The Role Of
The Innate Immune System
In Inflammatory Bowel Disease

We have met the enemy and he is us

Pieter van Lierop

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De Rol Van Het Aangeboren Immuunsysteem In Inflammatoire Darmziekte

De vijand is in ons

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CONTENTS

Chapter 1

The role of the innate immune system in the pathogenesis of IBD

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INTESTINAL HOMEOSTASIS

In 1676 Antonie van Leeuwenhoek discovered small one-cell consisting microorganisms. These "animalcules", as van Leeuwenhoek called his discovery, are now known as bacteria and are as old as 4 billion years. In the human intestine an ecosystem has developed in which bacteria and eukaryotic cells live in a mutual beneficial relationship. The intestinal tract is colonized with over 10^{14} bacteria and over 400 species. These commensal bacteria live in symbiosis with the human species, helping with the extraction of nutrients from ingested food and suppressing the growth of pathogenic counterparts.[1, 2]

In order to maintain intestinal homeostasis, the mucosal immune system plays a crucial role. On the one hand the immune system has to recognize invading pathogenic bacteria and mount appropriate pro-inflammatory immune responses in order to protect the host from these bacteria. On the other hand, extensive pro-inflammatory immune responses have to be restricted to pathogens only. In turn, an aberrant immune response upon for example food antigens (celiac disease) or commensal bacteria (Inflammatory Bowel Disease (IBD)) could lead to tissue damage and chronic inflammation.

INFLAMMATORY BOWEL DISEASE

IBD is described as a chronic inflammatory response of the gastrointestinal tract with a prevalence of roughly 5-15 per 100.000.[3] To date IBD includes two disease entities: Crohn's disease (CD) and ulcerative colitis (UC). CD was described for the first time as regional ileitis in 1932 by Crohn, Ginzburg and Oppenheimer in New York. Around the same time, reports on UC were published by Lockhart-Mummery in London.

CD and UC are distinguished based upon clinical and histopathological differences. Although these two entities differ in multiple ways (e.g. phenotype and drug responsiveness) both diseases are thought to result from an aberrant immune response to commensal bacteria. Therefore CD and UC could well be part of a broad spectrum of intestinal inflammatory conditions.

Traditionally, exaggerated responses by mucosal T- and B-cells (the *adaptive* immune system) have been regarded as the key contributors to the pathogenesis of IBD.[4] The recent discovery however, of *innate* defects that are associated with IBD have refocused the attention to this arm of the immune system.[5, 6] The innate immune system plays a crucial role in the development of IBD. Cells of this immune system are the first that come in contact with the microbiota in the intestinal lumen.

MICROBIOTA:

Bacterial host cross-talk

The human intestine contains about 10^{14} micro-organisms that critically contribute to tissue homeostasis, host metabolism and other beneficial processes. On the other hand, it has been shown that IBD may, in contrast, result from aberrant cross talk between the mucosal immune system and this vast population of commensal bacteria.[7]

IBD as infectious disease

The first reports of diseases suggestive of IBD contained descriptions of a condition named hyperplastic tuberculosis of the intestine. Subsequently, various case reports were published on patients suffering from what was believed to be intestinal tuberculosis, but acid-fast organisms could not be isolated.[8] To date, stating that IBD is caused by a pathogenic microorganism is at least controversial. Amongst the many proposed pathogens, *Mycobacterium avium paratuberculosis* (MAP) has gained some support.[9-11] MAP was originally identified in cows suffering from an intestinal inflammation called Johne's disease that shows histopathological similarities with Crohn's disease. Although the association of MAP with CD has gained support, a causative role for MAP in the CD pathogenesis has not been established. Specifically, it can be argued that MAP is a secondary invader rather than a true pathogen.[12-14]

Along the same lines, other microorganisms such as *paramyxovirus* (measles), *Chlamydia spp.*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Listeria monocytogenes* and *Helicobacter spp.* have been implicated in part, based on the detection of specific antibodies (Table 1).[15-30] More in particular, an increase of adhesive and invasive of *E. coli* strains has been associated with CD.[21, 31, 32] Enhanced invasiveness may result from the expression of certain microbial components such as flagellin.[33] Moreover, flagellin has been identified as a microbially derived immunodominant antigen that is associated with CD and is involved in intestinal inflammation in various mouse models.[34-38] In analogy with the distinct association between *Helicobacter pylori* and peptic ulcer disease, many attempts have been made to establish a similar mucosal paradigm for IBD. Based on the available data however, it seems more likely that these diseases do not result from primary infections but that microbial invasion and subsequent immune responses are secondary to inflammation and intestinal barrier disruption.[39]

A role for non-pathogenic luminal microbiota

It has been widely accepted that luminal microbiota play an important role in the pathogenesis of IBD (figure 1). Substantiation comes from various IBD-animal models that are dependent on intestinal colonization.[40-43] In humans, surgical deviation of inflamed intestine has shown to ameliorate inflammation that is thought to result from

Table 1, IBD associated microbiota and antibodies
Partly adapted from [173]

Bacteria	Reference:
Mycobacterium avium paratuberculosis	9-11, 174, 175
Listeria monocytogenes	23, 176
Escherichia. Coli	20-27, 177
Enterotoxigenic Bacteroides fragilis	178-180
Klebsiella oxytoca	181
Pseudomonas maltophilia	182, 183
Chlamydia spp.	17-19, 184
Streptococci spp.	23
Helicobacter spp	30, 49
Yersinia pseudotuberculosis	185
Cytomegalovirus	28
Saccharomyces cerevisiae	29
Paramyxovirus	15, 16
Mycoplasma pneumoniae	17
Coxiella burnetii	17

Antibodies:	Reference:
Saccharomyces cerevisiae (gASCA)	186-188
Klebsiella pneumoniae	189, 190
Outer membrane porins (OmpC)	186, 191, 192
Pseudomonas fluorescens-associated sequence I2 (anti-I2)	193, 194
Flagellin (CBir1)	34-38

diminished microbial exposure.[44] Further support comes from the apparent efficacy of certain antibiotics in the treatment of IBD.[45-48]

Colitogenic vs. protective microbial strains

Although no specific pathogens have been isolated and mucosal responses seem to be directed towards a wide range of microbial antigens, the question remains whether specific groups of bacteria are still important in the pathogenesis of IBD. On the other hand, it seems likely that certain bacteria or bacterial factors are important for the induction of tolerance and therefore protection from inflammation. In certain animal models, various segmented filamentous bacteria fail to induce colitis whereas in contrast, colonization with *Helicobacter muridarum* results in an accelerated development of IBD.[49] In other models it was shown that the phenotype of intestinal inflammation depends on the species of commensal bacteria that were used. Furthermore, it was shown that the location of disease depends on the specific bacterial strain that is used for colonization.[50]

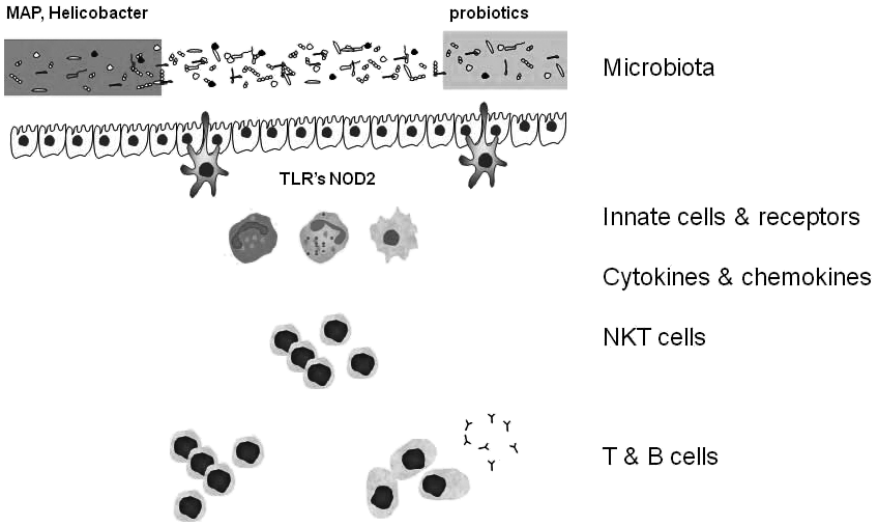

Figure 1. The role of the microbiota and the innate immune system in the pathogenesis of IBD
Commensal bacteria can evoke immune responses via TLR or NOD signaling. Innate cells respond through the production of cytokines and chemokines to attract other immune cells (e.g. neutrophils, macrophages). Dendritic cells can sample bacteria and activate T and B cells. For each level of this paradigm both protective as well as contributive mechanisms are discussed.

Molecular techniques now enable us to characterize microbiota in fecal streams and colonic biopsy specimens. New findings however, have greatly complicated this field of research; as such, it has been established that bacteria are very capable of altering the expression of capsule polysaccharides that are directly associated with immunogenicity.[51, 52] In other words, a specific microbe may suppress the immune system under certain conditions[53, 54], while a genetically identical microorganism may lead to inflammation in other circumstances.[46, 55] The driving force behind this microbial adaptation may well be the host immune system itself as has been shown recently.[46] In these mouse studies, luminal microbes attained a colitogenic phenotype in a genetically susceptible host. Intriguingly, these bacteria could now provoke colitis in naïve animals.[46] These studies may in fact suggest that in IBD patients, we should treat the microbes along with the immune system.

Finally, there is the issue of the use of probiotics as IBD treatment, based on the concept that the assumed disbalance between pathogenic and commensal bacteria could be restored with the administration of these bacteria. Although some progress has been made into understanding the working mechanisms[56] and some efficacy has been seen in certain IBD patients.[57-60] Much more research is needed before these interventions can be applied in large patient cohorts. The fact that certain microbial fermentation products such as butyrate can suppress inflammation through inhibition of NF-κB activation, may eventually lead to the discovery of certain specific beneficial microbial species.[61-64]

PATTERN RECOGNITION RECEPTORS

Sensing luminal microbes

Pattern recognition receptors (PRR) such as toll-like receptors (TLR) continuously recognize bacteria (figure 1). Subsequently, activation of PRR mediated pathways leads to chemokine and cytokine production by immune cells or production of antimicrobial molecules by Paneth cells.

TLR are transmembrane proteins which contain a leucine-rich domain and a conserved cytoplasmic domain.[65] TLR activation results in nuclear translocation and activation of the pro-inflammatory transcriptionfactor NF-κB that is associated with the production of mediators. [66-68] Moreover, in antigen presenting cells (e.g. dendritic cells), TLR signaling leads to the induction of co-stimulatory molecules and subsequent activation of adaptive immune cells such as T-lymphocytes.[65] In turn, activated T cells can exert their function e.g. cytokine production.

Nucleotide-binding oligomerization domain (NOD) or Catterpillar receptors are intracellular receptors that can trigger similar NF-κB dependent pathways. Microbial muramyl dipeptide (MDP), the smallest bioactive component of peptidoglycan (PGN) that is a component of all bacteria, has been identified as the specific ligand for NOD2.[69, 70] Different mutations in the NOD2 gene, located on chromosome 16q12, have been shown to associate with CD. Specifically, NOD2-mutations are thought to alter the susceptibility and location of the disease.[5, 6, 71-73]

Recently, a third class of PRRs has been identified: the inflammasome. The inflammasome is an intracellular protein complex that initiates the release of caspase-1. In concert with NF-κB phosphorylation upon TLR signaling, activation of the inflammasome will result in secretion of the pro-inflammatory cytokines IL-1β and IL-18.[74, 75] One of the key constituents of the inflammasome is the NALP3 molecule and NALP3-mutations are associated with inflammatory disease.[76, 77] Interestingly, flagellin was identified as a specific ligand for NALP3 reiterating the potential importance of this specific microbial ligand for inflammation.

The exact function and interactions of all these PRRs, and how signaling through these receptors eventually leads to an increased and chronic inflammatory responses is strongly debated.[78] In short, it is not known how these ligands actually reach their respective PRRs and what would be the net effect of activation of various receptors at the same time. A first step will be the identification of specific cellular receptors and transporters that mediate the uptake of the microbial ligands and assess if they are involved in the IBD pathogenesis.

LOSS OR GAIN OF INNATE IMMUNE FUNCTION:

To date, two main hypotheses address how defects in the innate immune system could contribute to the IBD pathogenesis:

I: *Loss of function*: upon recognition of microbial ligands there is a diminished responsiveness, i.e. less production of mediators such as chemokines and cytokines. In turn, this will lead to suboptimal recruitment and function of innate cells such as neutrophils and macrophages. In this condition, otherwise harmless commensal microbiota will increasingly penetrate and translocate the intestinal mucosa. Finally, this will lead to chronic attraction and activation of adaptive immune cells e.g. B- and T-lymphocytes, thus chronic inflammation.

II: *Gain of function*: defects in innate cells will lead to hyper-responsiveness upon microbial stimulation. In this case, despite normal levels of ligand availability, excessive chemokine production will directly cause inflammation by recruited innate (e.g. neutrophils and macrophages) and adaptive cells (e.g. B and T cells).

Before assessing the actual contribution of these two functional innate defects to the IBD pathogenesis we will first address the principles of microbial sensing:

Regulating PRR sensitivity: ligand exposure and PRR expression

Theoretically, activation of PRRs depends on the availability of ligands and can be controlled by altering receptor expression or through the induction of various intracellular regulators.[79] Intestinal epithelium expresses low amounts of TLR at the apical surface under healthy conditions.[80-86] An altered expression of TLR3 and 4 has been suggested in IBD and for some murine colitis models. As such, TLR4 was described to be upregulated, whereas cytoplasmic TLR3 was downregulated in active CD.[81, 87] As TLR5 is mainly expressed at the basolateral side of colonic epithelial cells, activation of this receptor may depend on regulation of the exposure to its ligand flagellin. Indeed, expression of TLR5 by the IEC seems to remain unchanged in IBD but disruption of the integrity of the epithelial barrier during IBD can lead to an enhanced translocation of flagellin and subsequent cellular activation. In addition, flagellin exposure after disruption of the epithelial barrier in murine models indeed results in an exacerbation of colitis.[85, 88-92]

Regulating PRR sensitivity: expression of regulators

A next step for the regulation of PRR sensitivity is through the production of intracellular regulators. Induction of colitis in mice that lack SIGIRR, an intracellular inhibitor in the TLR–NF-κB pathway, results in excessive epithelial damage and increased severity of an experimental colitis.[93-95] GRIM-19 is another regulator of NOD2 dependent NF-κB activation. A recent study suggested that inflamed mucosa of IBD patients expresses less GRIM-19 in comparison to mucosa of non-IBD patients.[96] To date, no mutations in the

GRIM-19 gene have been linked to IBD.[97] Finally, CD patients have been found to have an increased number of mutations in the gene encoding for PPARγ that is implicated in regulation of TLR signalingγ.[98, 99] Furthermore, PPARγ expression seems to be reduced in UC patients.[100]

Notably, even well controlled animal models seem to yield conflicting results; some recent studies mention enhanced susceptibility to inflammation in the absence of TLR signaling,[101-103] while other studies establish a contributive role for TLR signaling.[104, 105] Recently, various novel regulatory mediators and associated mechanisms have been described: Tollip, sTLR2, IRAK-M, A20 and SLPI.[85, 106-113]

In summary of this part, it is important to stress that to date, it has been impossible to estimate the relative contribution of each of these regulatory mechanisms. The complexity of this innate network, with the wide range of ligands and increasing number of TLRs, NODs, inflammasome components and regulators may in fact need computational analyses to establish its contributions to health and inflammation.

Diminished PRR signaling in IBD, loss of function

The association between mutations in the intracellular microbial pattern receptor NOD2 and Crohn's disease (CD) have been attributed to a loss of function of the innate immune system in these patients. NOD2 is expressed by monocytes and epithelial cells such as the Paneth cells (Pc).[114, 115] Pc are predominantly located in the terminal ileum, within the crypts of Lieberkühn. Upon microbial stimulation, PC produce mediators such as anti-microbial molecules, e.g. α-defensins (cryptdins in mice).[116-121] A defective PC function in NOD2 mutated patients could contribute to the IBD through a failure of microbial regulation at the mucosal surfaces. Patients with CD were found to have a diminished expression of α-defensins that was most pronounced in CD patients with a NOD2 mutation.[122, 123] As PC are mainly found in the terminal ileum it is not surprising that genotypic defects in NOD2 seem to be linked to a disease phenotype with severe inflammation at that specific location.

Substantiation for this concept comes from animal models; NOD2 deficient mice show impaired PC responses upon challenge with the NOD2 ligand MDP and a failure to eradicate certain pathogens.[124] These studies explain how a defect in clearance of certain bacteria may lead to a perpetuative, albeit insufficient, mucosal immune response and therefore chronic inflammation.

Recently, other epithelial derived anti-microbial products such as β-defensins and cathalicidin have been suggested to protect the host from the enhanced microbial pressure that occurs during mucosal inflammation. Indeed, local administration of cathalicidin was shown to ameliorate colitis in mice.[125] Anti-microbials could therefore provide new therapeutic options in the IBD treatment.

A second innate cell type that may contribute to the chronic inflammation in IBD is the mucosal macrophage (figure 1). Similar to the mechanism for Paneth cells, a loss of function in macrophages has been described.[126] Furthermore, monocyte-derived macrophages from CD patients with a NOD2 mutation produced less of the chemoattractant IL-8 upon stimulation with the NOD2 ligand MDP. In turn, this could lead to a reduced and delayed recruitment of neutrophils, and suboptimal clearance of bacteria from the intestine, again leading to chronic inflammation. Similar mechanisms have been proposed for macrophages that are deficient in the expression of other PPRs such as TLR4.[127, 128]

Enhanced PRR signaling in IBD, gain of function

Chemokines are among the first mediators that innate cells produce upon stimulation. Various researchers have been able to link the expression of various chemokines (ENA-78, CXCL-8, MCP-1, MCP-2, MCP-3, MIP1α, MIP1β, MIP-3α) in colonic epithelium to the severity of the inflammation in IBD, that is suggestive of enhanced innate responsiveness.[129-134]

A strong case for enhanced PRR signaling in IBD was offered by research performed in NOD2 deficient mice. Microbial peptidoglycan (PGN) can be recognized by the extracellular receptor TLR2 as well as the intracellular NOD2 protein. When macrophages derived from NOD2 deficient mice were stimulated with PGN, this resulted in an overexpression of pro-inflammatory cytokines compared to wild-type macrophages.[135] It was suggested that NOD2 may act as a negative regulator for TLR2 signaling. This way, defective signaling through NOD2 may lead to exaggerated TLR2 sensitivity through the absence of dampening mechanisms. In humans, in was confirmed that in the absence of NOD2 signaling, TLR2 *activation* can result in enhanced expression of pro-inflammatory cytokines upon stimulation.[135-138]

Finally, Damen et al. showed that buccal epithelial cells derived from pediatric Crohn's disease patients secrete higher amounts of CXCL-8, MIG, and IP-10 in comparison to adult Crohn's patients or age-matched UC patients.[139] From these studies it was concluded that pediatric CD may represent a specific subset of patients that is incapable of maintaining a physiological state of epithelial hyporesponsiveness to microbial stimulation.[140]

BRIDGING INNATE AND ADAPTIVE:

Next to aberrant innate immune responses to luminal bacteria by epithelial cells, Paneth cells and macrophages, other non-adaptive cells have been implicated in the IBD pathogenesis: goblet cells[141, 142], neutrophils[143], eosinophils[144], mast cells[145] and NKT cells (figure 1).[146-149]

All these immune-cells seem to play a significant role in maintaining the typical non-inflammatory state of the healthy intestinal mucosa but consequently contribute to inflammation in susceptible hosts.

Dendritic cells

Dendritic cells (DC) are key innate immune cells that play a crucial role in the recognition and processing of microbial and viral products. These cells are highly capable of picking up antigens via PRR, leading to activation of these cells. Activation leads to a functional maturation of the DC and migration to the draining lymph node. Next, antigens are presented to the cells of the adaptive immune system leading to a consequent adaptive immune response.

Intestinal DC can contribute to inflammation through the production of pro-inflammatory cytokines such as TNF-α.[46] Next, DC are involved in antigen presentation and activation of B- and T-lymphocytes e.g. through the production of specific cytokines.[150] Mucosal dendritic cells are present within the isolated follicles of the gut and the lamina propria where they can sample bacteria in the lumen.[151, 152] In human IBD and different IBD models, DC have been shown to display a more activated phenotype and an enhanced production of pro-inflammatory cytokines.[87, 139, 153-159] It is believed that activation of intestinal DC is a prerequisite for the activation of the pathogenic T cells that are key to the pathogenesis of IBD (figure 1). Next, it is the type of mediators that DC produce that will determine the subtype of effector T cell, e.g. Th1, Th2 or Th17. Each of these specific T cell subsets has been implicated in specific IBD types.[160] As such, Th1 and Th17 cells are thought to play a role in CD, whereas UC is associated with a mixed Th1/Th2 profile.[147] Whether the required activated DC phenotype for specific Th subset differentiation results from particular microbial exposure or is associated with genetic defects that directly affect DC function remains to be elucidated. One of the determining factors could be *Thymic stromal lymphopoietein* (TSLP) that is produced by the IEC upon TLR activation and gives rise to Th2 differentiation.[161-164] As such, CD could result from an impaired TSLP production by the IEC.[165]

The capacity to serve as innate cells as well as initiators of adaptive responses have emphasized the role of DC in the immunological cascade that contributes to mucosal tolerance as well as inflammation.

GENETICS

Genetic involvement in the pathogenesis of IBD first came from epidemiological studies. First-degree relatives from IBD patients have a 2-5 times increased risk in developing CD or UC. In monozygotic twins the rate of concordance increases up to 60% for CD and

almost 20% for UC. As the rate of concordance is not 100% in monozygotic twins, this indicates that IBD is inherited as a complex genetic basis.[4]

Multiple genes have been linked to IBD over the years. These genes were associated with activity of disease, disease phenotype or were specifically associated with either adult or pediatric IBD.[166-171] Prominent was the discovery of IBD associated genetic variability in NOD2 (CARD15), which directed research to the interaction between microbiota and the innate immune system.[5, 6, 71-73] Although these genetic studies have brought great discoveries regarding heritability and genetic risk factors the functional immunological consequences of genetic variability is difficult to establish.

Furthermore, it is becoming clear that IBD patients represent a very heterogeneous group that may need a novel classification beyond that of CD and UC. Given the distinct disease phenotype and associated immune pathology in children for example, it seems warranted that early onset inflammatory bowel disease can be seen as a specific disease entity.[172]

OUTLINE OF THIS THESIS

Inflammatory bowel disease (IBD) is thought to result from an aberrant intestinal immune response to commensal bacteria in a genetically susceptible host. In this thesis we investigated the interaction between the innate immune system and the microbiota at various levels.

As described in chapter 1, loss of function as well as gain of function of the innate immune system has been suggested to result in intestinal inflammation. Whether loss of function or gain of function is detectable in an individual will depend on the disease type, duration and state of disease, cell type and other factors. Due to this complexity, we argued in **chapter 2** that results regarding the function of immune cells in patients from one study cannot be extrapolated to IBD in general.

In **chapter 3** we first assessed the interaction between the innate and the adaptive immune system in a mouse model for IBD. Innate immune cells are crucial in the establishment of colitis. Here we showed that inhibition of T-cell function by tacrolimus results in a diminished recruitment of neutrophils and monocytes that is associated with lower colitis scores.

Dendritic cells (DC) play a key role in the recognition of various microbial products in the intestine. In **chapter 4** we assessed the origin and phenotype of the pro-inflammatory DC in the pathogenesis of colitis. For this purpose we studied the phenotype of the DC during the different phases of colitis development in a mouse model for Crohn's disease, TNBS colitis.

In **chapter 5** we further investigated the role of DC in the pathogenesis of IBD. We studied the intrinsic capacity of DC of pediatric IBD patients to secrete the pro-inflammatory cytokines Interleukin (IL)-12 and IL-23 upon specific microbial stimulation. We assessed whether the cytokine production was related to the phenotype of IBD, duration of disease and treatment of disease.

Paneth cells (Pc) play an important role in the protection against microbial invasion into the intestinal crypts and in the regulation of microbial intestinal composition in general. In **chapter 6** we assessed the role of PC derived TNF-α, a key innate pro-inflammatory cytokine, in intestinal inflammation in pediatric IBD patients and celiac disease patients. We further investigated the regulation of the pro-inflammatory cytokine TNF-α in Pc in mice and *in vitro*.

Aberrant tolerance to microbial products has been proposed in the pathogenesis of multiple inflammatory conditions. In **chapter 7** we investigated the acquisition of innate microbial tolerance in IBD patients and healthy controls by analyzing responsiveness of monocytes upon repetitive LPS stimulation. This process of endotoxin tolerance is an important protection mechanism to handle continuous microbial triggers, as seen in the intestine.

Optimal treatment of IBD aims at the induction and preservation of clinical remission. However, drug responsiveness varies between patients. In **chapter 8** we analyzed immune processes and drug responsiveness by investigating RNA gene expression profiles of pediatric IBD patients with inactive disease and control individuals.

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Chapter 2

Defective acute inflammation in Crohn's disease

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DEFECTIVE ACUTE INFLAMMATION IN CROHN'S DISEASE

Daniel Marks and colleagues[1] suggest that patients with Crohn's disease have a general defect in the innate mucosal immune response, including diminished interleukin 8 production. We are not convinced.

Various studies show an increased number of neutrophils—probably associated with the respective induction of specific chemokines, such as interleukin 8—rather than a diminished neutrophilic influx.[2] Moreover, intestinal epithelial cells are an important source of interleukin 8, in ulcerative colitis as well as in Crohn's disease.[3] Taken together, these data indicate a limitation to the general constitutional abnormality in Crohn's disease as postulated by Marks and colleagues.

By contrast with Marks and colleagues' findings, we have shown increased release of interleukin 8 (on stimulation and at basal levels) by buccal epithelial cells derived from children with Crohn's disease when compared with healthy controls and children with ulcerative colitis.[4] Furthermore, monocyte-derived dendritic cells from our children with inflammatory bowel disease did not show this striking difference in chemokine production. Whether epithelial cells represent a cell type with a particular responsiveness that might not be seen in myeloid cells such as macrophages remains to be clarified. Finally, childhood Crohn's disease might represent a specific disease entity. Indeed, reports suggest that, in comparison with adults, children with the disease have a higher number of associated mutations, different kinetics, and differences in location of inflammation and responsiveness to immunosuppression.[5]

Therefore, we suggest that the differences in immune responses found by Marks and colleagues do not necessarily represent a general mucosal paradigm for Crohn's disease. We argue that their results cannot be extrapolated to other involved immune cells or to specific patients such as children with Crohn's disease.

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Chapter 3

T-cell regulation of neutrophil infiltrate at the early stages of a murine colitis model

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ABSTRACT

Background & Aims: T-cells are a main target for anti-inflammatory drugs in inflammatory bowel disease. As the innate immune system is equally implicated in the pathogenesis of these diseases, T-cell suppressors may not only inhibit T-cell-dependent production of pro-inflammatory mediators but also affect innate immune cell function. Specifically, these drugs may impair innate immune cell recruitment and activation through inhibition of T-cells or act independent of T-cell modulation. We explored the extent of immune modulation by the T-cell inhibitor tacrolimus in a murine colitis model.
Methods: We assessed the effects of tacrolimus on TNBS colitis in wild type and Rag2-deficient mice. Severity of colitis was assessed by means of histological scores and weight loss. We further characterized the inflammation using immunohistochemistry and by analysis of isolated intestinal leukocytes at various stages of disease.
Results: Tacrolimus treated wild type mice were less sensitive to colitis and had fewer activated T-cells. Inhibition of T-cell function was associated with strongly diminished recruitment of infiltrating neutrophils in the colon at the early stages of this model. In agreement, immunohistochemistry demonstrated that tacrolimus inhibited production of neutrophil chemo-attractants CXCL1 and CXCL2. Rag2-deficient mice displayed enhanced baseline levels of lamina propria neutrophils that was moderately increased in TNBS colitis and remained unaffected by tacrolimus.
Conclusion: Both the innate and the adaptive mucosal immune system contribute to TNBS colitis. Tacrolimus suppresses colitis directly through inhibition of T-cell activation and by suppression of T-cell-mediated recruitment of neutrophils.

INTRODUCTION

Inhibition of T-cell mediated pro-inflammatory immune responses has been a successful strategy in the treatment of inflammatory bowel disease (IBD).[1-3] However, various defects in the function of innate immune cells have been implicated in the pathogenesis of these diseases as well. As such, a lack of responsiveness to bacterial stimuli of macrophages and Paneth cells has been linked to a failure of microbial eradication at the mucosal surfaces.[4-6] In contrast to this lack of responsiveness, it has been established that innate immune cells may instead display exaggerated responses to bacterial stimuli.[7-11]

Innate responsiveness is tightly regulated through the control of expression of innate receptors such as Toll-like proteins and nucleotide-binding oligomerization domain containing 2 (NOD2) is strongly dependent on the presence of a range of both extracellular as well as intracellular mediators.[12] In addition, interactions between adaptive and innate immune cells have been shown to regulate innate sensitivity that is crucial to maintain the physiological non-inflammatory state of the intestinal mucosa. Specifically, activation of lamina propria (LP) $CD4^+$ T lymphocytes regulate epithelial integrity and functions e.g. susceptibility to microbial stimulation.[13-16] Moreover, activated T-cells regulate recruitment of innate cells through the production of chemokines such as CXCL8 (IL-8) or the murine analogues CXCL1 (KC) and CXCL2 (MIP-2) and through interactions with other chemokine producing immune cells such as macrophages and epithelial cells.[14, 17]

To specifically address the interplay between innate and adaptive cells in colitis we applied a classical T-cell dependent mouse model. Trinitro-benzene sulphonic acid (TNBS) induced colitis has been widely used as a murine model for Crohn's disease.[18, 19] The occurrence of hapten-specific antibodies, the efficacy of anti-IL-12 treatment and studies of TNBS colitis in mouse models that are deficient at certain checkpoints of T-cell activation have established a contributive role for T- and B-cells.[20-24] Further substantiation for a crucial contribution of T-cells to this colitis model was established by showing the disease was transferable by injection of T-cells derived from colitic mice into WT recipients.[25, 26] The fact that TNBS induced intestinal inflammation is diminished in Rag-deficient mice may suggest the involvement of the adaptive immune system in regulating innate immune cells in TNBS colitis.[27] In all cases, TNBS colitis has been associated with neutrophil infiltration in the early phases of disease.[24, 28]

Tacrolimus (FK506) has strong T-cell inhibiting capacities and was originally used in the prevention of allograft rejection in tissue transplantation.[29] The main working mechanism is closely related to that of cyclosporin A. Through the inhibition of calcineurin, the phosphatase activity of the transcription factor 'nuclear factor of activated T-cells' (NFAT) is reduced, resulting in the inhibition of T-cell activation.[2, 30, 31] Tacrolimus has been used as treatment of IBD and efficacy has been shown in various colitis models.[2, 32-40]

To specifically address T-cell mediated control of innate infiltration in TNBS colitis, we applied tacrolimus in wild type (WT) and Rag2-deficient mice ($Rag^{-/-}$). Pathways of T-cell activation were further explored by using a mouse model for diphtheria toxin (DT) - inducible short-term depletion of dendritic cells (CD11c-DTR).

MATERIALS AND METHODS

Animals

All animal procedures and protocols were performed with the approval of local institutional animal studies ethics committee.

Male 6-8 week old BALB/c mice were purchased from Charles River (Maastricht, The Netherlands). BALB/c $Rag^{-/-}$ mice were generated from BALB/c Rag2/Il2rg double-knockout mice on a mixed background by outcrossing (10X) $Rag^{-/-}$ onto a BALB/c background. BALB/c Rag2/Il2rg double-knockout mice were generated at the Erasmus MC, Rotterdam, The Netherlands.

BALB/c CD11c-DTR transgenic mice have been reported previously.[41] DO11.10 mice were obtained from Charles River. All animals were housed in isolator cages with water and a standard pellet diet ad libitum (Hope Farms, Woerden, The Netherlands).

Induction of colitis

Colitis was induced as previously described.[42] In brief, mice were sensitized with 2,5% TNBS (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 50% EtOH by skin painting (100 µl). At day seven after sensitization 100 µl containing 2.5 mg of TNBS in 50% ethanol was administered intrarectally (i.r.) under Isoflurane anesthesia. Control mice received 100 µl of 50% EtOH. Body weight, stools and general health status were monitored daily. At indicated time points, mice were sacrificed and the colon was removed for further analyses. Severity of colitis was scored using parameters as described in table 1.

Table 1. Criteria for microscoping scoring of severity of colitis.*

Score	Symptom
0-1-2	Hyperaemia
0-1-2	Bowel wall thickening
0-1-2	Destruction of normal architecture
0-1-2	Goblet cell depletion
0-1-2	Ulceration
0-1-2	Mononuclear cell influx / celllar infiltration

Distal colonic sections were scored for the listed parameters and scores were summed.
*Adapted from Mc Cafferty et al. with minor modifications.[57]

Inhibition of T-cell function

To establish the appropriate dose of tacrolimus (Astellas Pharma, Leiderdorp, The Netherlands), the drug was tested in an ovalbumin (OVA)-specific T-cell system.[43] WT mice received 1x10^7 $CD4^+$ DO11.10 T-cells, labeled with 5,6-carboxysuccinimidyl-fluoresceine-ester (CFSE) (Molecular Probes, Leiden, The Netherlands), by intravenous (i.v.) injection. After 24 hours, mice were treated with 70 mg OVA protein grade V (Sigma Aldrich) per intragastric gavage (i.g.). T-cell divisions were assessed on cells isolated from the draining mesenteric lymph node using flow cytometry at 72 hours. A daily dose of 0.125 mg/ml, 200 µl intra-peritoneally (i.p.) of tacrolimus dissolved in NaCl 0.9% resulted in complete inhibition of T-cell proliferation.

Depletion of dendritic cells

Dendritic cell (DC) depletion was achieved by intra-peritoneal (i.p.) injection of transgenic (Tg) CD11c-DTR mice with 200 µl of diphtheria toxin (DT) at 4 ng/g body weight[41] one day prior to colitis induction. As a control, non-Tg littermates received an equal dose of DT.

Isolation and characterization of colonic leukocytes

Complete colon including the caecum was resected for isolation of lamina propria mononuclear cells (LPMC) as described.[44] To characterize lamina propria leukocytes, cells were incubated with anti-CD16/CD32 (2.4G2). Cells were stained with PE/Texas Red labeled anti-CD45 (30-F11) (Invitrogen), FITC-labeled anti-Ly6C (AL-21), PerCP-labeled CD11b (MAC-1α), APC-labeled CD11c (HL3) (BD Pharmingen, Breda, The Netherlands), Alexa700-labeled anti-MHC-II (M5/114.15.2), PE-Cy7-labeled anti-CD8α (53-6.7) (Biolegend, Uden, The Netherlands) and APC-Cy7-labeled anti-GR-1 (RB6-8C5) (e-Bioscience) for 30 minutes at 4°C. Cells were measured on a FACS Canto II cytometer with FACS Diva software (BD). Data were analyzed using Flowjo software (Tree Star).

Histology

Distal colon sections (5 mm) were fixed in 4% PFA in PBS and embedded in paraffin. Four µm sections were stained with hematoxylin (Vector Laboratories, Burlingame, CA) and eosin (Sigma-Aldrich). For immunohistochemistry (IHC), sections were deparaffinized and endogenous peroxidases were quenched with 3% H_2O_2 in methanol for 20 min. Antigen retrieval was achieved by microwave treatment in citrate buffer (10 mM pH 6.0). Sections were blocked for 1 hour in either 1% blocking reagent (Roche, Almere, The Netherlands) in PBS or 10 mM Tris, 5mM EDTA, 0.15M NaCl, 0.25% gelatine, 0.05% Tween-20, 10% normal mouse serum, 10% normal rabbit serum, pH 8. Antibody incubation was performed overnight at 4°C with rabbit anti-myeloperoxidase (MPO; Rockland, Gilbertsville, PA) diluted 1: 1000, anti-mouse CXCL1, anti-mouse CXCL2 (R&D systems,

Abingdon, United Kingdom) using a concentration of 2µg/ml and biotinylated anti-TNP (BD Pharmingen) diluted 1:500. Immunoreactions were detected using respectively biotinylated secondary goat-anti-rabbit or rabbit-anti-goat serum with the Vectastain ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Sections were counterstained with Hematoxylin.

Immuno-fluorescence microscopy

Cytospins of isolated colonic LP leukocytes were fixed for 10 min in 100% methanol. Antibodies for CD3 (rabbit anti-human CD3; Dako, Heverlee, Belgium) and CXCL1 or CXCL2 (goat anti-mouse chemokine, R&D Abingdon, UK) were incubated overnight. Secondary antibodies anti-rabbit Alexa fluochrome 555 and anti-goat Alexa fluochrome 488 (Invitrogen) were used to detect CD3 and CXCL1 or CXCL2 respectively. Cytospins were incubated with Prolong gold (Vector) containing DAPI (Sigma).

RNA isolation, reverse transcription and Real Time quantative PCR

RNA was isolated from colonic tissue with RNeasy midi kit (Qiagen #75144) according to the manufacturers' instructions. Messenger RNA expression was analyzed in duplicate, normalized against cyclophilin as an internal control gene. Results are expressed as relative messenger RNA expression using the ΔCt method.[45] Sequences of the IFNγ primer: forward primer: 5'-CAAAAGGATGGTGACATGA-3', reverse primer: 5'-GGGTTGTTGACCTCAAACT-3'. Sequences used of the cyclophilin primer: forward primer: 5'-AACCCCACCGTCTTCT-3', reverse primer: 5'-CATTATGGCGTGTAAAGTCA-3'.

T-cell stimulation assay

Human whole blood was centrifuged (10 min, 260g) and serum was removed. Using a Lymphoprep^{tm} gradient (Axis-Shield, Oslo, Norway) lymphocytes were isolated. $CD4^+$ T-cells were isolated using negative depletion for $CD4^+$ cells using a $CD4^+$ T-cell isolation kit (MACS, Miltenyi Biotec, Utrecht, The Netherlands). Cells were incubated in 96 wells plates ($2*10^5$ cells / 200 µl) in IMDM medium containing 10% human serum, 100 U/ml penicillin and 100 µg/ml streptomycin with 1 µg/ml CD3/CD28 T-cell expander Dynal beads (Invitrogen) for 72 hours with various concentrations of tacrolimus as indicated. CXCL8 concentrations were determined in the supernatant by ELISA (BD Biosciences).

Statistical analysis

Changes in weight, histology score, cell numbers and cytokines levels were statistically analyzed using an unpaired Student's t-test when appropriate. A p-value of <0.05 was considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM) of n=5 to 8 mice per group (as indicated). Experiments have been performed at least twice.

RESULTS

TNBS colitis development is associated with colonic neutrophil influx

We characterized the involvement of adaptive and innate immune cells in TNBS colitis during the first days of disease. Induction of TNBS colitis resulted in a decrease of body-weight within 24 hours and reached maximum levels at 72 hours ($p<0.001$) (figure 1a). Histology showed maximum colonic inflammation (Table 1) within 24 hours after colitis induction compared to EtOH controls ($p<0.01$) and remained at constant levels during the first three days (figure 1b). The rapid development of colitis strongly implicated involvement of innate immune cells. Therefore, we assessed MPO expression on colonic sections to evaluate neutrophil infiltration into the intestinal tissues. TNBS treated animals showed neutrophils in the colon LP at 24 hours, suggesting the involvement of these cells in the development of TNBS colitis (figure 1c).

To characterize the infiltrating cells in more detail, we isolated whole leukocyte populations from the colonic LP at indicated time points for analysis by flowcytometry. Total numbers of neutrophils ($CD45^+GR1^+$) and monocytes ($CD45^+CD11b^+CD11c^-$) in the LP increased compared to EtOH controls at day 1 and day 3 ($p<0.01$) (figure 1d,e). Total numbers of eosinophils ($CD45^+CD11b^+GR1^{int}FSC^{low}SSC^{high}$), DCs ($CD45^+CD11c^+MHC\text{-}II^+$) and T- ($CD45^+CD3^+$) and B-cells ($CD45^+CD19^+$) did not differ compared to control animals (data not shown). However, we detected increased percentages of $CD3^+CD4^+CD25^+$ (activated) T-cells in the mesenteric lymph nodes of TNBS treated mice (figure 1f). Percentages of $CD3^+CD4^+CD25^+FoxP3^+$ regulatory T-cells did not increase during TNBS colitis (data not shown).

These data show that TNBS colitis development is associated with neutrophil influx within 24 hours in the colonic LP and an increase of activated effector T-cells within the draining lymph node.

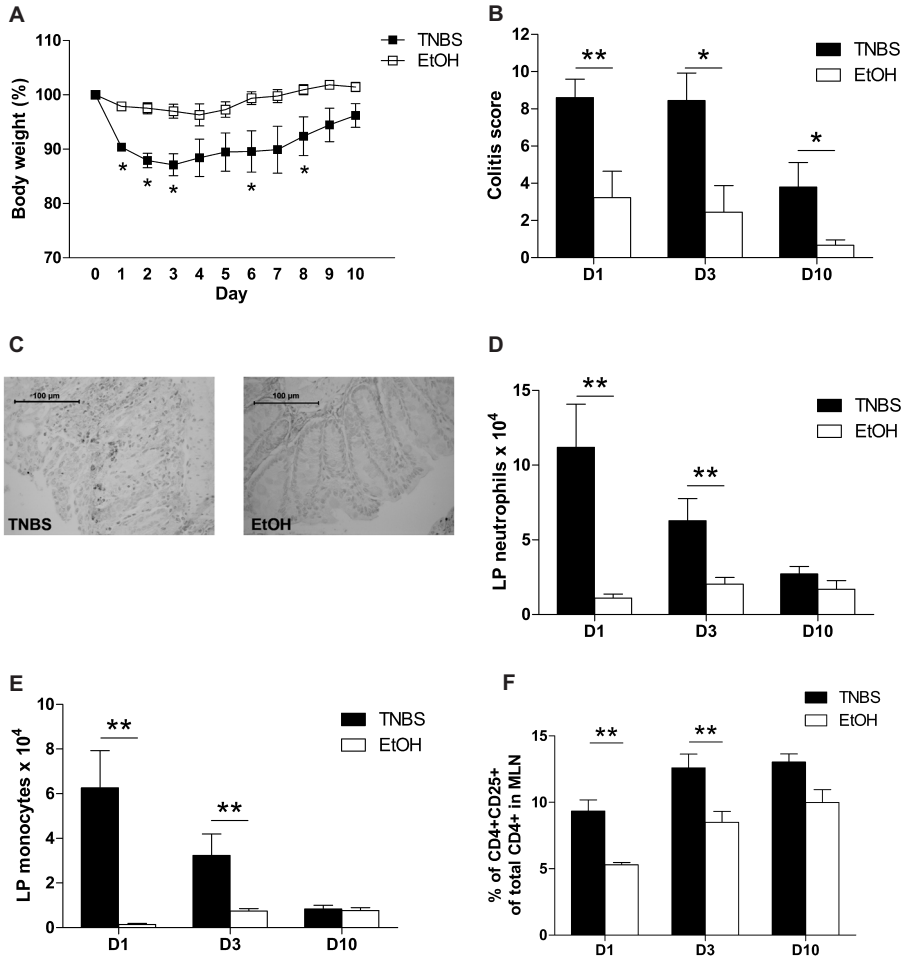

Figure 1. TNBS colitis development is associated with colonic neutrophil influx.
(A) Weight loss and (B) severity of colitis in TNBS colitic WT mice. (C) MPO staining, indicating neutrophils in colonic tissue at 24 hours after TNBS colitis induction (left) en EtOH control (right). (D,E) Total number of infiltrating neutrophils (D) and monocytes (E) in the colonic LP after TNBS administration, analysed by flowcytometry. (F) Percentages of activated T-cells ($CD4^+CD25^+$) of total $CD4^+$ T-cell in the mesenteric lymph node.
(A-F) n=7 per group, **p<0.01, *p<0.05, error bars represent SEM.

Adaptive immune cells are essential for colonic neutrophil recruitment

We next investigated the involvement of adaptive immune cells in the recruitment of neutrophils by applying TNBS colitis in $Rag^{-/-}$ mice. Numbers of colonic LP neutrophils were increased in untreated $Rag^{-/-}$ mice compared with WT animals ($p<0.05$) (figure 2a). Upon TNBS colitis induction, $Rag^{-/-}$ mice develop a mild colitis (figure 2b) that is associ-

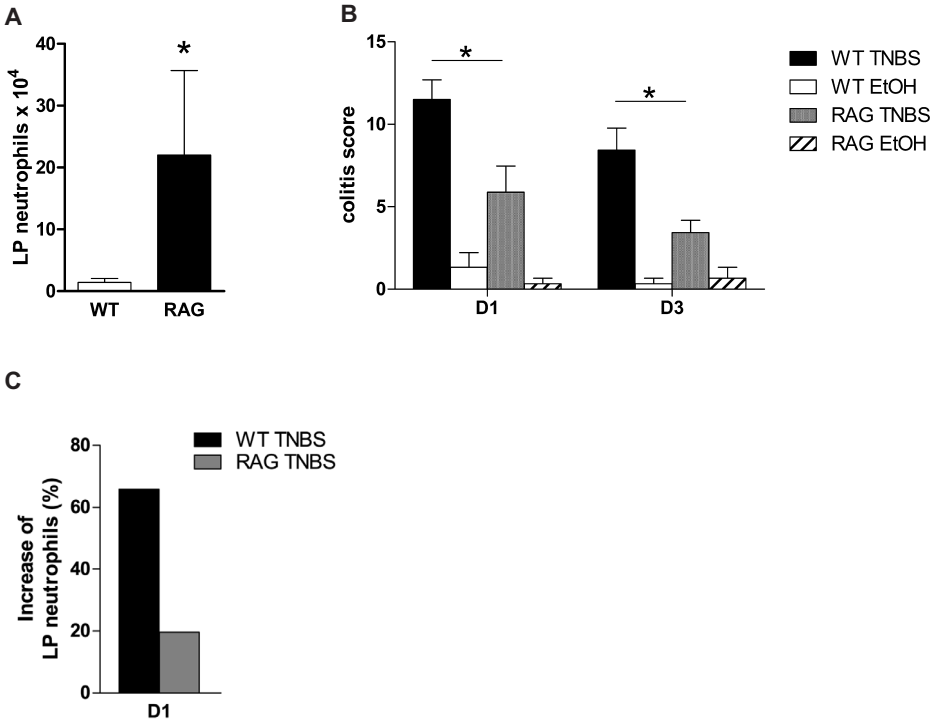

Figure 2. Adaptive immune cells are essential for colonic neutrophil recruitment
(A) Absolute numbers of neutrophils in the colonic LP under baseline conditions in WT and $Rag^{-/-}$ mice. (B) Colitis score of TNBS colitic $Rag^{-/-}$ mice as compared to WT mice. (C) Mean increase of neutrophils in the colonic LP in TNBS colitis as compared with EtOH controls in WT and $Rag^{-/-}$ mice. (A) n=4 per group. (B,C) n=7 for TNBS, n=3 for EtOH, *p<0.05, error bars represent SEM.

ated with a moderate increase of lamina propria neutrophils (19.6%) in comparison with WT animals (65.8%) (figure 2c).

Tacrolimus reduces severity of colitis in WT mice

To specifically address the effect of T-cell inhibition on innate inflammation, we analyzed the effect of the T-cell inhibitory drug tacrolimus on colitis development. To this aim, we first established a model for tacrolimus mediated T-cell suppression. The optimum dose of 0.125 mg/ml for T-cell inhibition was established as described in materials & methods (figure 3a). Mice were injected daily with 200 µl 0.125 mg/ml tacrolimus i.p. starting one day prior to colitis induction. Tacrolimus administration did not affect TNBS associated weight loss (figure 3b). However, histology scores were markedly decreased in the tacrolimus treated animals at 24 and 72 hours ($p<0.01$; $p<0.05$) (figure 3c).

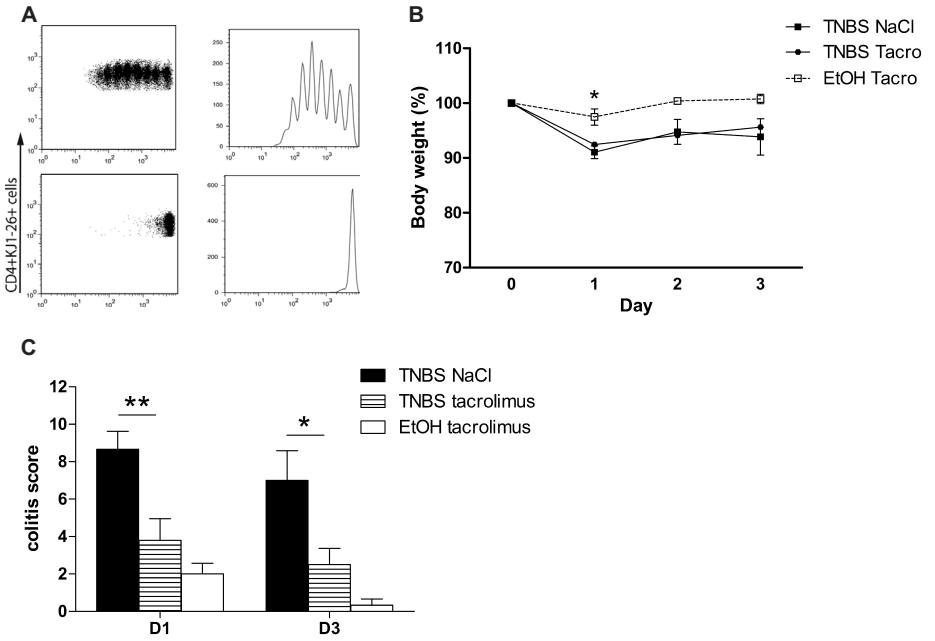

Figure 3. Tacrolimus reduces severity of colitis in WT mice.
(A) Proliferation of OVA-specific T-cells ($CD3^+CD4^+KJ1\text{-}26^+$) in the mesenteric lymph node of control (top) and tacrolimus treated WT mice (bottom). (B,C) Weight loss (B) and severity of colitis (C) of tacrolimus treated mice upon TNBS administration.
(A-C) n=7 for TNBS, n=5 for EtOH. (D,E) n=5, **p<0.01, *p<0.05, error bars represent SEM.

Tacrolimus inhibits neutrophil influx and pro-inflammatory cytokine production in TNBS colitis

To assess whether suppression of T-cell proliferation by tacrolimus affected the infiltration of innate cells during TNBS colitis, we performed immunohistological staining for MPO. Colon sections of tacrolimus treated colitic animals showed a decrease in MPO positive cells in the LP compared to controls (figure 4a). In agreement, the number of neutrophils in the LP was reduced in tacrolimus treated mice at 24 hours after colitis induction (p=0.01) (figure 4b). Numbers of other immune cells (monocytes, eosinophils, DCs, T- and B-cells) were not affected by tacrolimus treatment (Supplementary figure 1). However, tacrolimus treatment was associated with a lack of increase of activated T-cells within the mesenteric lymph nodes at 24 and 72 hours of disease induction (figure 4c).

To further explore the immunosuppressive effects of tacrolimus in TNBS colitis we determined expression of the pro-inflammatory cytokine IFN-γ in colonic tissue by qPCR. Expression of IFN-γ was diminished upon tacrolimus treatment at 24 hours after colitis induction (p<0.05) (figure 4d).

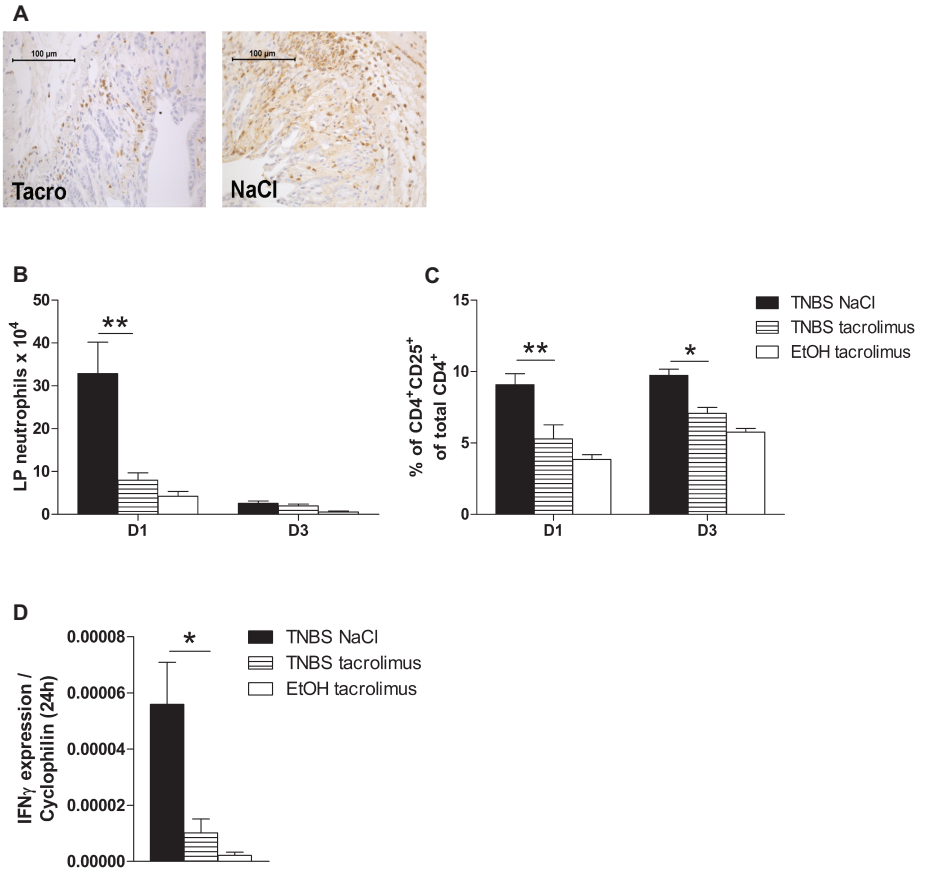

Figure 4. Tacrolimus inhibits neutrophil influx and pro-inflammatory cytokine production in TNBS colitis. (A) Representative colonic section obtained at 24 hours after TNBS induction in tacrolimus treated (left) and control (right) animals. Brown staining indicates MPO positive cells. (B) Total number of neutrophils in the colon during TNBS colitis in tacrolimus treated and control animals. (C) Percentages of activated T-cells in mesenteric lymph nodes of tacrolimus treated and control animals. (D) Colonic levels of IFNγ determined by qPCR. (A-D) n=7 per group, **p<0.01, *p<0.05, error bars represent SEM.

Tacrolimus has no direct effect on neutrophils in moderate colitis in Rag$^{-/-}$ animals

To exclude a direct effect of tacrolimus on innate cell function, we investigated whether tacrolimus could directly affect neutrophil influx in mice in the absence of a functional adaptive immune system. Therefore, we applied tacrolimus in TNBS colitic Rag$^{-/-}$ mice. Tacrolimus treatment had no effect on the mild form of colitis that occurs in Rag$^{-/-}$ mice after TNBS induction (figure 5a). In addition, numbers of colonic neutrophils were not affected by tacrolimus in Rag$^{-/-}$ colitic mice, indicating that tacrolimus does not directly inhibit neutrophil influx in TNBS colitis (figure 5b).

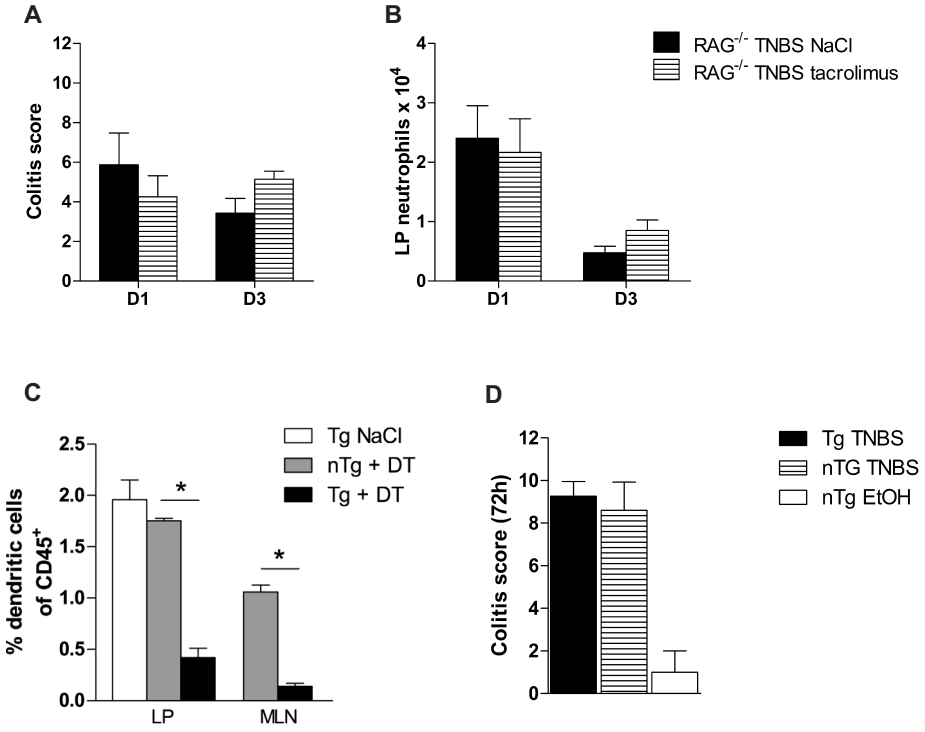

Figure 5. Tacrolimus has no effect on colitis severity and neutrophil numbers in Rag$^{-/-}$ mice.
(A,B) Effects of tacrolimus on severity of colitis (A) and on the number of neutrophils in the colonic LP (B) in TNBS colitic Rag$^{-/-}$ mice. (C) Percentage of DCs (CD11c^{+}MHC-II^{+}) in the MLN and colonic LP of total CD45^{+} cells in Tg (DT sensitive) and non-Tg (DT non-sensitive) CD11c-DTR mice at 24 hours after DT i.p. injection. (D) Severity of colitis determined by histological scores of Tg TNBS treated animals compared with non-Tg TNBS treated animals at 72 hours post TNBS induction.
(A,B) n=7 per group (C,D) n=5 per group, **p<0.01, *p<0.05, error bars represent SEM.

As tacrolimus has also been suggested to inhibit dendritic cell antigen presentation[46] we first explored contributions of DCs to TNBS colitis using CD11c-DTR mice. To this aim, DCs were depleted 24 hours prior to colitis induction by i.p. injections of DT. This treatment resulted in a 90% reduction of MLN DCs and a ~70% reduction of DCs in the colonic LP (figure 5c). This level of DC reduction had no effect on histological colitis scores (figure 5d). We next stained colonic tissue with antibodies directed to the trinitrophenyl (TNP)-hapten modified self–antigen complex.[20] At 24 hours after TNBS colitis induction, the hapten was detected in epithelial cells (Supplementary figure 2). As such, other antigen presenting cells, such as IEC, could well be involved in the initial antigen presentation to T-cells. Together with the DC depletion data, this suggests that tacrolimus may not have inhibited colitis through suppression of DC mediated antigen presentation.

Tacrolimus affects innate recruitment through T-cell suppression

As the recruitment of neutrophils is regulated via chemo-attractants we performed IHC staining for the neutrophil chemo-attractants CXCL1 (KC) and CXCL2 (MIP-2), mouse equivalents of CXCL8. Both chemokines were abundantly present in leukocytes within the LP and within the intestinal epithelial cells (IEC) of TNBS colitic WT mice (figure 6a). The expression of CXCL1 and CXCL2 was almost completely absent in tacrolimus treated animals (figure 6b). Immune fluorescence staining for CXCL1 or CXCL2 and T-cells (CD3) on isolated LP leukocytes from TNBS treated mice confirmed that T-cells are capable of producing neutrophil attracting chemokines (figure 6c).

Next, we assessed *in vitro* whether tacrolimus could affect chemokine production by T-cells. Hereto, we analyzed chemokine production of stimulated human peripheral T-cells in the presence of a dose range of tacrolimus. The production of CXCL8 diminished by tacrolimus in a dose dependent manner ($p<0.001$) (figure 6d).

DISCUSSION

T-cell activation has been regarded as a crucial contribution to the pathogenesis of IBD through the release of various mediators e.g. cytokines that are implicated in mucosal inflammation. Furthermore, these cells may also contribute through regulating recruitment and activation of other immune cells such as neutrophils. We explored the effects of the T-cell inhibitor tacrolimus on innate infiltration in a murine colitis model. First, we established that the early phases of TNBS colitis are characterized by an influx of mainly MPO positive cells. Further analysis by flowcytometry of colonic LP leukocytes identified neutrophils and monocytes as the main infiltrating populations (figure 1).

To address the contribution of adaptive immune cells to colitis, we decided to induce TNBS colitis in Rag2-deficient mice. We show that $Rag^{-/-}$ mice develop only a moderate TNBS colitis that is accompanied by reduced neutrophil recruitment in comparison to WT mice. As we found that $Rag^{-/-}$ mice exhibit enhanced baseline levels of neutrophils within the lamina propria we were unable to properly compare these mice to WT animals. We therefore explored T-cell mediated control of innate inflammation, by treating WT mice with the T-cell inhibiting drug tacrolimus starting one day prior to TNBS colitis induction. These experiments revealed that T-cell inhibition by tacrolimus results in diminished severity of colitis that is accompanied by a strong reduction in neutrophil infiltration and diminished pro-inflammatory cytokine levels (figure 3,4). These data indicate that T-cells contribute to mucosal inflammation through recruitment of innate cells.

To exclude a direct suppressive effect of tacrolimus on innate cells,[47,48] we treated TNBS colitic mice $Rag^{-/-}$ mice with tacrolimus, as these mice are not capable of mounting adaptive immune responses. Treatment of these $Rag^{-/-}$ mice with tacrolimus did not result in

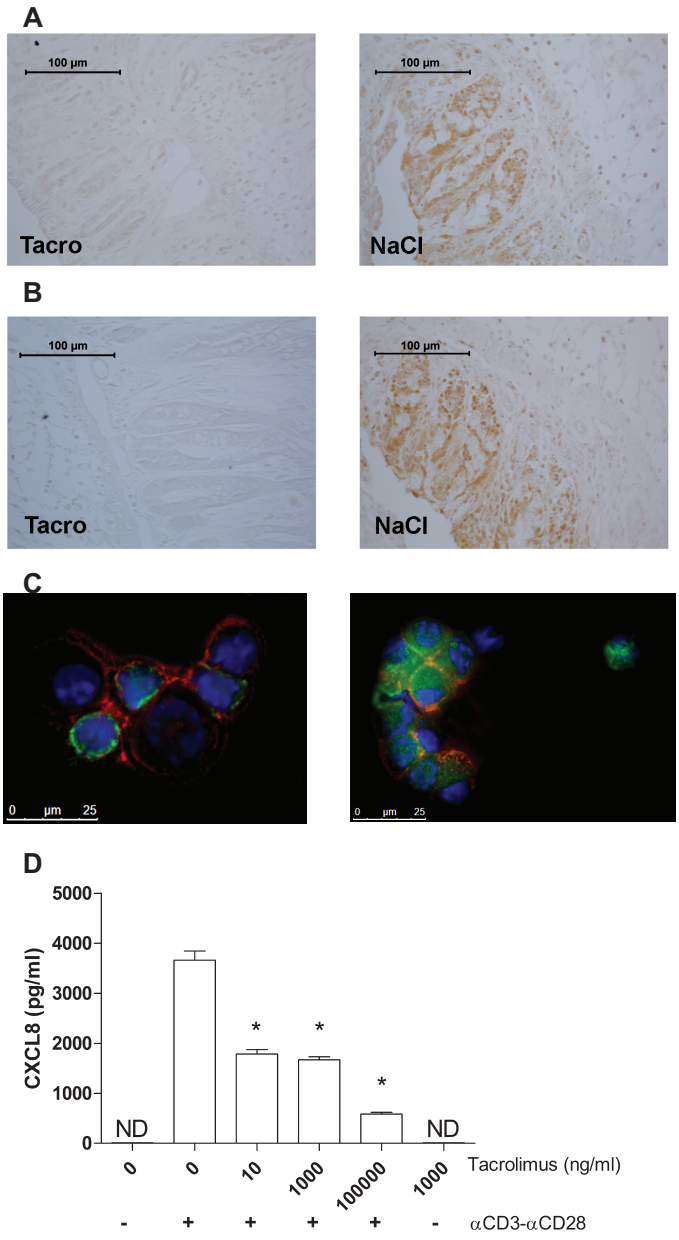

Figure 6. Tacrolimus affects neutrophil attracting chemokine production through T-cell suppression. Representative example of expression of neutrophil attracting chemokines CXCL1 (A) and CXCL2 (B) on colonic sections of TNBS treated mice at 24 hours after tacrolimus treatment (left) and NaCl controls (right). (C) Immuno-fluorescence staining of CXCL1 (left) or CXCL2 (right) in red and CD3 in green on isolated colonic LP leukocytes from colitic mice. (D) Production of CXCL8 by human $CD4^+$ T-cells after αCD3-αCD28 stimulation with various concentrations of tacrolimus. CXCL8 is determined in the supernatant at 72 hours (A,B,C) n=7 per group (D) n=3 per variable, ND:non detected, *p<0.001, error bars represent SEM.

any reduction of colitis severity or inhibition of neutrophil infiltration (figure 5). These data indicate that -at this dose- tacrolimus does not inhibit neutrophil recruitment in the absence of adaptive immune cells.

Chemokines involved in neutrophil recruitment, e.g. CXCL1 and CXCL2, can be produced by different cells that are present within the intestinal tract.[7, 49-51] We detected CXCL1 and CXCL2 within LPMC and within the IEC. Double staining of CD3 (T-cells) and these chemokines identified T-cells as a potential source of these neutrophil attracting chemokines.

Tacrolimus treatment was associated with a robust down-regulation of the production of these chemokines in colitis on histology. These analyses suggest that tacrolimus treatment inhibits neutrophil recruitment through suppression of T-cell mediated chemokine production. Indeed, similar mechanisms for tacrolimus have been proposed in a model of acute lung injury, where neutrophil recruitment in lung tissue could be effectively inhibited by tacrolimus.[52] Moreover, it has been shown that T-cells can produce substantial amounts of CXCL2 or CXCL8 in mice and humans respectively.[14,17] Our *in vitro* experiments confirm a dose dependent suppression by tacrolimus of CXCL8 production by human T-cells.

Next, T-cells can induce chemokine production by other cells as well. For example, it has been shown that primed T-cells are capable of inducing chemokine production by epithelial cells.[14] We show that tacrolimus treatment is associated with diminished expression of CXCL1 and CXCL2 both in cells that are present within the LP and in the IEC (figure 6). These data establish that tacrolimus suppresses chemokine production by T-cells and, indirectly, by other cells such as IEC and macrophages.

As tacrolimus has also been suggested to inhibit dendritic cell antigen presentation[46] we explored contributions of DCs to TNBS colitis. DC depletion by diphteria toxin in CD11c-DTR mice resulted in a 90% reduction of MLN DCs and a ~70% reduction of DCs in the colonic LP that is comparable to recent reports using similar interventions in another experimental colitis model.[53] This level of DC reduction had no effect on histological colitis scores (figure 5). As it has been suggested that the intestinal epithelial cells may act a potent antigen presenting cells[54, 55] we next stained colonic tissue with antibodies directed to the trinitrophenyl (TNP)-hapten modified self–antigen complex.[20] At 24 hours, the hapten was strongly detected in epithelial cells. These data suggest that IEC could be involved in the initial antigen presentation to T-cells. Given the strong inhibition of tacrolimus on TNBS colitis development, this suggests that this drug may not have inhibited colitis through suppression of DC mediated antigen presentation. Future studies may therefore aim at assessing whether tacrolimus has direct immunosuppressive effects on antigen presenting cells other than classical $CD11c^+$ DC.

Next to adaptive immune cells e.g. T-cells, neutrophils have been identified as effector cells in IBD, responsible for epithelial injury and dysfunction leading to tissue damage.

This mechanism is supported by reports that describe successful treatment of murine colitis by chemokine inhibitors.[56] In accordance, T-cell inhibition in IBD patients may not only result in inhibition of the release of cytotoxic mediators by these cells, but also from diminished T-cell dependent neutrophil recruitment to the inflamed intestine. Given the broad spectrum of action of such T-cell inhibitors, one could argue that these drugs may be a first choice during fulminant inflammation in IBD.

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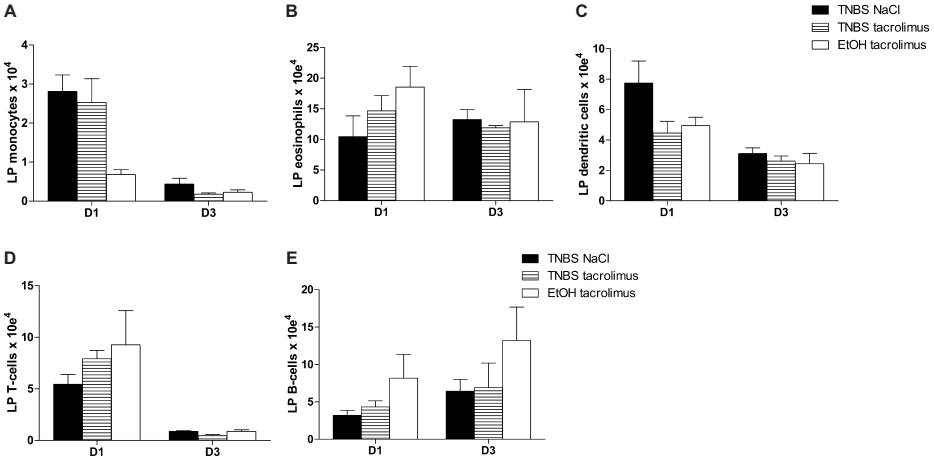
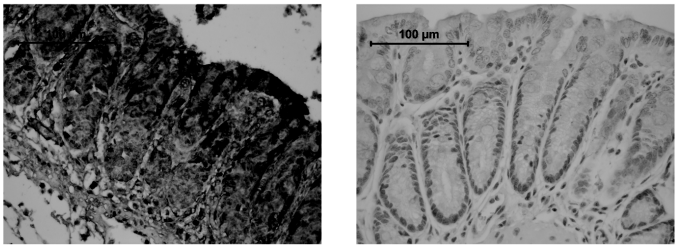
SUPPLEMENTARY FIGURES



1. Tacrolimus does not affect total numbers of monocytes, eosinophils, DCs, T-cells or B-cells in the colonic LP.
(A-E) Number of monocytes (A), eosinophils (B), DCs (C), T-cells (D) and B-cells (E) in the colonic LP during TNBS colitis in tacrolimus treated mice, determined by flowcytometry. n=7 per group, error bars represent SEM.



2. TNP is present throughout the intestinal epithelial layer and the LP after TNBS administration.
Representative example of TNP (brown) stained colonic section at 24 hours after TNBS induction (left) and ethanol control (right).

Chapter 4

A role for Ly6C^{+} monocyte-derived dendritic cells in TNBS colitis

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SUMMARY

Resident lamina propria dendritic cells play an important role in maintenance of tolerance to harmless antigens. Recent new insights have revealed that in such steady-state conditions resident lamina propria DC can be divided into non-overlapping subtypes with distinct functions on the basis of surface marker expression. However, it is unclear whether a subtype of these DC or newly recruited inflammatory antigen presenting cells drive intestinal inflammation upon loss of tolerance. To determine which DC are predominantly involved in antigen presentation during colonic inflammation, we have characterized the role of DC subtypes during hapten-induced colitis. Within 1 day post colitis induction and influx of Ly6c$^+$ monocytes occurred in the lamina propria. On day 3 of inflammation an increase in the percentage of monocyte-derived Ly6c$^+$ CD11b$^+$ DC in the mucosa draining lymph nodes occurred. Transient depletion of resident CD11c$^+$ cells in CD11c-DTR mice effectively reduced CD11c+ DC in the mucosa draining LN on day 3 of colitis but did not affect Ly6c$^+$ CD11b+ DC nor influenced the severity of disease. Mucosal administration of fluorescently labeled ovalbumin on day 2 of colitis established that Ly6c$^+$ CD11b$^+$ DC were associated with antigen and effectively drove proliferation of OVA specific D011.10 cells. These data infer that monocyte infiltration during intestinal inflammation gives rise to a new subtype of monocyte-derived DC that can present antigen and may support T-cell division.

INTRODUCTION

Mononuclear phagocytes including macrophages and dendritic cells (DC) are highly abundant in the lamina propria of the intestine. These cells exert a broad range of immune functions in both innate and adaptive immunity ranging from phagocytosis and killing, stimulation and recruitment of other immune cells[1, 2] to antigen presentation and T cell differentiation after migration to the local lymph nodes (LN).[3-5] Recent identification of non-overlapping subtypes of antigen presenting cells (APC) with distinct functions has led to pivotal progress in understanding the intricacies of APC function in lamina propria. Three major subsets of intestinal lamina propria APC can be distinguished; the $CD11c^+$ DC that are divided into $CD11c^+$ $CD103^+$ $CD11b^-$ $CX3CR1^-$ DC and $CD11c^+$ $CD103^-$ $CD11b^+$ $CX3CR1^+$ DC as well as the macrophage like CD11c-low $CD11b^+$ $CX3CR1^+$ F4-80^+ cells.[6-8]

$CD11c^+$ $CD103^+$ $CD11b^-$ $CX3CR1^-$ DC which originate from a common DC progenitor migrate from the lamina propria to the draining mesenteric lymph nodes (MLN) during normal steady state conditions.[4, 9] These $CD103^+$ DC induce the expression of gut homing receptors CCR9 and α4β7 on adaptive T-cells, and are involved in immune regulation via the induction TGF-β and retinoic acid dependent induction of $FOXP3^+$ Tregs.[10-12] In contrast, $CD11c^+$ $CD103^-$ $CD11b^+$ $CX3CR1^+$ DC which originate from $Ly6C^+$ monocytes fail to migrate to MLN.[4, 9] Instead these DC are closely associated with the epithelium and are capable of extending processes across the epithelium into the intestinal lumen.[13, 14] As such, $CD11c^+$ $CD103^-$ $CD11b^+$ $CX3CR1^+$ DC may play an important role in local sampling and uptake of luminal microbes and stimulate T cell responses to a lesser extent.

$CD11c^{low}$ $CD11b^+$ $CX3CR1^+$ F4-80^+ lamina propria macrophages also originate from $Ly6C^+$ monocytes.[9] These macrophages spontaneously secrete IL-10 and are hyporesponsive to TLR stimulation.[6] Furthermore, they were reported to induce $FOXP3^+$ Tregs in the presence of TGF-β, and suppress the action of the $CD11b^+$ lamina propria DC.[6]

Epithelial cell derived factors are suggested to steer DC differentiation and condition their acquisition of particular functions.[3] For example, epithelial cells imprint the ability to metabolize vitamin-A, which allows these cells to generate gut tropic $CCR9^+$ $Foxp3^+$ cells.[15, 16] $CD11c^+$ $CD103^-$ $CD11b^+$ $CX3CR1^+$ DC may escape conditioning or be insensitive to this imprinting and instead have the ability to drive inflammatory T-cell responses. These $CD103^-$ cells could act as sentinels for the presence of pathogenic antigens or bacteria.[4, 17]

Dysregulation of intestinal homeostasis, as occurs in inflammatory bowel disease, may be associated with shifts in balance between these populations of APC. As such, inflammation could be induced by pro-inflammatory APC that are already present at steady state but become dominant and are activated after contact with microbial patterns and pro-inflammatory cytokines. For example, genetically engineered mice transiently

lacking CX3CR1$^-$ lamina propria APC were found to be more sensitive to DSS colitis due to a predominance of TNF secreting CX3CR1$^+$ DC.[7] Alternatively, new subpopulations of inflammatory DC could be recruited to the intestine during inflammation. Here we report that infiltrating Ly6c$^+$ monocytes critically predominate in lamina propria and give rise to a new subtype of monocyte derived DC that can present antigen and supports T-cell division in the mucosal draining MLN during trinitrobenzene sulfonic acid (TNBS) colitis in WT mice.

MATERIALS & METHODS

Animals

Specific pathogen-free BALB /c mice (8 – 10 weeks) were purchased from Charles River (Maastricht, The Netherlands). DO11.10 transgenic (Tg) mice, which have a Tg TCR specific for the ovalbumin (OVA) 323 – 339 peptide, and CD11c-DTR transgenic mice on a BALB/c background[18] were bred at our own facility. All mice were kept under routine animal housing conditions and experiments were approved by the animal experimental committee of the Erasmus MC.

Induction of colitis

Colitis was induced as previously described.[19] In brief, mice were sensitized with 2.5% TNBS (Sigma-Aldrich, The Netherlands) in 50% ethanol (EtOH) by skin painting (100 µl). At day seven after sensitization 100 µl of 2.5% TNBS in 50% EtOH was administered intrarectally (i.r.) under isoflurane anesthesia. Control mice received 100 µl of 50% EtOH. Body weight, stools and general health status were monitored daily. At indicated time points, mice were sacrificed and the colon and draining lymph nodes were removed for further analyses.

Isolation and purification of lymph node and colonic APC

At various time points after colitis induction the MLN and iliac lymph nodes (ILN) were isolated and digested using Liberase Blenzyme 3 (Roche, The Netherlands) in the presence of DNAse I (Roche) for 30 – 35 min at 37°C. At the same time colonic lamina propria mononuclear cells (LPMC) were isolated as previously described.[20]

Depletion of dendritic cells

Two days after colitis induction CD11c$^+$ DC were depleted in CD11c-DTR mice by an intra-peritoneal (i.p.) injection of 200 µl of diphtheria toxin (DT) at 4 ng/g body weight in 0.9% saline. As a control, non-Tg or wildtype (WT) littermates were treated with an equal dose of DT.

Flow cytometry

To characterize lamina propria, MLN- and ILN-derived leukocytes, cells were first incubated with anti-CD16/CD32 (2.4G2) (a kind gift of Bioceros, The Netherlands) to block non-specific binding of antibodies. Next, cells were stained with PE labeled anti-CD45 (30-F11), PE-Cy7-labeled anti-F4-80 (BM8), Alexa700-labeled anti-MHCII (M5/114.15.2), PE-Cy7-labeled anti-CD8 (53-6.7) (Biolegend, Uden, The Netherlands), FITC-labeled anti-Ly6C (AL-21), PerCP-labeled anti-CD11b (MAC-1), APC-labeled anti-CD11c (HL3), PE-labeled anti-CD103 (M290) (BD Parmingen), PE-labeled anti-CCR7 (4B12), APC-Cy7-labeled anti-GR-1 (RB6-8C5) or APC-Cy7-labeled anti-F4-80 (BM8) (e-Bioscience, Frankfurt, Germany) for 30 minutes at 4°C. Cells were measured on a FACS Canto II cytometer with FACS Diva software (BD). Data were analyzed using Flowjo software (Tree Star Inc, Ashland, OR).

OVA-tracking

OVA (Calbiochem, San Diego, CA) was coupled with alexa-647-conjugated carboxylic acid succinimidylester (Invitrogen, The Netherlands) according to manufacturers' instructions. Two days after colitis induction mice received 100 µl (17 mg/ml) alexa-647-labeled OVA i.r. or 300 µl intragastrically (i.g.). Presence of OVA-alexa-647 was studied in the different DC subsets from the MLN by comparison with cells from MLN of mice treated with non-labeled OVA.

Histology

Distal colon sections (5 mm) were fixed in 4% PFA in PBS and embedded in paraffin. Four µm sections were stained with hematoxylin (Vector Laboratories, Burlingame, CA) and eosin (Sigma-Aldrich). Severity of colitis was scored using parameters as previously described.[21]

Adoptive transfer and CFSE labeling

Lymph nodes and spleens were isolated from DO11.10 mice and single-cell suspensions were prepared, enriched for $CD4^+$ T cells as described earlier[22] and labeled with CFSE (Molecular Probes, Invitrogen) to follow their division profiles *in vivo*. Each mouse received 1.10^7 $CD4^+$ $KJ1.26^+$ cells in 100 µl saline by intravenous injection one day after induction of colitis. The next day mice were treated i.r. with 35 mg in 100 µl or i.g. with 70 mg in 200ul PBS. OVA-induced proliferation was studied in the ILN and MLN four days after colitis induction.

Statistical analysis

Changes in weight, histology score, cell numbers and cytokines levels were statistically analyzed using an unpaired Student's t-test when appropriate. A p-value of < 0.05 was

considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM) of n=5 to 8 mice per group (as indicated). Experiments have been performed at least twice.

RESULTS

Increased numbers of CD11b$^+$ Ly6c$^+$ APC in lamina propria and MLN during TNBS colitis

In order to investigate whether shifts in predominance of different APC populations occur during TNBS colitis in mice, we characterized APC subsets based on expression of CD11c and CD11b in the colonic lamina propria as well as the draining lymph nodes at various time points after induction of TNBS colitis.

I.r. administration of TNBS in pre-sensitized mice causes an acute colitis characterized by weight loss and a high disease activity score (figure 1a). This colitis peaks at day 3 after induction, and mice slowly recovered showing only minimal signs disease activity at day 10. Within one day after induction of colitis a strong increase in the CD11c$^-$ CD11b$^+$ cells accounting for up to 50% of the CD45$^+$ lamina propria cells was observed (figure 1b). By comparison, in EtOH controls this CD11c$^-$ CD11b$^+$ APC population consisted of approximately 5% of the CD45$^+$ lamina propria cells. Further characterization of the CD11c$^-$ CD11b$^+$ APC population revealed that the majority of the cells are Ly6c$^+$, suggesting that these cells derive from recently migrated monocytes (figure 1c). During the course of disease this CD11c$^-$ CD11b$^+$ APC population remained significantly increased as illustrated by elevated levels at day 3 and returned to normal levels at day 10 after TNBS challenge (figure 1b).

To see how the intestinal inflammation affects APC subsets in the draining lymph nodes, we isolated and analyzed the MLN at different time points after colitis induction. Three days after colitis induction the percentage of CD11c$^+$ MHCII$^+$ DC within the total CD45$^+$ leukocyte population was significantly increased in the MLN (figure 1d). This coincided with strong increase in the percentage of CD11c$^+$ CD11b$^+$ DC population, which could already be detected at day 1. Similarly to the cells in the lamina propria, the majority of the CD11c$^+$ CD11b$^+$ DC subset expressed Ly6c suggesting these cells are monocyte-derived DC. To corroborate that the CD11c$^+$ MHCII$^+$ CD11b$^+$ Ly6C$^+$ DC are monocyte-derived DC, surface expression of known mo-DC markers was performed.[23] Flow cytometry of the CD11c$^+$ MHCII$^+$ CD11b$^+$ Ly6C$^+$ DC population in the MLN of TNBS colitis mice revealed that the cells were negative for CD103 and positive for F4-80. Moreover, a small percentage of cells was CCR7 positive, indicative of recent migration into the node (figure 1e). The same phenotype was detected when Ly6C$^+$ DC were studied in the ILN (data not shown).

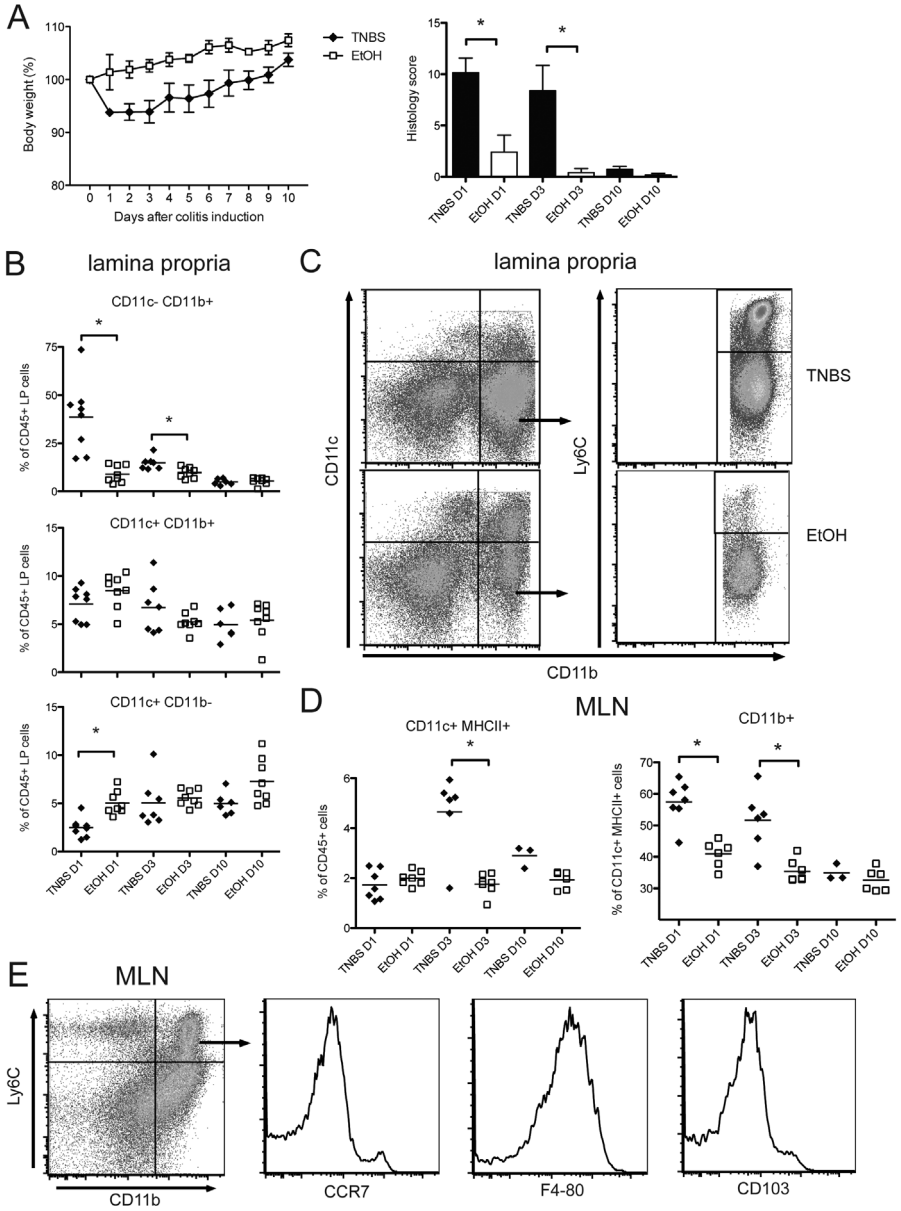

Figure 1. TNBS colitis is associated with monocyte influx into the lamina propria and DC into the LN. A: Weight loss and severity of colitis were determined at 1, 3 and 10 days after induction of colitis. B: $CD45^+$ leukocytes were isolated from colon lamina propria and the changes in APC subsets characterized using flowcytometric analysis of CD11b and CD11c expression. C: Expression of Ly6C was used to discriminate between resident macrophages and recruited monocytes within the $CD11c^-$ $CD11b^+$ population of lamina propria cells. D: Changes in $CD11c^+$ $MHCII^+$ DC in MLN and the DC subpopulation expressing CD11b were determined during the course of colitis. E: The population of $Ly6C^+$ $CD11b^+$ $CD11c^+$ $MHCII^+$ DC detected in the MLN at day 3 was analyzed for the expression of CCR7, F4-80 and CD103. * Statistically significant ($P < 0.05$)

On day 10 the percentage of CD11c$^+$ CD11b$^+$ DC in MLN of TNBS colitis mice was similar to the percentage in control EtOH mice although the percentage of CD11c$^+$ MHC-II$^+$ DC within the total CD45$^+$ leukocyte population remained higher than in EtOH controls.

From these data we conclude that CD11c$^-$ CD11b$^+$ Ly6c$^+$ monocytic cells infiltrate the lamina propria within one day after TNBS colitis induction. Moreover, at day 3 there is a strong increase in the proportion of CD11C$^+$ CD11b$^+$ Ly6c$^+$ DC in the MLN.

Depletion of CD11c$^+$ cells does not affect the course of TNBS colitis

To assess the contribution of the CD11c$^+$ DC in TNBS colitis a transient depletion of the cells was performed by i.p. injection of DT in CD11c-DTR mice. Previously we reported that CD11c$^+$ DC depletion with DT injection before TNBS colitis induction did not affect disease severity.[21] As the peak increase of CD11c$^+$ MHCII$^+$ DC in MLN occurred at 3 days post TNBS challenge the DT treatment was now performed on day 2 after TNBS challenge. As shown in figure 2a, the depletion of CD11c$^+$ cells did not affect disease severity and progression.

DT injection on day 2 after TNBS challenge was effective as, on day 3, the CD11c$^+$ cells in the MLN were reduced by 50% in CD11c-DTR transgenic mice when compared to non-transgenic controls, (figure 2b). In the colonic lamina propria complete depletion of the CD11c$^+$ CD11b$^-$ DC was obtained (figure 2c). Crucially, in both lamina propria and MLN the majority of the residual CD11c$^+$ DC were CD11b$^+$ Ly6C$^+$ suggesting that this population of DC was not affected by DT injections. Possibly, these cells have escaped depletion because they were monocytes that were CD11c$^-$ at the time of DT injection. In agreement, analysis of the lamina propria after DT treatment revealed that on day 3 after colitis induction the monocyte influx was not affected by CD11c-depletion in the CD11c-DTR mice (figure 2c). These data revealed that CD11c$^+$ DC depletion did not affect severity TNBS colitis. However, as CD11c$^+$ Ly6c$^+$ CD11b$^+$ cells were not depleted these monocyte-derived DC may play a crucial role in the disease.

Ly6C$^+$ DC are the main population containing mucosally administered antigen in colitic mice

To see whether the Ly6C$^+$ DC could be involved in the presentation of antigens we administered OVA-alexa-647 i.g. on day 2 of colitis. On day 3 of colitis OVA-647 positive CD11c$^+$ MHCII$^+$ DC were detected in the MLN. Moreover, the majority of OVA-647 positive DC had the Ly6C$^+$ phenotype (figure 3). Similarly, i.r. administration of OVA-647 on day 2 led to OVA-647$^+$ Ly6C$^+$ CD11c$^+$ MHCII$^+$ DC in the colon draining ILN (data not shown).

In sum, CD11c$^+$ MHCII$^+$ CD11b$^+$ Ly6C$^+$ DC in MLN of TNBS colitis mice can contain mucosally administered antigen.

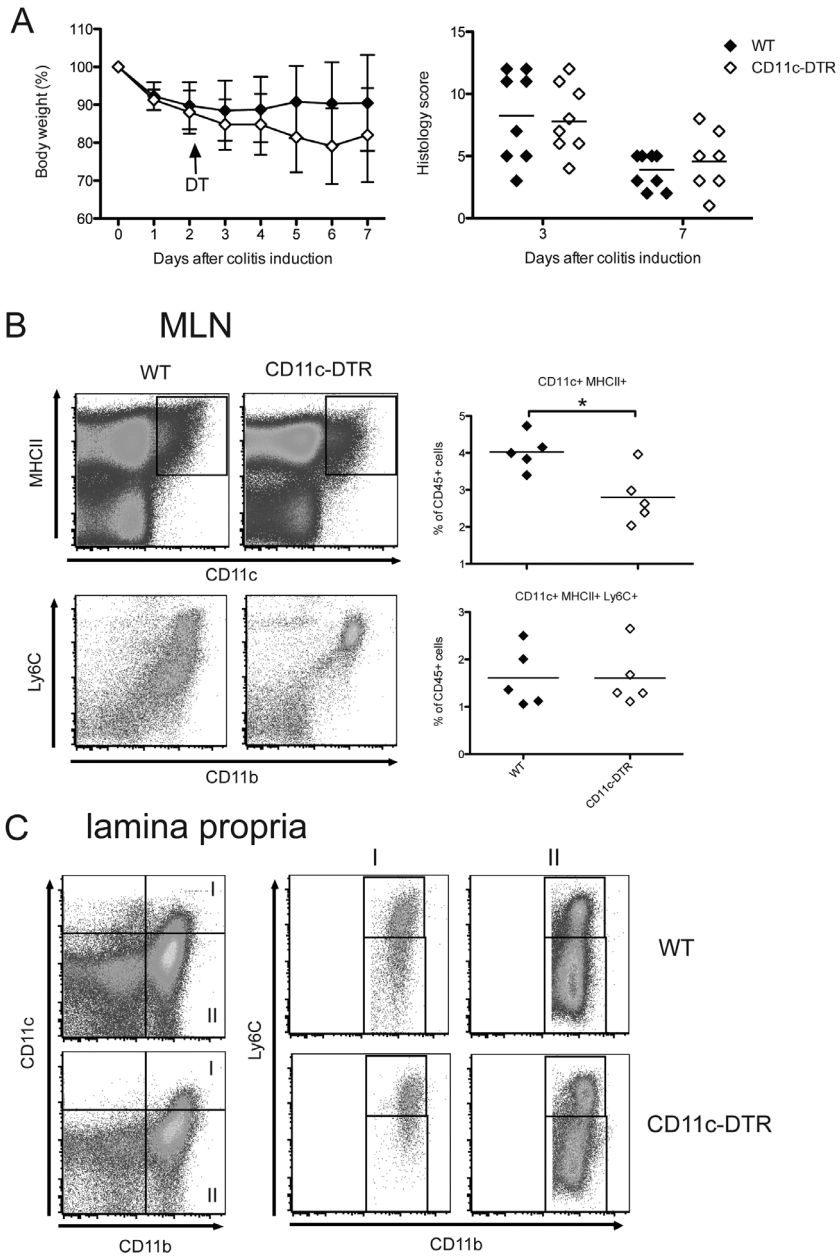

Figure 2. DC depletion did not affect severity of TNBS colitis and did not deplete Ly6C+ cells. A: CD11c$^+$ DC were depleted in CD11c-DTR mice using an i.p. injection of DT 2 days after the induction of colitis. The course of TNBS colitis was assessed using weight loss and colitis score. B: The CD11c$^+$ MHCII$^+$ DC and the CD11b$^+$ Ly6C$^+$ subpopulation were analyzed in the MLN of mice one day after the DC depletion (3 days after colitis induction) using flow cytometry. C: The effect of DC-depletion on the lamina propria APC subsets was studied using the expression of CD11c and CD11b. In addition the expression of Ly6c within the CD11b$^+$ populations (I+II) was assessed (C). * Statistically significant ($P < 0.05$)

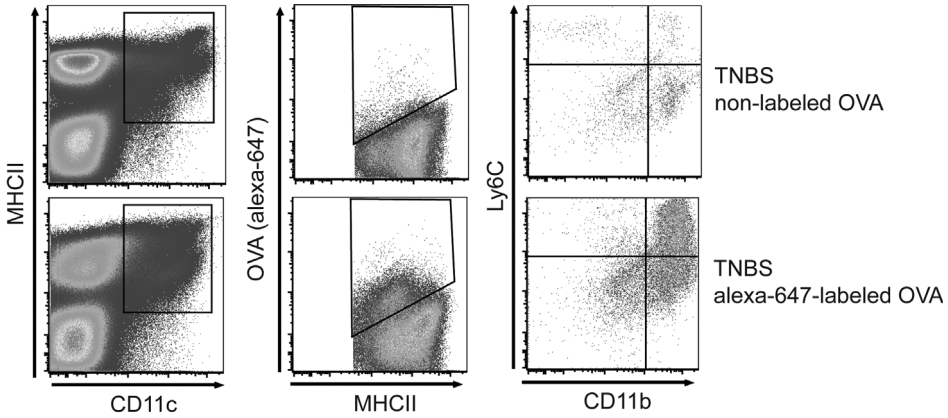

Figure 3. Ly6C$^+$ DC are the main population containing mucosally administered antigen in colitic mice
Mice received OVA-labeled with Alexa Fluor-647 (or non-labeled OVA as control) in 200 µl saline i.g. 2 days after induction of colitis. One day after antigen administration the MLN were isolated and the expression Ly6C and CD11b was studied within the CD11c$^+$ MHCII$^+$ OVA-Alexa-647$^+$ population.

Mucosal administration of antigen induces enhanced Ag-specific T-cell proliferation

To see how the presence of this subset within the lymph node affects the adaptive T-cell response during TNBS colitis, it was determined whether colonic OVA application induced differential proliferation of OVA-specific T-cells in TNBS colitis mice versus EtOH controls. Thereto, an adaptive transfer of CFSE-labeled DO11.10 T-cells was performed on day 1 of TNBS colitis. On day 2 of colitis mice were given OVA i.r. and the OVA-specific T-cell response was analyzed on day 3 of colitis. As depicted in figure 4A, the percentage of proliferating OVA specific T-cells is strongly increased compared to that seen in EtOH controls in the ILN and MLN 3 days after antigen exposure (figure 4a). In agreement with their vigorous proliferation profile, the cells expressed high levels of the activation marker CD25+ and low levels of the lymph nodes homing receptor CD62L (figure 4b).

These data show that the predominance of OVA-containing Ly6C$^+$ CD11b$^+$ DC in the MLN is associated with enhanced T-cells proliferation and activation.

DISCUSSION

In this study we show that within one day after induction of TNBS colitis Ly6c$^+$ monocytes infiltrate the intestinal lamina propria followed by a Ly6c$^+$ CD11b$^+$ DC increase in the draining lymph nodes. As the Ly6C$^+$ DC are the major population containing mucosally delivered antigen and support T-cell division in vivo these cells have the capacity to contribute to the adaptive inflammatory T-cell response. In agreement, upon depletion

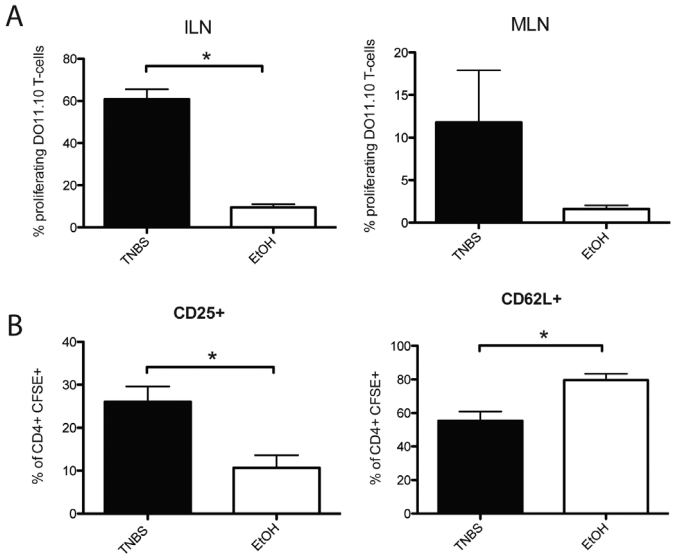

Figure 4. TNBS colitis is associated with increased DO11.10 proliferation after mucosal delivery of OVA. Mice received 1 x10^7 CSFE-labeled $CD4^+$ $KJ1.26^+$ DO11.10 T-cells i.v. one day after colitis induction. The next day OVA was administered to the mucosa either i.r (ILN) or i.g. (MLN). Three days after OVA administration the ILN and MLN were isolated and single-cell suspensions were stained for the presence of $CD4^+$ $KJ1.26^+$ DO11.10 T-cells. A: CFSE profiles of $CD4^+$ $KJ1.26^+$ T cells were used to determine the percentage of proliferating cells in ILN and MLN. B: The expression of activations markers CD25 and the lymph node homing receptor CD62L in the ILN were studied within the $CD4^+$ $KJ1.26^+$ DO11.10 T-cell population.

of classical $CD11c^+$ DC while maintaining $Ly6c^+$ DC the course of colitis was unaffected arguing that this new population of inflammatory APC has an essential function in disease development.

Our data show that a new subpopulation of $Ly6c^+$ inflammatory DC is recruited to the MLN during TNBS colitis. Under steady state conditions these monocyte-derived DC are not detectable in lymphoid organs.[24] Instead, these cells require inflammatory signals for their recruitment.[25] Our observation that the $Ly6C^+$ DC were the major population containing mucosally administered OVA-antigen, combined with the enhanced proliferation of OVA-specific T-cells in mice suffering from colitis, argue that these $Ly6C^+$ DC have a role in antigen presentation. This is in line with the function of protein presenting $Ly6C^+$ DC that arise after vaccination with protein in the presence of alum.[26] On the other hand, $Ly6C^+$ DC are often referred to as inflammatory DC or TNF/iNOS-producing (Tip)-DC that arise during infections.[1, 23] Tip-DC are generally poor antigen presenters and contribute to immune responses through the production of antimicrobial mediators and by providing help to B cells for antibody production.[27] Further experiments are currently performed to assess the innate and adaptive function of the $Ly6C^+$ MLN-DC in our TNBS colitis model.

Indirect evidence for the role of the Ly6C$^+$ DC in TNBS colitis comes from our observation that depletion of classical DC in CD11c-DTR mice while maintaining Ly6c$^+$ DC did not affect the severity of disease. This is in contrast to the recent observation that depletion of classical DC could enhance DSS colitis severity.[28] The differences between these findings could not be attributed to the timing of depletion as neither depletion before challenge nor depletion on day 2 affected disease severity.[21] Differences in the mechanisms of colitis induction between the hapten TNBS and the toxic DSS polymer, location and chronicity of the inflammation between DSS and TNBS colitis may be the cause for differential effects in these models.

Although the Ly6C$^+$ MLN-DC are likely to be derived from a Ly6C$^+$ monocyte population it is questionable whether these cells have migrated from the lamina propria as only a very small proportion of the cells expressed the chemokine receptor CCR7. Alternatively, the Ly6+DC have differentiated locally from monocytes that are recruited to the LN as has been described for influenza infection.[29]

The strong increase in the number of Ly6C$^+$ monocytes in lamina propria TNBS colitis agrees with previous reports that CD11b$^+$ macrophages infiltrate the lamina propria during chronic colitis in IL-10 deficient mice.[30] This monocytic infiltration is likely to be chemokine dependent as CCL2 is increased in laminia propria after colitis induction.[31] Similarly, infection with Toxoplasma gondii leads to a strong CCR2 dependent recruitment of Ly6C+ monocytes[32] and oral infection with Salmonella induces recruitment of Ly6C+ monocytes that produce high levels of TNF and iNOS.[33]

Interestingly, multiple studies have shown that depletion of macrophages effectively inhibit colitis induction.[30, 34] However, as the depleting agents MS-Cl2MDP and saporin-conjugated anti-CD11b are likely to also affect both monocytes and monocyte-derived DC these studies should be re-evaluated.

In conclusion, our findings that intestinal inflammation during TNBS colitis is associated with a newly recruited subset of monocyte-derived DC have important consequences for understanding the mechanisms that lead to loss of tolerance to bacterial flora as seen in inflammatory bowel disease.

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Chapter 5

Production of IL-12p70 and IL-23 by monocyte-derived dendritic cells in children with inflammatory bowel disease

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INTRODUCTION

In the past, analysis of the mucosal T cell phenotype largely supported the concept of Crohn's disease (CD) as a T helper 1 (Th1) disease, driven by interleukin-12 (IL-12) and characterized by the production of the signature cytokine interferon (IFN)-gamma, and ulcerative colitis (UC) as an atypical Th2 disease characterized by production of cytokines such as IL-13.[1] The antigen presenting dendritic cells have been identified as key players in their capacity to drive this T cell polarization (Rescigno).[2,3]

The discovery of the Th17 lineage, driven by IL-23 (as well as IL-6 and transforming growth factor-β) and characterized by IL-17 production has led to re-evaluation, and the realization that a number of inflammatory conditions including CD, may actually be Th17 dependent.[2,3]

While re-examining the concepts of the various T cell phenotypes in inflammatory bowel disease (IBD), it has become apparent that our understanding of the complex cytokine networks regulating these cells is far from complete. Overall, we are unable to determine the relative contribution of each cytokine to the initiation and perpetuation of inflammation in IBD.[2] We know that IL-12 (containing a p35 and a p40 subunit) and IL-23 (containing a p19 and a p40 subunit) are both produced in large quantities, along with other cytokines, by activated antigen-presenting cells (APCs), such as dendritic cells (DCs). *In vitro*, their secretion can be studied using artificial innate ligands, which activate receptors such as Toll-like receptors (TLRs) and *Nod2*-receptors. It is evident that there are significant differences in the regulation of transcription of the component subunits (p19, p35 and p40), and indeed the kinetics and time-course of secretion differ, raising the possibility that they act differentially in acute and chronic inflammation.[7]

In addition, the promiscuity of p40 is far from unique in the IL-12 family of cytokines; the recent demonstration of the combination of p35 with EBI-3 (itself a component of IL-27) to form the regulatory cytokine IL-35 illustrates how much more we have to learn in the field of cytokine regulation.[8] The activation status of the immune cells and the relative synergy between multiple TLR signals determine how different pathogen-associated molecular patterns (PAMPs) elicit different cytokine-driven T lymphocyte phenotypes.

The ability of an APC to secrete a cytokine does not confirm a relevant biological role, as postsecretory effects are modified by the relative expression of the appropriate receptor on the target cell, and by the local presence of other cytokines and regulatory T cells, which are capable of modifying T cell responses to cytokines.[2] Kugathasan et al. compared T cell clones derived from the colonic mucosa of pediatric patients with newly diagnosed CD with those from patients with established disease or infective colitis.[9] They were able to examine the relative secretion of T cell cytokines, and the susceptibility to modulation by APC-derived cytokines. Overall, they found significant differences

between T cells from early and late disease. Early disease was characterized by a strongly polarized Th1 response indistinguishable from that evoked in infective colitis, expressing high levels of the specific IL-12 receptor β2 subunit and IFN-γ, and sensitivity to cytokine modulation. In contrast, clones from established disease produced more IL-4 and IL-10, and demonstrated relative insensitivity to the effects of modulation when cultured with cytokines. Of note, clones derived from early disease had the ability to upregulate markedly the secretion of the immunoregulatory cytokines IL-10 and IL-4 in response to the appropriate stimulus.

This study raises a number of important questions regarding the role of cytokines in shaping the mucosal infiltrate in IBD, and the potential for disease modifying therapies. In particular, given the apparent insensitivity of T cells in established disease (at least to the cytokines used), it will be important to establish the additional mechanisms active in controlling the mucosal adaptive response, such as co-stimulatory molecules, microbial antigens and the effects of regulatory T cells.[10] Perhaps the most intriguing question is whether targeting the disease early (in immunological terms), when cytokines are still dominant players in shaping the immune response and establishing chronicity, might offer greater potential to modify the course of disease than intervening late with anti-cytokine therapy.[2]

This concept is supported by clinical observations of greater efficacy with biological therapies when used in early IBD. Further studies looking at the earliest immunological events in IBD are needed to guide the rational selection of the most appropriate cytokine target, as are longitudinal studies of the balance of cytokines in the evolution of chronic IBD.

One of the remaining questions is, what will determine the development of these typical effector T cells: the expression of specific T cell receptors or the production of differentiating cytokines by antigen presenting cells? In this chapter, we directly addressed whether the capacity of dendritic cells to produce IL-12 and/or IL-23 could be linked to a specific subset of pediatric IBD patients.

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PRODUCTION OF IL-12P70 AND IL-23 BY MONOCYTE-DERIVED DENDRITIC CELLS IN CHILDREN WITH INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) patients represent a heterogeneous group of patients that may need novel classification beyond just Crohns' disease (CD) and ulcerative colitis (UC). Notably, based on patient specifics such as genetics, disease location, immune responses and drug responsiveness it seems likely that early onset inflammatory bowel diseases represents a specific disease entity.[1] Consequently, various disease associated effector T cells have been identified, likely generated under the control of cytokines that are produced by antigen-presenting cells. In their recent publication, Kugathsan et al. demonstrate that the level of IL12β2 (IL-12 receptor) expression by mucosal T cells may be a major determinant during the initial manifestations of the CD for the development of the typically associated mucosal Th1 cytokine profile.[2] In this case, the presence of a specific T cell receptor correlated with the development of early CD. Similar mechanisms have been proposed for another recently discovered effector T cell subset. As such, T cell differentiation into interleukin-17 producing cells (Th17) may, in part, depend on T cell expression of the IL-23 receptor.[3] Specifically, germline variations of IL23R have been implicated in conferring protection to ileal CD.[4] The question now is, what will determine the development of these typical effector T cells; the expression of specific T cell receptors or the production of differentiating cytokines by antigen presenting cells? In our studies we directly addressed whether the capacity of dendritic cells to produce IL-12 and/or IL-23 could be linked to a specific subset of pediatric IBD patients.

To this aim, we cultured monocyte-derived dendritic cells (MoDC) obtained from 17 children with Crohn's disease (age 6.9–18 yrs, median 14.1 yrs) and 18 children with ulcerative colitis (age 3.2–17.2 yrs, median 13.3 yrs).[5] After harvesting, the moDCs (2x105 cells per well, 200 µl) were stimulated overnight with various microbial ligands at different concentrations: lipopolysaccharide (E.Coli, serotype 055:B5 L-4005, Sigma-Aldrich, Schnelldorf, Germany), zymosan (Sigma-Aldrich) and pam3cys (EMC Microcollections GmbH, Tubingen, Germany). Cell supernatants were collected and the production of IL-12p70 and IL-23 was determined by means of Cytometric Bead Array (BD Biosciences, San Jose, USA) and enzyme-linked immunoabsorbent assay (coating Affinity Purified anti-human IL-23p19 [clone eBio473P19], detection anti-human IL-12p40/70 [clone C8.6; eBioscience]) respectively. For statistical analyses, Pearson correlation coefficients and Spearman rank-correlation coefficients were calculated both for the original values and the logarithmic transformed values. Furthermore, Analysis of Variance (Anova) and Linear Mixed Models with a unstructured correlation matrix for the residuals to account for dependencies (due to repeated measurements) were used.

Within the two patient groups, there was a high variability in IL-12p70 and/or IL-23 production upon microbial stimulation (figure 1a and b). The levels of either IL-12 or

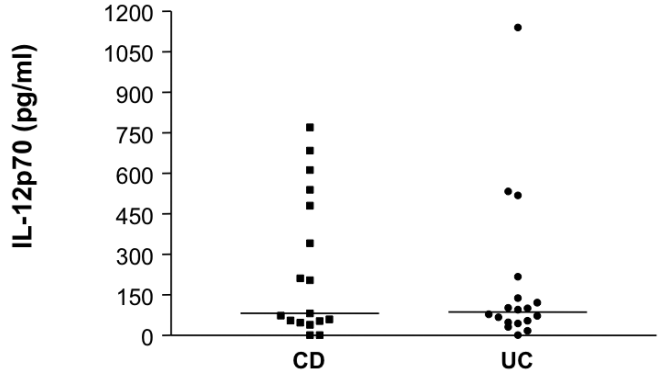

Figure 1a: High variability in the production of IL-12p70 by moDCs derived from children with Crohn's disease (CD) vs. ulcerative colitis (UC). moDCs were stimulated overnight with LPS at 0.1 µg/ml.

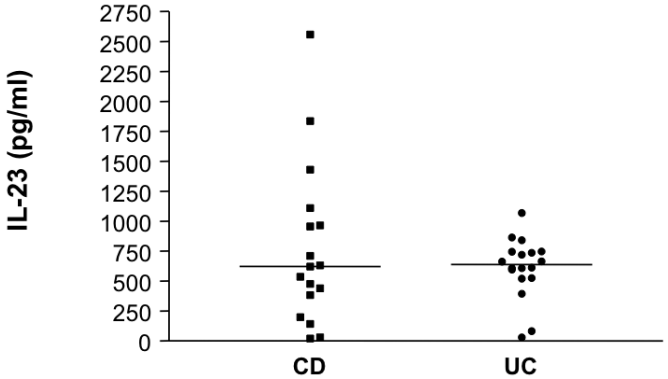

Figure 1b: High variability in the production of IL-23 by moDCs obtained from children with Crohn's disease (CD) vs. ulcerative colitis (UC). moDCs were stimulated with LPS 0.1 at µg/ml.

IL-23 could not be related to disease activity (expressed by pediatric Crohn's disease activity index [PCDAI] or Lightiger colitis activity index [LCAI] respectively), disease location, or medication. There was no difference in cytokine production by moDCs when we compared early to late IBD (for CD as well as for UC). There was a clear dose dependent relation between the production of IL-12 or IL-23 and for all the used microbial ligands. As such, we could subcategorize the patient moDCs into high, intermediate, or low responders, irrespective of the used microbial ligand (data not shown).

These data indicate that neither of the specific IBD subsets (CD or UC) may be associated with a ''hardwired'' predisposition for IL-12 or IL-23 production. We did not find any relation between IL-12p70 and IL-23 levels (figure 2), confirming recent reports that the production of these two cytokine family members is differentially regulated. We conclude that the intrinsic capacity of MoDC to produce IL-12p70 and/or IL-23 is not associated with a specific subtype or stage of inflammatory bowel disease. We therefore

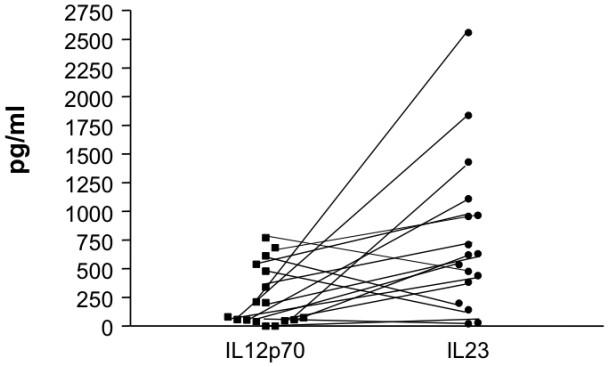

Figure 2: No relation between the production of IL-12p70 and IL-23 by moDCs. moDCs were derived from children with Crohn's disease and stimulated with LPS at 0.1 mg/ml.

hypothesize that the presence of specific mucosal T effector cells that have been identified in CD or UC results from the local amount of particular microbial ligands and T-cell expression of specific receptors such as IL-12β2 and IL-23R.

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Chapter 6

Production of TNF-α by Paneth cells is associated with intestinal inflammation

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ABSTRACT

Background: Intestinal Paneth cells (Pc) produce anti-microbial defensins, lysozyme and pro-inflammatory TNF-α. Impaired Pc defensin production has been associated with intestinal inflammation. Elucidation of the mechanisms that regulate TNF-α production by Pc may identify a novel contributive pathway of mucosal inflammation.

Methods: With immunohistochemistry, we determined lysozyme and TNF-α expression by Pc of 74 pediatric Inflammatory Bowel Disease (IBD) patients, 20 celiac disease patients and 27 healthy controls.

Next, we explored differences in the regulation of lysozyme and TNF-α production in an *in vitro* model using a human monocyte cell-line.

Finally, we examined jejunal segments derived from IL-$10^{-/-}$ mice in SPF and germ-free conditions and LPS-insensitive mice (CH3/HeJ) for TNF-α and lysozyme expression.

Results: Lysozyme was equally expressed by Pc in all patients and controls. In contrast, 45% of the Crohn's disease patients, 37% of the ulcerative colitis patients and 35% of the celiac disease patients expressed TNF-α in Pc. 19% of healthy duodenum was positive for TNF-α in Pc.

In vitro analysis showed that TNF-α production by a monocyte cell line could be induced by LPS and suppressed by IL-10 and not by IL-22. Under similar conditions, LPS, IL-10 and IL-22 had no effect on lysozyme expression as determined by quantitative PCR.

In line with this finding, we observed enhanced expression of TNF-α in Pc of IL-$10^{-/-}$ mice compared to WT mice that was absent in germ-free IL-$10^{-/-}$ mice. Finally, CH3/HeJ LPS-insensitive mice expressed less Pc-derived TNF-α compare to CH3/HeN LPS sensitive mice.

Conclusion: In humans, Pc TNF-α levels are associated with mucosal inflammatory diseases. In mice, TNF-α production by Pc is dependent on microbial stimulation and regulated by IL-10. Under comparable conditions, Pc lysozyme levels remain unaffected. These data indicate differential regulation of TNF-α and lysozyme production by Pc.

INTRODUCTION

Paneth cells (Pc) play an important role in the regulation of intestinal microbial homeostasis in the small intestine. Pc release anti-microbial peptides such as lysozyme and defensins, in order to prevent bacterial invasion into the intestinal crypts.[1-4] Impaired production and release of these peptides has been associated with bacterial overgrowth and inflammatory intestinal diseases such as Crohn's disease (CD).[5, 6]

Next to lysozyme and defensins, tumor necrosis factor-α (TNF-α) is also produced by Pc.[7] Augmented production of TNF-α by hematopoietic cells has been associated with multiple inflammatory conditions including inflammatory bowel disease (IBD). Over-expression of Pc-derived TNF-α has been suggested to contribute to necrotizing enterocolitis.[8]

Production of TNF-α in hematopoietic cells can be inhibited by interleukin (IL)-10.[9, 10] Along these lines, IL-10 knock-out mice spontaneously develop colitis that is thought to depend on increased levels of TNF-α.[11, 12] Furthermore, polymorphisms in the IL-10 promotor and IL-10 receptor have been associated with IBD in humans, indicating an important regulatory and anti-inflammatory role for IL-10.[13-15] To date, it is not known how induction and control of TNF-α by Pc is regulated.

To identify a potential role for Pc derived TNF-α in mucosal inflammation we determined expression of both lysozyme and TNF-α in intestinal biopsies from pediatric patients with IBD or celiac disease and controls. Next, we studied various transgenic mice to determine the relative contribution of microbial signaling and IL-10 to Pc TNF-α production and regulation.

MATERIALS & METHODS

Patients

Biopsies of 44 pediatric Crohn's disease patients and 30 ulcerative colitis patients were included in this study, as well biopsies derived from 20 patients with celiac disease and 27 controls. All biopsies were performed for diagnostic purposes and none of the patients received medication at the time of biopsy. IBD patients were diagnosed by endoscopy, histo-pathological and clinical characteristics according to the Porto criteria.[16] Biopsies of celiac disease patients were all scored Marsh III.[17, 18] Control biopsies showed no sign of inflammation. All biopsies were fixed in paraformaldehyde and embedded in paraffin. Histological scores and pathological characteristics of the sections were performed by a blinded pathologist.

Animals

Specific pathogen-free (SPF) 10-week-old female BALB/c mice were purchased from Charles River (Saint Aubin Lee´s Elbeuf, France). SPF 10-week-old female LPS-unresponsive C3H/HeJ mice and LPS-responsive C3H/HeN mice[19, 20] were purchased from CLEA Japan, Inc. (Tokyo, Japan). SPF 8-to 10-week old IL-10-deficient C57BL/6 mice, formally designated 'IL-10tm1Cgn' (I. J. Bristol, M. Mähler, E. H. Leiter, J. P. Sundberg: IL-10tm1Cgn, an IL-10 gene-targeted mutation; JAX notes 471, The Jackson Laboratory, Bar harbor, ME, USA, 1997) (obtained from M. Mähler, Hanover, Germany), and their wild-type (WT) littermates were bred at the animal facility of the Erasmus MC (Rotterdam, the Netherlands) and maintained in isolator cages with water and a standard pellet diet (Hope Farms, the Netherlands) ad libitum.

Immunohistochemistry

Sections (4mm) were routinely stained with hematoxylin (Vector Laboratories, CA) and eosin (Sigma-Aldrich, The Netherlands) to study morphological alterations.

For immunohistochemistry sections were dewaxed and endogenous peroxidases were quenched with 3% H2O2 in methanol for 20 min. Antigen retrieval for lysozyme P (for human and mouse sections) was obtained after microwave treatment in citrate buffer (10 mM pH 6.0) and for TNF-α after treatment with 0.1 % pepsin in 0.01 N HCl for 7 minutes at 37°C. Sections were blocked for 1 hour in either 1% blocking reagent (Roche, The Netherlands) in PBS or 10 mM Tris, 5mM EDTA, 0.15M NaCl, 0.25% gelatine, 0.05% Tween-20, supplemented with either 10% normal mouse plus normal rabbit serum or 10% normal human serum, pH 8. Antibody incubation was performed overnight at 4°C with rabbit anti-lysozyme P (DakoCytomation, Denmark) diluted 1:50, goat anti-mouse TNF-α (Santa Cruz Biotechnology, CA) diluted 1:200, mouse anti-human TNF-α (ImmunoTools, Germany) diluted 1:100. Immunoreactions were detected using respectively biotinylated secondary goat anti-rabbit, rabbit anti-goat or horse anti-mouse serum with the Vectastain ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich).

Alls sections were counterstained with Hematoxylin (Vector Laboratories) and pictures were taken by a Leica DM 5500 microscope with 40x and 100x lens.

In vitro assays

THP-1 cells were maintained in RPMI1640 medium (Gibco) containing 10% heat inactivated fetal calf serum in 5% CO2 at 37°C. For each experiment, THP-1 cells were seeded in 96-well flatbottom plates at a density of 2x105 cells/well. THP-1 cells were treated with 1µg/ml LPS (E.coli 055:B5, Sigma) alone or in combination with either 10ng/ml rhIL10 (ImmunoTools, Germany) or 10ng/ml rhIL22 (ImmunoTools). Supernatant and cells were harvested after 6h of stimulation. TNF-α production was measured by ELISA (hTNF-α,

BD) and lysozyme expression was analyzed by qPCR on RNA that was isolated using a RNA isolation kit (Qiagen). cDNA was generated by standard methods and amplified using an ABI Prism 7700 Sequence detector in the presence of SYBR Green according to manufacturer's protocol (DyNAmo HS SYBR Green qPCR kit, New England Biolabs). We used GAPDH primers as an internal control and specific primers for hLysozym. Primer sequences: hGAPDH-F: GTCGGAGTCAACGGATT, hGAPDH-R: AAGCTTCCCGTTCTCAG, hLysozym-F: ACCCCAGGAGCAGTTAAT and hLysozym-R: GCCACCCATGCTCTAAT.

Statistical analysis

Production of TNF-α and lysozyme was statistically analyzed using an unpaired Student's t-test when appropriate. A p-value of < 0.05 was considered statistically significant. Data are presented as the mean ± standard error of the mean (SEM).

RESULTS

Human Pc express higher levels of TNF-α in inflammatory conditions

To investigate whether Pc-derived TNF-α is involved in human intestinal inflammation, we obtained biopsies from duodenum, terminal ileum and the distal part of the colon of newly diagnosed IBD patients. Next to H&E staining, these biopsies were stained for lysozym and TNF-α. We observed PC metaplasia in 65% of the IBD patients (CD 66%, ulcerative colitis (UC) 63%), confirming earlier reports (Table 1).[21]

We found that all Pc from IBD patients as well as controls expressed lysozyme at similar levels (figure 1a). Immunohistochemical staining for TNF-α showed that 42% of the IBD

Table 1. TNF-α expression in Pc of IBD patients, celiac disease patients and controls

	Total	Metaplasia	%
IBD	74	48	64.8
CD	44	29	65.9
UC	30	19	63.3
	Total	**TNF-α**	**%**
IBD	74	31	41.9
CD	44	20	45.5
UC	30	11	36.7
Celiac disease	20	7	35.0
Control	27	5	18.5

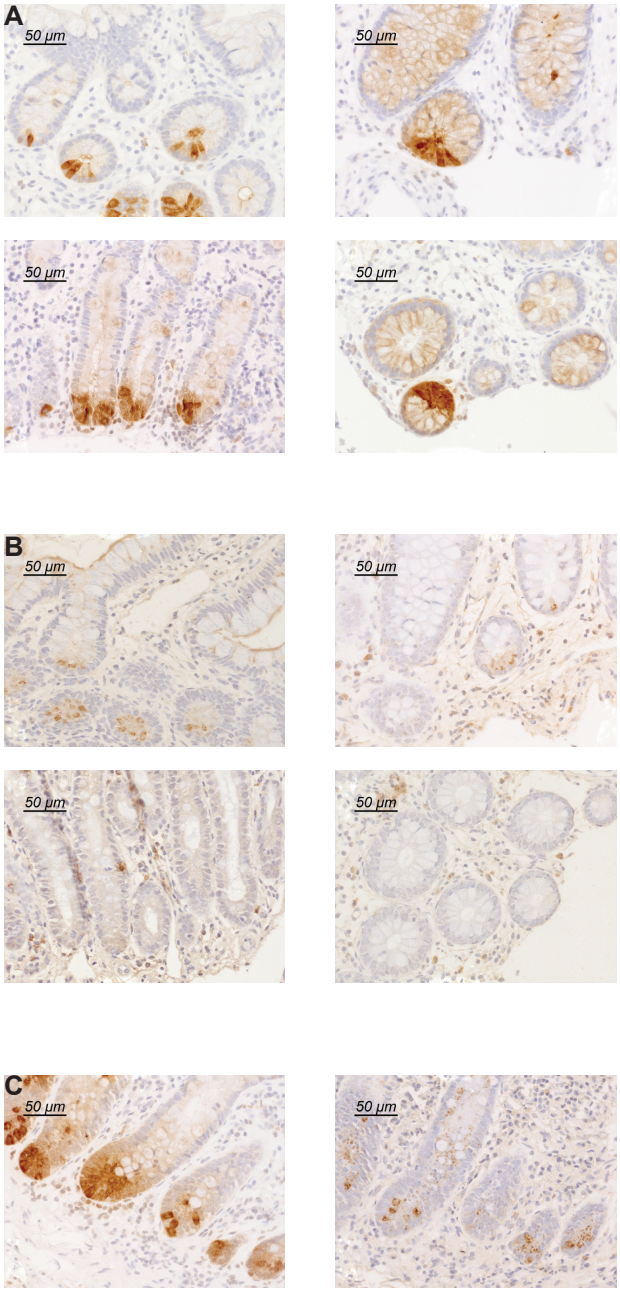

Figure 1. Paneth cells express TNF-α irrespective of location
(A-C) Representative examples of immunohistochemical staining for lysozyme (A) and TNF-α (B) on intestinal sections of pediatric IBD patients. (A) Paneth cells (Pc) are present in small intestinal (left) and colonic (right) sections from pediatric IBD patients. Brown staining indicated lysozyme expression. (B) TNF-α (brown) in the Pc is expressed in a subset of patients. (C) Representative small intestinal section of a celiac disease patient. Brown staining indicates lysozyme (left) and TNF-α (right) expression.

patients contained TNF-α expressing Pc (Table 1). Within the group of IBD patients, Pc of CD and UC patients displayed equal positivity for TNF-α (45% and 37%, respectively, Table 1). Examples of immunohistochemical staining for TNF-α are shown in figure 1b. Intriguingly, TNF-α positivity in the Pc was irrespective of the location of the Pc. Patients with colonic metaplastic Pc showed similar expression (on / off) for TNF-α as compared with Pc in the small intestine. In contrast, only 19% of healthy controls showed TNF-α expression in the Pc of duodenal biopsies, indicating that increased TNF-α expression in Pc is associated with mucosal inflammatory diseases.

To determine whether the induction of TNF-α expression in the Pc was specific for IBD, we analyzed duodenal biopsies from celiac disease patients with high level of intestinal inflammation (Marsh III score). We found that in 35% of these celiac disease patients, duodenal biopsies contained TNF-α expressing Pc (figure 1c, Table 1). These results showed that Pc-derived TNF-α was irrespective of intestinal location and not specific for IBD, but rather associated with intestinal inflammation in general. In contrast to TNF-α, lysozyme was constitutively expressed in Pc of both patients and controls, suggesting differential regulation of lysozyme and TNF-α in the Pc.

Lysozyme and TNF-α are differentially regulated *in vitro*

Our finding that lysozyme was constitutively expressed in all Pc and TNF-α expression was only observed in Pc of a subset of patients, suggests that lysozyme and TNF-α expression are differentially regulated in the Pc.

As there is no Pc cell line available, we determined lysozym and TNF-α expression in the human monocytic cell line THP-1. THP-1 cells have been shown to produce lysozyme as well as TNF-α upon lipo-polysaccharide (LPS) stimulation.[22] Furthermore, it has been shown that IL-10 can downregulate TNF-α production in hematopoietic cells.[9, 10]

We stimulated THP-1 cells with LPS alone (1μg/ml) or in addition of recombinant human (rh) IL-10 or, as a negative control, the IL-10 family member rhIL-22 (each 10ng/ml). Supernatant and cells were harvested after 6h of stimulation. TNF-α production was measured by ELISA and lysozyme expression was analyzed by qPCR. We found that stimulation with LPS induced the expression of TNF-α, while expression of lysozym remained stable. Addition of rhIL-10 partially inhibited the induction of TNF-α but did not affect lysozyme expression. As expected, the addition of rhIL-22 did not have any effect on either TNF-α or lysozyme expression (figure 2). These data confirm that TNF-α production is induced by LPS and down-regulated by IL-10, whereas lysozym expression is constitutive.

Lysozyme and TNF-α are differentially regulated *in vivo*

Our *in vitro* experiment indicated a direct role for IL-10. As we were unable to stain IL-10 or its receptor on our human biopsies, we further expanded on our *in vitro* data by exam-

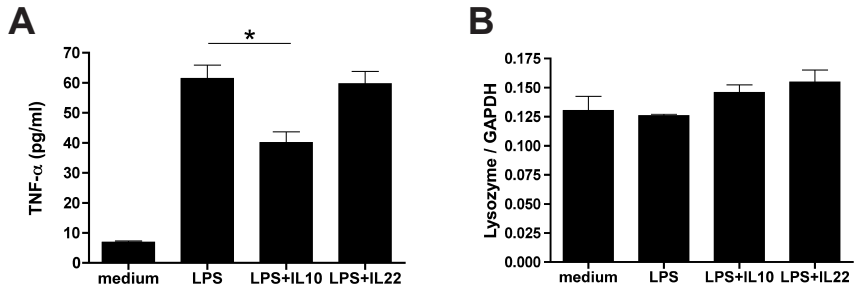

Figure 2. Lysozyme and TNF-α are differentially regulated *in vitro*
(A,B) TNF-α and lysozyme production by THP-1 cells 6 hours after stimulation with LPS (1μg/ml) alone or in addition of recombinant human IL-10 or the IL-10 family member IL-22 (each 10ng/ml). Production of TNF-α was measured by ELISA (A), whereas lysozyme was analyzed by qPCR (B). Error bars represent SEM, *p<0.05

ining IL-10-deficient mice. In our facility these mice spontaneously develop colitis at 20 weeks of age. To compare expression of Pc-derived TNF-α and lysozyme in non-inflamed mice, we used 9 weeks old IL-$10^{-/-}$ mice and age-matched WT mice.

We found strong TNF-α staining in Pc of IL-$10^{-/-}$ mice compared to WT controls (figure 3a,b,c). In contrast, both type of mice expressed similar levels of lysozyme (figure 3d,e). These results confirm that, also in an *in vivo* model, IL-10 inhibits TNF-α expression in Pc and has no effect on the expression of lysozyme under similar conditions.

It has been well established that microbial exposure stimulates production of TNF-α in various immune cells. To investigate potential microbial regulation of TNF-α in the Pc *in vivo*, we analyzed the expression of TNF-α in IL$10^{-/-}$ mice that were maintained under germ-free conditions. In sharp contrast with IL$10^{-/-}$ mice that were maintained under spf conditions, these mice did not express TNF-α in the Pc (figure 3f) indicating a role for microbial stimulation in Pc TNF-α production.

We found similar expression levels of lysozyme when comparing germ-free IL$10^{-/-}$ mice to IL$10^{-/-}$ mice that were kept at SPF conditions (figure 3g). Together with the data shown in figure 3a and 3b, these data confirm that microbial exposure is crucial for the expression of Pc-derived TNF-α, whereas lysozyme is constitutively expressed.

TNF-α expression is dependent on LPS signaling

Next, we determined lysozyme and TNF-α expression in Pc within the terminal ileum of LPS-insensitive mice (CH3/HeJ) and controls (CH3/HeN) kept under spf conditions. CH3/HeJ mice are deficient for toll-like receptor 4 (TLR4), the specific receptor for LPS, and are therefore insensitive to LPS stimulation.

By counting TNF-expressing Pc in both type of mice we determined that Pc from CH3/HeJ mice expressed less TNF-α compared to CH3/HeN controls, indicating that the

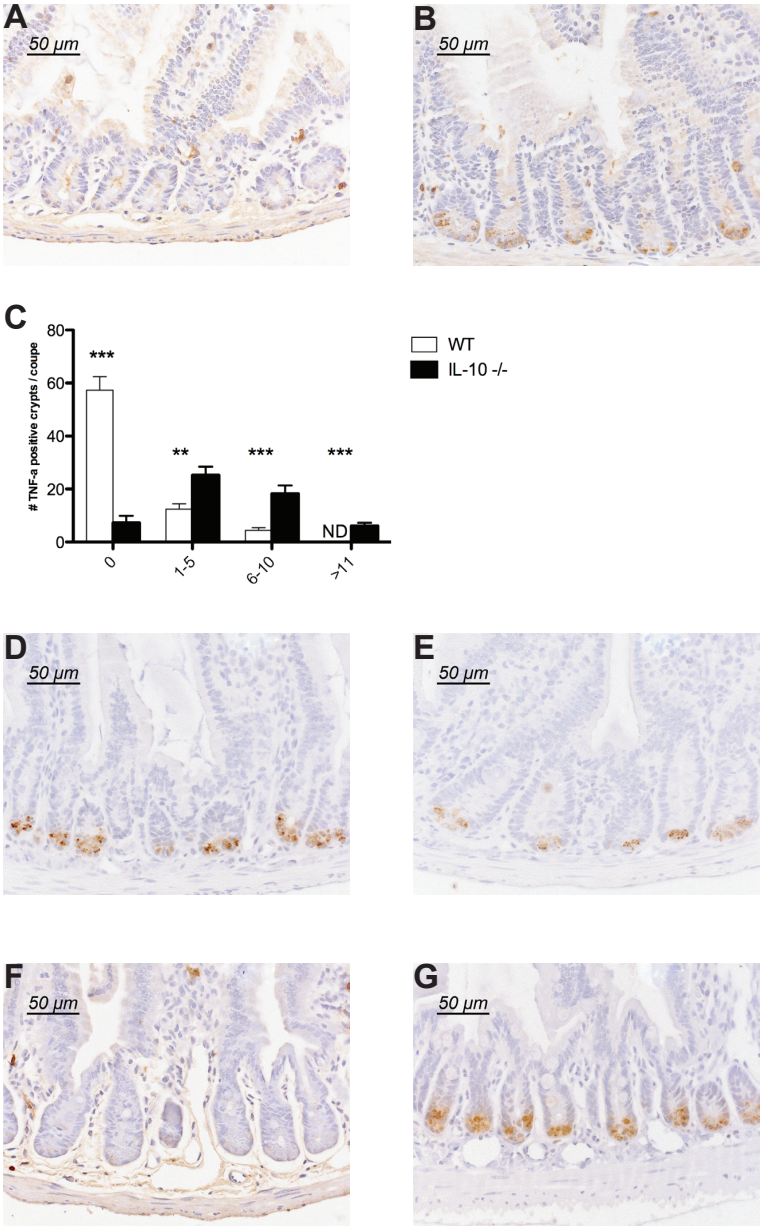

Figure 3. Lysozyme and TNF-α are differentially regulated *in vivo*
(A-E) Representative examples of immunohistochemical staining for TNF-α (brown, A,B) and lysozyme (brown, D,E) on small intestinal sections of wild type (WT) mice (A,D) and interleukin (IL)-10-deficient mice (B,E) in SPF conditions. (C) Quantification of TNF-α expression in the crypts of WT and IL-$10^{-/-}$ mice depicted as number of TNF-α positive crypts per section. (F,G) Expression of TNF-α (F) and lysozyme (G) in small intestinal sections of IL-$10^{-/-}$ mice that were kept under germ-free conditions.
Error bars represent SEM, *** $p<0.001$, ** $P<0.01$, ND = not detected, n=9 mice per group for mice in SPF conditions. N=3 mice per group in germ free conditions.

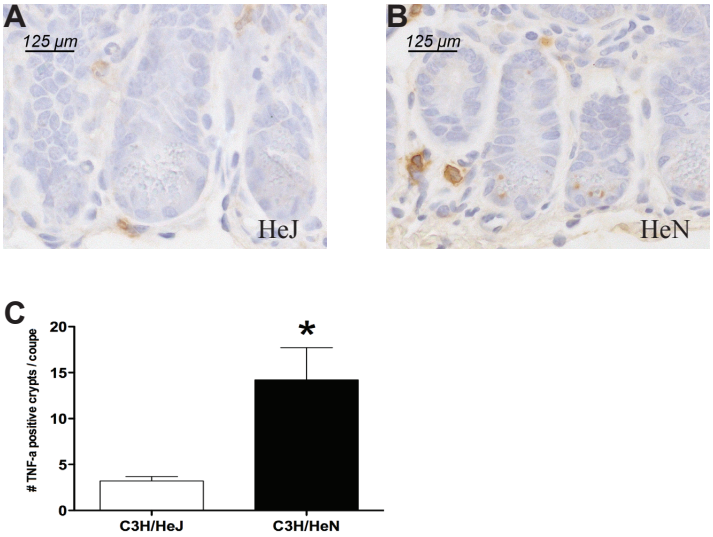

Figure 4. Impaired LPS signaling results in aberrant TNF-α expression in the Pc
(A,B) Representative examples of TNF-α expression in TLR4-deficient CH3/HeJ (A) and CH3/HeN controls (B). (C) Number of TNF-α positive crypts per section in LPS insensitive (CH3/HeJ) and controls (CH3/HeN). Error bars represent SEM, * $p<0.05$, n=5 mice per group.

production of TNF-α in the Pc is at least partially regulated by LPS ($p<0.05$) (figure 4). This confirms the observation that Pc-derived TNF-α production is driven by TLR4 signaling.

DISCUSSION

We found enhanced levels of Pc TNF-α in patients with mucosal inflammatory diseases. In order to explore the regulation of Pc derived TNF-α, we analyzed the expression of TNF-α *in vitro* and *in vivo*. In mice, TNF-α production by Pc was dependent on microbial stimulation and regulated by IL-10. Under comparable conditions, Pc lysozyme levels remained unaffected, indicating differential regulation of TNF-α and lysozyme production by Pc.

IL-10 plays an important role in the regulation of TNF-α in hematopoietic cells. Binding of IL-10 to its receptor leads to the phosphorylation of suppressor of cytokine signaling-3 (SOCS3) via activation of signal transducer and activator of transcription-3 (STAT3). This in turn leads to the inhibition of pro-inflammatory genes expression. Whether IL-10 exerts a comparable function in the Pc is unknown. However, as epithelial cells express STAT3, a similar mechanism could be responsible for the regulation of TNF-α expression by Pc.[23-25]

Although we were unable to detect IL-10 or the IL-10 receptor by immunohistochemistry on our human biopsies, insufficient IL-10 expression, as has been found in a subset of IBD patients[15], could result in augmented production of TNF-α by Pc. Nonetheless, these polymorphisms are rare in IBD and not identified in celiac disease patients. Therefore, aberrant IL-10 signaling may partly explain the difference in TNF-α expression by Pc between controls and patients with mucosal inflammatory diseases.

Microbial exposure was crucial for the induction of TNF-α in the Pc in our mouse experiments.

Even in IL10$^{-/-}$ mice, colonization with SPF microbiota is required for Pc TNF-α production. By showing that TLR4-deficient CH3/HeJ mice express diminished levels of TNF-α we established a role for LPS-TLR4 signaling as a prerequisite for Pc TNF-α production (figure 3,4). Indeed, toll-like receptor stimulation by microbial products has been shown to initiate pro-inflammatory immune responses in epithelial cells that may include Pc.[26] Interestingly, we observed no difference between Pc derived TNF-α expression in the small intestine as compared with metaplastic Pc in the colon. As the bacterial load varies widely in these two locations, differences in microbial stimulation are not a sufficient explanation for the enhanced expression of TNF-α in Pc of these patients. Moreover, in celiac disease patients, all patients had a Marsh III score but clearly differed in Pc TNF-α expression, thereby excluding local mucosal inflammation as a dominant determinant. These findings indicate that the expression of TNF-α by Pc is in part intrinsically regulated. In this model, host genetics, systemic conditions e.g. inflammation as well as local factors may determine this Pc function. In this small patient cohort, we were not able to establish differences in local infiltrations or systemic inflammation (data not shown).

In contrast to TNF-α, lysozyme was constitutively expressed under all tested conditions. A lack of IL-10 or absence of microbial stimulation had no effect on the expression of lysozyme in the Pc, suggesting differential regulation of TNF-α and lysozyme. As lysozyme was constitutively expressed in germ free mice, this suggests that the production of lysozyme is largely independent of microbial triggers. However, release of lysozyme has been shown to be dependent on interactions with glycolipid and independent on LPS stimulation.[4, 27] The exact role of microbial induced lysozyme production by Pc therefore remains to be elucidated.

Finally, differences in the regulation of TNF-α expression and lysozyme in the Pc could be explained by potential differences in epigenetic control of the genes encoding for these proteins. A recent paper established the existence of tolerazible genes (including genes encoding for TNF-α) and non-tolerazible genes (e.g. anti-microbial genes).[28] These findings can explain how TNF-α production may become down-regulated under conditions that do not affect lysozyme translation. Although enhanced TNF-α expression is associated with mucosal inflammatory diseases as IBD, celiac disease (this study) and necrotizing enterocolitis[8], it remains to be explored whether Pc derived TNF-α contrib-

utes to intestinal inflammation. A contributing role is supported by our finding that Pc express enhanced levels of TNF-α prior to the development of colitis in IL-10$^{-/-}$ mice. Future studies are needed to expand on our findings and the potential pro-inflammatory role of Pc in mucosal inflammation.

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Chapter 7

Tolerance to LPS is in increased in inflammatory bowel disease

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Submitted

ABSTRACT

Introduction: Despite continuous microbial exposure within the intestine, the mucosal immune system is able to maintain a state of tolerance to the resident microbiota. Impaired immune responses to intestinal microbial products are thought to contribute to inflammatory bowel disease (IBD). Repetitive microbial stimulation of monocytes from healthy individuals leads to tolerance evidenced by a reduced release of inflammatory cytokines. We assessed whether acquisition of innate microbial tolerance in IBD patients differs from healthy controls by analyzing responsiveness of monocytes upon repetitive lipopolysaccharide (LPS) stimulation.

Materials & Methods: Blood-derived monocytes of 20 pediatric patients with inactive IBD (10 Crohn's disease, 10 ulcerative colitis) and 14 pediatric controls were stimulated with various doses of LPS. After 18h the cells were re-stimulated with 10ng/ml LPS to assess the reduction in LPS responsiveness. The production of the pro-inflammatory cytokine TNF-α was determined.

Results: Monocytes from patients with inactive IBD and controls produced comparable levels of TNF-α upon initial LPS stimulation. However, when cells from Crohn's disease and ulcerative colitis patients were primed with low-dose LPS, LPS re-stimulation resulted in considerably lower levels of TNF-α compared to controls.

Conclusion: This study shows that blood-derived monocytes from pediatric IBD patients become more tolerant to LPS upon repetitive stimulation. This intrinsic immune defect may contribute to the etiopathogenesis of IBD.

INTRODUCTION

Innate immune defects have been implicated in the pathogenesis of inflammatory bowel disease (IBD).[1-5] We have shown that in pediatric Crohn's disease patients, epithelial cells derived from the buccal mucosa display enhanced responsiveness to microbial stimulation.[4] On the other hand, dendritic cells derived from the same group of patients did not show any differences in immune responses compared to controls.[6] Similar to most of the published studies, these experiments involved the use of a single microbial stimulus but not repetitive stimulation. As such, it remains unclear whether these experiments represent the more physiological situation of chronic microbial exposure within the intestinal mucosa.

Furthermore, while the cytokine production by immune cells upon microbial stimulation varies widely in controls and IBD patients, absolute concentrations of cytokines are not directly associated with disease activity.[6] Therefore it seems more likely that altered cytokine responses within patients determine the outcome of following immune responses, rather than the absolute value upon a single microbial stimulus.

It is well established that repetitive microbial stimulation of innate cells can lead to a state of non-responsiveness denoted as microbial tolerance.[7-9] Innate defects in IBD may be accounted for by this mechanism of impaired microbial tolerance.

We assessed sensitivity to the induction of lipopolysaccharide (LPS)-tolerance in blood-derived monocytes of IBD patients with inactive disease by applying repetitive LPS stimulation Our data indicate that monocytes from IBD patients display enhanced unresponsiveness to repetitive LPS stimulation compared to controls.

MATERIAL AND METHODS

Patients

A cohort of 19 pediatric IBD patients, containing 10 Crohn's disease (CD) patients and 9 ulcerative colitis patients (UC), was included in this study, as well as 14 healthy control donors (Table 1). The control group consisted of age-matched orthopedic patients in the same age group with no underlying inflammatory or intestinal diseases. The patients were diagnosed with IBD by endoscopy, histo-pathological and clinical characteristics according to the Porto criteria.[10] Disease activity was determined using the PCDAI and PUCAI indexes for Crohn's disease and ulcerative colitis, respectively.[11, 12] Only patients with inactive or mild active disease (PCDAI / PUCAI < 30) and with written parental informed consent were included. Approval for this study was obtained by the local ethics committee.

Table 1, Patient characteristics

	Crohn's disease	Ulcerative Colitis	Controls
Number of patients	10	9	14
Male (n)	6	3	9
Age of Diagnose (y)	13,3 (9-16)	10,3 (3-15)	
Age (y)	15,1 (11-17)	15,2 (15-16)	13,7 (10-18)
PCDAI	6,5 (0-30)		
PUCAI		5,5 (0-20)	
Azathioprine	7	3	0
Mesazaline	0	7	0
Prednisolone	0	1	0
Infliximab	1	0	0
Methotrexate	2	0	0

Isolation of peripheral blood monocytes

Venous blood was collected in EDTA-tubes and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over a Lymphoprep density gradient according to standard protocols. First, 20 ml of whole blood was centrifuged (450G, 10 min, room temperature) to remove plasma. The remaining blood was diluted 1:1 with PBS, placed over 15 ml Lymphoprep, centrifuged (1140G, 20 min, room temperature, without brake) and the lymphocyte-containing interface was collected. After washing with PBS, PBMC were incubated with MACS $CD14^+$ separation beads (Miltenyi) according to manufacturer's protocol. CD14 positive cells were isolated by magnetic separation and washed several times with PBS before use. Purity of monocytes (~95%) was analyzed by flowcytometry.

Flowcytometry

To assess cell purity and viability, cells were incubated with anti-CD16/CD32 (2.4G2) to block FC-receptors, stained with CD14-Fitc (BD) and 7AAD and measured on a FACS Canto II cytometer with FACS Diva software (BD). Data were analyzed using Flowjo software (Tree Star).

Desensitization assay

After isolation, $CD14^+$ cells were incubated in a flatbottom 96-well plate (Falcon, $2x10^5$ cells / 200 ml) in Iscove's Modified Dulbecco's Media (IMDM, Gibco) containing 10% Human serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were incubated at 37°C /5% CO_2 to adhere for 18 hours. Next, adherent monocytes were washed once with medium and stimulated in triplo with or without 0.1 or 10 ng/ml LPS (E.coli, serotype 055:B5, Sigma) for 6h (sensitization phase). Supernatant was collected and stored at -80°C until further analysis. Monocytes were then incubated in fresh medium for another

18h at 37°C, washed and either or not re-stimulated for 6h with a second dose of LPS (10 ng/ml).

Level of tolerance

Production of TNF-α in the supernatant was determined by means of ELISA (BD) according to manufacturer's protocol. The assay has a detection limit of 10 pg/ml. To quantify the level of LPS tolerance, TNF-α production of unprimed monocytes was compared to primed cells by calculating the slope of the TNF-α concentrations in the supernatant after re-stimulation with LPS. We used LOG10 transformed values in order to correct for the variance of TNF-α production.

The slope for each individual was calculated using the formula: y=ax, in which 'a' stands for the slope. Y equals the difference in TNF-α production (LOG10) of unprimed and primed samples. For X the arbitrary value of 2 was used. A steeper slope indicated enhanced LPS tolerance.

Statistical analysis

LPS responses were statistically analyzed with a non-parametric T-test. Correlations between data sets were analyzed with Spearman correlation coefficients. P-values <0.05 were considered significant.

RESULTS

Monocytes from IBD patients and healthy controls respond similar to primary LPS stimulation

We investigated the primary response to LPS stimulation of monocytes from IBD patients and controls. Monocytes were either unprimed or primed with 0.1 or 10 ng/ml LPS for 6 hours and production of TNF-α was determined by means of ELISA. The majority of unprimed monocytes did not produce detectable levels of TNF-α within this time frame (figure 1a). Cells that were primed with LPS showed a range of TNF-α production, confirming earlier reports (figure 1b,c).[2, 5, 6] We conclude that monocytes from IBD patients and controls produced similar amounts of TNF-α upon priming with high- or low-dose LPS.

Monocytes produce low levels of TNF-α upon re-stimulation with LPS

In order to investigate the effect of chronic LPS stimulation on the innate immune response, LPS-primed monocytes from patients and controls were re-stimulated with high-dose LPS (10ng/ml). Monocytes that were unprimed produced high levels of TNF-α upon stimulation with LPS (figure 2a), comparable to levels of TNF-α as shown in figure 1c.

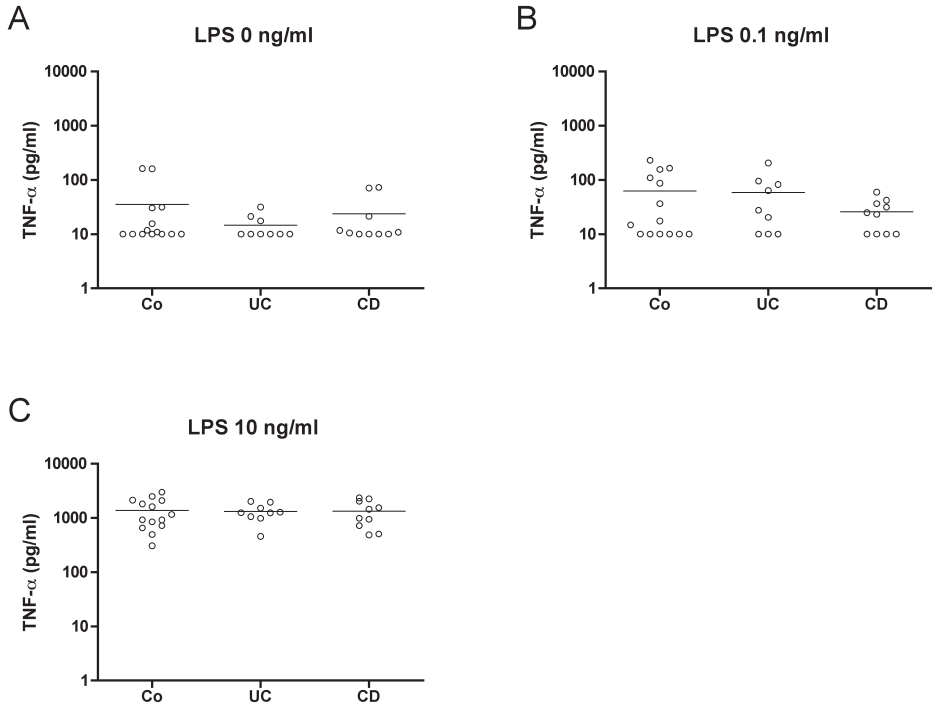

Figure 1. Monocytes from IBD patients and healthy controls respond similar to initial LPS-stimulation TNF-a production of peripheral blood-derived monocytes of controls (Co), Crohn's disease (CD) and ulcerative colitis (UC) patients. TNF-α was determined in the supernatant 6 hours after stimulation without LPS (A), 0.1 ng/ml LPS (B) and 10 ng/ml LPS (C).

In UC patients, priming with low-dose LPS resulted in significantly reduced TNF-α production by monocytes compared to controls ($p<0.05$), whereas TNF-α production in CD patients was not significantly different from controls, although a trend could be observed ($p=0.06$, figure 2b).

In contrast, monocytes that were primed with high-dose LPS were almost insensitive to re-stimulation with LPS, reflected by the lack off or very low amounts of TNF-α production (figure 2c).

These results indicate that priming with LPS of monocytes from patients as well as controls results in a diminished secondary response upon re-stimulation. This effect was most prominent when cells were primed with a high concentration of LPS. Responses to priming with low-dose LPS differed between IBD patients and controls, suggesting enhanced responsiveness upon low-dose LPS in IBD patients.

Monocytes from IBD patients display enhanced LPS-tolerance

The level of TNF-α production by monocytes showed ample variation upon initial LPS stimulation as well as upon re-stimulation. Therefore, comparing the average responses

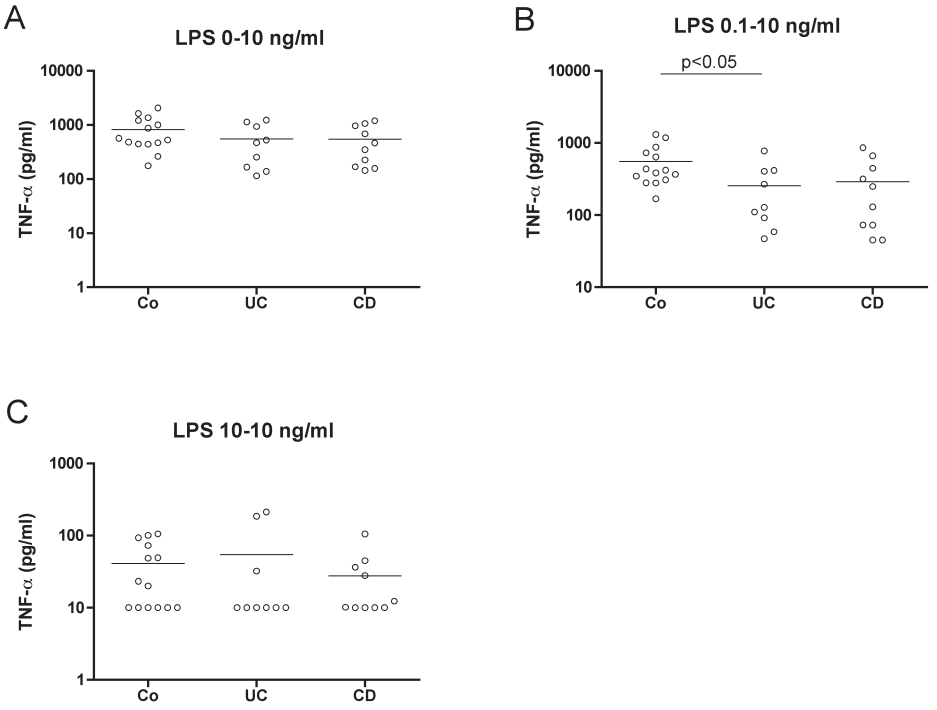

Figure 2. Monocytes produce low levels of TNF-α upon re-stimulation with LPS
TNF-α production of peripheral blood-derived monocytes of controls (Co), Crohn's disease (CD) and ulcerative colitis (UC) patients upon re-stimulation with high-dose LPS. Cells were primed without LPS (A), 0.1 ng/ml LPS (B) and 10 ng/ml LPS (C) for 6 hours. Cells were washed, incubated with fresh medium for 18 hours and re-stimulated in fresh medium for 6 hours with 10 ng/ml LPS. TNF-α was measured in the supernatant 6 hours after the last stimulation with LPS.

per group is not sufficient to elucidate differences between individuals. In order to quantify the level of tolerance for each individual, we determined the relative reduction in TNF-α production by calculating the slope between unprimed and primed monocytes (see Materials and Methods). Steeper slopes represent more robust LPS tolerance. We compared the secondary response upon priming with low- or high-dose LPS, respectively (figure 3).

Cells of CD as well as UC patients that were primed with low-dose LPS, displayed steeper slopes compared to controls ($p<0.01$, figure 3a). However, when cells were primed with high-dose LPS, all individuals displayed comparable slopes and therefore comparable levels of LPS-tolerance (figure 3b).

This indicates that – depending on the dose of LPS at priming – monocytes from IBD patients are less responsive to a secondary LPS stimulus compared to controls.

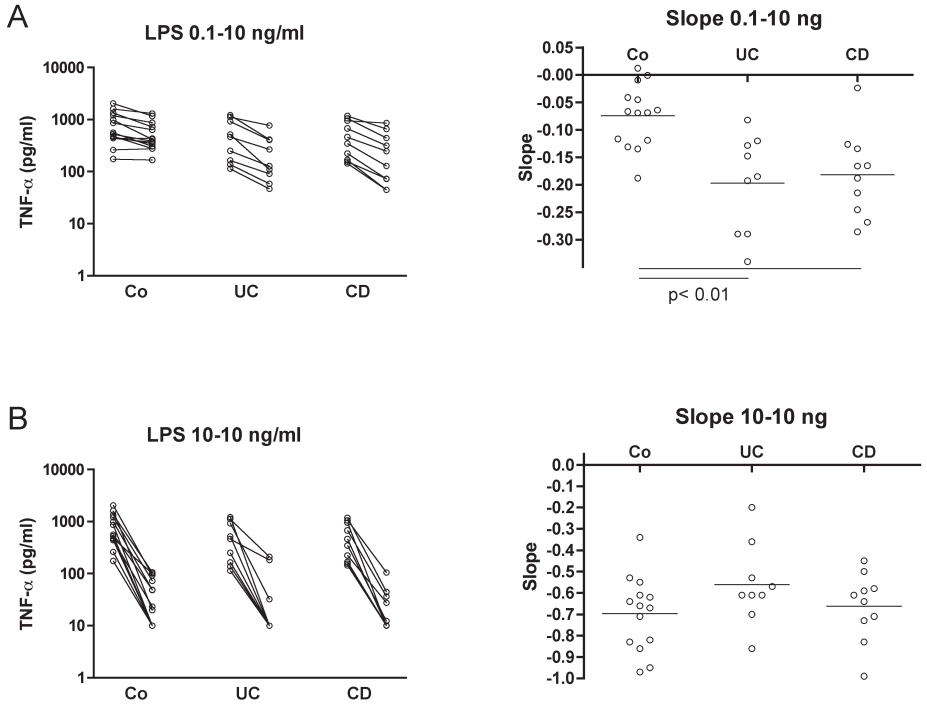

Figure 3. Monocytes from IBD patients display enhanced LPS-tolerance
The level of LPS tolerance was determined by quantifying the extent of diminished TNF-α production. The slope was calculated between TNF-α levels of un-primed and primed cells with low-dose LPS (0.1 ng/ml) (A, left panel) and high-dose LPS (10 ng/ml) (B, left panel) for each patient. All cells were re-stimulated with high-dose LPS (10 ng/ml). The level of tolerance was determined by the steepness of the slope (right panels).

DISCUSSION

Defective interactions between the innate immune system and the intestinal microbiota are strongly implicated in the pathogenesis of IBD.[13, 14] Both augmented as well as diminished responsiveness of innate hematopoietic and non- hematopoietic cells have been proposed as contributors to this mucosal inflammation.[1-5] Interpretation of this apparent paradoxical data is even more complex as most studies concern single cell stimulation assays that seem to overlook the mechanism of innate tolerance that is induced by repetitive stimulation. For example, tolerance to LPS is long known as a physiological strategy to limit inflammatory responses and involves a complex network of regulatory proteins that interfere with pro-inflammatory gene expression.[7, 9]

To address a possible contribution of altered innate tolerance to the IBD pathogenesis we developed a tolerization assay using peripheral blood-derived monocytes. Here we show that monocytes of pediatric IBD patients produce comparable levels of TNF-α upon initial LPS stimulation (priming). Depending on the dose of LPS at priming, the production of TNF-α is diminished in IBD patients as well as in controls upon a secondary stimulus (figure 2). In short, higher doses of LPS in the priming phase led to a steeper decline in TNF-α production upon a secondary challenge.

TNF-α production upon low-dose LPS exposure by monocytes was comparable between the three groups, ranging from 60 to 1300 pg/ml. To be able to quantify the degree of tolerance induction (i.e. desensitization) for LPS on secondary challenge we calculated the slope between the level of TNF-α production of unprimed and primed monocytes for each individual patient. In this way we determined significant differences in LPS responsiveness of individual patients compared to controls, while the average responsiveness was not significantly different. Pediatric IBD patients displayed a steeper slope compared to controls when cells were primed with low-dose LPS (figure 3). These data reveal the subtle differences that may be overlooked in more robust experimental set-up e.g. by using a higher dose of LPS at the sensitization phase. Indeed, when cells were primed with high-dose (10 ng/ml) LPS, monocytes became equally tolerized in both IBD patients and controls.

The patients we included in this study had a disease activity ranging from inactive to mildly active disease based upon PCDAI and PUCAI scores. Most of the patients had inactive disease (Table 1), indicating that the differences in tolerization between IBD patients and controls represent an intrinsic defect. Furthermore, the level of tolerance was irrespective of PCDAI or PUCAI score, IBD subtype (CD vs UC) or clinical inflammatory parameters (CRP and ESR), suggesting that defects are independent of disease activity (data not shown). Within our cohort, patients were treated with different drugs (Table 1). Azathioprine was used in the majority of CD patients, whereas mesazaline was mainly used in UC patients. In our studies we could not associate slopes, which represent individual levels of tolerance, to specific drugs used.

IBD has been associated with various mutations in genes that play a particular role in innate microbial sensing. Intriguingly, monocytes have been shown to become tolerant to TLR2 stimulation by peptidoglycan upon stimulation with the NOD2 ligand muramyl dipeptide. Consequently, defective tolerance to specific TLR2 stimulation was detected in monocytes from CD patients with a homozygous mutation in the CD-associated molecule NOD2 that seems to be in contrast with our findings.[2] Of note, NOD2 mutated cells are incapable of responding to MDP. Furthermore, patients with a homozygous NOD2 mutation are very rare (2%) in our hospitalized pediatric IBD population. We

therefore hypothesize that yet unidentified other gene-defects may be involved in the mechanisms involved in this enhanced LPS tolerance in our tested population.

In this study we showed enhanced tolerance upon repeated LPS stimulation in pediatric IBD patients. Augmented tolerance could potentially lead to a net deficit in antimicrobial defense upon chronic exposure. We hypothesize that this condition may result in disturbed homeostatic interactions between the commensal microbiota and the mucosal immune system.[14] Subsequently, this may result in enhanced local microbial pressure and the activation of adaptive immune cells, including T- and B-lymphocytes, ultimately leading to chronic inflammation.

Interestingly, a recent study showed a similar increased LPS-tolerance in peripheral-derived monocytes from patients with cystic fibrosis. These cells displayed an impaired pro-inflammatory immune response, a high phagocytic phenotype and diminished capacity of antigen presentation upon tolerization. The authors speculate that this locked-up tolerance contributes to the high level of recurrent infections associated with this disease.[15] In contrast with these findings, we did not find differences in the primary response to LPS stimulation (figure 1).

Given the clear differences between these two disease entities we speculate that enhanced ability to become tolerant may represent a broad paradigm that can be associated with various chronic diseases. Although similar (unidentified) gene mutations within these diseases may play a role, it seems more likely that this enhanced tolerance may result from shared elements of these two pathologies. As such, IBD as well as CF may be associated with enhanced and chronic exposure of immune cells to microbes.[16]

Endotoxin tolerance is regulated through the production of a range of regulatory proteins that are produced upon stimulation and interfere with the pro-inflammatory cascade. Other mechanisms of control include epigenetic modifications of genes involved in cellular activation upon microbial stimulation.[17] Future studies in well-defined cellular subsets, derived from specific cohorts of IBD patients, may help elucidate the mechanisms underlying enhanced tolerance in IBD.

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Chapter 8

Subcategorization of quiescent pediatric IBD patients by immune gene expression analysis of peripheral cells

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ABSTRACT

Introduction: To date, optimal treatment of inflammatory bowel disease aims at the induction and preservation of clinical remission. Establishment of remission is confirmed by clinical and laboratory parameters as described in scoring systems both for pediatric ulcerative colitis and pediatric Crohn's disease. A detailed analysis of the immune status during this inactive state of disease may provide a useful tool to subcategorize this phase of the of disease and to potentially refine maintenance treatment in the future.

Methods: By using affymetrix GeneChips, we analysed gene expression profiles of peripheral blood leucocytes from pediatric quiescent inflammatory bowel disease patients and control individuals. We performed (un)supervised clustering analysis of inflammatory bowel disease-associated genes applying Ingenuity® pathway software to identify specific molecular profiles that may enable patient stratification.

Results: Pediatric inflammatory bowel disease patients with inactive disease display heterogeneously distributed gene expression profiles that are significantly distinct from controls. Different clusters of IBD patients displayed specific immunological gene expression profiles and pathways, which were not correlated to medication, diagnosis of ulcerative colitis or Crohn's disease or disease activity. Furthermore, in depth analysis of medication (azathioprine and mesalazine) associated gene profiles revealed distinct response patterns between individual patients.

Conclusion: This study indicates that the immune status of clinical remission in inflammatory bowel disease can be differentiated from that of healthy controls. Moreover, medication associated genetic profiling of inflammatory bowel disease patients reveals differences in expression between patients despite comparable inflammatory parameters. Future analyses may thus allow for patient subcategorization for tailored maintenance therapy.

INTRODUCTION

Inflammatory bowel disease (IBD) is known as a chronic inflammatory disease of the intestine. Although multiple treatment options have been used to diminish inflammation, to date no curative interventions have been described. Therefore, the major aim of treatment is the induction and preservation of clinical remission. Furthermore, it is becoming clear that IBD patients represent a heterogeneous group that may need a novel classification beyond that of Crohn's disease (CD) and ulcerative colitis (UC). As such, disease location, RNA expression, genotype and demographic parameters may help discriminating subsets within this patient population. Given the distinct disease phenotype and associated pathological changes in children compared to adults, pediatric onset disease can be seen as such a specific subset of IBD.[1-4].

Clinical remission in IBD is defined as the absence of symptoms of disease, that is expressed as the pediatric crohn's disease activity index (PCDAI) or pediatric ulcerative colitis activity index (PUCAI). PCDAI or PUCAI scores <10 defines remission and scores from 10-30 represent mild active disease.[5,6] The PCDAI describes the overall disease activity and includes laboratory findings and overall wellbeing, whereas the PUCAI only involves clinical parameters. During remission, inflammatory processes can still be present on a macroscopic as well as a microscopic level.[12] Specifically, subclinical immune activation might still be present despite the absence of clinical symptoms. A detailed analysis of the immune status during quiescent disease may provide a useful tool to subcategorize the type of disease and to potentially refine maintenance treatment in the future.

Inflammation in IBD results from an aberrant immune responses to harmless antigens, including the intestinal microbiota, resulting in mucosal inflammation.[7] In the IBD pathogenesis, defects have been identified ranging from initial microbial recognition by innate immune cells to aberrant activation of T lymphocytes.[8-10] As a consequence, remission may involve various immune mechanisms, such as diminished T helper cell activation and suppressed microbial stimulation of innate cells.[11]

Next, various drugs are prescribed in order to maintain remission. For example, azathioprine is widely used in CD and mesalazine in UC. However, drug responsiveness differs widely between patients. Mutations in the genes encoding for the enzymes that metabolize these drugs have been shown to predict drug responsiveness and toxicity.[13] Consequently, due to differences in drug responsiveness as well as the heterogeneity of the IBD subtypes, it is difficult to identify patients that will or will not respond to specific medication. Although enhanced general inflammatory markers (e.g. CRP and ESR) are associated with imminent exacerbations[14], and severity of disease may be predicted based upon a panel of genetic polymorphisms[15], the usefulness of these parameters for individual patients seems limited.[16]

As such, in depth analysis of the overall RNA expression profiles of the specific genes involved in specific drug responses may help predict drug responsiveness in IBD.

To assess the immune status during remission in pediatric IBD, we analyzed RNA gene expression profiles of peripheral blood leucocytes (PBL) obtained from pediatric IBD patients with inactive to mild active disease and control individuals. By using unsupervised clustering analysis, supervised analysis of expression of IBD-associated genes as well as Ingenuitiy® pathway analysis, we detected marked differences in immunological gene expression when comparing pediatric IBD patients with inactive disease compared to healthy controls. These data indicate that the immune status during clinical remission differs from that of healthy controls. Moreover, detailed analysis of the genetic pathways involved in azathioprine and mesalazine responses revealed two distinct profiles. Expression of associated genes revealed differences between the two groups, suggesting differences in medication responsiveness for each group.

MATERIAL AND METHODS

Patients

A cohort of 45 pediatric IBD remission patients containing 25 Crohn's disease patients and 20 ulcerative colitis patients were included in this study, as well as 14 age matched controls (table 1 patient characteristics). The control group consisted of age-matched orthopedic patients with no underlying inflammatory or intestinal diseases. All IBD patients were diagnosed by endoscopy, histo-pathological and clinical characteristics according to the Porto criteria.[17] Disease activity was determined using the PCDAI and PUCAI indexes for Crohn's disease and ulcerative colitis, respectively.[5, 6] Only patients with inactive or mild active disease (PCDAI / PUCAI < 30) and with a written parental informed consent were included. The majority of patients had a score below 10 (Table 1). Patient approval for this study was obtained by the local ethics committee.

Isolation and Quality Control of RNA

Venous blood (2ml) was collected in PAXgene tubes (PreAnalytiX, Hombrechtikon, Switzerland) and stored at –20°C until RNA extraction. Total cellular RNA was extracted using the PAXgeneTM blood RNA kit (Qiagen, Mississauga, Canada) according to the manufacturer's protocol. RNA levels, quality and purity were assessed with the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent). The samples did not show RNA degradation or contamination with DNA.

Table 1 Patient characteristics

IBD	IBD (n=45)	%		Control (n=14)	%	
total	45			14		
male	19	42.2		8	57.1	
female	26	57.8		6	42.9	
Crohn's disease	25	55.6				
Colonic involvement	18	72.0				
Ulcerative colitis	20	44.4				
	Mean	**Range**	**SD**	**Mean**	**Range**	**SD**
Age (years)	15.7	11-18	1.2	13.7	10-18	2.3
Age CD	15.8	11-18	1.5			
Age UC	15.6	14-17	0.9			
Age of onset (years)	12.1	3-16	3.1			
Age of onset CD	13.1	9-16	2.2			
Age of onset UC	11.2	3-15	3.6			
PCDAI	5.7	0-30	8.8			
PUCAI	5.2	0-30	9.0			
ESR	12.4	2-55	11.2			
ESR CD	14.2	2-55	12.8			
ESR UC	10.7	2-35	9.4			
CRP	2.9	0-17	3.3			
CRP CD	3.7	0-17	4.0			
CRP UC	2.1	1-8	1.9			
Medication CD	**#**	**%**				
Azathioprine	15	65.2				
Inflixmab	2	8.7				
Prednisolone	3	13.0				
Mesalazine	4	17.4				
Methotrexate	5	21.7				
None	1	4.3				
Medication UC	**#**	**%**				
Azathioprine	5	23.8				
Inflixmab	0	0.0				
Prednisolone	1	4.8				
Salazopurine	3	14.3				
Mesalazine	16	76.2				
Methotrexate	0	0.0				
None	1	4.8				

Gene Expression Profiling and Quality Control

Samples were analyzed by Affymetrix U133 2.0 plus GeneChips as described in.[18] The GeneChip contains 54,675 probe sets, representing approximately 39,000 genes. We used 10 µg of total RNA to prepare antisense biotinylated RNA. Single-stranded complementary DNA (cDNA) and double-stranded cDNA was synthesized according to the manufacturer's protocol (Invitrogen Life Technologies) with the use of the T7-(deoxythymidine)24-primer (Genset). In vitro transcription was performed with biotin-11-cytidine triphosphate and biotin-16-uridine triphosphate (Perkin–Elmer) and the MEGAScript T7 labeling kit (Ambion). Double-stranded cDNA and complementary RNA (cRNA) was purified and fragmented with the GeneChip Sample Cleanup Module (Affymetrix). Biotinylated RNA was hybridized to the GeneChip (45°C for 16 hours). Staining, washing, and scanning procedures were carried out according to the manufacturer's protocol (Affymetrix). All GeneChips were visually inspected for irregularities. The R package affyQCreport was used for quality control and indicated high quality and overall comparability of samples.

Data Normalization, Statistical Analysis, and Visualization

Data was normalized as previously described.[18] In short, scanned array images of all samples were normalized. To visualize the clustering of the samples principal component analysis (PCA) was used. The normalized datafile was transposed and imported into Biowisdom/OmniViz version 6.0.3 (Biowisdom, Inc) for further analysis.

Group analysis was performed using Pearsons Rank correlation, student's T-test, One –way ANOVA and Chi-square test when appropriate. A p value <0.05 was considered significant.

Unsupervised cluster analysis was performed in Biowisdom/Omniviz using Pearson's correlation in the Correlation View. Supervised analysis was based on SAM (Significance Analysis of Microarrays).[19] Cut-offs values for significantly expressed genes were chosen based on the false discovery rate (FDR) and the fold change (FC). Functional annotation of the SAM results was done using Ingenuity® Pathway Analysis (Ingenuity, Mountain View, CA)

RESULTS

Gene expression profiles of quiescent pediatric IBD patients are heterogeneously distributed and independent of CD or UC diagnosis

We analyzed the overall gene expression profiles of PBL using PCA. Samples that display comparable expression profiles show clustering in the PCA. Figure 1 illustrates the PCA of controls and pediatric IBD patients, in which control samples appear in a separate

cluster (blue) and IBD patients show heterogeneous distribution. CD (red) and UC (green) patients with quiescent disease did not cluster separately based on overall gene expression profiles.

These results indicate that, based upon overall gene expression profiles, IBD patients show distinct profiles compared to controls. Although the phenotype of CD and UC patients differed, sub-classification between CD and UC was not seen, indicating overlap in gene expression profiles between these two subsets.

IBD patients can be stratified based on 3-fold difference in gene expression level relative to average levels

With the use of unsupervised ordering, samples were classified into subgroups that identified subsets of IBD patients. We generated a Pearson's correlation plot that included

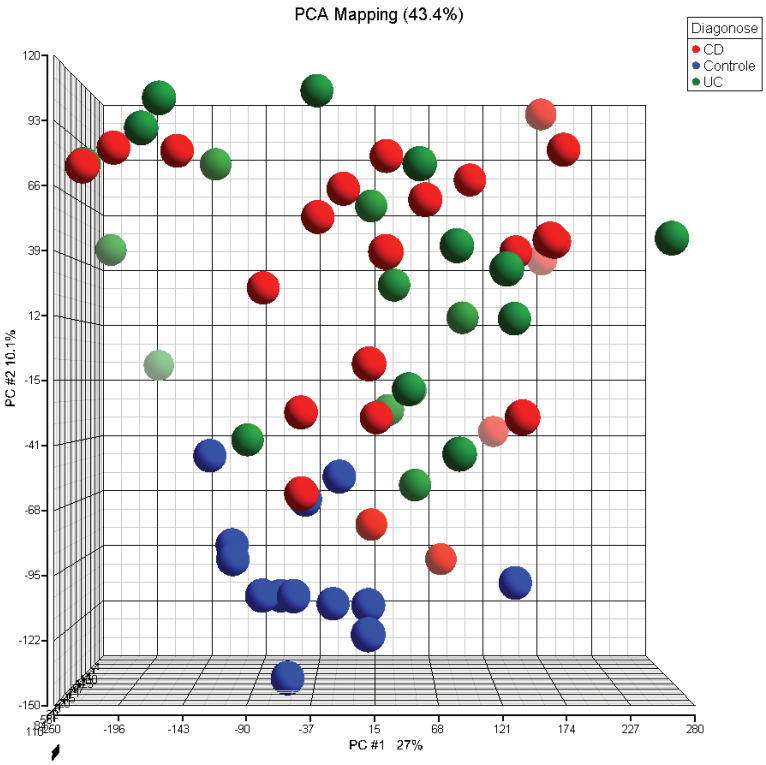

Figure 1. Gene expression profiles of quiescent pediatric IBD patients
General gene expression profile of PBL was analyzed using principal components analysis (PCA). Samples that display a comparable expression profile show clustering on the PCA. Control samples appear in a separate cluster (blue), whereas IBD study patients appear in a heterogeneous distribution. CD (red) and UC (green) patients with quiescent disease could not be separated based on overall gene expression profiles.

probe sets with a 3-fold difference in gene expression level relative to the geometric mean, reflecting up- or down-regulation (figure 2). In total, 2957 records met these criteria and correlation between the samples was analyzed on the basis of similarities in gene expression profiles. Samples that show positive correlation in gene expression profiling are indicated in red, whereas negative correlation between samples are illustrated in blue. This Pearson's correlation plot revealed three distinct groups, which we annotated as group A, B and C. All control samples were located in group A, whereas IBD patients were heterogeneously distributed over all three groups. The clustering of patients was not due to a specific disease-related parameter as we did not observe any correlation with diagnosis of CD or UC, severity of disease as determined by PCDAI or PUCAI, age of

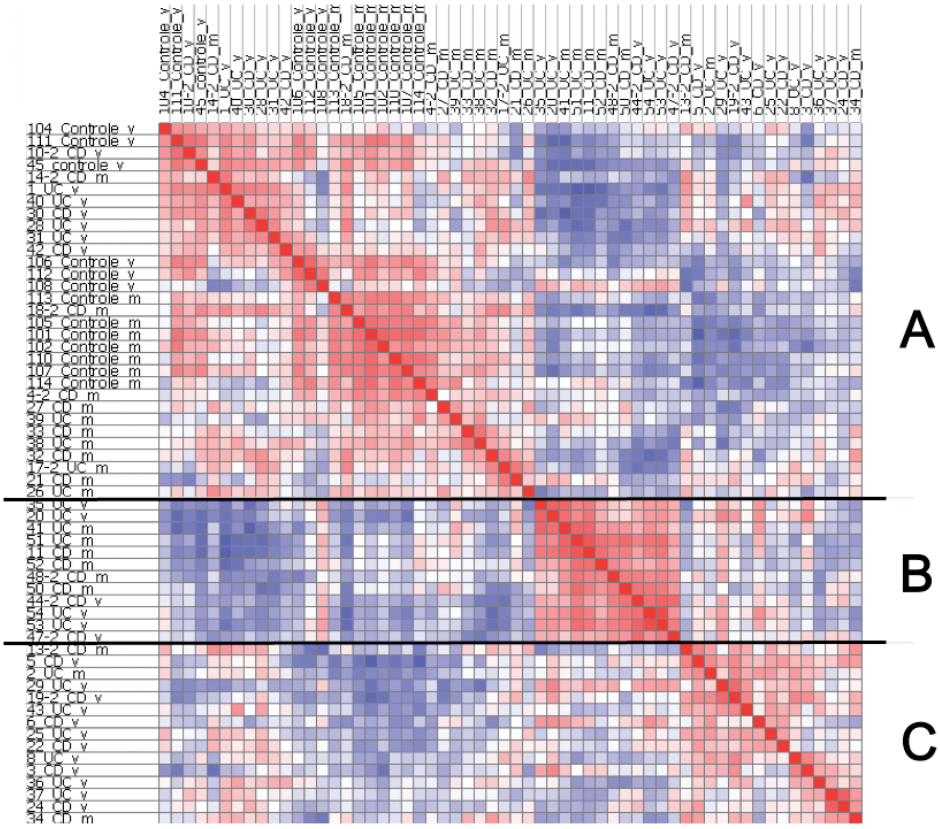

Figure 2. Correlation view of quiescent IBD patients involving 2957 probe sets.
The correlation visualization displays pair wise correlations between the samples. The colors of the cells relate to Pearson's correlation coefficient values, with deeper colors indicating higher positive (red) and negative (blue) correlations. This Pearson's correlation plot revealed three distinct groups (A, B, C). Group A, containing controls and IBD patients, and group B and C, which both contain solely IBD patients.

onset, CRP, ESR, or medication. However, group C contained significantly more women compared to groups A and B (p=0.018, Suppl. table 1).

These results indicate that the IBD population of patients with clinically inactive disease can be subdivided into three groups with distinct gene expression profiles. This distinction of groups A,B and C is made on expression of genes irrespective of function. CD and UC patients are equally distributed and only part of the IBD patients are comparable to controls.

Different clusters of quiescent IBD patients display specific immunological gene expression profiles

As quiescent IBD patients displayed differential gene expression profiles compared to non-diseased controls and the immune system has been shown to play an essential role in IBD pathogenesis, we analyzed the expression of IBD-related immune genes (suppl table 2) in the three groups in a supervised manner.

First, we determined for each group probe sets differences with a >1.5 fold higher or lower expression compared to controls. Next we determined which of these probes sets were significantly different between IBD patients and controls by SAM analysis. The IBD patients in group A displayed significantly different expression for 31 immune-associated probe sets, whereas group B and C contained 151 and 161 probe sets, respectively. Table 2 shows the immune-related genes that were SAM significant for each group compared to controls.

This analysis indicates that within the three selected groups there is a distinction from controls on the basis of immune profile. Although the majority of IBD patients are in clinical remission, IBD associated genes were found to be active and differed significantly from controls.

In order to investigate which immunological genes were significantly expressed for each group, we analyzed significant differences in the immunological gene expression between all groups and controls. We found that for group A, only two immunological probe sets were differentially expressed compared to controls and the two other groups. Group B displayed specific gene expression for 75 probe sets, whereas in group C 26 probe sets were distinct from controls as well as from the two other groups (table 3).

These results indicate that although the clinical phenotype of IBD patients and controls is comparable, there are still significant differences in immunological gene expression between IBD patients and controls as well as among IBD patients. The correlation plot as depicted in figure 2 identified three groups based upon differences in overall gene expression profiles. Further supervised analysis demonstrated that these groups also differed in the expression of immunological genes that are associated with IBD.

Table 2A, Top up-regulated genes IBD vs Control

	Top Up-regulated		
A vs Control	**Symbol**	**Entrez Gene Name**	**Log Ratio**
	BCL2L1	BCL2-like 1	1,122
	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	0,799
	MUC5AC	mucin 5AC, oligomeric mucus/gel-forming	0,716
	SOD2	superoxide dismutase 2, mitochondrial	0,715
	CSTA	cystatin A (stefin A)	0,634
	PIK3C3	phosphoinositide-3-kinase, class 3	0,631
	SOS2	son of sevenless homolog 2 (Drosophila)	0,620

	Top Up-regulated		
B vs Control	**Symbol**	**Entrez Gene Name**	**Log Ratio**
	POLR2K	polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	2,424
	IL8	interleukin 8	2,352
	IFI44	interferon-induced protein 44	2,254
	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	2,062
	CSTA	cystatin A (stefin A)	2,018
	IFNGR1	interferon gamma receptor 1	1,857
	ANXA1	annexin A1	1,815
	HMGB1 (includes EG:3146)	high-mobility group box 1	1,786
	NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	1,752
	PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	1,665
	LRRK2	leucine-rich repeat kinase 2	1,526
	TGFBR1	transforming growth factor, beta receptor 1	1,515
	IL15	interleukin 15	1,510
	ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1,460
	JAK2	Janus kinase 2	1,440
	TAF7	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 55kDa	1,428
	TLR4	toll-like receptor 4	1,390
	PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	1,388
	NRIP1	nuclear receptor interacting protein 1	1,372
	PRKACB	protein kinase, cAMP-dependent, catalytic, beta	1,336
	MAP3K7	mitogen-activated protein kinase kinase kinase 7	1,332
	HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	1,308
	TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa	1,295
	ZNF91	zinc finger protein 91	1,287
	SRGN	serglycin	1,263
	MAPK6	mitogen-activated protein kinase 6	1,248

Table 2A (continued)

SLC26A2	solute carrier family 26 (sulfate transporter), member 2	1,246
S100P	S100 calcium binding protein P	1,209
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	1,202
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	1,197
LYZ	lysozyme (renal amyloidosis)	1,191
KAT2B	K(lysine) acetyltransferase 2B	1,171
HSPA14	heat shock 70kDa protein 14	1,139
STAT1	signal transducer and activator of transcription 1, 91kDa	1,124
CREB1	cAMP responsive element binding protein 1	1,122
HLTF	helicase-like transcription factor	1,119
GTF2B	general transcription factor IIB	1,050
SLPI	secretory leukocyte peptidase inhibitor	1,001
TAF2	TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 150kDa	0,953
ACAT1	acetyl-Coenzyme A acetyltransferase 1	0,948
NCOA2	nuclear receptor coactivator 2	0,947
PTGER4	prostaglandin E receptor 4 (subtype EP4)	0,934
RRAS2	related RAS viral (r-ras) oncogene homolog 2	0,912
GTF2H5	general transcription factor IIH, polypeptide 5	0,910
GTF2A1	general transcription factor IIA, 1, 19/37kDa	0,909
POLR2B	polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	0,897
MAPK8	mitogen-activated protein kinase 8	0,887
PPP3CA	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	0,887
SMAD4	SMAD family member 4	0,862
MED14	mediator complex subunit 14	0,838
GTF2E1	general transcription factor IIE, polypeptide 1, alpha 56kDa	0,831
TAF5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa	0,827
PTPN2	protein tyrosine phosphatase, non-receptor type 2	0,826
TAF9B	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa	0,826
SOD2	superoxide dismutase 2, mitochondrial	0,809
TAF1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa	0,796
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	0,749
C13ORF31	chromosome 13 open reading frame 31	0,748
TLR1	toll-like receptor 1	0,738
HLA-DRA	major histocompatibility complex, class II, DR alpha	0,728
S100A9	S100 calcium binding protein A9	0,723
ISG15	ISG15 ubiquitin-like modifier	0,721
FEZ2	fasciculation and elongation protein zeta 2 (zygin II)	0,715

Table 2A (continued)

	WDR92	WD repeat domain 92	0,712
	SRI	sorcin	0,711
	MAP2K4	mitogen-activated protein kinase kinase 4	0,704
	PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	0,701
	IFI16	interferon, gamma-inducible protein 16	0,699
	SMAD2	SMAD family member 2	0,697
	BTN2A1	butyrophilin, subfamily 2, member A1	0,690
	GTF2H2	general transcription factor IIH, polypeptide 2, 44kDa	0,687
	SLC22A4 (includes EG:6583)	solute carrier family 22 (organic cation/ergothioneine transporter), member 4	0,681
	SOS2	son of sevenless homolog 2 (Drosophila)	0,665
	TAF12 (includes EG:6883)	TAF12 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 20kDa	0,664
	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	0,661
	SUMO1	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)	0,655
	HLA-DRB1	major histocompatibility complex, class II, DR beta 1	0,653
	FGFR1OP (includes EG:11116)	FGFR1 oncogene partner	0,646
	MAPK14	mitogen-activated protein kinase 14	0,642
	KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	0,631
	TAF1A	TATA box binding protein (TBP)-associated factor, RNA polymerase I, A, 48kDa	0,630
	SUMO4	SMT3 suppressor of mif two 3 homolog 4 (S. cerevisiae)	0,627
	PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	0,615
	CCNH	cyclin H	0,611
	PPP2CA	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0,595

Top Up-regulated

C vs Control	**Symbol**	**Entrez Gene Name**	**Log Ratio**
	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	2,126
	CXCL10	chemokine (C-X-C motif) ligand 10	1,944
	CSTA	cystatin A (stefin A)	1,810
	BCL2L1	BCL2-like 1	1,578
	S100P	S100 calcium binding protein P	1,522
	IL1R2	interleukin 1 receptor, type II	1,382
	KAT2B	K(lysine) acetyltransferase 2B	1,278
	CCL2	chemokine (C-C motif) ligand 2	1,215
	IFNGR1	interferon gamma receptor 1	1,210
	IFI44	interferon-induced protein 44	1,189
	TLR5	toll-like receptor 5	1,180

Table 2A (continued)

PBX1	pre-B-cell leukemia homeobox 1	1,173
SLC22A4 (includes EG:6583)	solute carrier family 22 (organic cation/ergothioneine transporter), member 4	1,167
POLR2K	polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	1,163
CCL8	chemokine (C-C motif) ligand 8	1,118
FKBP5	FK506 binding protein 5	1,104
MAPK14	mitogen-activated protein kinase 14	1,094
SRGN	serglycin	1,087
ITLN1	intelectin 1 (galactofuranose binding)	1,078
ANXA1	annexin A1	1,061
WDR92	WD repeat domain 92	1,058
JAK2	Janus kinase 2	1,010
LYZ	lysozyme (renal amyloidosis)	0,994
CD163	CD163 molecule	0,972
S100A9	S100 calcium binding protein A9	0,972
TLR4	toll-like receptor 4	0,965
GYPB	glycophorin B (MNS blood group)	0,960
IL1RN	interleukin 1 receptor antagonist	0,942
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	0,869
STAT1	signal transducer and activator of transcription 1, 91kDa	0,868
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	0,823
CA4	carbonic anhydrase IV	0,819
SLPI	secretory leukocyte peptidase inhibitor	0,804
HMGB1 (includes EG:3146)	high-mobility group box 1	0,791
SELENBP1	selenium binding protein 1	0,755
MXI1	MAX interactor 1	0,749
IL15	interleukin 15	0,712
SOS2	son of sevenless homolog 2 (Drosophila)	0,712
CHP	calcium binding protein P22	0,700
SOD2	superoxide dismutase 2, mitochondrial	0,694
FHL2	four and a half LIM domains 2	0,671
MT1H	metallothionein 1H	0,668
TAF7	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 55kDa	0,636
LRRK2	leucine-rich repeat kinase 2	0,633
STAT5B	signal transducer and activator of transcription 5B	0,632
GTF2H5	general transcription factor IIH, polypeptide 5	0,631
MAP3K1	mitogen-activated protein kinase kinase kinase 1	0,620
IL18RAP	interleukin 18 receptor accessory protein	0,606

Table 2A (continued)

	TTLL3	tubulin tyrosine ligase-like family, member 3	0,602

Table 2B, Top down-regulated genes IBD vs Control

A vs Control	Symbol	Entrez Gene Name	Log Ratio
	NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	-1,072
	TRA@	T cell receptor alpha locus	-0,931
	TRD@	T cell receptor delta locus	-0,908
	CD3G	CD3g molecule, gamma (CD3-TCR complex)	-0,889
	GNAS	GNAS complex locus	-0,878
	SLC26A2	solute carrier family 26 (sulfate transporter), member 2	-0,864
	PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	-0,845
	C11ORF30	chromosome 11 open reading frame 30	-0,839
	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	-0,825
	ZNF91	zinc finger protein 91	-0,790
	NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	-0,769
	CCL5	chemokine (C-C motif) ligand 5	-0,760
	EP300	E1A binding protein p300	-0,759
	TAF3	TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 140kDa	-0,745
	MUC8	mucin 8	-0,699
	CD247	CD247 molecule	-0,663
	TGFBR1	transforming growth factor, beta receptor 1	-0,645
	CCR5	chemokine (C-C motif) receptor 5	-0,609
	SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	-0,606
	KAT2B	K(lysine) acetyltransferase 2B	-0,597
	CUX1	cut-like homeobox 1	-0,591
	SLC20A1	solute carrier family 20 (phosphate transporter), member 1	-0,589

Top Down-regulated

B vs Control	Symbol	Entrez Gene Name	Log Ratio
	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	-1,745
	HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	-0,882
	NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	-0,870
	MUC8	mucin 8	-0,790
	CCL5	chemokine (C-C motif) ligand 5	-0,756
	GNAS	GNAS complex locus	-0,726
	NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	-0,714
	POU2F1	POU class 2 homeobox 1	-0,712
	SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	-0,698

Table 2B, continued

	MAP2K7	mitogen-activated protein kinase kinase 7	-0,692
	C11ORF30	chromosome 11 open reading frame 30	-0,662
	ATG16L1	ATG16 autophagy related 16-like 1 (S. cerevisiae)	-0,647
	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	-0,633
	NCOR2	nuclear receptor co-repressor 2	-0,615
	NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	-0,600
	NCOA1	nuclear receptor coactivator 1	-0,592
	TAF11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	-0,587

Top Down-regulated

C vs Control	Symbol	Entrez Gene Name	Log Ratio
	NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	-1,459
	NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	-1,395
	CD3G	CD3g molecule, gamma (CD3-TCR complex)	-1,225
	GNAS	GNAS complex locus	-1,219
	CCL5	chemokine (C-C motif) ligand 5	-1,141
	C11ORF30	chromosome 11 open reading frame 30	-1,117
	CD247	CD247 molecule	-1,112
	PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	-1,081
	SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	-1,068
	TAF3	TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 140kDa	-1,025
	TGFBR1	transforming growth factor, beta receptor 1	-1,008
	TRA@	T cell receptor alpha locus	-0,978
	UBE2I	ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)	-0,944
	EP300	E1A binding protein p300	-0,942
	SLC26A2	solute carrier family 26 (sulfate transporter), member 2	-0,922
	NCOR2	nuclear receptor co-repressor 2	-0,916
	SLC20A1	solute carrier family 20 (phosphate transporter), member 1	-0,916
	TAF11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	-0,888
	NFATC1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	-0,883
	ZNF91	zinc finger protein 91	-0,874
	TRD@	T cell receptor delta locus	-0,861
	IL16	interleukin 16 (lymphocyte chemoattractant factor)	-0,860
	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	-0,839
	IGL@	immunoglobulin lambda locus	-0,808
	HLA-DQB1	major histocompatibility complex, class II, DQ beta 1	-0,803
	NCOA1	nuclear receptor coactivator 1	-0,803

Table 2B, continued

POU2F1	POU class 2 homeobox 1	-0,801
CFD	complement factor D (adipsin)	-0,798
PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide	-0,792
C5ORF56	chromosome 5 open reading frame 56	-0,782
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1	-0,767
MUC8	mucin 8	-0,763
MED1	mediator complex subunit 1	-0,762
IGH@	immunoglobulin heavy locus	-0,743
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	-0,733
CXCL5	chemokine (C-X-C motif) ligand 5	-0,712
SMAD4	SMAD family member 4	-0,701
CUX1	cut-like homeobox 1	-0,698
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	-0,698
JAK1	Janus kinase 1	-0,679
PIK3CD	phosphoinositide-3-kinase, catalytic, delta polypeptide	-0,669
NOLC1	nucleolar and coiled-body phosphoprotein 1	-0,668
RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	-0,665
GUSB	glucuronidase, beta	-0,661
SMAD3	SMAD family member 3	-0,660
IL12RB1	interleukin 12 receptor, beta 1	-0,647
MAP3K14	mitogen-activated protein kinase kinase kinase 14	-0,635
IL32	interleukin 32	-0,633
HLA-DOA	major histocompatibility complex, class II, DO alpha	-0,628
SMARCA2	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2	-0,627
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	-0,622
CREBBP	CREB binding protein	-0,621
CCR5	chemokine (C-C motif) receptor 5	-0,618
CD14	CD14 molecule	-0,601
SCAP	SREBF chaperone	-0,589
POLR2A	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa	-0,587
ZGPAT	zinc finger, CCCH-type with G patch domain	-0,586

Table 3A, Top up-regulated genes Group vs Control AND Groups

A vs Control AND Group B,C	Symbol	Entrez Gene Name	Log Ratio
	FCGR1A	Fc fragment of IgG, high affinity Ia, receptor (CD64)	0,799
	CSTA	cystatin A (stefin A)	0,634

Top Up-Regultated

B vs Control AND Group A,C	Symbol	Entrez Gene Name	Log Ratio
	POLR2K	polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	2,424
	IL8	interleukin 8	2,352
	IFI44	interferon-induced protein 44	2,254
	IFNGR1	interferon gamma receptor 1	1,857
	ANXA1	annexin A1	1,815
	HMGB1 (includes EG:3146)	high-mobility group box 1	1,786
	NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	1,752
	PIK3C2A	phosphoinositide-3-kinase, class 2, alpha polypeptide	1,665
	LRRK2	leucine-rich repeat kinase 2	1,526
	TGFBR1	transforming growth factor, beta receptor 1	1,515
	IL15	interleukin 15	1,510
	ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	1,460
	TAF7	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 55kDa	1,428
	PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	1,388
	NRIP1	nuclear receptor interacting protein 1	1,372
	PRKACB	protein kinase, cAMP-dependent, catalytic, beta	1,336
	MAP3K7	mitogen-activated protein kinase kinase kinase 7	1,332
	HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	1,308
	TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa	1,295
	ZNF91	zinc finger protein 91	1,287
	MAPK6	mitogen-activated protein kinase 6	1,248
	SLC26A2	solute carrier family 26 (sulfate transporter), member 2	1,246
	NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	1,202
	NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	1,197
	HSPA14	heat shock 70kDa protein 14	1,139
	CREB1	cAMP responsive element binding protein 1	1,122
	HLTF	helicase-like transcription factor	1,119
	GTF2B	general transcription factor IIB	1,050
	TAF2	TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 150kDa	0,953

Table 3A, continued

	ACAT1	acetyl-Coenzyme A acetyltransferase 1	0,948
	PTGER4	prostaglandin E receptor 4 (subtype EP4)	0,934
	RRAS2	related RAS viral (r-ras) oncogene homolog 2	0,912
	GTF2A1	general transcription factor IIA, 1, 19/37kDa	0,909
	POLR2B	polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	0,897
	MAPK8	mitogen-activated protein kinase 8	0,887
	SMAD4	SMAD family member 4	0,862
	MED14	mediator complex subunit 14	0,838
	GTF2E1	general transcription factor IIE, polypeptide 1, alpha 56kDa	0,831
	TAF5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa	0,827
	TAF9B	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa	0,826
	PTPN2	protein tyrosine phosphatase, non-receptor type 2	0,826
	TAF1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa	0,796
	HLA-DRA	major histocompatibility complex, class II, DR alpha	0,728
	FEZ2	fasciculation and elongation protein zeta 2 (zygin II)	0,715
	SRI	sorcin	0,711
	PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	0,701
	BTN2A1	butyrophilin, subfamily 2, member A1	0,690
	TAF12 (includes EG:6883)	TAF12 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 20kDa	0,664
	HLA-DRB1	major histocompatibility complex, class II, DR beta 1	0,653
	MAP2K4	mitogen-activated protein kinase kinase 4	0,638
	TAF1A	TATA box binding protein (TBP)-associated factor, RNA polymerase I, A, 48kDa	0,630
	SMAD2	SMAD family member 2	0,606
	C11ORF30	chromosome 11 open reading frame 30	0,605
	PPP2CA	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	0,595

Top Up-Regultated

C vs Control AND Group A,B	**Symbol**	**Entrez Gene Name**	**Log Ratio**
	IL1R2	interleukin 1 receptor, type II	1,382
	CCL2	chemokine (C-C motif) ligand 2	1,215
	IFNGR1	interferon gamma receptor 1	1,210
	IFI44	interferon-induced protein 44	1,189
	TLR5	toll-like receptor 5	1,180
	PBX1	pre-B-cell leukemia homeobox 1	1,173
	POLR2K	polymerase (RNA) II (DNA directed) polypeptide K, 7.0kDa	1,163
	FKBP5	FK506 binding protein 5	1,104

Table 3A, continued

ITLN1	intelectin 1 (galactofuranose binding)	1,078
ANXA1	annexin A1	1,061
CD163	CD163 molecule	0,972
MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	0,869
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	0,823
HMGB1 (includes EG:3146)	high-mobility group box 1	0,791
MXI1	MAX interactor 1	0,749
TGFBR1	transforming growth factor, beta receptor 1	0,749
IL15	interleukin 15	0,712
TAF7	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 55kDa	0,636
LRRK2	leucine-rich repeat kinase 2	0,633

Table 3B, Top down-regulated Group vs Control AND Groups

	Top Down-regulated Symbol	**Entrez Gene Name**	**Log Ratio**
A vs Control AND Group B,C	None detected		
	Top Down-regulated Symbol	**Entrez Gene Name**	**Log Ratio**
B vs Control AND Group A,C	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	-1,745
	Top Down-regulated Symbol	**Entrez Gene Name**	**Log Ratio**
C vs Control AND Group A,B	HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	-0,839
	TRA@	T cell receptor alpha locus	-0,978
	NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3	-1,395

Ingenuity pathway analysis reveals group-specific involvement of immunological pathways

In our IBD population multiple IBD-associated genes showed a significantly different expression as compared with controls as well as between groups (table 2 and 3). In order to investigate the possible interaction of these different immunological genes, we ana-

Table 4 Top canonical pathway Group A/B/C vs Control

	Name	p-value	ratio
A vs Control	Glucocorticoid Receptor Signaling	1.01E-23	16/280 (0,057)
	Role of NFAT in Regulation of the Immune Response	1.75E-16	11/194 (0,057)
	Systemic Lupus Erythematosus Signaling	1.11E-15	10/150 (0,067)
	iCOS-iCOSL Signaling in T Helper Cells	6.51E-13	8/115 (0,07)
	T Cell Receptor Signaling	7.77E-13	8/110 (0,073)
B vs Control	Glucocorticoid Receptor Signaling	9.26E-92	61/280 (0,218)
	Estrogen Receptor Signaling	1.46E-42	29/121 (0,24)
	Colorectal Cancer Metastasis Signaling	3.56E-22	22/247 (0,089)
	IL-4 Signaling	2.63E-21	15/72 (0,208)
	B Cell Receptor Signaling	1.97E-20	18/155 (0,116)
C vs Control	Glucocorticoid Receptor Signaling	5.66E-72	52/280 (0,186)
	IL-4 Signaling	2.65E-19	14/72 (0,194)
	Dendritic Cell Maturation	1.60E-18	17/173 (0,098)
	RAR Activation	1.26E-17	17/178 (0,096)
	Systemic Lupus Erythematosus Signaling	1.41E-16	15/150 (0,1)

lyzed which related immunological pathways were associated with the immunological genes for each group. Therefore, IBD associated genes that were expressed differently between groups (A,B and C) and controls (table 2) were analyzed by Ingenuity pathway analysis. Pathways for each group that were distinct from controls are depicted in table 4 and involved immunological pathways at all levels of the immune cascade.

In all groups, genes of the glucocorticoid receptor signalling pathway showed different expression compared with controls. In the two groups B and C, IL-4 signalling was one of the pathways that differed strongly from the control group.

Next, to study which pathways were most distinctive for each group individually, we analyzed the associated immune pathways from table 3. Associated immunological pathways that were distinct between the three IBD groups are listed in table 5. Again, the glucocorticoid receptor signalling pathway was one of the significant pathways that was distinct for each group.

This in depth Ingenuity pathway analysis indicated that in clinical IBD remission patients, active immune processes are still present that can be clustered into two different subsets.

Table 5 Top canonical pathways Group A/B/C vs Group and Controls

	Name	p-value	ratio
A vs B,C and controls	Role of PKR in Interferon Induction and Antiviral Response	4.05E-3	1/46 (0,022)
	Fcg Receptor-mediated Phagocytosis in Macrophages and Monocytes	9.21E-3	1/104 (0,01)
	Systemic Lupus Erythematosus Signaling	1.24E-2	1/150 (0,007)
	Dendritic Cell Maturation	1.44E-2	1/173 (0,006)
	Role of NFAT in Regulation of the Immune Response	1.59E-2	1/194 (0,005)
B vs A,C and controls	Glucocorticoid Receptor Signaling	1.89E-51	34/280 (0,121)
	Estrogen Receptor Signaling	9.51E-28	18/121 (0,149)
	Colorectal Cancer Metastasis Signaling	4.28E-14	13/247 (0,053)
	HMGB1 Signaling	8.82E-14	10/98 (0,102)
	LPS-stimulated MAPK Signaling	3.98E-13	9/80 (0,112)
C vs A,B and controls	Glucocorticoid Receptor Signaling	5.41E-18	12/280 (0,043)
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.37E-7	5/135 (0,037)
	Communication between Innate and Adaptive Immune Cells	5.03E-5	3/90 (0,033)
	LXR/RXR Activation	5.51E-5	3/86 (0,035)
	Colorectal Cancer Metastasis Signaling	9.92E-5	4/247 (0,016)

IBD patient variation of RNA gene expression patterns involved in drug responsiveness

As we found differences in immunological genetic pathways between patients and medication-responsiveness varies widely between patients, we wanted to investigate whether heterogeneity existed in the patient group treated with a particular anti-inflammatory drug. For this purpose we analyzed RNA expression profiles of patients treated with azathioprine or mesalazine (Table 6, 7). The two drugs are widely used in the treatment of CD and UC respectively.

Table 6 Azathioprine associated genes

Gene Title	Gene Symbol	Chromosomal Location
ATP citrate lyase	ACLY	chr17q12-q21
ATPase, class II, type 9A	ATP9A	chr20q13.2
calreticulin	CALR	chr19p13.3-p13.2
CD1c molecule	CD1C	chr1q22-q23
CD27 molecule	CD27	chr12p13
GTPase activating protein (SH3 domain) binding protein 2	G3BP2	chr4q21.1
hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	HPRT1	chrXq26.1
integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	ITGA4	chr2q31.3

Table 6 (continued)

Gene Title	Gene Symbol	Chromosomal Location
interleukin 21 receptor	IL21R	chr16p11
lactate dehydrogenase A	LDHA	chr11p15.4
lactate dehydrogenase B	LDHB	chr12p12.2-p12.1
lactate dehydrogenase B	LDHB	chr12p12.2-p12.1
lactate dehydrogenase C	LDHC	chr11p15.5-p15.3
MYC-associated zinc finger protein (purine-binding transcription factor)	MAZ	chr16p11.2
Phosphoribosyl pyrophosphate amidotransferase	PPAT	chr4q12
platelet-derived growth factor alpha polypeptide	PDGFA	chr7p22
tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	chr3q26

Table 7, Mesalazine associated genes

Gene Title	Gene Symbol	Chromosomal Location
acyl-CoA synthetase short-chain family member 2	ACSS2	chr20q11.22
alkaline phosphatase, intestinal	ALPI	chr2q37.1
alkaline phosphatase, liver/bone/kidney	ALPL	chr1p36.12
alkaline phosphatase, placental (Regan isozyme)	ALPP /// ALPPL2	chr2q37
alkaline phosphatase, placental-like 2	ALPPL2	chr2q37
arachidonate 12-lipoxygenase	ALOX12	chr17p13.1
arachidonate 5-lipoxygenase	ALOX5	chr10q11.2
cartilage intermediate layer protein 2	CILP2	chr19p13.11
cartilage intermediate layer protein, nucleotide pyrophosphohydrolase	CILP	chr15q22
conserved helix-loop-helix ubiquitous kinase	CHUK	chr10q24-q25
gamma-glutamyltransferase 1	GGT1	chr22q11.23
gamma-glutamyltransferase 2/3	GGT1 /// GGT2 /// GGT3P /// GGTLC2 /// GGTLC3	chr22q11.21 /// chr22q11.22 /// chr22q11.23
gamma-glutamyltransferase 5	GGT5	chr22q11.23
gamma-glutamyltransferase 6	GGT6	chr17p13.2
gamma-glutamyltransferase 7	GGT7	chr20q11.22
gamma-glutamyltransferase light chain 1	GGTLC1	chr20p11.1
inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	IKBKB	chr8p11.2
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	chr14q13
peroxisome proliferator-activated receptor gamma	PPARG	chr3p25
prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	PTGS1	chr9q32-q33.3

Table 7 (continued)

Gene Title	Gene Symbol	Chromosomal Location
prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	chr1q25.2-q25.3
superoxide dismutase 2, mitochondrial	SOD2	chr6q25.3
V-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	RELA	chr11q13

First we included patients with azathioprine from our cohort. 21 patients were included which were treated with azathioprine. As in figure 2, we performed a Pearson's correlation plot, revealing two subsets (figure 3a). This indicated that there is heterogeneity between patients treated with azathioprine.

Next, using Ingenuity pathway analysis, we analyzed the expression of genes that are known to be responsive to azathioprine (Table 6, figure 3a). These known genes are depicted in a treescape figure next to the correlation plot. Up-regulated genes as compared to the mean are shown in red, down-regulated genes are shown in green. Although all patients were treated with azathioprine, associated genes showed differences in expression, indicating variation in drug responsiveness between groups.

We performed an identical analysis for patients with mesalazine. Figure 3b shows the Pearson correlation plot of 16 patients with mesalazine. Known genes involved in the working mechanism of mesalazine are depicted in a treescape figure on the left and in table 7. As with azathioprine, patients with mesalazine form two groups. Again, the genetic responsiveness on mesalazine showed significant different response patterns between patients (figure 3b). These data indicate that while in remission, patients have detectable differences in therapy responsiveness.

DISCUSSION

To date treatment of IBD is regarded successful if clinical remission is achieved. This clinical remission is determined by low scores on the PCDAI and PUCAI for CD and UC respectively. Although clinical remission is achieved, macroscopic as well as microscopic inflammatory markers can still be present and potentially contribute to future relapse.[20-22] Therefore, clinical remission cannot merely be regarded as the absence of disease activity.

Bioinformatics based integration of molecular and clinical information has proven to be a crucial for the discovery of new disease pathways relevant to identify formation of subclasses of various diseases.[18] Using gene expression arrays in IBD, multiple genes have been discovered which are associated with activity of disease, phenotype (e.g. CD

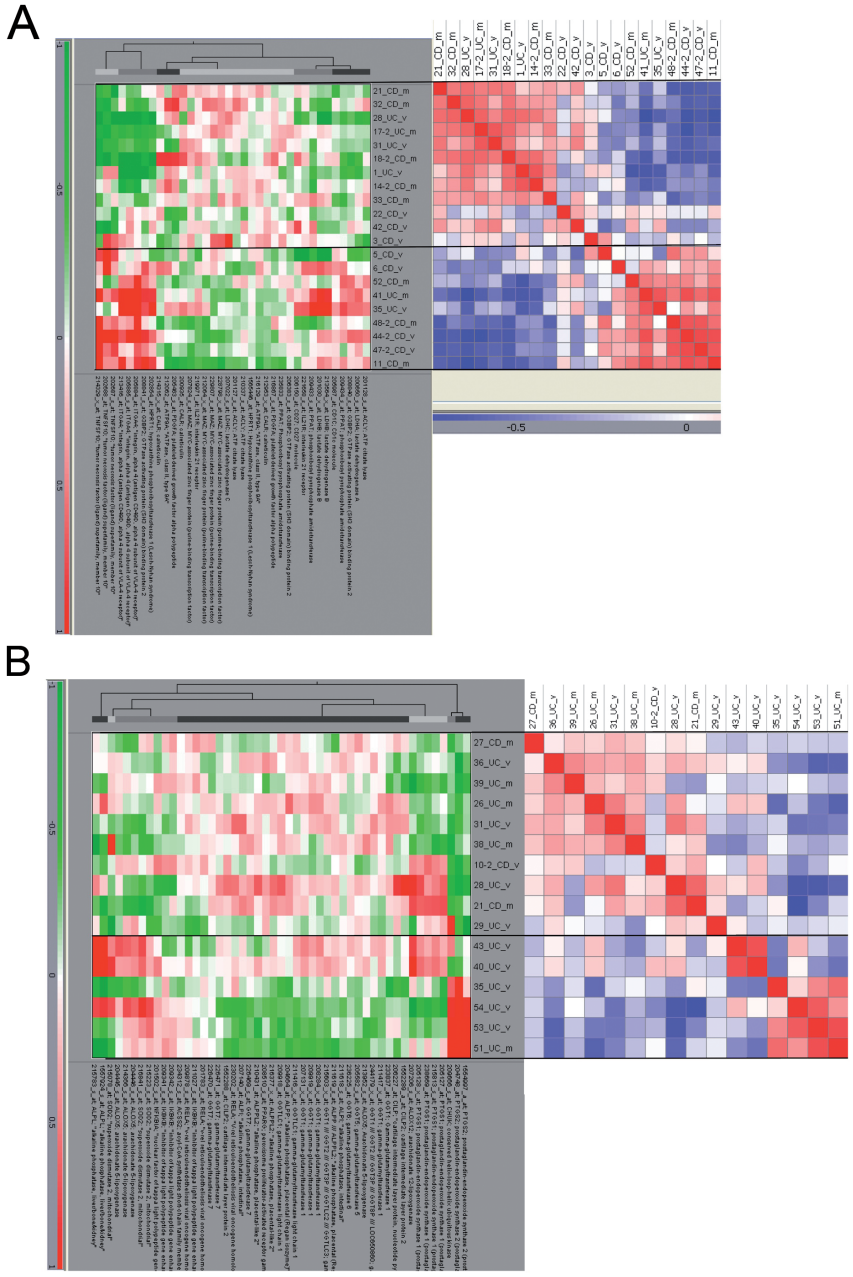

Figure 3. RNA gene responsiveness to mediation differs between IBD patients
A,B, right panel: The correlation visualization displays pair wise correlations between patients with azathioprine (A) and mesalazine (B). The colors of the cells relate to Pearson's correlation coefficient values, with deeper colors indicating higher positive (red) and negative (blue) correlations. This Pearson's correlation plot revealed two distinct groups. Left panel: treescape figure for SAM significant azathioprine (A) and mesalazine (B) targeted genes. The level of gene expression is shown from red (increase) to green (decrease) by a factor of at least 4 relative to the geometric means of the control samples.

and UC) and adult and pediatric IBD.[23-28] However, most of these studies were performed using supervised analyzes in order to find associated genes for CD vs UC or disease activity. This study is, to our knowledge, the first to study paediatric quiescent IBD patients including unsupervised analyses that revealed substantial differences between patients and controls.

Using gene expression arrays of PBL, we have shown that although quiescent pediatric IBD patients display a clinical remission phenotype, the gene expression profile is diverse and distinct from controls in the majority of patients (figure 1). Interestingly, CD patients as well as UC patients did not form separate clusters within the PCA, suggesting overlap of gene expression between these two subsets in this phase of disease.

Using unsupervised analysis on the genes with a three-fold expression form the geometric mean, quiescent pediatric IBD patients could be divided into three groups (figure 2). Differences in gene expression between groups revealed multiple significant records. Although non-immune genes were overrepresented in these three groups, significant differences in immunological gene expression and consequent pathways were present between controls as well as between groups (table 2-5).

We established that immune pathways are still involved in IBD patients with inactive disease. We found differences between IBD patients groups and associated pathways (table 4, 5). Genes involved in the glucocorticoid receptor signalling pathway were abundantly expressed in group B and C. As these groups were most distinct from controls (figure 2), this suggests that this pathway plays an important role in the distinction between controls and IBD patients.

Due to the limited number of patients and the heterozygous phenotype of IBD, we could not establish correlations with clinical characteristics or duration of disease. Therefore, we are unable to tell whether these groups will result from differences in earlier disease activity or may help predict the course of clinical disease including exacerbations. A larger prospective study has to be performed in order to answer these questions.

However, we did observe specific differences in gene responsiveness upon medication (figure 3). Patients with azathioprine as well as patients with mesalazine, two important anti-inflammatory drugs in the treatment of CD and UC respectively, could be divided into two groups based upon the expression of medication-associated genes. As our patient groups are limited, we were unable to link these data to clinical responsiveness at this stage.

Given the ethical limitations of performing biopsies in paediatric IBD patients with quiescent disease. we decided to focus on expression arrays of peripheral blood cells. Numerous studies have shown potential immunological differences in peripheral derived immune cells from IBD patients in the active stages of the disease.[26, 29-33] Continuous

monitoring of our IBD patients did not reveal a correlation between group subset and clinical phenotype (e.g. relapse) (data not shown). However, as shown in figure 3, by analyzing the RNA expression levels of the target specific genes for azathioprine and mesalazine, we were able to detect differences in medication responsiveness between patients. Whether these differences may help predict potential relapses and subsequently give rise to specific therapeutic interventions remains to be elucidated.

This study indicates that remission in IBD is associated with, gene expression profiles that can be separated from controls. We conclude that in spite of clinical remission various pathways are still active that include genes involved in inflammatory responses. Finally, subsets of expression profiles exist in the genes involved in azathioprine and mesalazine responses.

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Supplementary table 1, Groups - Patient characteristics

	Group A		Group B		Group C	
	#	%	#	%	#	%
	18		12		15	
Male	11	61.1	6	50	2	13.3
Female	7	38.9	6	50	13	86.7
CD	10	55.6	6	50	8	53.3
UC	8	44.4	6	50	7	46.7
	Mean (SD)	SD	**Mean (SD)**	SD	**Mean (SD)**	SD
PCDAI/PUCAI	2.2	3.6	8.9	3.1	7.5	3.5
ESR (mm/h)	10.9	2.6	10.6	4.2	15.9	2.1
CRP (mmol/l)	2.6	0.6	2.0	0.5	4.0	1.2
Age (y)	16.2	0.3	15.0	0.4	15.7	0.2
Age onset (y)	13.5	0.7	11.7	1.4	12.7	0.5

Supplementary table 2

Please link to: http://snuif.com/tabellen.pdf

Chapter 9

Summary and discussion

SUMMARY AND DISCUSSION

The human intestine contains about 10^{14} micro-organisms that critically contribute to tissue homeostasis, host metabolism and other beneficial processes. Despite a continuous microbial exposure within in the intestine, the mucosal immune system is able to maintain a state of tolerance to the microbiota. An aberrant immune response of the intestinal immune system results in a defective tolerance in the intestine, which leads to a breakdown of intestinal homeostasis. This in turn, can lead to chronic intestinal inflammation or inflammatory bowel disease (IBD).

IBD includes two disease entities: Crohn's disease (CD) and ulcerative colitis (UC). To date, CD and UC are distinguished based upon clinical and histopathological differences. CD is characterized by a transmural granulomatous inflammation, which can occur throughout the gastrointestinal tract. In contrast, in UC the colon is affected by a superficial continuous ulcerative inflammation.

From an immunological perspective, exaggerated responses by mucosal T and B cells (the adaptive immune system) have traditionally been regarded as the key contributors to the pathogenesis of IBD. However, discoveries of innate defects that are associated with IBD have refocused the attention to this arm of the immune system.

Two hypotheses could explain how a defective innate immune system could lead to IBD. An aberrant innate immune response can result from a *loss of function*, which leads to sub-optimal recruitment and function of innate cells. Consequently, harmless commensal microbiota will increasingly penetrate and translocate the intestinal mucosa. In turn, this will lead to chronic attraction and activation of adaptive immune cells. On the other hand, *gain of function* of the innate immune response may lead to hyper-responsiveness upon microbial stimulation. In this case, excessive chemokine production of innate immune cells will directly cause inflammation by recruited innate and adaptive cells.

Is has become clear that IBD represents a plethora of disease entities that result from a large range of defects, including aberrant innate immune responses.

In this thesis we try to answer some of the open questions concerning the pathogenesis of IBD and defects in the innate immune system. In **chapter 1**, we give an overview on the recent literature of the role of the innate immune system in the pathogenesis of IBD. Specifically, we describe how exaggerated as well as a defective innate immune responses by various immune cells can lead to IBD.

As the pathogenesis of IBD is complex, we argue in **chapter 2** that results from one study cannot be extrapolated to IBD in general. Macrophages of adult CD patients were found to produce less of the pro-inflammatory cytokine IL-8 upon microbial (LPS) stimulation.[1] However, we have shown previously an enhanced production of this chemokine by buccal epithelial cells of pediatric IBD patients[2], which illustrates the immune differences between various immune cells within an individual IBD patient.

Although innate defects play a crucial role in the pathogenesis of IBD, drugs that affect the adaptive immune system are widely used in the treatment of IBD. Inhibition of T-cell function leads to remission in the majority of IBD patients. As the innate immune system is strongly implicated in the pathogenesis of IBD, we hypothesized that T-cell suppressors may not only inhibit T-cell-dependent production of pro-inflammatory mediators, but also – indirectly – affect innate immune cell function.

In **chapter 3** we describe the extent of the T-cell inhibiting drug tacrolimus on the function of the innate immune system. The usage of a murine model for CD, TNBS-induced colitis, enabled us to characterize the various immune cells during the early development of colitis. Within 24 hours after the induction of colitis, recruitment of innate immune cells, specifically neutrophils and monocytes, into the intestinal lamina propria was observed. At the same time, numbers of T- and B-cells (adaptive immune cells) remained stable. However, by inhibiting T-cell function by the administration of tacrolimus, we could diminish colitis to almost control levels. This effect of tacrolimus was T-cell dependent, as we did not observe any effect of this drug in TNBS colitic mice without an adaptive immune system.

This study illustrated that at the early phases of colitis development, adaptive immune cells play an important role in the recruitment of innate immune cells. As described in chapter 1, the innate immune system is crucial in the establishment of intestinal homeostasis. As patients with IBD are being treated with T-cell inhibiting drugs during remission, the side effect of these drugs could be a diminished recruitment of innate immune cells. In turn, this *loss of function* could lead to bacterial overgrowth and immune activation.

Although we did not study the effect of tacrolimus in human IBD, the question remains whether the recruitment or function of innate immune cells should be restored during the remission phases of IBD.

Resident lamina propria dendritic cells (DC) play an important role in maintenance of tolerance to harmless antigens. However, it is unclear whether subtypes of these DC or newly recruited inflammatory antigen presenting cells (APC) drive intestinal inflammation upon loss of tolerance. In **chapter 4** we characterized the role of DC subtypes during TNBS-induced colitis. In the establishment of colitis development we observed an

influx of Ly6c$^+$ monocytes in the lamina propria. Hereafter, an increase in the percentage of monocyte-derived Ly6c$^+$CD11b$^+$ DC in the mucosal draining lymph nodes occurred. These DC were capable of presenting antigens from the intestine to adaptive immune cells in the draining lymph nodes. In accordance to our study described in chapter 3, these data indicate that monocytic infiltration during intestinal inflammation gives rise to a new subtype of monocyte-derived DC that can present antigens and may support T-cell division. In mice, blocking of migration of CD11b$^+$ cells ameliorates colitis. Whether this treatment could be applied in human IBD remains to be investigated.

DC play an important role in the differentiation of naive T-cells to T-helper cells (e.g. Th1, Th2 and Th17) and regulatory T-cells. Formerly, CD was known as a Th1 disease, while UC was characterized by a Th2 immuno-type. Nowadays, this sub-classification is not as strict. It is unknown what exactly determines the differentiation between these phenotypes. The differential expression of a subset of T-cell receptors as well as specific cytokine production by antigen presenting cells may be involved in the proliferation and differentiation of various subtypes of pro-inflammatory T-cells.

In **chapter 5**, we analyzed the cytokine responses of monocyte-derived DC from pediatric IBD patients. The production of the pro-inflammatory cytokines IL-12 and IL-23 was analyzed and varied widely within the group of patients. We did not find any correlation between disease activity, disease location, medication and the production of IL-12 or IL-23. Specifically, we found no relation between early and late onset of IBD or whether the patients were diagnosed with CD or UC. However, we found a clear dose-dependent relationship between the production of these two cytokines and the type of microbial ligand that was used. In conclusion we hypothesized that the differences in T-cell subsets originate from the local amount of specific microbial ligands and expression of receptors such as IL-12b2 and IL-23R on the T cells.

Both augmented as well as diminished responsiveness of innate cells have been proposed as contributors to the mucosal inflammation in IBD. Indeed as indicated in chapter 2, we found that buccal epithelial cells derived from pediatric CD patients, showed enhanced responsiveness. However, as we described in chapter 5, DC derived from the same group of patients displayed no difference in response patterns between controls and IBD patients. Moreover, we showed that absolute concentrations of cytokines were not directly associated with disease activity.

In **chapter 6** we developed an assay using chronic stimulation of peripheral blood-derived monocytes to represent a more physiological condition of chronic microbial exposure within the intestinal mucosa. In this study we showed that monocytes from IBD patients and controls that were stimulated with a single challenge of LPS, produced comparable amounts of the pro-inflammatory cytokine TNF-α, confirming our obser-

vations in chapter 5. When we challenged these monocytes a second time with high dose LPS, these cells became tolerant. This process is known as endotoxin tolerance and operates as a self-protective mechanism during phases of excessive inflammation.

Interestingly, we showed that pediatric IBD patients with inactive disease – both CD and UC patients –displayed enhanced endotoxin tolerance compared to controls. As these patients showed no clinical sign of active disease, our data suggest that this enhanced endotoxin tolerance results from intrinsic aberrancies in the mechanisms that regulate innate tolerance. Indeed, IBD has been associated with various mutations in genes that play a particular role in innate microbial sensing that could be extrapolated to the mechanisms of innate tolerance.[3-5]

Augmented tolerance could potentially lead to a net deficit in antimicrobial defense upon repetitive exposure, which may result in disturbed homeostatic interactions between the intestinal microbiota and the mucosal immune system. Subsequently, this may result in enhanced local microbial pressure and the activation of adaptive immune cells, ultimately leading to chronic inflammation.

Paneth cells (Pc) play an important role as innate immune cells in the regulation of intestinal microbial homeostasis in the small intestine. When stimulated, Pc release anti-microbial peptides (e.g. lysozyme and defensins) in order to limit bacterial invasiveness in the intestinal crypts. Pc dysfunction leads to impaired production and release of these peptides and has been associated with bacterial overgrowth and inflammatory intestinal diseases. TNF-α, a key pro-inflammatory cytokine in the pathogenesis of IBD, is expressed by Pc as well. In hematopoietic cells, TNF-α is induced by microbial stimulation and tightly regulated via anti-inflammatory molecules, including IL-10. However, the role and regulation of this cytokine in Pc is not known.

In **chapter 7** we analyzed the regulation of Pc-derived TNF-α expression and compared it to the expression of lysozyme. We showed that over-expression of Pc-derived TNF-α is associated with IBD and celiac disease, while lysozyme was constitutively expressed. Further *in vitro* research indicated a direct regulation of TNF-α by microbial stimulation (via LPS) and IL-10. These factors had no effect on lysozyme production, indicating differential regulation of TNF-α and lysozyme. We confirmed these results in two mouse models, IL10-deficient (IL-$10^{-/-}$) and LPS-sensitive mice. IL-$10^{-/-}$ mice showed enhanced expression of Pc-derived TNF-α, confirming a role for IL-10 in the regulation of Pc-derived TNF-α. Dependency of microbial stimulation was confirmed in germfree IL-$10^{-/-}$ mice and LPS insensitive mice. These mice showed no TNF-α (germfree) or less TNF-α (LPS insensitive) expression in the Pc, while lysozyme was constitutively expressed in these conditions. Enhanced production of TNF-α by Pc may contribute to the establishment of chronic intestinal inflammation.

In **chapter 8** we analyzed RNA gene expression profiles of peripheral blood cells from pediatric IBD patients with inactive disease. First we showed that pediatric IBD patients with inactive disease displayed altered gene expression profiles compared to controls. Moreover, we did not detect a sub-classification for CD and UC patients, indicating genomic overlap between these two phenotypes. Further analysis revealed three subclasses based upon RNA expression profiles. Different immunological genes were transcribed in each of these groups, indicating that IBD-associated genes were still active and significantly different from controls, despite the fact that the majority of IBD patients were in clinical remission. This differential gene expression could reflect baseline immunological differences between IBD patients and controls, as well as between individual IBD patients.

Most CD patients are treated with azathioprine, whereas the majority of UC patients is treated with mesalazine. In the majority of patient, these two types of drugs are efficient in establishing and maintaining remission. However, drug responsiveness varies between patients. We investigated the basis for variations in drug responsiveness by analyzing the expression levels of the target-specific genes for azathioprine and mesalazine. Based on specific gene expression profiles of the pediatric IBD patients that were treated with azathioprine and/or mesalazine we were able to identify two specific clusters. Whether these differences may help predict potential relapses and subsequently give rise to specific therapeutic interventions remains to be elucidated.

CONCLUSIVE REMARKS & FUTURE STUDIES

The discoveries of defects in IBD that are typically associated with innate immune cells have refocused research to this arm of the immune response. In spite of the abundant evidence that T- and B-cells contribute to the IBD pathogenesis, it is conceivable that future IBD therapies will specifically aim at targeting specific bacteria or molecules involved in microbial sensing. In this respect, it is of particular interest that pediatric or early onset IBD seems to be specifically associated with defects of the innate immune system.[6] Finally, many of the proposed mechanisms may only apply for certain subsets of patients or occur simultaneously in the same patient. The real challenge therefore, will be to develop novel (molecular) tools that will enable us to subcategorize patients into more specific IBD subsetss of IBD other than just CD or UC. Tools for the identification of the specific disease subtype in a certain patient and at a specific phase of disease will eventually generate a range of novel patient-tailored interventions.

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SAMENVATTING EN DISCUSSIE

In de darm bevinden zich circa 10^{14} micro-organismen die een belangrijke bijdrage leveren aan de homeostase, het metabolisme en andere gunstige processen. Ondanks dat de darm permeabel is voor verschillende voedingstoffen en bacteriële producten, leidt dit normaal gesproken niet tot chronische ontsteking. De complexe immuunregulatie die voorkomt dat het mucosale immuunsysteem overreageert op de in de darm aanwezige commensale bacteriën staat bekend als mucosale tolerantie. Een disfunctionerende immuunrespons kan resulteren in een verbreking van de tolerantie. Uiteindelijk kan dit leiden tot een chronische ontsteking in de darm en tot een inflammatoire darmziekte (IBD).

Binnen IBD wordt onderscheid gemaakt tussen twee fenotypes: de ziekte van Crohn (CD) en colitis ulcerosa (UC). Dit onderscheid wordt gemaakt op basis van klinische en histopathologische gronden. CD wordt gekarakteriseerd door een transmurale granulomateuze ontsteking, welke door de gehele darm kan voorkomen. Daarentegen is bij UC alleen het colon aangedaan door een oppervlakkige continue ulceratieve ontsteking.

Vanuit een immunologisch perspectief werden overreacties van mucosale T- en B-cellen (het verworven immuunsysteem) gezien als de belangrijkste oorzaken in de pathogenese van IBD. Echter, door de ontdekking van defecten in het aangeboren (innate) immuunsysteem die geassocieerd zijn met IBD, is de aandacht van het verworven immuunsysteem verlegd naar dit aangeboren immuunsysteem.

Op twee manieren kan een disfunctionerend aangeboren immuunsysteem leiden tot IBD. Een *verzwakte functie* van het aangeboren immuunsysteem heeft een suboptimale aantrekking en functie van aangeboren immuuncellen tot gevolg. Dit kan leiden tot een overgroei en penetratie van bacteriën in de darm, hetgeen resulteert in de aantrekking en activatie van cellen van het verworven immuunsysteem en zo tot een chronische ontsteking in de darm.

Ook een *versterkte functie* van het aangeboren immuunsysteem kan uitmonden in een disfunctionerende immuunrespons op microbiële stimulatie. In dit geval zorgt een overproductie van chemokines door de cellen van het aangeboren immuunsysteem direct voor ontsteking door versterkte aantrekking en activatie van aangeboren en verworven immuuncellen.

Het is duidelijk geworden dat IBD uit een veelvoud van ziektetypen bestaat die elk het gevolg zijn van een meervoud van defecten, inclusief die van het aangeboren immuunsysteem.

In dit proefschrift proberen wij een antwoord te vinden op enkele van de nog openstaande vragen met betrekking tot de pathogenese van IBD en defecten van het aangeboren immuun systeem. In **hoofdstuk 1** geven wij een overzicht van de literatuur waarin de rol wordt beschreven van het aangeboren immuunsysteem in de pathogenese van IBD. Wij beschrijven hoe een versterkte als ook een verzwakte functie van de verschillende aangeboren immuuncellen kan leiden tot IBD.

Aangezien de pathogenese van IBD complex is, bediscussiëren wij in **hoofdstuk 2** dat gevonden studieresultaten niet zonder meer mogen worden geëxtrapoleerd naar IBD in zijn algemeenheid. In dit specifieke geval produceerden macrofagen van volwassenen met CD minder interleukine (IL)-8 na stimulatie.[1] Echter, onze groep liet zien dat epitheliale cellen van het wangslijmvlies van kinderen met CD dit chemokine juist in verhoogde mate produceerden.[2] Dit voorbeeld illustreert de verschillen in immuunrespons tussen verscheidene immuuncellen en tussen patiënten met IBD.

Ondanks dat defecten van het aangeboren immuunsysteem een belangrijke rol spelen in de pathogenese van IBD worden medicijnen die aangrijpen op het verworven immuunsysteem in veelvoud gebruikt bij de behandeling van dit ziektebeeld. Inhibitie van de functie van T-cellen leidt tot remissie in een groot deel van de patiënten met IBD. Aangezien het aangeboren immuunsysteem een cruciale rol speelt in de pathogenese van IBD, was onze hypothese dat suppressie van T-cellen niet alleen resulteert in een inhibitie van de productie van pro-inflammatoire mediatoren door deze cellen, maar dat ook – indirect – aangegrepen wordt op de functie van aangeboren immuuncellen.

In **hoofdstuk 3** beschrijven we de reikwijdte van het T-cel inhiberende medicijn tacrolimus op de functie van het aangeboren immuunsysteem. Door gebruik te maken van een muismodel voor CD, TNBS colitis, konden wij de verschillende immuuncellen die betrokken zijn bij het ontstaan van colitis karakteriseren. Binnen 24 uur na de inductie vond er rekrutering plaats van aangeboren immuuncellen (neutrofielen en monocyten) in de lamina propria van de darm, hetgeen resulteerde in colitis. Tegelijkertijd bleven T- en B-cellen (het verworven immuunsysteem) in aantallen gelijk. Inhibitie van de T-celfunctie door het geven van tacrolimus zorgde voor een zeer sterke reductie van de influx van monocyten en neutrofielen en leidde hiermee tot een reductie van colitis. Dit effect van tacrolimus was T-cel afhankelijk, aangezien we geen effect zagen van dit medicijn op TNBS colitis in muizen zonder verworven immuunsysteem.

Deze studie illustreerde dat in de vroege fase van de ontwikkeling van colitis ook verworven immuuncellen een belangrijke rol spelen in de aantrekking van aangeboren immuuncellen. Zoals beschreven in hoofdstuk 1 is het aangeboren immuunsysteem cruciaal voor het in stand houden van de intestinale homeostase. Aangezien IBD patiënten behandeld worden met T-cel inhiberende medicijnen, ook ten tijde van remissie, zou

een bijwerking van deze medicatie kunnen zijn dat de aantrekking van aangeboren immuuncellen wordt verminderd. Deze verzwakte functie van het aangeboren immuunsysteem zou kunnen leiden tot bacteriële overgroei, immuunactivatie en exacerbatie van colitis. Hoewel we het effect van tacrolimus niet hebben bestudeerd in humane IBD, is de vraag of ten tijde van remissie de rekrutering of activatie van aangeboren immuuncellen in zekere mate zouden moeten worden gewaarborgd.

Dendritische cellen (DC) zijn aangeboren antigeen presenterende cellen en van groot belang in de totstandkoming van tolerantie tegen de commensale microben. Onduidelijk is of ten tijde van het verbreken van tolerantie intestinale inflammatie gedreven wordt door een subtype van de in de darm aanwezige DC of door nieuw gerekruteerde antigeen presenterende cellen. Om een antwoord op deze vraag te vinden, bestudeerden wij in **hoofdstuk 4** de rol van DC tijdens TNBS colitis.

Bij het ontstaan van colitis zagen wij een influx van $Ly6c^+$ $CD11b^+$ monocyten in de lamina propria. Hierna volgde een toename van DC in de drainerende lymfklieren, welke gevormd waren uit deze $Ly6c^+$ $CD11b^+$ monocyten. Deze DC waren in staat antigenen vanuit de darm op te pikken en aan te bieden aan T-cellen in de lymfklier. Deze studie, volgend op de data van hoofdstuk 3, laat zien dat ten tijde van inflammatie monocyten de darm infiltreren en uitgroeien tot nieuwe DC in de lymfklier. Vanuit eerdere studies in muizen is bekend dat blokkade van monocyten en macrofagen kan leiden tot een vermindering van colitis. Of deze behandeling ook zou kunnen worden toegepast bij IBD moet echter nog worden onderzocht.

DC spelen een belangrijke rol in de differentiatie van naïeve T-cellen naar T-helper cellen (zoals Th1, Th2 en Th17) en regulatoire T-cellen. Vroeger werd CD beschouwd als een ziekte van Th1 cellen, terwijl UC gekarakteriseerd werd door een Th2 fenotype. Tegenwoordig is deze classificatie minder stringent. Het is onduidelijk wat de differentiatie van deze twee fenotypes bepaalt. Zowel de differentiële expressie van een subset van T-celreceptoren, als ook productie van specifieke cytokines door antigeen presenterende cellen kunnen zorgen voor de proliferatie en differentiatie van verschillende subtypes van pro-inflammatoire T-cellen.

Om de rol van cytokines die door DC worden geproduceerd te onderzoeken, analyseerden wij in **hoofdstuk 5** de cytokine-respons van DC van pediatrische IBD patiënten. De productie van interleukine (IL)-12 en IL-23 werd gerelateerd aan het type IBD, de ziekteduur en de behandeling.

De productie van deze twee cytokines kende een grote spreiding binnen onze patiënten. Er was geen relatie tussen ziekteactiviteit, lokalisatie van de ontsteking of medicatie en de productie van IL-12 en IL-23. Bovendien vonden we voor zowel CD als UC geen relatie tussen korte of langdurige ziekteactiviteit. We vonden echter wel een duidelijke

dosis-respons relatie tussen de productie van deze twee cytokines en het type gebruikte microbiële ligand.

Dit onderzoek toonde aan dat noch CD, noch UC is geassocieerd met een predispositie in de productie van een van deze cytokines. Daarbij was het vermogen van de DC om IL-12 of IL-23 te produceren niet gecorreleerd met de ziekteactiviteit. Bovendien konden we geen verband aantonen tussen IL-12 en IL-23, hetgeen suggereert dat deze twee cytokines differentieel gereguleerd worden.

Concluderend is onze hypothese dat de verschillende T-cel subsets voortkomen vanuit de lokale hoeveelheid van specifieke microbiele liganden en de expressie van receptoren op de T-cel, zoals IL-12β2 en IL-23R.

Zoals in beschreven hoofdstuk 1 en 2 kunnen zowel een verzwakte als ook een versterkte functie van het aangeboren immuunsysteem bijdragen aan de mucosale inflammatie in IBD. In hoofdstuk 2 beschreven wij dat epitheelcellen van het wangslijmvlies van kinderen met CD een versterkte immuunreactie lieten zien. Echter, zoals in hoofdstuk 5 beschreven, lieten DC afkomstig van dezelfde groep patiënten geen verschil zien in responspatronen tussen controles en IBD patiënten. De vraag is nu of een enkele microbiële stimulatie wel te vergelijken is met de fysiologische conditie van chronische microbiële blootstelling in de intestinale mucosa.

In **hoofdstuk 6** hebben wij een assay ontwikkeld waarin chronische stimulatie van uit bloed afkomstige monocyten kan worden geanalyseerd. Voor iedere patiënt afzonderlijk berekenden wij de verschillen in reactie op deze chronische stimulatie. In dit onderzoek lieten wij zien dat monocyten van pediatrische IBD patiënten in vergelijking met controles een vergelijkbare hoeveelheid van het pro-inflammatoire cytokine TNF-α produceerden op een enkele bacteriële stimulus (LPS), waarmee we onze bevinding in hoofdstuk 5 bevestigden.

Monocyten die wij voor een tweede maal stimuleerden met hoge dosis LPS werden tolerant voor deze stimulus. Dit proces, dat bekend staat als endotoxine tolerantie, is bekend sinds de vijftiger jaren van de vorige eeuw en werkt als een zelfbeschermingsmechanisme tijdens fases van excessieve inflammatie, zoals sepsis.

Interessant genoeg zagen wij dat pediatrische IBD patiënten met een inactieve ziekteactiviteit een versterkte tolerantie lieten zien in vergelijking met controles. Aangezien deze patiënten geen ziekteactiviteit hadden, suggereert dit dat deze versterkte tolerantie het gevolg is van intrinsieke defecten in het mechanisme dat zorg draagt voor aangeboren tolerantie.[3-5]

Een versterkte tolerantie zou kunnen leiden tot een verminderde verdediging tegen de continue blootstelling aan micro-organismen in de darm. De daaropvolgende bacteriële overgroei en penetratie leidt vervolgens tot een verstoorde homeostase en tot activatie van T- en B-cellen en chronische inflammatie.

Panethcellen (Pc) spelen een belangrijke rol als aangeboren immuuncellen in de regulatie van intestinale microbiële homeostase in de darm. Door de secretie van antimicrobiële peptiden door Pc, zoals lysozym en defensines, wordt de intestinale crypt en de daar aanwezige stamcel beschermd tegen bacteriële invasie. Disfunctie van de Pc leidt tot verminderde productie en secretie van deze peptiden en is geassocieerd met bacteriële overgroei en intestinale inflammatoire ziekten.

Ook produceren Pc TNF-α, een belangrijk pro-inflammatoir cytokine betrokken in de pathogenese van IBD. In hematopoietische cellen wordt TNF-α geproduceerd na microbiële stimulatie en wordt productie gereguleerd via anti-inflammatoire cytokines, zoals IL-10. De rol en de regulatie van TNF-α in de Pc is echter niet bekend.

In **hoofdstuk 7** analyseerden wij de regulatie van Pc-TNF-α. In dit hoofdstuk lieten we zien dat overexpressie van TNF-α in de Pc geassocieerd is met IBD en coeliakie. *In vitro* analyse liet een directe regulatie zien van TNF-α door microbiële stimulatie (LPS) en IL-10. Deze twee factoren hadden geen invloed op de productie van lysozym, hetgeen suggereert dat TNF-α en lysozym differentieel gereguleerd worden.

Wij bevestigden deze resultaten in twee muismodellen. TNF-α in de PC kwam tot verhoogde expressie in IL-10 deficiënte (IL-$10^{-/-}$) muizen, hetgeen de regulerende rol van IL-10 bevestigd. Door gebruik te maken van steriele (bacterie-vrije) IL-$10^{-/-}$ muizen konden wij de initiërende rol van de microbiota op de productie van TNF-α door Pc bevestigen. Steriele muizen produceerden geen TNF-α in de Pc, terwijl lysozym onder dezelfde condities tot normale expressie kwam.

Een overproductie van TNF-α door Pc zou kunnen bijdragen tot het ontstaan van ontsteking in de darm, zoals wordt gezien bij onze pediatrische IBD patiënten.

In **hoofdstuk 8** analyseerden wij de RNA-expressieprofielen van pediatrische IBD patiënten met een inactieve ziekteactiviteit. Als eerste lieten wij zien dat deze patiënten een ander genetisch profiel hadden in vergelijking met controle patiënten. Daarbij was er geen subclassificatie zichtbaar tussen CD en UC, hetgeen duidt op een genetische overlap tussen deze twee fenotypes. Verdere analyse, gebaseerd op RNA-expressieprofielen, liet drie subgroepen zien. Ondanks dat de patiënten in remissie waren, kwamen in elke groep verschillende immunologische genen tot expressie. Dit betekent dat IBD-geassocieerde genen nog steeds actief zijn ten tijde van remissie en de expressie verschilt van controle patiënten en tussen patiënten met IBD. Basale immunologische verschillen tussen IBD patiënten en controle patiënten, als ook tussen IBD patiënten onderling, kunnen op deze manier verder worden onderzocht.

Behandeling van CD en UC vindt in een groot aantal gevallen plaats met respectievelijk azathioprine en mesalazine. Bij de meeste patiënten zijn deze twee medicijnen effectief voor de inductie en het in stand houden van remissie. De mate van responsiviteit verschilt echter tussen patiënten.

Wij onderzochten de basis voor variatie in de responsiviteit door analyse van de RNA-expressieprofielen van de specifieke aangrijpingsgenen voor azathioprine en mesalazine. Op basis van deze specifieke genexpressieprofielen van onze pediatrische IBD patiënten, konden wij twee specifieke clusters onderscheiden. Het is de vraag of deze verschillen in de toekomst mogelijk potentiële exacerbaties kunnen voorspellen en zo kunnen leiden tot meer specifieke therapeutische interventies.

CONCLUSIE EN TOEKOMSTIGE STUDIES

De ontdekking van defecten in het aangeboren immuunsysteem die geassocieerd zijn met IBD heeft de aandacht van het verworven immuunsysteem verlegd naar het aangeboren immuunsysteem.

Ondanks het overvloedige bewijs dat T- en B-cellen een belangrijke rol spelen in de pathogenese van IBD, is het voorstelbaar dat toekomstige therapieën voor IBD vooral zullen aangrijpen op specifieke bacteriën of op de moleculen die de microben herkennen. In dit licht is het van belang om te weten dat pediatrische IBD specifiek geassocieerd is met defecten van het aangeboren immuun systeem.[6]

Tenslotte kunnen de hierboven beschreven mechanismen mogelijk alleen van toepassing zijn op specifieke groepen van patiënten of kunnen meerdere van deze mechanismen tegelijkertijd binnen één patiënt aanwezig zijn.

De uitdaging ligt er daarom in om nieuwe moleculaire technieken te ontwikkelen die het mogelijk maken om patiënten beter te classificeren dan alleen in CD of UC. De technieken die onderscheid maken in fenotypes van verschillende patiënten op verschillende fases van de ziekte zullen uiteindelijk leiden tot nieuwe methodes om IBD beter te behandelen.

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CURRICULUM VITAE

Pieter van Lierop werd op 19 oktober 1977 geboren te Tilburg. Na het behalen van zijn middelbare schooldiploma aan het Gymnasium Haganum te Den Haag in 1996, startte hij met de studie geneeskunde aan de Katholieke Universiteit Leuven. In het daarop volgende jaar werd deze studie voortgezet aan de Rijksuniversiteit Groningen. De gastro-enterologie trok in deze tijd zijn aandacht. Tijdens zijn studie was hij werkzaam als student-assistent bij het levertransplantatie team en verrichte hij wetenschappelijk onderzoek naar de excretie van ongeconjugeerd bilirubine (in Groningen onder leiding van Prof.dr. H.J. Verkade en vervolgens in Boston, Verenigde Staten, onder leiding van Prof.dr. M.C. Carey).

Na zijn artsexamen (2004) begon hij als assistent kindergeneeskunde op de chirurgische intensive care van het Erasmus MC – Sophia Kinderziekenhuis (hoofd: Prof.dr. D. Tibboel), waarna hij in december 2004 startte met zijn promotieonderzoek "The Innate immune system In Inflammatory Bowel Disease" onder supervisie van Prof.dr. E.E.S. Nieuwenhuis op het laboratorium kindergeneeskunde van het Erasmus MC – Sophia Kinderziekenhuis. Dit heeft geleid tot het voor u liggende proefschrift. Vanaf april 2009 is hij begonnen aan de opleiding tot kinderarts vanuit het UMC Utrecht - Wilhelmina Kinderziekenhuis (opleider Dr. J. Frenkel).

Pieter van Lierop is getrouwd met Elseline Snuif en heeft twee kinderen: Siebren en Feiko.

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A role for Ly6C$^+$ monocyte-derived dendritic cells in TNBS colitis
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Manuscript in preparation
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Production of TNF-α by Paneth cells is associated with intestinal inflammation
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Subcategories of quiescent pediatric IBD patients by immune gene expression analysis of peripheral cells
Pieter van Lierop, Sigrid Swagemakers, Sabine Middendorp, Merel van Pieterson, Lilian de Ruiter, Rolien Raatgeep, Ytje Simons-Oosterhuis, Wilfred van IJcken, Janneke Samsom, Lizzy de Ridder, Johanna Escher, Peter van der Spek, Edward Nieuwenhuis
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