Caucasian IBD patient population. Similar to findings in a mixed Northern American population, the allele frequency was elevated in UC patients, providing further evidence that this polymorphism is associated with disease susceptibility for this subtype of IBD. Such elevated frequency of the –94 ATTG-deletion was not observed in CD. The association found in UC appeared to be a little stronger in men. Interestingly, patients (men and women together) homozygous for the –94 ATTG-deletion allele had a significantly younger age of onset of disease.
It has to be noted that the studied population is relatively small, and the results have not been corrected for multiple testing. Furthermore, the phenotypical description of the patients is exploratory, because it is impossible to predict the development of disease. Although this could lead to an underestimation of patients with the more severe forms of disease, we find significant association with the −94 delATTG polymorphism in our UC patient group, in concordance with the results from Karban et al. On the other hand, a recently published study by Oliver et al. did not find evidence for association of this polymorphism in a white Spanish population (Oliver et al. (2005). The discrepancies between these studies could be explained by differences in environmental factors for the different populations, but a more likely explanation could be that the haplotypic context of the Spanish population is different from the North European and North American populations. Indeed, the white North American populations studied in genome screens identifying the 4q region (Cho et al. (1998); Rioux et al. (2000) descend largely from the North European Caucasian populations, in which the same region was also identified 17,18. Of note, part of the population described by Hampe was recruited from the Academic Medical Center in Amsterdam, and thus virtually from the same population as ours, and the population described by Vermeire was recruited from the Flemish Belgian population, which is relatively closely related to our Amsterdam population.

The exact mechanism underlying the \textit{NFKB1} related disease susceptibility remains unknown, but one can think of several explanations. The deletion of the −94 ATTG leads to a less strong activation of NF-κB1 transcription, which seems contra intuitive to the fact that especially in UC, high levels of NF-κB activation are seen. However, if the diminished \textit{NFKB1} transcription would lead to less available p50, fewer inhibitory p50/p50 homodimers can be formed. Fewer blocking homodimers could then lead to a stronger activation of transcription of inflammatory genes, leading to the strong NF-κB induced immune response, as seen in UC.

Another potential explanation is that low levels of p50 lead to a poor innate immune response. Bacteria that would have normally been handled by this first line of defense are then able to provide for ongoing antigenic signals and subsequently cause an on-going inflammation, as is seen in mice lacking \textit{Nfkb1} 21-23. On the other hand, mice that express enhanced levels of p50 also show chronic inflammation and higher susceptibility to bacterial infections 9. Clearly, any change in the delicate balance of NF-κB signalling can lead to an alteration in immune response which in is associated with enhanced susceptibility to colitis.

In summary, we found that the allele frequency of the −94 ATTG deletion was significantly higher in UC patients compared to healthy controls, but not in CD patients, and patients homozygous for the −94 ATTG deletion had a younger age of onset. Our findings confirm the previously found association between this polymorphism and susceptibility to UC in an independent study population. Studies to determine the physiological effect of this polymorphism are warranted.
Chapter 6

**IL12B** and **IRF1** gene polymorphisms and susceptibility to celiac disease

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D. Seegers and M. E. A. Borm contributed equally to this study

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Summary
Celiac disease (CeD) is a common gastrointestinal disorder resulting from permanent intolerance to wheat gliadins and related proteins in rye and barley. In addition to the strong genetic association with HLA-DQ2 and HLA-DQ8, a genetic region on chromosomes 5 (CELIAC2) has been identified that harbors a susceptibility gene for CeD. The gene(s) responsible for this association, however, remains to be identified. In the present study we evaluated polymorphisms in the genes encoding interleukin-12 p40 (IL12B) and interferon regulatory factor 1 (IRF1). Both genes are located in the CELIAC2 region, and have key roles in inducing interferon (IFN)-γ secreting T helper 1 (Th1) cells, one of the immunological hallmarks of CeD. The frequencies of an TaqI gene polymorphism in the 3′ UTR of IL12B and a HinfI gene polymorphism in the 3′ UTR of IRF1 were studied in 258 Dutch CeD patients and 237 ethnically matched healthy controls. The transmission of the polymorphic variants from parents to affected child was determined in 123 families with at least one affected child. The frequencies of the IL12B TaqI gene polymorphism and the IRF1 HinfI gene polymorphism did not differ significantly between patients and controls. In addition, in the family study, no deviation from the expected transmission from parents to affected child of any of the polymorphic variants was found. The IL12B TaqI and the IRF1 HinfI gene polymorphisms do not appear to be involved in susceptibility to CeD. Further studies on the factors that drive the Th1 immunopathology in CeD are required.
Introduction
Celiac disease (CeD) is an immune-mediated disorder of the small intestine caused by permanent intolerance to gluten, a dietary protein found in wheat, rye and oats. CeD affects up to 1 in 150–300 people in western European and Northern American populations. Genetic factors play a key role in CeD susceptibility. This genetic component can in part be attributed to a strong association with the HLA genes. The primary HLA association in most cases of celiac disease is with DQ2, and in a minority of patients the association is with DQ8. However, since DQ2 occurs at a high prevalence in the general Caucasian population, it is anticipated that other, as yet unknown genes are involved in disease susceptibility. Indeed, linkage studies have provided evidence for the existence of additional genetic risk factors. Two chromosomal regions have been identified in more than 1 genome-wide screen, and there is general agreement that these regions harbor CeD susceptibility genes. They are located on chromosome 5q31-33 (designated CELIAC2) and on 2q33 (designated CELIAC3).

Immunologically, CeD appears to be a T helper 1 (Th1) mediated disorder, characterized by high expression of interferon-gamma (IFN-γ) mRNA and increased numbers of IFN-γ positive cells at lesional sites. What drives these high IFN-γ responses during the inflammatory response in the small intestine is unknown. Accumulating evidence from in vivo and in vitro experimental test systems supports the notion that interleukin-12 (IL-12) is required for optimal in vivo Th1 responses. Active IL-12 exists as a disulfide-linked heterodimer of 35-kDa (p35) and 40-kDa (p40) subunits, each encoded by separate genes. Given its strong capacity to induce IFN-γ secretion, IL-12 is a potential candidate gene for CeD. In addition, the gene encoding the p40 chain (IL12B) resides within CELIAC2 on chromosome 5q31-33. IL12B is highly conserved among human subjects. However, an A→C polymorphic site is found in the 3′ untranslated region (UTR) at position 1188, which creates a TaqI cutting site. This polymorphism has recently been the focus of considerable interest since it was found to be associated with susceptibility to both multiple sclerosis and type I diabetes mellitus, and has an effect on IL-12 secretion in vitro. It must be noted, however, that the genetic association data have been inconclusive thus far for both diseases, emphasizing the importance of replicating genetic association studies in independent data sets.

Interferon regulatory factor (IRF)-1 is a member of the IRF family of transcription factors that are involved in the regulation of genes that control cell growth, differentiation, and death. In addition, IRF-1 is involved in several steps of the immune response that may be pertinent to CeD, including the polarization of the cytokine response in CD4+ T cells. Thus, Irf1 deficient mice (−/−) fail to mount Th1 responses, which can be attributed to impaired IL-12 production in these mice. Indeed, IRF-1 binding sites are present in the IL12B promoter. The gene for Irf1 is located on chromosome 5q31. A G→A polymorphism at position 1688 in the 3′-untranslated region of the gene was recently described. In the present study we investigated the involvement of both gene polymorphisms in CeD susceptibility in the Dutch population. This was accomplished by studying these polymorphisms in a case–control study as well by studying the transmission of alleles from parents to affected children.
Materials and methods

Patients and controls
A total of 258 Dutch celiac patients (70 males and 188 females) and 237 Dutch controls (113 males and 124 females) were included in the study. The mean age at presentation of disease was 39 years (range 1–75 years), and did not differ from the mean age of the control group (38 years; range 6–87 years). For the transmission disequilibrium test (TDT), a total of 123 trios (parents and affected child) were available. The affected children from the trios were included in the case–control group. All patients and controls were unrelated and Caucasian. Histological analysis of patients was performed at the time of diagnosis prior to initiating a gluten-free diet. The histological features were classified according to the modified Marsh classification\(^27\), with a slight modification of Marsh III. Marsh III is characterized by subtotal villous atrophy (VA) and significant intraepithelial lymphocytosis (> 30 lymphocytes per 100 epithelial cells). In order to define the severity of the histological features of subtotal villous atrophy more precisely, we have further classified the Marsh III type lesion into three subgroups based on architectural changes: Marsh IIIa (partial VA), characterized by a villous-crypt ratio of less than 1/1; Marsh IIIb (subtotal VA), characterized by recognizable villi in an otherwise flat mucosa, and Marsh IIIc (total VA), characterized by a nearly total absence of villi.

TaqI RFLP analysis
Genotyping for the TaqI polymorphism in the 3′ UTR of IL12B was performed as previously described\(^28\). In short, 50 ng of genomic DNA was PCR amplified using primers that cover the TaqI restriction site. After PCR amplification, the 233-bp product was cut with the TaqI restriction enzyme for 16 h at 65 °C. The products were visualized on an ethidium bromide-stained 2% agarose gel.

Hinf I SNP analysis
Genotyping for the HinfI polymorphism in the 3′ UTR of IRF1 was performed as previously described\(^26\). In short, PCR amplification of 50 ng of genomic DNA was performed using primers that cover the G to A transition at position 1688. This transition, however, does not confer the restriction enzyme recognition site. A HinfI site was introduced by altering the 3′ end of the primer to generate the enzyme recognition site, as shown in bold script (forward primer: 5′TCTCGTTCCAAAGAAGACTAC3′; reverse primer: 5′CTCTGAGGCTGAGGGAGATA3′). In the presence of the G allele at position 1688, this results in the HinfI cutting site, whereas this site is lost when the A allele is present. After PCR amplification, the 114-bp product was digested with the HinfI restriction enzyme for 10 h at 37 °C, resulting in an uncut product for IRF-1*A and 94- and 20-bp products for IRF-1*G, which were visualized on an ethidium bromide-stained 4.5% agarose gel.

Statistical analysis
Data are presented as genotype and allele frequencies for each gene polymorphism. For the comparison of genotype and allele frequencies, the χ² test was performed using the GraphPad Instat® software package (Graphpad Software, San Diego, CA). A p-value < 0.05 was considered statistically significant.
Associations with the two gene polymorphisms were subsequently determined using the transmission disequilibrium test. This test scores the transmission of alleles from heterozygous parents to affected children by comparing the transmitted alleles vs. the untransmitted alleles. Because both gene polymorphisms under investigation are biallelic, this test assumes a 50% transmission of each allele if the polymorphism is not associated with the disease.

Results

Case–control study

**IL12B**

The A→C base pair substitution at position 1188 in the 3′ UTR of *IL12B* results in the creation of a *Taq*I cutting site. All genotypes were in Hardy–Weinberg equilibrium. As can be seen from Table 1, no significant differences were seen in the genotype distributions between patients and healthy controls. We subsequently stratified patients according to the genotype distributions between patients and healthy controls. This resulted in 75 patients with Marsh IIIA, 119 with Marsh IIIB and 64 with Marsh IIIC. No association with the *Taq*I gene polymorphism was found either when patients were subdivided according to this classification. Finally, no differences between genotype and allele frequencies were found when males and females were analyzed separately (results not shown).

<table>
<thead>
<tr>
<th>Table 1. Genotypes of the <em>IL12B</em> TaqI RFLP in 258 Dutch celiac patients and 237 healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>Patients</td>
</tr>
<tr>
<td>Controls</td>
</tr>
</tbody>
</table>

AA indicates absence of the *Taq*I restriction site; CC indicates homozygous for the presence of the *Taq*I restriction site, and AC indicates heterozygous.

**IRF1**

The single base pair mismatch reverse primer used for the determination of the genotype frequencies of the *IRF1* polymorphism introduces a *Hinf*I restriction enzyme cutting site in the *IRF1*^G^ genotype. Table 2 shows the genotype distribution in 252 Dutch patients and 237 controls. The populations were in Hardy–Weinberg equilibrium, and the genotype and allele frequencies were similar between males and females. No association was found with this polymorphism when patients were compared with controls. When patients were classified according to the modified Marsh III classification, no association with the *IRF1* polymorphism was found either (results not shown).
Table 2. Genotype of the IRF1 Hinf I RFLP in 252 Dutch celiac patients and 237 healthy controls

<table>
<thead>
<tr>
<th>Total Genotype</th>
<th>Genotype AA</th>
<th>Genotype AG</th>
<th>Genotype GG</th>
<th>Allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>A</td>
</tr>
<tr>
<td>Patients</td>
<td>252</td>
<td>24 (9.5)</td>
<td>120 (47.6)</td>
<td>108 (42.9)</td>
</tr>
<tr>
<td>Controls</td>
<td>237</td>
<td>31 (13.0)</td>
<td>96 (40.5)</td>
<td>110 (46.5)</td>
</tr>
</tbody>
</table>

AA indicates absence of the Hinf I restriction site; GG indicates homozygous for the presence of the Hinf I restriction site, and AG indicates heterozygous.

Family study

From 123 CeD families, the parents were genotyped for the polymorphisms in IL12B and IRF1. From these 123 trios, there were 66 informative transmissions of heterozygous parents to affected children for the IL12B polymorphism, and 114 informative transmissions for the IRF1 polymorphism. For both polymorphisms, no significant deviation from the expected transmission rate of 50% was observed, as is shown in Table 3.

Table 3. Transmission of the infrequent allele vs. transmission of the frequent alleles from heterozygous parents to affected children

<table>
<thead>
<tr>
<th>IL12B*%C (%)</th>
<th>IL12B*A (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL12B 66 informative transmissions</td>
<td>31 (47)</td>
<td>35 (53)</td>
</tr>
<tr>
<td>IRF1*%A (%)</td>
<td>IRF1*%G (%)</td>
<td></td>
</tr>
<tr>
<td>IRF1 114 informative transmissions</td>
<td>53 (46.5)</td>
<td>61 (53.5)</td>
</tr>
</tbody>
</table>

IRF1*A and IL12B*C represent the infrequent alleles, whereas IRF1*G and IL12B*A represent the common alleles.

Discussion

Genetic factors play an important role in the susceptibility to CeD. In particular, the role of the HLA-DQ genes in CeD is well established, contributing an estimated 40% of the genetic risk of CeD. In order to identify non-HLA genetic factors related to CeD, several genome-wide mapping studies have been conducted. Several independent studies identified linkage with a region on chromosome 5q31-33 and it is now generally accepted that this region, designated CELIAC2, harbors a gene involved in CeD susceptibility. Among the many genes that are located in this region, the gene encoding the IL-12 p40 chain is of particular interest given its role in priming T cells to IFN-γ producing T helper 1 cells. It is now well established that CeD is associated with increased IFN-γ secretion at lesional sites. Thus, in a study by, it was shown that IFN-γ mRNA in mucosal biopsies was
increased more than 1000-fold in cases of untreated disease. Interestingly, however, in the same study, only minute amounts of IL-12 p40 mRNA expression were found in the same biopsies. A possible explanation for the discrepancy between IL-12 p40 and IFN-γ expression may be that T cells primed with a soluble protein in the presence of IL-12 are rendered capable of secreting IFN-γ independently of IL-12 on subsequent antigen stimulation. Therefore, the discrepancy between IFN-γ mRNA levels and IL-12 p40 mRNA does not exclude a role for a dysregulated IL-12 response in the susceptibility to CeD. In the present study, however, we found no significant association between IL-12B genotypes and susceptibility to celiac disease. Also, no significant associations were found when patients were classified according to the severity of the disease. Our findings are in accordance with those of a previous study, where no association with this polymorphism was found in a group of celiac patients from Norway and Italy. Based on the results of these two studies involving three European populations, the role of this polymorphism in susceptibility to CeD can be excluded. It must be noted, however, that it cannot be entirely excluded that other IL12B variants may contribute to the cause of this immune-mediated T_{h}1 disease. Given its potent and pluripotent role in the immuneresponse, IRF1 is another positional and functional candidate gene for CeD. IRF1 is induced by several exogenous and endogenous factors, including cytokines, and in turn induces the induction of a large variety of genes. Of particular relevance for CeD, IRF-1 is a potent inducer of IFN-α, which in turn has been shown to be capable of inducing enhanced T_{h}1 responses and crypt cell hyperplasia in explant cultures of human fetal gut. Another target of interest is the IL12B gene, since IRF-1 directly affects IL-12 production by macrophages, whereas IL-12 in turn can induce IRF-1 through STAT4. Thus, IRF-1 has a significant role in T_{h}1 differentiation. Since IRF1 has not been studied previously in CeD, we determined whether a Hinf I RFLP in the 3′ UTR of IRF1 is involved in CeD susceptibility. Based on the results of the present study, it can be concluded that it is very unlikely that this gene is involved in susceptibility to CeD. Having found no association with IL12B and IRF1 in the pathogenesis of CeD, we need to consider other cytokines that are involved in T_{h}1 differentiation. In this regard, the T_{h}1-inducing cytokine IL-23 (which consists of the IL-12 p40 chain linked to a p19 chain, not to the IL-12 p35 chain) has been shown, at least in murine systems, to stimulate T_{h}1 responses in memory cells more effectively than does IL-12. Thus, it is possible that it is more important than IL-12 in sustaining T_{h}1 inflammation. Whether IL-23 plays a role in CeD, however, remains to be seen, and this is currently under investigation.

Acknowledgements G.B. is a fellow of the Royal Netherlands Academy of Arts and Sciences. J.B. received a fellowship from the NUFFIC, The Hague.
References


Chapter 7

Dose dependent interaction of NOD2 with TLR2 in human monocytes

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Abstract
The mechanism by which mutations in Nucleotide-binding Oligomerization Domain (NOD2), predispose to Crohn’s disease (CD) is still poorly understood. Previous studies in mice have shown that NOD2 signaling inhibits Toll-Like Receptor (TLR)-2 responses. So far, this effect could not be reproduced in humans. Making use of monocytes isolated from normal NOD2 expressing subjects as well as NOD2 deficient CD patients, we tested the effect of NOD2 signaling on TLR responses after stimulation with different doses of the NOD2 ligand MDP, together with suboptimal doses of TLR ligands. Culture supernatants and nuclear extracts were isolated, and NF-κB activation and production of the pro-inflammatory cytokines TNF-α, IL-6, IL-12p40 and IL-12p70 were measured. Monocytes from individuals without NOD2 mutations or heterozygous for any of the NOD2 mutations respond to stimulation with MDP in a dose dependent fashion, although this response is weak compared to TLR stimulation. Simultaneous addition of TLR ligand and low doses of MDP to the culture medium resulted in a significant increase in the NF-κB activation as well as cytokine production, as compared to TLR stimulation alone. However, addition of higher doses of MDP in combination with TLR2 signaling led to a striking downregulation of the responses, whereby cytokine levels returned to baseline production. These results were reflected in the NF-κB activation. Importantly, this downregulation of TLR responses by high dose MDP was not seen in monocytes from NOD2−/− patients. The results show for the first time that human monocytes respond to combined TLR2/NOD2 signaling in a biphasic fashion. This mechanism is likely a safety mechanism to prevent exaggerated antibacterial immune responses in the gut to high or perpetuating bacterial load. This regulatory mechanism is absent in NOD2−/− deficient patients and may therefore underlie the onset of IBD in this group of patients.
Introduction

The innate immune system forms the first line of defense against microbial pathogens. Essential in the detection of pathogens is the family of Toll Like Receptors (TLRs) which recognize conserved microbial structures, such as bacterial cell wall components like lipopolysaccharide (LPS, recognized by TLR4), lipoproteins and lipoteichoic acid (LTA, recognized by TLR2), double stranded RNA (dsRNA, recognized by TLR3) or single stranded viral RNA (recognized by TLR7) \(^1\). Although the various TLRs signal via a range of adaptor molecules and different downstream kinases, the signaling ultimately result in an NF-κB dependent activation of inflammatory genes \(^1\). Next to TLRs, which are all membrane bound receptors (whether or not on the cell surface or in intracellular vesicles), there are also various cytosolic receptors involved in the innate immune response. One of those is nucleotide-binding oligomerization domain 2 (NOD2). NOD2 is a sensor of bacteria-derived muramyl dipeptide (MDP) \(^3\). It is a member of the NOD-leucine-rich repeat (LRR) protein family, which comprises of proteins that all consist of a C-terminal ligand recognition domain, a central NOD domain, and an N-terminal protein-protein interaction domain(s) \(^5\). Members of the NOD-LRR protein family are known to be involved in regulation of apoptosis \(^7\) and NF-κB activation \(^8\). NOD2 has also been shown to display NF-κB activating properties, after binding of its ligand, MDP \(^3\). NOD2 has been shown to be primary expressed in monocytic cells, dendritic cells and granulocytes \(^6, 9\), and to a lesser extent in intestinal epithelial cells, especially Paneth cells, \(^10, 11\), and T cells, in which it can be upregulated after TNF-α or LPS administration \(^9, 12\).

Mutations in the LRR of NOD2 are implicated in an increased risk of developing Crohn’s disease (CD), \(^13, 14\) a chronic inflammatory disorder of the gastrointestinal tract. From epidemiological studies throughout the Western world, homozygosity for NOD2 mutations leads to a 20-40 times higher chance of developing CD, where the risk of developing the disease is approximately four times higher in individuals that are heterozygotic \(^15\).

It is shown that NOD2 activation can act synergistically with TLR activation, leading to a stronger immune response, but this potentiating effect is lost in cells from CD patients with mutations in NOD2 \(^3, 16-23\). This effect is seen at the level of immune cells, whereas in the intestinal Paneth cells the NOD2 mutation leads to impaired production of α-defensins, which could lead to an overgrowth of the commensal-bacterial population \(^12, 24\). Indeed, in a mouse model deficient for cryptdins, (the murine ortholog for α-defensins), it has been found that these animals are highly susceptible for bacterial infections, especially after oral administration, and not through intravenous or intraperitoneal routes \(^25\). Furthermore, NOD2 has been shown to directly activate the IL-1β converting enzyme (ICE) \(^26\), which is needed for the conversion of pro-IL-1β into the functional IL-1β \(^27\). Subsequently, it has been shown in studies using human PBMCs that NOD2 deficiency leads to a decreased IL-1β expression \(^28, 29\).

Together, these data point to an important role of NOD2 in host defense at the level of various cell types and factors, but the importance of each effect from the mutation on associated risk with CD is not yet clear.

Recently it has been implicated by Watanabe et al. that NOD2 activation leads to an inhibition of specifically TLR2 signaling in mice \(^30\). MDP, as a derivative of the TLR2 ligand peptidoglycan, is also
recognized by TLR2, so that both NOD2 and TLR2 recognize the same bacterial substance, which could provide an explanation for why NOD2 interact with specifically this TLR. In the absence of NOD2, the lack of efficient dampening of the TLR2 response could conceivably lead to an unwanted strong immune response, which ultimately could lead to CD. In a follow up study by the same group, it was shown that NOD2 deficient mice actually need TLR2 signaling in order to develop colitis. They showed that Nod2 mice, adaptively transferred with OVA specific CD4+ T cells, develop a transient T H1 driven colitis when infected with a recombinant E.Coli strain, expressing OVA (ECOVA), and that this colitis induction by ECOVA can be prevented either by introducing a TLR2 deficiency in the mice, or by addition of anti IL-12p40 monoclonal antibodies. These findings further substantiate the initial finding that there is a close interaction between NOD2 and TLR2 signaling involved in downregulation of the immune response.

Since the observations of Watanabe et al. were only seen in mice, we wanted to expand these studies to human cells. Monocytes isolated from individuals with or without mutations in NOD2 were stimulated with different doses of TLR ligands, combined with different doses of NOD2 stimulation. We found that strong NOD2 stimulation specifically affects the TLR2 signaling in a downmodulating fashion, and that this effect was not seen in NOD2 deficient patients.

Materials and Methods

Preparation of human monocytes

After approval of the Medical Ethical Committee of the Free University Medical Center, Crohn’s disease (CD) patients and healthy volunteers with known NOD2 statuses (See Supplementary Table 1) were recruited from our outpatient clinic. Written informed consent was obtained from all individuals before venous blood was drawn. Human peripheral blood mononucleated cells (PBMCs) were isolated from 50 ml of heparinized peripheral blood by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences). Monocytes were isolated from the PBMCs using a 52% gradient of Percoll (Amersham Biosciences). Purity of monocytes was assessed using Flow cytometric analysis of the cell surface antigen CD14, using a PC5-labelled anti CD14 mAb (IOTest, Beckman Coulter). Monocytes were cultured in triplicate in 96 wells flat-bottom plates (10⁵ cells/well) in 100 µl RPMI (Invitrogen), supplemented with 1% Penicillin/Streptomycin (Invitrogen). The cells were first allowed to rest overnight in culture medium, and then stimulated for 3 hours or 1 day (in separate plates). Cells were stimulated with muramyl dipeptide (MDP, InvivoGen) Lipopolysaccharide from Salmonella enterica serotype Enteritidis (LPS, Sigma), Pam₃CSK₄ (Pam, InvivoGen) or peptidoglycan from S. Aureus (PGN, BioChemika) poly I:C (InvivoGen) or Imiquimod (InvivoGen). Cell viability was checked using trypan blue staining.
Measurement of cytokines

ELISA assays for IL-6, TNF-α (DuoSet ELISA Development System, R&D Systems), IL12p40 and IL12p70 (OptEIA Sets, BD Biosciences) were performed according to manufacturers recommendations with culture supernatant in 1:4, 1:10, 1:20, and 1:50 dilutions for IL-12p70, TNF-α, IL-12p40, and IL-6, respectively.

NF-κB activation

Nuclear extracts were isolated from the monocytes using the Nuclear Extraction kit, and translocation was assessed using the NF-κB Family detection kit (both Active Motiv), according to manufacturers recommendations. Briefly, after 20 minutes of stimulation, supernatant was removed, the cells were washed with ice cold PBS with phosphatase inhibitors, and cells lysates were prepared in a hypotonic buffer and detergent, nuclei were separated from the lysates by high speed centrifugation and lysed. After centrifugation, the concentration of nuclear extract in the supernatants was determined by using a BCA protein assay (Pierce). Translocation was assessed with the ELISA-based NF-κB Family detection kit. The appropriate binding antibodies were coated on the plates and 5 µg of nuclear extract was used to detect the subunits. Measurements were done in an ELISA plate reader (Bio-Kinetics Reader Microplate EL 312e, Bio-Tek Instruments, USA) at 570 nm for the BCA assay and at 450 nm for the NF-κB Translocation assay.

Statistical Analysis

Results were described as means and minimum/maximum values. Statistical differences were calculated using Graphpad Prism 4 (GraphPad Software Inc., San Diego). Two-tailed p-values for differences in cytokine production were calculated using the Wilcoxon rank test for paired values and were considered to be significant at the p < 0.05 level.
Results

Genotype dependent effects on cytokine production after TLR2 and TLR4 stimulation combined with NOD2 stimulation

To study the effect of NOD2 co-stimulation on TLR responses, we isolated monocytes from 5 healthy donors and 15 Crohn’s disease patients divided in 3 groups of 5 carrying 0, 1, or 2 mutant NOD2 alleles, respectively.

After isolation and an overnight rest the cells were stimulated with suboptimal doses of TLR ligands, together with different doses of MDP. Culture supernatants were harvested after 3 or 24 hours and the production of TNF-α, IL-6, and IL-12 was determined.

The dose of MDP needed for optimal stimulation differed from person to person, and was in the range from 1 to 25 µg/ml MDP. For clarity of the graphs, we show here only the dose that resulted in the highest cytokine production for that particular individual together with the response without MDP (TLR stimulation only), as well as the response with the maximal dose of MDP (100 µg/ml).

Figure 1. TNF-α production in relation to NOD2 genotype in monocytes stimulated with Pam3CSK4 in conjunction with different doses of MDP

Monocytes were isolated from 5 healthy controls as well as from a total of 15 Crohn’s disease patients with different NOD2 genotypes. Cells were allowed to rest overnight, and stimulated for 3 hours with pure synthetic TLR2 ligand (Pam3CSK4; 50 pg/ml) and MDP (0 or 100 µg/ml and an intermediate dose ranging between 1-25 µg/ml). Cell culture supernatants were harvested and analyzed by ELISA for cytokine production. Each panel includes 5 different donors stimulated with the 3 different MDP doses. Panel A, healthy controls; B, Crohn’s disease patients without NOD2 mutation; C, heterozygous NOD2 deficient patients; D, homozygous NOD2 deficient patients.
Table 1  Average values of cytokine production by NOD2 competent monocytes

|        | 0 MDP absolute value (range) (pg/ml) | 1-25 MDP absolute value (range) (pg/ml) | p value compared to 0 MDP | 100 MDP absolute value (range) (pg/ml) | p values compared to 0 MDP and 1-25 MDP resp.
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<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TLR2</td>
<td>692.6 (28-2761)</td>
<td>1625 (176-4073)</td>
<td>0.002</td>
<td>665.8 (85-1857)</td>
<td>0.9/0.004</td>
</tr>
<tr>
<td>TLR4</td>
<td>996.7 (43-3063)</td>
<td>1679 (161-5862)</td>
<td>0.0098</td>
<td>1703 (260-5582)</td>
<td>0.004/0.7</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>543.7 (100-2217)</td>
<td>1661 (518-5640)</td>
<td>0.004</td>
<td>634 (109-1883)</td>
<td>0.32/0.0078</td>
</tr>
<tr>
<td>TLR4</td>
<td>1513 (100-4223)</td>
<td>2328 (441-5716)</td>
<td>0.0117</td>
<td>2461 (521-8396)</td>
<td>0.006/0.7</td>
</tr>
<tr>
<td>IL-12p70</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>140.4 (15-320)</td>
<td>242 (23-693)</td>
<td>0.23</td>
<td>166.1 (0-569)</td>
<td>0.8/0.004</td>
</tr>
<tr>
<td>TLR4</td>
<td>109.6 (0-522)</td>
<td>174.4 (0-630)</td>
<td>0.04</td>
<td>216.6 (13-961)</td>
<td>0.0098/0.4</td>
</tr>
<tr>
<td>IL-12p40</td>
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<td></td>
<td></td>
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<tr>
<td>TLR2</td>
<td>7883 (1611-18610)</td>
<td>11870 (2432-30650)</td>
<td>0.04</td>
<td>8312 (546-22280)</td>
<td>0.8/0.002</td>
</tr>
<tr>
<td>TLR4</td>
<td>5521 (1381-15800)</td>
<td>10600 (2481-23330)</td>
<td>0.03</td>
<td>11540 (2347-22830)</td>
<td>0.5/0.1</td>
</tr>
</tbody>
</table>

Shown in Figure 1 is the TNF-α response of the monocytes from different groups of CD patients and healthy controls after stimulation with 50 ng/ml of the TLR2 ligand Pam₃CSK₄. Adding the intermediate dose of MDP resulted in substantial upregulation of TNF-α responses in 13 out of 15 individuals (either patients or healthy controls) carrying at least one wildtype allele. Overall, the mean TNF-α response among these individuals increased from 865 (range: 28-2761) pg/ml after Pam₃CSK₄ stimulation only to 1556 (range: 176-4073) pg/ml after Pam₃CSK₄ + 1-25 µg/ml MDP, p<0.0001 (Table 1). Strikingly, adding 100 µg/ml MDP resulted in a marked reduction of TNF-α responses as compared to the intermediate dose and at this concentration of MDP, TNF-α values returned to, or below the baseline level that was seen after Pam₃CSK₄ stimulation alone (754 pg/ml [range: 85-1857], p = 0.0002 as compared with the intermediate dose).

Table 2  Average values of cytokine production by NOD2 heterozygous monocytes

|        | 0 MDP absolute value (range) (pg/ml) | 1-25 MDP absolute value (range) (pg/ml) | p value compared to 0 MDP | 100 MDP absolute value (range) (pg/ml) | p values compared to 0 MDP and 1-25 MDP resp.
<table>
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<tbody>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>1221 (428-1674)</td>
<td>1420 (513-2169)</td>
<td>0.06</td>
<td>931.8 (591-1349)</td>
<td>0.3/0.3</td>
</tr>
<tr>
<td>TLR4</td>
<td>1964 (931-2642)</td>
<td>2375 (920-3495)</td>
<td>0.12</td>
<td>2354 (1041-3731)</td>
<td>0.3/1</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>1237 (356-2150)</td>
<td>1440 (813-2277)</td>
<td>0.25</td>
<td>1157 (515-2923)</td>
<td>1.1/0.6</td>
</tr>
<tr>
<td>TLR4</td>
<td>1297 (273-2581)</td>
<td>1785 (875-3063)</td>
<td>0.12</td>
<td>1886 (907-3405)</td>
<td>0.12/0.4</td>
</tr>
<tr>
<td>IL-12p70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>27 (0-98)</td>
<td>81.4 (0-276)</td>
<td>0.25</td>
<td>31.6 (0-103)</td>
<td>1.1/0.12</td>
</tr>
<tr>
<td>TLR4</td>
<td>73.6 (19-115)</td>
<td>155.4 (110-224)</td>
<td>0.06</td>
<td>134 (12-256)</td>
<td>0.3/0.8</td>
</tr>
<tr>
<td>IL-12p40</td>
<td></td>
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</tr>
<tr>
<td>TLR2</td>
<td>8459 (4211-14080)</td>
<td>15700 (8027-30650)</td>
<td>0.06</td>
<td>9377 (2453-22280)</td>
<td>1/0.06</td>
</tr>
<tr>
<td>TLR4</td>
<td>7323 (1169-15800)</td>
<td>10850 (3865-23330)</td>
<td>0.06</td>
<td>8975 (2233-22830)</td>
<td>0.06/0.8</td>
</tr>
</tbody>
</table>
The magnitude of this response was particularly evident in individuals carrying two wildtype alleles and less pronounced in heterozygous individuals, which is indicative of a gene-dosage effect. Indeed, as shown in Table 2, the increase in TNF-α secretion in heterozygous individuals was only marginal (from a mean of 1221 pg/ml to 1420 pg/ml), whereas in wildtype individuals the mean production increased from 692 pg/ml to 1625 pg/ml (Table 1).

As expected, adding MDP did not result in significant upregulation of TNF-α in monocytes from NOD2 deficient patients. Thus, in this group of individuals mean TNF-α stimulation alone was 783 pg/ml (range: 351-1313), and 914 pg/ml (range 357-1991) after stimulation with Pam3CSK4 + 1-25 µg/ml MDP (p = 0.6). Adding the highest dose of MDP did not result in a significant increase in TNF-α production either (mean 1029 pg/ml [range: 355-2104]; p = 0.2) (Table 3, Figure 1d). So, in sharp contrast to the findings seen in individuals carrying at least 1 NOD2 wildtype allele, no up- and downregulation by MDP of the TLR2 induced response was seen in NOD2-/- individuals.

Table 3 Average values of cytokine production by NOD2-/- monocytes

<table>
<thead>
<tr>
<th></th>
<th>0 MDP absolute value (range)</th>
<th>1-25 MDP absolute value (range)</th>
<th>100 MDP absolute value (range)</th>
<th>p value compared to 0 MDP</th>
<th>p values compared to 0 MDP and 1-25 MDP resp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>783 (351-1313)</td>
<td>914 (357-1991)</td>
<td>1029 (355-2104)</td>
<td>0.6</td>
<td>0.2/0.4</td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
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</tr>
<tr>
<td>TLR4</td>
<td>2789 (454-5083)</td>
<td>3340 (499-6275)</td>
<td>3412 (523-6693)</td>
<td>0.6</td>
<td>0.6/0.6</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>TLR2</td>
<td>794 (322-2112)</td>
<td>1125 (402-3260)</td>
<td>1380 (320-4051)</td>
<td>0.12</td>
<td>0.37/0.625</td>
</tr>
<tr>
<td>TLR4</td>
<td>3321 (1670-6845)</td>
<td>4620 (1700-10689)</td>
<td>4952 (3481-9800)</td>
<td>0.12</td>
<td>0.12/0.625</td>
</tr>
<tr>
<td>IL12-p70</td>
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</tr>
<tr>
<td>TLR2</td>
<td>89 (59-156)</td>
<td>84 (34-138)</td>
<td>126 (67-188)</td>
<td>0.8125</td>
<td>0.12/0.12</td>
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<tr>
<td>TLR4</td>
<td>457 (61-1670)</td>
<td>527 (112-1837)</td>
<td>498 (134-1715)</td>
<td>0.06</td>
<td>0.3/0.4</td>
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<td>IL12-p40</td>
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<tr>
<td>TLR2</td>
<td>3135 (513-3663)</td>
<td>2727 (2362-4034)</td>
<td>2918 (2661-3850)</td>
<td>0.8125</td>
<td>0.6/0.4</td>
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<tr>
<td>TLR4</td>
<td>9054 (669-3063)</td>
<td>8417 (372-23416)</td>
<td>8830 (1210-25903)</td>
<td>0.8125/0.8125</td>
<td></td>
</tr>
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</table>

To exclude the possibility that the high dose of MDP is in fact lethal for the cells, cell viability was determined and was found not to be different between the intermediate and high MDP dose. In addition, as shown in Figure 2, the decrease in cytokine production after the high dose of MDP is only seen in Pam3CSK4/MDP co-stimulation, and not in LPS/MDP co-stimulation. Thus, cells from functional NOD2 expressing individuals that received LPS as a TLR4 stimulus combined with the high dose of MDP did not show the remarkable reduction in TNF-α production (mean 1910 pg/ml [range:161-5862]) as compared to the intermediate dose of LPS/MDP (mean 1920 pg/ml [range 260-5582]; p = 0.8, Table 1).

Taken together, these findings indicate that NOD2 signaling influences TLR2 signaling in a biphasic fashion, first augmenting the response, and at a higher dose, diminishing the response. This response is TLR2 specific, independent from disease status and is abrogated in NOD2-/- individuals.
Figure 2. TNF-α production in relation to NOD2 genotype in monocytes stimulated with LPS in conjunction with different doses of MDP.

Monocytes were isolated from 5 healthy controls as well as from a total of 15 Crohn’s disease patients with different NOD2 genotypes. Monocytes were allowed to rest overnight, and were stimulated for 3 hours with TLR4 ligand (LPS, 10 pg/ml) and MDP (0 or 100 µg/ml and an intermediate dose ranging between 1-25 µg/ml). Cell culture supernatants were harvested and analyzed by ELISA for cytokine production. Each panel includes 5 different donors stimulated with the 3 different MDP doses. Panel A, healthy controls; B, Crohn’s disease patients without NOD2 mutation; C, heterozygous NOD2 deficient patients; D, homozygous NOD2 deficient patients.

In addition to TNF-α, we also tested the IL-6 response (Figure 3). The same biphasic response to MDP stimulation combined with Pam3CSK4 as seen for TNF-α production was seen for IL-6 in NOD2 competent individuals. After an initial increase in IL-6 production after addition of the intermediate dose of MDP (698 pg/ml [range100-2217] after Pam3CSK4 alone as compared to 1592 pg/ml [range: 518-5640] after Pam3CSK4 + 1-25 µg/ml MDP, p = 0,001), a significant reduction in IL-6 production was seen when the highest dose of MDP was compared with the intermediate doses (783pg/ml [range: 109-2923] p = 0,004). Little effect was seen in the NOD2−/− monocytes (794 pg/ml [range: 322-2112] after Pam3CSK4 alone, 1125 pg/ml [402-3260] after Pam3CSK4 + 1-25 µg/ml MDP; p = 0,1, and 1380 pg/ml range: 320-4051] after Pam3CSK4 + 100 µg/ml MDP; p = 0,4 as compared to the intermediate dose of MDP, Table 3). Again, these effects on IL-6 production were not seen in the co-stimulations of MDP in conjunction with LPS (Figure 3 and Tables 1-3).
Figure 3. IL-6 production in relation to NOD2 genotype in monocytes stimulated with Pam₃CSK₄ in conjunction with different doses of MDP.

Monocytes were isolated from 5 healthy controls as well as from a total of 15 Crohn’s disease patients with different NOD2 genotypes. Monocytes were allowed to rest overnight, and were stimulated for 3 hours with pure synthetic TLR2 ligand (Pam₃CSK₄; 50 pg/ml) and MDP (0 or 100 µg/ml and an intermediate dose ranging between 1-25 µg/ml). Cell culture supernatants were harvested and analyzed by ELISA for cytokine production. Each panel includes 5 different donors stimulated with the 3 different MDP doses. Panel A, healthy controls; B, Crohn’s disease patients without NOD2 mutation; C, heterozygous NOD2 deficient patients; D, homozygous NOD2 deficient patients.

In the original study by Watanabe et al. it was demonstrated that Nod2 deficient mice exhibited increased IL-12 responses as compared with wildtype mice. In a next series of experiments we therefore determined IL-12 secretion in human monocytes, after 24 hours of stimulation with the different co-stimulation regimes. Contrary to the findings in mice, no increased secretion of IL-12 was found in NOD2⁻/⁻ individuals. In fact, as can be seen from Figures 4 and 5, a similar pattern as seen with TNF-α and IL-6 responses was found for this cytokine. Thus, IL-12p40 increased from 8075 pg/ml (range: 1611-18611) after Pam₃CSK₄ alone to 13148 pg/ml (range: 2432-30648) after 1-25µg/ml MDP (p < 0.0001) and returned to 8667 pg/ml (546-22283) pg/ml after 100 µg/ml MDP for IL-12p40 (p < 0.0001) in individuals carrying at least one wildtype allele. For IL-12p70 these values were 102 pg/ml (range: 0-326) after Pam₃CSK₄ alone, 188 pg/ml (range: 0-693) after 1-25 µg/ml MDP and 121 pg/ml (range: 0-569) after 100 µg/ml MDP, p = 0.0043, Table 4??). It must be noted however that similar to the findings in mice, the IL-12p70 responses were generally low. Again, no measurable effect of MDP on the IL12p40 and IL12p70 production was found in monocytes from NOD2⁻/⁻ patients. Similarly, this biphasic response was not seen after simultaneous stimulation with LPS and MDP.
Monocytes were isolated from 5 healthy controls as well as from a total of 15 Crohn’s disease patients with different NOD2 genotypes. Monocytes were allowed to rest overnight, and were stimulated for 24 hours with pure synthetic TLR2 ligand (Pam3CSK4; 50 pg/ml) and MDP (0 or 100 µg/ml and an intermediate dose ranging between 1-25 µg/ml). Cell culture supernatants were harvested and analyzed by ELISA for cytokine production. Each panel includes 5 different donors stimulated with the 3 different MDP doses. Panel A, healthy controls; B, Crohn’s disease patients without NOD2 mutation; C, heterozygous NOD2 deficient patients; D, homozygous NOD2 deficient patients.
This indicates that also for IL-12, NOD2 interacts in a biphasic manner with TLR2. Furthermore, the fact that these cells continue producing IL-12p40 and IL-12p70 after 24 hours of incubation with high doses of MDP in conjunction with LPS is indicative of the fact that the high concentration of MDP is not toxic.

**Table 4 Average values of cytokine production by monocytes expressing at least one functional NOD2 allele**

<table>
<thead>
<tr>
<th></th>
<th>0 MDP absolute value (range) (pg/ml)</th>
<th>1-25 MDP absolute value (range) (pg/ml)</th>
<th>p value compared to 0 MDP</th>
<th>100 MDP absolute value (range) (pg/ml)</th>
<th>p values compared to 0 MDP and 1-25 MDP resp.</th>
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<td>TNF-α</td>
<td>TLR2  865 (28-2761) 1556 (176-4073)</td>
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<td>TLR4  1319 (43-3063) 1910 (161-5862)</td>
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<td>0.0009</td>
<td>0.0034/0.8</td>
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<td>IL-6  783,3 (0-2217) 1592 (518-5640)</td>
<td>783 (109-2923)</td>
<td>0.0005</td>
<td>0.5/0.004</td>
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<td></td>
<td>TLR4  1548 (0-4223) 2160 (441-5716)</td>
<td></td>
<td>0.0007</td>
<td>0.0007/0.5</td>
<td></td>
</tr>
<tr>
<td>IL-12p70</td>
<td>TLR2  102 (0-326) 188 (0-693)</td>
<td>121 (0-569)</td>
<td>0.078</td>
<td>0.9/0.0043</td>
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<tr>
<td></td>
<td>TLR4  97 (0-522) 168 (0-630)</td>
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<td>0.0012</td>
<td>0.0043/0.67</td>
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</tr>
<tr>
<td>IL-12p40</td>
<td>TLR2  8075 (1611-18611) 13148(2432-30648)</td>
<td>8667 (546-22283)</td>
<td>0.0026</td>
<td>0.7/0.0001</td>
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<tr>
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<td>TLR4  6722 (1169-15799) 10765 (2481-23328)</td>
<td>9829 (2333-22826)</td>
<td>0.0015</td>
<td>0.09/0.25</td>
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</table>

**NF-κB activation**

In addition to the cytokine responses, we also assessed the NF-κB activation. For this purpose, cells from a wild-type donor, a NOD2 heterozygous donor and a NOD2 deficient donor were stimulated with either Pam₃CSK₄, Pam₃CSK₄ and a low dose of MDP (1 µg/ml), and Pam₃CSK₄ with the high dose of MDP (100 µg/ml). Cells were stimulated for 20 minutes after which nuclear extracts were isolated. The amount of translocated NF-κB subunits was then determined. As can be seen from Figure 6, for the wildtype, and to a lesser extent for the heterozygous donor, the p50 subunit exhibited a similar pattern as the cytokine responses, e.g. an elevated translocation was seen after simultaneous stimulation with Pam₃CSK₄ and a low dose of MDP, while adding a higher dose of MDP resulted in a less pronounced translocation. A similar pattern was seen for the p65 subunit. Again, this response was abrogated in NOD2⁻/⁻ individuals. This effect was not seen in the other tested subunit, c-Rel. In fact, c-Rel was hardly expressed by these cells, suggesting that the p50 and p65 units are the most important in NOD2 regulated activation.
Figure 6. Translocation of NF-κB subunits in relation to NOD2 genotype in monocytes. Monocytes were isolated from a non-mutated donor, a heterozygous donor and a NOD2 deficient donor.

Discussion

The strong genetic associations of the NOD2 gene and Crohn’s disease clearly point to an important role for alterations in innate immunity in the etiology of the disease. How the alterations in the NOD2 protein exert their effects is not completely clear, not in the last place because this sensor of the bacterial peptidoglycan component MDP is expressed in various cell types, including Paneth cells as well as macrophages and dendritic cells. Furthermore, activation of NOD2 results in activation of multiple pathways, including the NF-κB and MAPK pathways, leading to a variety of responses.

Several studies have made it clear that the NOD2 mutations found in man result in a loss of NF-κB signaling function leading to impaired cytokine production after NOD2 stimulation, and to an impaired costimulatory effect of NOD2 signaling on TLR mediated cytokine production. This seems contradictory to the elevated levels of NF-κB activation-dependent T\(_1\) cytokines, like TNF-α, IL-1β, IL-6, IL-12, IL-18, and IFN-γ found in the inflamed tissues from patients with Crohn’s disease.

In mice, deficiency for NOD2 does not lead to spontaneous intestinal inflammation, however they are more susceptible to dextran sodium sulfate (DSS) induced colitis and impaired antibacterial responses were found after oral exposure to pathogens. This suggests that in spite of various other bacterial sensing receptors, NOD2 is required for the optimal regulation of the antimicrobial response, possibly through expression of cryptdins by the Paneth cells. NOD2 has also been suggested to play...
a role as a negative regulator of TLR2 signaling, leading to excessive NF-κB dependent IL-12 production associated with the strong Th1 response seen in murine colitis. In contrast, in man no indications for a negative regulatory interaction between NOD2 and TLR2 exist, and all data on the simultaneous activation through NOD2 and TLRs, including TLR2, show a positive synergistic effect.

Our data presented in this paper may bridge this controversy and clearly show for the first time that TLR2 signaling and NOD2 signaling are linked, and that high doses of NOD2 ligands negatively interfere with TLR2 signaling. The NOD2 ligand MDP itself only marginally leads to upregulation of cytokine production, but stimulation with MDP in combination with TLR2 or TLR4 ligands dramatically enhances this production. In monocytes which are activated with TLR ligands and moderate levels of the MDP a synergistic effect is seen, correlating with earlier reports. However, when high doses of MDP are used this synergistic effect is lost in the case of TLR2 signaling, but not in the case of TLR4. Crohn’s disease patients with both NOD2 alleles mutated do not show this downregulation, whereas a gene dosage effect is seen in patients with one allele affected. The results clearly show that high doses of MDP do interact with TLR2 signaling in a negative fashion and that this is based on downregulation of the NF-κB pathways.

On could argue that the MDP concentration added is fairly high, and that the effect seen in cytokine production are not due to an active inhibition of the TLR2 signaling, but to toxic effects from the high MDP concentrations. The cells that received the high MDP dose however, remained viable, and could still produce high levels of cytokines in combination with TLR4 stimulation, even after prolonged periods of incubation, as was shown by the IL-12 production. Furthermore, the NOD2 deficient cells did not produce less cytokines after high dose treatment compared to intermediate dose treatment, indicating that the effects seen are indeed indicative of a NOD2-TLR2 interaction. The importance of the linkage between TLR2 and NOD2 signaling pathways, which we show here for the first time in man, may be related to the fact that both TLR2 and NOD2 recognize closely related structures with MDP being a derivative of the TLR2 ligand peptidoglycan.

The biphasic response seen with lower and intermediate ligand stimulation being synergistic and only at high ligand levels leading to negative regulation points to complex signaling pathways. The loss of regulatory function of the NOD2 molecule as inferred from our experiments can explain the excessive Th1 responses seen in IBD patients and the strong linkage of the mutation of NOD2 and disease. It remains to be seen whether this function is important at the initial stage of recognition of microbial pathogens or plays a role at later stages of inflammation. The biphasic effects of NOD2 stimulation on TLR2 signaling could also be reflected in the overall effects of NOD2 mutation on the pathogenesis of CD.

First, an increase in susceptibility to infection by commensal bacteria could be caused by a reduced barrier function of the intestinal epithelium, due to impaired α-defensin production by Paneth cells, or a reduced IL-1β responses to bacterial challenge combined with a less strong initial potentiating of TLR responses. This way NOD2 variants could lead to a weaker mucosal defense, leading to overgrowth of commensal bacteria.

Second, when there is an ongoing mucosal immune response, and the negative regulation of NOD2 on specifically TLR2 is lacking, this can result in an unwanted strong response. Without a proper
downmodulation of the immune response, this provides an activated immune setting for ongoing inflammation as seen in Crohn’s disease. That the expression of NOD2 can be induced in T cells under the influence of pro-inflammatory factors such as TNF-α\(^9,^{12}\), adds another level of complexity to the etiology of Crohn’s disease and suggests that NOD2 could also play a role in later stages of the disease when the adaptive immune system gets involved.

Clearly, NOD2 is a key player in innate immune responses of many cell types. The lack of an initial strong response, combined with subsequent insufficient TLR2 inhibition by due to NOD2 defects could be an attractive mechanism that plays an important part in CD pathogenesis. To determine the exact role(s) of NOD2 in the etiology of CD, it will be important to dissect the particular role of NOD2 and its interaction with TLR2 in the various cell types involved in the intestinal homeostasis, such as epithelial Paneth cells and hemopoietic immune cells.
References

Dose dependent interaction of NOD2 with TLR2 in human monocytes


### Supplementary Table 1 Genotypes of Crohn's disease patients

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<thead>
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<th>NOD2 heterozygous patients</th>
<th>Genotype</th>
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<tbody>
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</tr>
<tr>
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<td>L1007frs/+</td>
</tr>
<tr>
<td>C3</td>
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<tr>
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<td>G908/+</td>
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</table>

<table>
<thead>
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<th>NOD2 deficient patients</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>L1007frs/G908</td>
</tr>
<tr>
<td>D2</td>
<td>L1007frs/G908</td>
</tr>
<tr>
<td>D3</td>
<td>L1007frs/G908</td>
</tr>
<tr>
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<td>L1007frs/R702W</td>
</tr>
<tr>
<td>D5</td>
<td>L1007frs/L1007frs</td>
</tr>
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</table>
Chapter 8.1

Summary and Discussion
The studies described in this thesis were intended to contribute to a better understanding of the
genetic and immunological mechanisms underlying intestinal inflammation.
The predisposition to develop IBD, of which Crohn’s disease (CD) and ulcerative colitis (UC) are the
two major components, is largely genetically determined. The major difficulties in identifying these
factors rely on the fact that CD and UC are complex genetic diseases which means that there are
different genetic factors contributing to disease susceptibility. These susceptibility factors do not
necessarily have to occur in each patient; in fact, the strongest genetic risk factor identified so far
occurs only in a minority of patients. In addition, it is well appreciated that individual patients may vary
widely in clinical manifestation of the disease, referred to as clinical heterogeneity. Finally, disease is
greatly influenced by environmental factors, life style and gut flora. These factors combined have
hampered identification of the disease genes as well as understanding their role in disease
pathogenesis. The current knowledge on the etiology of IBD is summarized in Chapter 1.
The pitfalls in unraveling the pathogenesis of IBD can in part be circumvented by the use of animal
model systems. The advantage of these models is that they constitute a lower degree of genetic
complexity and in addition, disturbing environmental factors can be kept stable. However one has to
keep in mind that such models do not always fully reflect human disease, and that extrapolation has
to be done with caution. Nevertheless, these models have been successfully applied to IBD research
and have provided substantial information relevant to human disease. An overview of the various
models used in IBD is given in Chapter 2.
An important observation is that different strains of mice show different grades of susceptibility to
colitis, indicating that there must be underlying genetic differences that cause this variation. In our
search for novel genes involved in IBD, it is exactly the latter of these features of mice we applied in
the first part of our studies (Chapter 3).
In this study, we performed a genome screen on a G-protein α inhibitory chain 2 (Gαi2, encoded by
Gnai2) deficient mouse model for colitis. C3H/HeN mice homozygous for the Gnai2 deficiency, when
reared under specific-pathogen-free conditions, develop colitis as early as 6 weeks of age, whereas
Gnai2−/− C57BL/6J mice maintained under identical circumstances, are highly resistant.
The colitis in C3H/HeN Gnai2−/− mice is characterized by severe diarrhea and weight loss, frequently
accompanied by rectal prolapse and dilation of the colon. Microscopically, massive infiltrations of
lymphocytic cells are seen, accompanied by depletion of goblet cells. Also, there is formation of crypt
elongation, with high vascular density and transmural bowel wall thickening with ulceration. In
general, only the distal half of the colon is involved, with a sharp demarcation between the involved
and non-involved areas (See also Figure 2 in Chapter 3). Immunologically, the inflammation is
characterized by a Th1 type inflammation with markedly high levels of IL-12, and low levels of IL-10.
Gαi2 normally inhibits IL-12 production and thus in the absence of this molecule, IL-12 production and
the Th1 response is greatly exaggerated \(^1\). This could be one explanation as to why Gnai2−/− mice
develop colitis. A second possible explanation could lay within defects in B cell development. It has
been shown that Gnai2−/− mice display a reduction in specific types of B cells, that have the capacity to
produce IL-10. Indeed, Gnai2−/− mice show a decreased in vitro LPS-induced B cell IL-10 responses \(^2\).
By means of genome-wide microsatellite methodology we performed a genetic analysis to identify genetic susceptibility loci in *Gnai2<sup>−/−</sup>* (C3H/HeN x C57BL/6J) F2 generation intercrosses. This genetic screening procedure allowed identification of a highly significant susceptibility locus on chromosome 3, provisionally named *Gpdc1*. It is interesting to point out that exactly the same locus on chromosome 3 was identified in a different mouse model, the IL-10 deficient mouse model for colitis<sup>3</sup>, indicating that the gene(s) located in this quantitative trait locus (QTL) have a profound effect on susceptibility to intestinal inflammation in the presence of another gene defect. In addition to this QTL on chromosome 3, other loci on chromosomes 1 (*Gpdc2*), 9 (*Gpdc3*), and the X-chromosome were found.

Based on both their function in immune regulation or epithelial barrier function, there are several genes present in the identified loci that would make attractive candidate genes. The most prominent for the locus on chromosome 3 showing strongest linkage are the genes encoding the NF-κB p105 transcription factor (*Nfkb1*, also discussed in more detail below), epithelial growth factor (*Egf*), and guanylate binding protein-1 (*Gbp1*). The gene most interesting due to its location closest to the peak linkage in *Gpdc1*, as well as its eminently important role in immune activation is the gene encoding *Nfkb1*. NF-κB designates a group of critical transcription factors controlling various promoters of pro-inflammatory cytokines, cell-surface receptors, transcription factors, and adhesion molecules<sup>4</sup>. The fact that IL-12 is a cytokine that is under transcriptional regulation of NF-κB makes this gene of even more interest, since in the *Gnai2<sup>−/−</sup>* model, the IL-12 expression is clearly altered<sup>1</sup>. Clearly, any mutation affecting the *Nfkb1* gene would have major implications for a great variety of immunological and/or inflammatory processes, and could be explanatory for the strain differences in susceptibility in C57BL/6J mice and C3H/HeN mice.

To address this possibility, we sequenced the coding region of *Nfkb1* and aligned the sequences of both strains to each other and to the known sequences from GenBank. This study is described in Chapter 4, as an Addendum to Chapter 3. We found 5 single nucleotide polymorphisms (SNPs) of which one lays in the 3'-UTR of the gene whereas the remainder occur in the coding region. All the SNPs identified in the coding region of the gene are silent mutations, i.e. mutations that do not cause a change in amino acid composition of the protein.

It must be noted however that we only sequenced the cDNA, omitting the 24 introns, the promoter and the 3'UTR. Mutations in the promoter, the introns, and even the 3'UTR could be involved in generating different splice variants or the expression of the gene. Ongoing studies in congenic mouse strains in which the QTL found on chromosome 3 is being transferred from the susceptible strain onto the resistant strain and vice versa will be helpful to further discern whether *Nfkb1* is involved in colitis susceptibility in the *Gnai2<sup>−/−</sup>* model, and if so if it could affect the IL-12 production in such a way that it would explain the elevated levels seen in both murine colitis and human CD.

Indirect evidence that this gene might be important in the susceptibility to colitis comes from a recent study by Karban et. al.<sup>5</sup>, who found an elevated frequency for a mutation in the promoter of the human gene *NFKB1* in UC patients. These findings enthused us to investigate whether this polymorphism was also present in a group of Dutch Caucasian IBD patients from our outpatient clinic.

As described in Chapter 5, this study demonstrates that the allele frequency of the –94 ATTG-
deletion was also elevated in our cohort of UC patients, whereas this difference was not observed in CD. The association found in UC appeared to be a little stronger in men, and patients homozygous for the −94 ATTG-deletion allele (men and women together) had a significantly younger age of onset of disease.

The exact mechanism underlying the association between \textit{NFKB1} gene polymorphism and susceptibility to UC remains so far to be determined, but one can think of several explanations. NF-κB activation is found to be upregulated in IBD patients, in the intestinal mucosa of both CD and UC patients \textsuperscript{6, 7}. This is seemingly in contradiction with the higher prevalence of the promoter polymorphism, since the deletion variant associated with UC showed strongly diminished binding of nuclear proteins isolated from normal human colonic tissue and cell lines \textsuperscript{5}.

These effects can be explained in several ways. \textit{NFKB1} codes for the p105/p50 subunit of NF-κB complexes \textsuperscript{8}. To exert its effect, p50 binds to p65 to form biologically active heterodimers which translocate from the cytoplasm of the cell to the nucleus. Here, they bind to NF-κB binding sites in promoter sequences of genes coding for inflammatory proteins, and regulate their transcription. Alternatively, p50 is able to form homodimers which block transcription by binding to the NF-κB sites in the nucleus \textsuperscript{9}. Should the diminished \textit{NFKB1} transcription now lead to less available p50, fewer inhibitory p50/p50 homodimers can be formed, and this could in turn lead to a stronger activation of transcription of inflammatory genes, leading to the strong NF-κB induced immune response, as seen in UC.

Alternatively, low levels of p50 could lead to a poor innate immune response. Bacteria that normally would have been recognized and eliminated by this first line of defense are now providing sustained antigenic signals leading to an ongoing inflammation. For example, mice lacking Nlkb1 are highly susceptible to colitis induction \textsuperscript{10-12}. On the other hand, mice overexpressing NF-κB p50 also show chronic inflammation and higher susceptibility to bacterial infections \textsuperscript{13}. Clearly, NF-κB needs to be regulated very accurately, and any disturbance of the balance can lead to aberrant immune responses.

NF-κB activation is involved in many inflammatory processes and is mandatory for the transcription of many cytokines involved in both UC and CD. As described in Chapter 2, it is well known from animal models of intestinal inflammation that virtually any disturbance of cytokine responses can lead to an increased susceptibility to intestinal inflammation. Therefore, genetic disturbances in key regulatory elements that drive these responses are likely candidates. Recognizing the importance of a disturbed \textit{T} \textsubscript{n}1 mediated immune response in the etiology of intestinal inflammation, we expanded our studies on this subject. In \textbf{Chapter 6} we studied polymorphism in the transcription factor interferon regulatory factor 1 (\textit{IRF1}) and the IL-12 p40 chain (\textit{IL12B}) genes in the susceptibility to celiac disease (CeD).

CeD is an immune-mediated disorder of the small intestine caused by permanent intolerance to gluten, a dietary protein found in wheat, rye and oats \textsuperscript{14}. This disturbed immune response is characterized by a \textit{T} \textsubscript{n}1 mediated immune response, however, the driving mechanism behind this \textit{T} \textsubscript{n}1 response has not been completely unraveled. Similar to IBD, genetic factors play a key role in CeD susceptibility \textsuperscript{15}. This genetic component can in part be attributed to a strong association with the HLA genes, but similar to IBD, CeD is a complex genetic disorder. Several chromosomal regions have
been identified that harbor susceptibility genes to CeD, including a locus on chromosome 5q31-33 (designated \textit{CELIAC2})\textsuperscript{16-23}. This QTLs harbors, among others, the genes \textit{IRF1} as well as \textit{IL12B}. IRF-1 is a member of the IRF family of transcription factors, and IRF-1 binding sites are present in the \textit{IL12B} promoter\textsuperscript{24}.

Given the linkage to this region and the profound T\textsubscript{h}1 response found in CeD, both \textit{IRF1} and \textit{IL12B} would be likely candidates for susceptibility to CeD. We therefore studied previously described polymorphisms in these genes in a Dutch Caucasian population, however, we could not link these polymorphisms to an elevated susceptibility to CeD. This suggests that a primary defect in these genes is not responsible for the observed linkage with this region in CeD nor that they are at the basis of the exaggerated T\textsubscript{h}1 response found in both CD and CeD.

Finding a gene and a mutation that is linked to a particular disease is only the first step in unraveling disease pathogenesis. As alluded to earlier, mutations in \textit{NOD2} are strongly associated with susceptibility to Crohn’s disease. The exact mechanisms by which \textit{NOD2} variants lead to CD however remain to be defined. \textit{NOD2} is expressed in several cell types in the intestine, including Paneth cells, and cells from the myeloid lineage\textsuperscript{25-28}. Recognition of its ligand muramyl dipeptide (MDP) by \textit{NOD2} can lead to secretion of antimicrobial products by Paneth cells, and immune activation and cytokine secretion by macrophages and antigen presenting cells\textsuperscript{29, 30}. Lack of proper functioning of \textit{NOD2} has been shown to lead to a defective \textit{α}-defensin production in Paneth cells\textsuperscript{31, 32} and less production of pro-inflammatory cytokines\textsuperscript{29, 33-39}. This could in turn facilitate invading bacteria, and contribute to the ongoing inflammation as seen in IBD.

Another explanation was provided by Watanabe \textit{et al.} demonstrating that lack of NOD2 signaling in murine cells results in a lack of regulation of TLR2 mediated activation, resulting in an unwanted high production of IL-1\textsubscript{β} and IL-12, again leading to an exaggerated inflammatory response\textsuperscript{40, 41}. They demonstrated that NOD2 activation normally has an inhibitory effect on TLR2 mediated activation, in particular through inhibition of IL-12 responses. This effect is lost in NOD2 mutated mice, leading to a profound IL-12 response. This could provide a plausible explanation for the exaggerated IL-12 responses seen in CD patients.

Since it was unclear whether the latter explanation also applied to human disease, we tested this hypothesis in humans. As described in Chapter 7, we isolated and stimulated freshly isolated human monocytes from healthy controls and CD patients with or without mutations in the \textit{NOD2} gene. We found that cells with functional NOD2 respond in a biphasic manner to MDP. Thus, at a low or intermediate dose of MDP the cytokine production of the cells after TLR2 stimulation is enhanced. However when cells were incubated with higher doses of MDP, we saw a remarkable reduction in cytokine production after TLR2 stimulation. The biphasic effects of NOD2 stimulation were also reflected in NF-κB activation, as measured by NF-κB subunit translocation to the nucleus. In sharp contrast however, in monocytes from \textit{NOD2} mutated patients this initial potentiation was not downmodulated after addition of the high dose of MDP, indicating that also in humans, NOD2 is a regulating factor for TLR2 activation that can downmodulate cytokine production. We thus show here for the first time that human monocytes can respond in a biphasic manner, an effect that is lost in
Summary and Discussion

patients carrying two mutated NOD2 alleles. This study bridges the initial ‘loss of function’ theories based on human PBMC studies with the ‘gain of function’ theories based on murine studies. In summary it is clear from the many genetic and functional studies that inflammatory bowel disease is a complex affection and that its etiology is far from known. This not only stems from the fact that a multitude of genes have now been identified to be associated with the disease, but also because of the multitude of cell types involved, both at the level of the intestinal epithelium, but also at the level of the many players of the immune system. Better knowledge of the involvement and function of the various gene products associated with IBD on barrier function and immune system will help to unravel the underlying mechanisms and especially the coordinated action of geneticists and immunologists will lead to adequate prevention or therapy of the disease.

Figure 1 Overview of genes studied in this thesis

Simplified overview of the activation route in which all the genes studied in this thesis play a part. From bottom to top, IL-12 is a pro-inflammatory Th1 type cytokine that is formed by two subunits, IL-12p40 and IL-12p35. A promoter polymorphism in the gene coding for IL-12p40, IL12B was studied in Chapter 6, as well as IRF1. IRF1 encodes for the transcription factor IRF-1, that is involved in activating IL12B transcription. Likewise, IL-12 expression can also be induced by NF-κB. We determined the prevalence of a promoter polymorphism of the human form of NF-κBp50 (NFKB1) in IBD patients in Chapter 5, and sequenced the murine Nfkb1 in search for variant that could provide an explanation for the strain difference in colitis susceptibility between C57BL/6J and C3H/HeN mice (Chapter 4). This after we discovered a QTL on chromosome 3 in a Gnai2−/− colitis model, using the same strains. Nfkb1 resides in this QTL, close to the area that showed the strongest linkage with colitis susceptibility. Next to these genetic studies, we explored the theory that NOD2 signaling interferes with TLR2 signaling, and that this has its effects on specifically IL-12 production.

IKK, Inhibitor of nuclear factor kappa-B kinase; IL, interleukin; IRF, interferon regulatory factor; ISRE, interferon-stimulated responsive element; JNK, janus kinase; NEMO, NF-Kappa-B Essential Modulator; NF-κB, nuclear factor-κB; NOD2 nucleotide-binding oligomerization domain 2; QTL, quantitative trait locus; RIP-2, Receptor-Interacting Protein-2; STAT, Signal Transducer and Activator of Transcription; TLR2, Toll-like receptor 2.
References


Hoofdstuk 8.2

Nederlandse Samenvatting
IBD (Inflammatory Bowel Disease) is een verzamelnaam voor verschillende aandoeningen van het maagdarmkanaal, gekenmerkt door chronische ontstekingen van de darmwand. Genetische aanleg voor verstoringen van de darmwand-functie en het immuunsysteem spelen een belangrijke rol bij het ontstaan van de ziekte, maar hoe dit precies tot stand komt is nog grotendeels onbekend. Het doel van dit proefschrift was om meer inzicht te verkrijgen in de genetica achter deze ziekte en hoe verschillen in genexpressie het immuunsysteem kunnen beïnvloeden.

In hoofdstuk 1 worden aan de hand van een literatuuroverzicht de meest recente inzichten beschreven over het ontstaan van de ziekten, met de nadruk op de erfelijke component van IBD.

De belangrijkste ziekten binnen IBD zijn de ziekte van Crohn en Colitis Ulcerosa. De voornaamste symptomen van beide ziekten zijn buikpijn, diarree, occult bloedverlies (bloedverlies via de ontlasting), gewichtsverlies, koorts en vermoeidheid. Naast de klachten gerelateerd aan de ontsteking in de darmen komen bij IBD patiënten ook veel andere ontstekingsklachten buiten het maagdarmkanaal voor, zoals ontstekingen van de gewrichten (artritis), de ogen, de lever en de huid. Wereldwijd zijn er ongeveer drie miljoen patiënten, en de ziekten komen het meeste voor bij caucasische (blanke) mensen en Joden uit Europa en Noord-Amerika. Ondanks dat de symptomen grotendeels overeenkomen, zijn de ziekte van Crohn en Colitis Ulcerosa twee verschillende ziekten.

De ziekte van Crohn

De ziekte van Crohn is genoemd naar de Amerikaanse arts Burril B Crohn, die de ziekte voor het eerst in 1932 beschreven heeft. De ziekte van Crohn is een chronische ontsteking van het maagdarmkanaal. In de meeste gevallen is het laatste stuk van de dunne darm, het terminale ileum, aangedaan. Bij sommige patiënten zijn ook andere delen van de dunne darm en stukken van de dikke darm (het colon) ontstoken. Bij een kleine groep patiënten kunnen de ontstekingen zelfs in de slokdarm en de mond ontstaan. Typerend voor de ziekte van Crohn is dat aangetaste stukken darm worden afgewisseld met stukken gezonde darm. De ontstekingen geassocieerd met de ziekte van Crohn treffen niet alleen het darmsluimvlies, maar ook de onderliggende darmwand en spierlagen. De ziekte wordt meestal ontdekt op jong volwassen leeftijd, tussen het 15de en 35ste levensjaar, en komt iets meer voor bij vrouwen dan bij mannen. In Nederland komen er naar schatting ongeveer duizend patiënten per jaar bij. Vooralhun zijn er geen afdoende middelen om de ziekte te genezen.

De behandeling bestaat over het algemeen uit het eerst zo snel mogelijk wegnemen van de actieve ontsteking met krachtige ontstekingsremmende medicatie, zoals steroïden (prednison en aanverwante middelen), om te voorkomen dat er te veel schade aan de darmen ontstaat. Verder kunnen er medicijnen voorgeschreven worden om symptomen als diarree, misselijkheid en buikpijn te verhelpen. Indien noodzakelijk kan er een onderhoudsbehandeling voorgeschreven worden, met medicijnen die het afweersysteem zodanig tot rust brengen dat de ontstekingsreacties ook milder worden. In sommige gevallen van ernstige ziekte kan het noodzakelijk zijn dat ernstig aangedane stukken darm chirurgisch moeten worden verwijderd.
Colitis Ulcerosa

Colitis Ulcerosa is een chronische ontsteking van het slijmvlies van de dikke darm. De ziekte komt alleen in de dikke darm (colon) voor. Net zoals bij de ziekte van Crohn zijn er verschillende gradaties in ernst van de ziekte. In de meest milde gevallen is alleen het laatste gedeelte van de darm, de endeldarm aangedaan (proctitis). Bij de iets ernstigere vorm is ook de laatste helft van de dikke darm, die zich aan de linkerkant van het lichaam bevindt, ontstoken (left-sided colitis), en in de ernstigste gevallen is het complete colon ontstoken (pancolitis).

Jaarlijks wordt in Nederland naar schatting bij vijfhonderd nieuwe patiënten de diagnose gesteld. Meestal openbaart de ziekte zich tussen het 15e en 40e levensjaar, met een tweede piek tussen het 50e en 60e levensjaar. De ziekte komt bij mannen en vrouwen evenveel voor.

Net zoals bij de ziekte van Crohn, is er voor Colitis Ulcerosa nog geen genezende behandeling mogelijk. Ook Colitis Ulcerosa wordt voornamelijk bestreden met ontstekingsremmers en middelen om het afweersysteem te beïnvloeden. Wanneer deze behandeling niet het gewenste effect heeft, kan worden overgegaan tot het verwijderen van de dikke darm. Na het verwijderen van de darm is de ziekte verdwenen, aangezien er geen dikke darm slijmvlies meer is. De patiënt zal dan echter wel een stoma krijgen, of er kan een inwendig reservoir gemaakt worden van een stuk dunne darm, een zogenaamde ‘pouch’. Deze pouch functioneert dan als een soort endeldarm, en de patiënt kan de ontlasting normaal lozen via de anus, alleen wel met frequenter toiletbezoek.

Het ontstaan van de ziekten

Ondanks dat er de afgelopen jaren veel vooruitgang is geboekt in het begrip over het ontstaan van IBD, is het voorlopig nog niet bekend hoe de ziekten ontstaan. Wat wel duidelijk is geworden, is dat er meerdere factoren een rol spelen in het ontstaan. Deze kunnen worden onderverdeeld in een drietal groepen
1 Omgevingsfactoren
2 Microbiële factoren
3 Patiëntgebonden factoren

Omgevingsfactoren

Onder omgevingsfactoren verstaan we factoren zoals leefomgeving en leefgewoonten. Een logische gedachte zou zijn dat het dieet een belangrijke factor kan zijn voor het ontstaan van een ziekte die de darmen aantast. Echter, er is geen bewijs dat voeding van invloed is op het ontstaan van IBD. IBD patiënten hoeven strikt genomen dan ook geen speciaal dieet te volgen, maar in sommige gevallen verdragen patiënten sterk gekruid of erg vet voedsel minder goed, en hebben ze er baat bij voeding met deze stoffen te mijden.

Een leefgewoonte die wel aantoonbaar van invloed is op IBD is roken. Roken verergert de mate van ontsteking in de ziekte van Crohn, maar opvallend genoeg lijken patiënten met Colitis Ulcerosa soms baat te hebben bij roken. In deze patiënten lijkt roken, naast alle schadelijke effecten dat het
elders in het lichaam heeft, juist een beschermend effect te hebben tegen de ziekte. Welke stoffen in sigarettenrook precies verantwoordelijk zijn voor dit effect, en hoe deze aangrijpen, is nog onduidelijk.

**Microbiële factoren**

Ons maagdarmkanaal huisvest een zeer groot aantal bacteriën, ongeveer 100 biljoen (100.000.000.000.000), tien keer zoveel als het totaal aantal lichaamscellen. Het overgrote gedeelte van deze bacteriën is onschadelijk, en zelfs noodzakelijk bij het verteren van ons voedsel en het aanmaken van bepaalde vitamine die we zelf niet kunnen maken. Uit recent onderzoek blijkt dat de normale darmflora betrokken zou kunnen zijn bij het ontstaan van IBD. IBD patiënten hebben in sommige gevallen meer bacteriën in hun darmen dan gezonde personen, en antibiotica kuren kunnen een positieve invloed hebben op het beloop van de ziekte. Ook het consumeren van ‘goede’ bacteriën, in de vorm van probiotica in melkproduct en lijkt in sommige gevallen een gunstige effect te hebben op de ernst van de ziekte.

Er zijn echter ook micro-organismen met pathogene (ziekmakende) eigenschappen. Er is geen sluitend bewijs dat deze pathogenen IBD kunnen veroorzaken, maar er zijn wel aanwijzingen dat infectie met een bepaalde bacterie, *Mycobacterium tuberculosis*, vaker voor komt bij Crohn patiënten. Opvallend is hier om op te merken dat een nauw verwante vorm van deze bacterie, *Mycobacterium avium subspecies paratuberculosis*, bij runderen de ziekte van Johne’s veroorzaakt, een aandoening van de darmen die verassend veel overeenkomsten met de ziekte van Crohn vertoont. Het is echter nog de vraag of een infectie bij mensen met *M. tuberculosis* de ziekte van Crohn veroorzaakt, of dat het omgekeerde het geval is, en dat mensen met de ziekte van Crohn gevoeliger zijn voor infectie met deze bacterie.

**Patiëntgebonden factoren**

De manier waarop de externe omgevingsfactoren en microbiële factoren een bijdrage leveren aan het ontstaan van IBD is waarschijnlijk grotendeels afhankelijk van hoe een persoon op deze factoren reageert. Een belangrijke rol is hier weggelegd voor het afweersysteem, of immuunsysteem.

**Het immuunsysteem en IBD**

Het immuunsysteem verdedigt het lichaam tegen allerlei indringers van buitenaf. De omgeving waarin het dat moet doen bij IBD is echter uitermate complex. In ons voedsel bevinden zich ontelbare lichaamsvreemde stoffen, en onze darmen zitten vol met bacteriën die nodig zijn voor het goed functioneren van het verteringstelsel. Hieruit moet het immuunsysteem de schadelijke bacteriën herkennen en elimineren, terwijl het de onschadelijke stoffen en bacteriën met rust moet laten. Een te zwakke afweer kan er toe leiden dat pathogenen de vrije hand krijgen en infecties en ziekte veroorzaken, terwijl een te sterke afweerreactie en ontsteking schade aan het eigen weefsel kunnen veroorzaken. Het moge duidelijk zijn dat het immuunsysteem heel nauwkeurig gereguleerd moet worden, en dat iedere afwijking van die regulatie tot ziekte kan leiden.
Het immuunsysteem kan worden gezien als een leger met verschillende divisies, bestaande uit verschillende celtypes, alle met hun eigen functie.

De eerste divisie, het aangeboren immuunsysteem verzorgt de eerste verdediging. Deze tak van het immuunsysteem werkt relatief aspecifiek. Het herkent pathogenen op algemene kenmerken. Deze kenmerken zijn specifiek voor microbiologische organismen. Zo bestaat de celwand van bacteriën uit bepaalde moleculaire bouwstenen die niet bij menselijke cellen voorkomen, maar binnen het rijk der bacteriën algemeen gebruikt zijn. Deze stoffen worden ook wel Pathogenen geassocieerde Moleculaire Patronen (PAMP) genoemd. De ‘verkenner’ cellen van het aangeboren afweersysteem, de Antigeen Presenterende cellen (APCs) herkennen deze bouwstenen met behulp van receptoren, eiwitten op de celmembrana, die alleen de PAMPs herkennen, de Pathogen Recognizing Receptoren, ofwel PRRs. Een belangrijke groep van deze PRRs zijn de Toll-like receptoren (TLRs), waar hieronder nog op teruggekomen wordt. Wanneer een bacterieel product bindt aan deze receptoren, raakt de APC geactiveerd en kan hij een immuunrespons in gang zetten. Om de immuunrespons op te starten, en andere cellen van het immuunsysteem aan te trekken en aan te sporen tot reageren, kunnen de APCs signaalstoffen, cytokinen uitscheiden. In de immunologie worden tientallen verschillende cytokinen beschreven, die afhankelijk van hun eigenschap, andere afweercellen kunnen aantrekken en aansporen tot het aanvallen van pathogenen, of er juist voor zorgen dat de activiteit van afweercellen wordt gedempt zodat de immuunreactie niet te sterk wordt. Cytokinen spelen dus een uitermate belangrijke rol in de regulatie van de immuunrespons.

Afhankelijk van het soort pathogen, bijvoorbeeld of het een virus of een bacterie betreft, of dat de bacterie wel of niet de cellen binnendringt, worden er verschillende cytokinen uitgescheiden en wordt er een verschillende immuunrespons opgestart. Bij de ziekte van Crohn is dit een zogenoemde T helper 1 (Th1) respons. Een Th1 respons wordt ook wel een cel-gemedieerde respons genoemd. Belangrijk bij deze respons zijn macrofagen, die pathogenen kunnen ‘opeten’ en zo onschadelijk maken, en NK (natural killer) cellen en cytotoxische T cellen die door direct cel contact de pathogenen kunnen elimineren. De cytokinen die bij een Th1 respons horen, en die ook in hoge concentraties bij de ziekte van Crohn patiënten gevonden worden zijn interleukine 12 (IL-12) interferon-γ (IFN-γ) en Tumor Necrosis Factor-α (TNF-α).

Bij Colitis Ulcerosa is er sprake van een Th2 respons. Een Th2 respons wordt ook wel een humorale immuunrespons genoemd, omdat er bij dit type afweerreactie veel antilichamen gevormd worden, die zich in de lichaamsvloeistoffen (de humora) bevinden. De cytokinen die bij dit ziektebeeld horen zijn IL-13 en IL-4. De ziekte van Crohn en Colitis Ulcerosa verschillen dus naast lokalisatie en type ontsteking ook in type immuunrespons.

Naast de gespecialiseerde cellen van het afweersysteem heeft het darmslimvlies, het epitheel, ook een rol in de afweer tegen pathogenen. Allereerst zitten de cellen van het darmslimvlies zeer stevig tegen elkaar vast met speciale structuren, de tight junctions, zodat de bacteriën er niet tussendoor kunnen. Ten tweede kunnen de slijmbekercellen (ook wel Paneth cellen genoemd) die verantwoordelijk zijn voor het produceren van het mucus, de laag slijm die de darmwand bedekt, ook
antibacteriële stoffen uitscheiden. Deze antibacteriële stoffen, defensinen, moeten er voor zorgen dat de bacteriën niet te dicht bij de darmwand kunnen komen en zich daar niet kunnen nestelen en delen. De integriteit en functionaliteit van de darmwand is dus van groot belang bij het afslaan van aanvallen van pathogenen. Binnen IBD is ook aangetoond dat de barrièrefunctie van de darmwand aangetast kan zijn bij patiënten, waardoor de pathogenen makkelijker binnen zouden kunnen dringen en een ontsteking zouden kunnen veroorzaken.

Genetica en IBD

Normaal gezien reageert het immuunsysteem in de darm adequaat op aanvallen van pathogenen. De reactie is sterk genoeg om de pathogenen te verwijderen, en wordt daarna weer geremd, om te voorkomen dat een te lange en te sterke afweerreactie schade aan het eigen weefsel gaat aanrichten. Gezien de sterke ontstekingsreacties in IBD is het voor de hand liggend aan te nemen dat de regulatie van de immuunrespons bij IBD op zijn minst verstoord is. Zoals gezegd reageert ieder persoon anders op invloeden van buitenaf, en is de een gevoeliger voor infecties dan de ander, of dit nu komt door een verstoring van het immuunsysteem of van de barrièrefunctie van de darmwand. Dit verschil in gevoeligheid kan grotendeels veroorzaakt worden door erfelijke factoren.

Er zijn talrijke aanwijzingen dat IBD (ten dele) erfelijk bepaald is. Allereerst is er het feit dat de ziekten vaak binnen een familie voorkomen. Ongeveer 10 tot 20 % van de patiënten met IBD heeft een familielid met dezelfde ziekte. Ook binnen bepaalde etnische bevolkingsgroepen komt de ziekte meer voor, voornamelijk bij Joden en caucasiërs terwijl Aziaat en negroïden nauwelijks zijn aangedaan. Sterke aanwijzingen dat de ziekten genetisch bepaald zijn, komen uit studies waarin eeneiige en twee-eiige tweelingen werden vergeleken. Eeneiige tweelingen zijn genetisch identiek, terwijl twee-eiige tweelingen ongeveer de helft van hun genen delen. Het blijkt dat eeneiige tweelingen vaak allebei de ziekte hebben, terwijl het risico dat twee-eiige tweelingen allebei de ziekte hebben net zo groot is als voor normale broers en zussen.

Wat zijn nu precies de erfelijke factoren die IBD kunnen bepalen, en hoe kunnen we ze vinden? Alle erfelijke factoren liggen opgeslagen in het DNA. Het totaal van DNA wat iemand bij zich draagt noemen we het genoom. Het genoom bestaat uit 23 chromosomen paren, en ieder chromosoom is een lange code van 4 bouwstenen A(denosine), C(ytosine), G(uanine) en T(hymidine), die ‘basen’ worden genoemd. Op ieder chromosoom liggen enkele duizenden genen, die allen voor hun eigen eiwit coderen. Wanneer een gen ‘actief’ is, of ‘tot expressie komt’, zal het eiwit waarvoor het gen codeert aangemaakt worden door de cel. Welke genen, in welke cel, op welk moment tot expressie komen, hangt af van de soort cel, welke functie die cel heeft en welke informatie de cel op dat moment van buitenaf krijgt.

Net zoals mensen kunnen verschillen van oogkleur, zitten er soms kleine verschillen in de code (basenfolgorde) van een gen. Deze verschillen worden polymorphismen genoemd. Deze polymorphismen kunnen een invloed hebben op hoeveel een gen tot expressie komt (de hoeveelheid
Eiwit die wordt aangemaakt) of op de structuur van het eiwit (de functionaliteit van een eiwit). Deze veranderingen in genetische factoren kunnen iemand gevoeliger maken voor IBD. Hoe kunnen we nu op zoek gaan naar genetische factoren die van invloed zijn op het ontstaan van IBD?

Een eerste methode kan zijn om de basenvolgorde van een bepaald gen waarvan we het vermoeden hebben dat het een invloed zou kunnen hebben op de ziekte te bepalen en te gaan vergelijken tussen patiënten met IBD en gezonde personen. Genen die betrokken zijn bij de afweerreactie zijn bijvoorbeeld interessante kandidaten om dat mee te doen. Wanneer er een verschil in de DNA code gevonden wordt binnen dit gen, kan gekeken worden of deze variatie vaker bij IBD patiënten voorkomt. Wanneer dit het geval is, wil dat zeggen dat gen betrokken is bij de gevoeligheid voor de ziekte. Er zijn echter een goede 40.000 genen bekend, en om alle genen stuk voor stuk af te gaan is ondoenlijk.

Een andere methode die daarom gebruikt wordt is ‘genomic screening’. Bij deze techniek wordt het complete DNA vergeleken van leden van een familie waarbinnen er meerdere personen aangedaan zijn. Zo kunnen er hele stukken DNA (als het ware hoofdstukken binnen een boek) worden aangewezen die verschillen tussen gezonde en zieke personen. De genen die betrokken zijn bij de ziekte moeten zich binnen deze ‘hoofdstukken’ bevinden. We noemen deze hoofdstukken ook wel ‘gevoeligheids loci’. Op deze manier kan het aantal genen wat afgezocht moet worden naar polymorfismen drastisch verkleind worden.

Daarnaast bestaat de mogelijkheid om voor duizenden genen tegelijkertijd te bepalen in welke mate ze tot expressie komen. Door te kijken of er verschillen zijn in expressie tussen patiënten en gezonde personen, kunnen ook aanwijzingen gevonden worden welke genen betrokken zijn bij de gevoeligheid voor IBD.

Er zijn tot nog toe een aantal genen gevonden die in zekere mate betrokken zijn bij de gevoeligheid voor IBD. Een overzicht hiervan wordt gegeven in hoofdstuk 1. Er is slecht nog maar één gen gevonden dat onomstotelijk met IBD, en meer specifiek met de ziekte van Crohn geassocieerd is. Dit gen is NOD2. NOD2 codeert voor een PRR die bacteriële stoffen die zich binnen in de cel bevinden herkent. Mutaties of polymorfismen in dit gen komen bij ongeveer een kwart van de Crohn patiënten voor. Hoe het komt dat mensen met mutaties in dit gen ziek worden is nog niet bekend. Hier zal later nog op terug gekomen worden.

Ondanks de huidige vooruitgang van de techniek, en onze kennis omtrent de immunologie en genetica, is er nog niet veel bekend over het ontstaan van IBD. De reden waarom het uitermate moeilijk is gebleken om de oorzaken van IBD te vinden kan op verschillende vlakken gezocht worden. Ten eerste is de menselijke populatie uitermate divers. Iedereen heeft zijn geheel eigen unieke genetische code en eigen levensstijl, die de gevoeligheid voor en het beloop van de ziekte kunnen beïnvloeden. Ten tweede bestaat de mogelijkheid dat ‘de ziekte van Crohn’ en ‘Colitis Ulcerosa’ in feite verzamelnamen zijn voor een aantal ziekten met vergelijkbare symptomen, allen met hun eigen oorzaak, in plaats van dat ze slechts één ziekte vertegenwoordigen. Ten derde komen de polymorfismen in de genen waarvan is aangetoond dat ze in meer of mindere mate invloed hebben op de gevoeligheid voor de ziekte vaak voor bij een zeer klein percentage van de patiënten, en is het...
in veel gevallen waarschijnlijk een combinatie van genen die de gevoeligheid voor de ziekte bepaalt, en niet slechts één gen. Dit alles heeft tot gevolg dat studies in humane IBD patiënten uitermate ingewikkeld zijn. Daarnaast moeten zeer grote groepen patiënten bestudeerd worden voordat de complexe mix van meerdere genen ontveld kan worden, in combinatie met het bepalen welke omgevingsfactoren een rol spelen in het ontstaan van IBD.

Een manier om deze complexiteit te omzeilen is het gebruik van modellen om IBD te bestuderen. In hoofdstuk 2 wordt een overzicht gegeven van de beschikbare modellen voor IBD. Binnen het onderzoek naar IBD worden veel muismodellen gebruikt. Er zijn verscheidene voordelen aan het gebruik van muismodellen. Een groot voordeel van het gebruik van muismodellen is dat alle muizen van één stam genetisch identiek zijn. Heel wat eenvoudiger dan de complexe humane situatie. Daarnaast kunnen de omgevingsfactoren relatief eenvoudig constant gehouden worden. Tot slot is het relatief simpel grote aantallen dieren te bestuderen. Een nadeel van het gebruik van muismodellen is dat het om muizen gaat, en dat muizen niet hetzelfde zijn als mensen, en ook niet exact hetzelfde ziektebeeld ontwikkelen. Vandaar dat we in muizen ook spreken van de gevoeligheid voor intestinale ontsteking of colitis, in plaats van de gevoeligheid voor IBD. Een belangrijke observatie echter, is dat er gevoeligheidsverschillen tussen de verschillende muizenstammen bestaan. Dit wil zeggen dat er dus genetische verschillen moeten zijn tussen deze muizenstammen die verantwoordelijk zijn voor de gevoeligheid. Deze eigenschap kan gebruikt worden in de zoektocht naar de genen die verantwoordelijk zijn voor intestinale ontsteking. Wanneer een bepaald gen betrokken is bij de muizenvorm van de ziekte, is de kans groot dat dit gen ook betrokken is bij de humane vorm. In hoofdstuk 3 hebben we gebruik gemaakt van deze eigenschap.

Wanneer in twee verschillende muizenstammen het gen voor G-protein \( G_{\alpha}i2 \) (gecodeerd door het gen \( GnaI2 \)) kunstmatig wordt uitgeschakeld, ontwikkelt de ene stam ontstekingen in de dikke darm (colitis), terwijl de andere stam, de C57BL/6J stam, gezond blijft. Door nu het complete genoom van deze twee stammen te vergelijken hebben we een aantal gebieden (loci) kunnen identificeren die geassocieerd zijn met de gevoeligheid voor colitis. In deze loci moeten dus genen liggen die verantwoordelijk zijn voor het verschil in gevoeligheid voor colitis. Het gebied met de sterkste associatie lag op chromosoom 3. We hebben dit gebied \( Gpdc1 \) genoemd. Binnen \( Gpdc1 \) liggen verscheidene genen die, gebaseerd op hun functie, interessante kandidaatgenen zouden zijn voor de gevoeligheid voor colitis. Een daarvan is \( NfkB1 \). \( NfkB1 \) codeert voor de transcriptiefactor NF-\( \kappa \)Bp50, een eiwit dat betrokken is bij de regulatie van de expressie van een groot aantal genen die betrokken zijn bij de afweerreactie. Wanneer bijvoorbeeld een receptor op een APC een bacterie herkent, wordt via verschillende signaalwitten het NF-\( \kappa \)B complex geactiveerd, dat bestaat uit NF-\( \kappa \)Bp50 (\( NfkB1 \)) en NF-\( \kappa \)Bp65 (\( NfkB2 \)). Het NF-\( \kappa \)B complex verplaatst zich vanuit het cytoplasma naar de celkern, en bindt daar aan het DNA. Hierdoor wordt de cel aangezet om het gen waarbij NF-\( \kappa \)B gebonden heeft te activeren en zo de productie van deze stoffen te starten. Vaak zijn dit genen die voor afweerfuncties coderen,
In hoofdstuk 4 hebben we getracht om polymorfismen in Nfkb1 te vinden die het verschil in colitis gevoeligheid tussen de twee stammen zouden kunnen verklaren. We hebben daarvoor de basenvolgorde van het Nfkb1 gen van de C3H/HeN stam en de C57BL/6J stam bepaald en met elkaar vergeleken, om te zien of er verschillen in de sequentie zaten. We hebben echter geen verschillen kunnen vinden die tot een verandering in het eiwit zouden kunnen leiden, wat een veranderd functioneren van NF-κBp50 met zich mee zou kunnen brengen. Het Nfkb1 gen is echter een relatief groot gen, en het gebied in het genoom juist voor en juist achter het gen kan ook mutaties bevatten. Deze mutaties zouden weliswaar niet voor een veranderd eiwit kunnen zorgen, maar kunnen wel invloed hebben op de expressie van het gen.

Rond dezelfde tijd dat wij de muisvariant van het Nfkb1 gen bestudeerden, werd er een zogenoemd promoter polymorfisme beschreven in de humane variant van het NFKB1 gen. De promoter van een gen is een gebied in het genoom wat net voor het gen zelf ligt, wat van invloed is op de expressie van het gen. De machinerie van de cel die nodig is om een gen af te lezen heeft de promoter nodig om aan te binden om het gen daarna daadwerkelijk af te kunnen lezen. Dit promoter polymorfisme bestaat uit een deletie van een klein stukje van de promoter van NFKB1 (een –ATTG deletie, 94 basenparen voor de start van het gen) en bleek betrokken te zijn bij de gevoeligheid voor Colitis Ulcerosa in een noord Amerikaanse patiëntengroep. Omdat polymorfismen niet altijd in alle patiëntengroepen voorkomen besloten wij te kijken of wij dezelfde associatie met Colitis Ulcerosa in onze Nederlandse caucasische patiënten terug konden vinden. Dit bleek het geval. De deletie van een klein stukje van de promoter bleek vaker voor te komen in voornamelijk mannelijke UC patiënten, en gaf aanleiding tot het zich openbaren van de ziekte op een jongere leeftijd. Deze studie is beschreven in hoofdstuk 5.

Hoe het komt dat het polymorfisme in de promoter van NFKB1 leidt tot een verhoogde gevoeligheid voor Colitis Ulcerosa is nog niet duidelijk. De –ATTG deletie zorgt ervoor dat NFKB1 minder effectief afgeschreven kan worden. Echter, in IBD patiënten wordt juist een verhoogde NF-κB activering gevonden. Dit fenomeen kan op verschillende manieren verklaard worden. Naast dat NF-κBp50 tezamen met NF-κBp65 een complex kan vormen wat genexpressie kan activeren, kan NF-κBp50 ook aan zichzelf binden. Dit complex kan ook aan het DNA binden, maar heeft juist een remmende werking op de genexpressie. Wanneer er minder NF-κBp50 beschikbaar is, kan de verhouding tussen de activerende en remmende complexen verschuiven, en uiteindelijk toch tot een verhoogde staat van NF-κB activering leiden.

Anderzijds kan de verminderde productie van NF-κBp50 leiden tot een verminderde activering van afweerstoffen. Hierdoor kunnen de bacteriën een voorsprong krijgen op het immuunsysteem, en dit kan tot een ergere infectie leiden. Deze kan door het NF-κB defect niet goed worden opgeruimd, en blijft dus bestaan. Uiteindelijk zou er daardoor een continue staat van activering kunnen ontstaan.

Hoe de verhoogde gevoeligheid ook tot stand komt, het is duidelijk dat de afweerrespons strak gereguleerd moet worden. Alle genen die hierbij betrokken zijn kunnen dan ook als kandidaat gezien worden voor de gevoeligheid voor IBD. Dit blijkt ook wel uit de grote verscheidenheid aan diermodellen met verschillende gendefecten die allemaal tot colitis leiden. Een overeenkomst in deze
genen is echter wel dat ze over het algemeen op de één of andere manier iets te maken hebben met de regulatie van de productie van cytokinen.

In hoofdstuk 6 hebben we de rol van de transcriptiefactor IRF-1 en één van de twee ketens van het cytokine IL-12, namelijk IL-12p40, onderzocht in de gevoeligheid voor coeliakie. Coeliakie is een ontstekingsziekte van de dunne darm, veroorzaakt door een overgevoeligheid voor gluten, een stof die voorkomt in tarwe, rogge en gerst. Ondanks dat men weet tegen welke stof de afweerreactie gericht is, is de genetisch achtergrond van deze ziekte, net zoals bij IBD, nog grotendeels onbekend. De genen IRF1 en IL12B (coderend voor IL-12p40) liggen in een gebied dat in ander studies is aangeduid als een gevoeligheidslocus voor coeliakie, CELIAC2, op chromosoom 5. In de promotor van IL12B bevinden zich gebieden waar de transcriptiefactor IRF-1 aan kan binden. IRF1 kan dus de expressie van IL12B reguleren. Gezien het belang van het cytokine IL-12 in het aansturen van immuunresponsen, en de ligging van deze twee genen in een gebied dat als gevoeligheidslocus wordt aangeduid, zijn dit interessante genen om te bestuderen, en te kijken of eerder beschreven polymorfismen in deze genen betrokken zijn bij de gevoeligheid voor coeliakie. Echter, we konden in onze Nederlandse caucasische patiëntengroep geen aanwijzingen vinden dat deze genen betrokken zijn bij de gevoeligheid voor coeliakie.

Het vinden van een gen wat betrokken is bij de gevoeligheid voor een ziekte is slechts de eerste stap in het begrijpen van het ontstaan van de ziekte. Zo is ondanks het feit dat NOD2 het eerste gen is waarvan een duidelijke betrokkenheid beschreven is bij het ontstaan van de ziekte van Crohn, het nog verre van duidelijk hoe mutaties in NOD2 leiden tot de ziekte. NOD2 komt tot expressie in verschillende celtypen en is een receptor voor het bacteriële product muramyl dipeptide (MDP). In slijmbekercellen in de darmwand leidt herkenning van MDP door NOD2 tot het afscheiden van antibacteriële stoffen. Slecht functionerend NOD2 als gevolg van mutaties in het NOD2 gen zouden kunnen leiden tot minder productie van antibacteriële stoffen. Hierdoor zouden de bacteriën de kans kunnen krijgen een infectie in de darm te veroorzaken, die uiteindelijk tot de ziekte van Crohn zou kunnen leiden.

In bepaalde cellen van het afweersysteem, macrofagen en dendritische cellen, komt NOD2 ook tot expressie. Hier kan het herkennen van MDP door NOD2 de reactie van de cellen op de activerende signalen van andere receptoren, de TLRs, versterken en de cellen extra aanzetten tot het tot expressie brengen van cytokinen en het opstarten van een immuunrespons. Wanneer deze extra activering weg zou vallen, zouden de bacteriën ook in dit geval een voorsprong op het immuunsysteem krijgen, wat tot langdurige ontsteking kan leiden.

Een andere mogelijkheid is echter dat NOD2 signalering de TLR activering, met name de TLR2 activering, negatief reguleert, dus in feite remt. Het is aangetoond in muizen dat wanneer de NOD2 signalering wegvalt, de IL-12 productie niet meer geremd wordt, en dat deze remming alleen op TLR2 van toepassing is.

In hoofdstuk 7 wilden we testen of dit proces ook in humane cellen plaats kon vinden. Om dit te onderzoeken hebben we monocytair cellen uit het bloed van Crohn patiënten met en zonder
mutaties in NOD2 geïsoleerd en in kweek gebracht, alsmede cellen van gezonde controle personen, met normaal NOD2. Wanneer cellen van gezonde personen of IBD patiënten zonder NOD2 mutatie via hun TLR2 gestimuleerd werden tegelijkertijd met een lage of intermediaire doses van MDP, werd de TLR2 gemedieerde cytokine respons versterkt. Wanneer de dosis MDP verder verhoogd werd echter, werd er beduidend minder cytokine geproduceerd door de cellen. Dit remmende effect werd ook gezien in de NF-κB activering van de cellen. Cellen die geen functioneel NOD2 tot expressie konden brengen, vertoonden dit effect niet. Weliswaar werd de cytokine-productie wel enigszins verhoogd door het toevoegen van MDP, maar de remming van de TLR2 respons bij de hoge dosering van MDP werd niet gezien. Deze resultaten laten voor het eerst zien dat ook in menselijke cellen NOD2 de TLR2 activering negatief kan reguleren, en dat deze regulatie wegvalt in monocytaire cellen van patiënten die geen functioneel NOD2 hebben.

Samenvattend kunnen we stellen dat ondanks de vele genetische, immunologische en epidemiologische studies die gedaan zijn de complexe oorsprong van inflammatoire darmziekten nog verre van duidelijk is. Vele genen en vele celtypen kunnen betrokken zijn bij het ontstaan van IBD, en de functie van de genen hoeft niet noodzakelijkerwijs hetzelfde te zijn in verschillende celltypen. Een betere kennis van de functie van alle genen die geassocieerd zijn met de ziekten en hun rol in de immunologische achtergrond van IBD, tezamen met een betere herkenning en indeling van de verschillende subtypen van IBD, zal helpen bij te dragen aan het begrip van de ziekten, en uiteindelijk kunnen leiden tot een adequate preventie van of therapie voor de ziekte.
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Curriculum Vitae
