Crohn’s disease: Mucosal immunology and immune modulating therapy
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Chapter 1

Introduction
Introduction

Inflammatory Bowel Disease

The idiopathic inflammatory bowel disease (IBD) comprises two types of chronic intestinal inflammation: Crohn’s disease and ulcerative colitis. Patients suffer from chronic intestinal inflammation leading to bloody diarrhoea, weight loss and fatigue. Most patients are diagnosed between their second and fourth decade of life. This high incidence at young age impairs career development, reproduction and leads to high health care and economical costs. Patients need chronic medical treatment, however refractoriness and loss of response are major problems. Characteristically, Crohn’s disease can affect the entire gastro-intestinal tract, resulting in discontinuous transmural ulcers, mainly in the terminal ileum and right sided large intestine. Ulcerative colitis continuously affects the mucosa of the large intestine. Furthermore, IBD is characterized by extra-intestinal manifestations in about 25% of patients. Extra-intestinal manifestations include joints resulting in arthritis or arthropathy, eyes resulting in uveitis, and skin lesions such as erythema nodosum and pyoderma gangrenosum. These extra-intestinal manifestations may precede symptoms of intestinal inflammation or persist after this subsides. Interestingly, Crohn’s disease patients are at greater risk to develop other autoimmune diseases such as ankylosing spondylitis and psoriasis, indicating that similar pathophysiological mechanisms are involved. Indeed, therapeutic drugs blocking tumour necrosis factor (TNF-α) are effective in Crohn’s disease as well as ankylosing spondylitis and psoriasis.

In 1932, Burriill B. Crohn (1884 - 1983), along with the surgeons Leon Ginzburg and Gordon Oppenheimer, were the first to describe “Regional ileitis”, or Crohn's disease as it was called later on. Before 1960 prevalence rates were low and reports only originated from Europe and North America. In the following decades the prevalence increased mostly in North America and Europe. Since then the incidence of IBD has increased in every studied region all over the world. In developing countries IBD used to be rare, however since these nations have become industrialized and westernized, the incidence has augmented. Moreover, incidence is higher in urban regions than rural area's. And emigrants who migrate from a low prevalence area of IBD to a higher prevalence area increase their risk to develop IBD. These findings illustrate the importance of environmental factors in the aetiology of IBD.
Aetiology of IBD
The exact nature of the chronic intestinal inflammation is unknown, however multiple factors have been identified. IBD seems to result from the accumulation of genetic susceptibility and an inappropriate immune response to environmental factors, as is depicted in Figure 1. Genome-wide association studies (GWAS) identified several genetic polymorphisms associated with Crohn’s disease and ulcerative colitis. Though little is known about the effects of these polymorphisms, it has become clear that the disturbance of the delicate homeostasis of the intestinal immune system plays a central role. High levels of the pro-inflammatory cytokines TNF-\(\alpha\) and IFN-\(\gamma\) have been found in Crohn’s disease intestinal inflammation. Since these cytokines are mainly produced by T helper (Th)1 cells, Crohn’s disease was considered a Th1 mediated disease. By contrast, UC was considered to be Th2 mediated since active rectal disease shows increased levels of IL-4 and IL-13, when compared to patients in remission. Genome-wide association studies (GWAS) have shown an association between polymorphisms in the IL-23R and sensitivity or protection in certain autoimmune diseases, including Crohn’s disease, suggesting that at least in some instances the IL-23 axis is involved in pathology. IL-23 is produced by myeloid cells, including dendritic cells, and is important for maintenance of Th17 cells. Indeed both IL-17 and IL-22, but also IL-23 were found at high levels in Crohn’s patient’s sera and intestinal inflammatory sites. More recently it was shown that both IL-17 and IL-22 can be produced by many cell types including innate lymphoid cells (ILCs) raising the possibility that ILCs rather than Th17 cells are the major producers of these cytokines in IBD.
Part I: Innate lymphoid cells in inflammatory bowel disease

A novel family of immune cells
In the last decade, ILCs have emerged as a novel family within the innate immune system and appear to play a role in several inflammatory diseases. ILCs are characterized by a lymphoid morphology and the absence of specific antigen receptors. Conventional Natural Killer (cNK) cells are one of the two prototypic members of the ILC family. In humans there are two major subsets: CD56\textsuperscript{bright} NK cells dedicated to cytokine production, mainly IFN-\gamma, and CD56\textsuperscript{low} NK cells which are cytotoxic by secretion of granules containing perforin and granzyme.\textsuperscript{15,16} In 2010, a subset of CD56\textsuperscript{+} cells, dedicated to IL-22 production, was identified in the intestine.\textsuperscript{17} These CD56\textsuperscript{+} cells expressed the IL-7 receptor (CD127) and could respond to IL-23 to produce cytokines. Because their expression of CD56 and the natural cytotoxicity receptor (NCR) family member NKp44 these cells were initially called NK22 cells.\textsuperscript{18,19} Subsequent studies have made clear that the NK22 cells represent a lineage of cells distinct from NK cells.\textsuperscript{13,18,20,21} NK22 cells express CD127 and the transcription factor ROR\(\gamma\)t whereas cNK cells lack these markers. Moreover whereas NK22 cell are developmentally dependent on IL-7, cNK develop independently of this cytokine. Recently, a uniform nomenclature was proposed, distinguishing ILC subsets according to their phenotype and cytokine production profile, which is in analogy with the T helper cell family. ILCs are divided in group 1 (IFN-\gamma producing), group 2 (IL-5/IL-13 producing), and group 3 (IL-17/IL-22 producing).\textsuperscript{22} ILCs are characterized as lineage negative (Lin\(^{-}\) means no T cells, B cells or cells with a myeloid origin), CD45\(^{+}\) lymphocytes that depend on IL-7 for their development and survival and therefore express CD127, with the exception of cNK cells.\textsuperscript{23}

Group 1 ILCs are abundant in the intestine of Crohn’s disease patients
Group 1 ILCs comprises cNK cells, CD127\(^{-}\) and CD127\(^{+}\) ILC1. The two latter ILC1 subsets differ in phenotype and anatomical localisation: CD127\(^{-}\) ILC1 reside in the gut epithelium whereas CD127\(^{+}\) ILC1 are located in the lamina propria of the intestine.\textsuperscript{24,25} Both ILC1 subsets have been described to outnumber IL-22 producing ILC3 in the inflamed intestine of Crohn’s disease patients.\textsuperscript{24,25} CD127\(^{-}\) ILC1 respond to danger signals (IL-12 and IL-15) from surrounding cells and act against pathogens that elicit these signals.\textsuperscript{26} CD127\(^{+}\) ILC1 produce IFN-\gamma in response to IL-12 and IL-18 and increased numbers have been observed after the onset of colitis in DSS models.\textsuperscript{24} Before birth, thus before bacterial colonization, no ILC1 have been identified.\textsuperscript{24} Therefore ILC1 most likely play a role in immunity against intestinal pathogens. Nevertheless, the exact function of these cells remains to be elucidated. Most likely, due to the high numbers and findings in colitis models, ILC1 contribute to either the onset and/or the chronic inflammatory state of IBD patients.

cNK cells are present throughout the intestine, and in healthy state most are CD56\textsuperscript{bright}.\textsuperscript{26} Even though NK cells were first discovered in the 1970s, their role in the pathogenesis of IBD is enigmatic. Cytotoxic NK cells have been shown to be enriched in the colonic lamina propria of IBD patients. Suggesting their pro-inflammatory role, azathioprine, an effective
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immunosuppressant for the treatment of IBD, inhibits proliferation of these NK cells and induces apoptosis of resting NK cells.\(^{27}\)

**Group 2 ILCs could play an essential role in ulcerative colitis**

Group 2 ILCs or ILC2 produce type 2 cytokines: IL-4, IL-5, and IL-13 to protect the host from extracellular pathogens. ILC2 express the receptors for response to IL-25 (IL-17RB), IL-33 (ST2), prostaglandin D2 (CRTH2) and TSLP (a receptor complex of TSLPR and CD127).\(^{28,29}\) The transcription factors ROR\(\alpha\) in mice and GATA3 in human are key regulators of ILC2 development, survival and function.\(^{30,31}\) ILC2 have been shown important in the first response after intestinal helminth infection and airway tissue repair after influenza infection.\(^{32,33}\) Nevertheless, by secretion of large amounts of especially IL-5 and IL-13, these ILC2 seem to be able to mediate allergic asthma and it's exacerbations.\(^{34,35}\) Also nasal polyps of patients suffering from chronic rhinosinusitis, are packed with ILC2.\(^{29}\) ILC2 are present in the lamina propria of human adult healthy intestine.\(^{29}\) However, so far nothing is known about the potential role of ILC2 in the intestine of UC patients. In the Tbx21\(^{-/-}\); Rag\(^{-/-}\) ulcerative colitis (TRUC) disease model, colitis is caused by IL-17 secreting ILC3. No ILC2 were described to aggravate disease in this model.\(^{36}\) A different colitis model, induced by oxazolone did result in more IL-13 producing ILC2, and confirmed higher numbers of NK-T cells.\(^{37}\) The presence of NK-T cells in ulcerative colitis is ambiguous. One group showed a decrease in CD161\(^{+}\) NK-T cells in inflamed mucosa of ulcerative colitis patients, whereas others showed an increase of CD4\(^{+}\)CD161\(^{+}\)CD1d\(^{+}\) NK-T cells producing abundant IL-13.\(^{10,38}\) In vitro the presence of IL-13, enhanced cytotoxic activity and depletion of CD161\(^{+}\) cells from the lamina propria led to decreased IL-13 production.\(^{10}\) Especially this depletion of CD161\(^{+}\) cells from the lamina propria could have included both NK-T cells and ILC2, decreasing IL-13. Furthermore, IL-13 production is induced by binding of IL-33 to it's receptor ST2. It has become clear that both ST2, and its ligand IL-33, are highly expressed in the intestinal mucosa of ulcerative colitis patients.\(^{39,40}\) In serum from UC patients soluble ST2, a decoy receptor for IL-33, correlates with UC disease severity.\(^{41}\) In the gut, the main sources of IL-33 are epithelial cells and fibroblasts. The receptor, ST2, is present on cells in the lamina propria, like mast cells and basophils, but also macrophages, Th2 cells and ILC2. Clinical trials investigating therapeutic efficacy of anti-IL13 antibodies for ulcerative colitis patients are currently being performed.\(^{12}\)

**Group 3 ILCs and their diverse functions in tissue homeostasis**

ILC3 are considered to express ROR\(\gamma\)t and c-Kit, distinguishing them from other ILC subsets.\(^{42}\) The group of ROR\(\gamma\)t\(^{+}\) ILCs contain lymphoid tissue inducer cells (LTi) and ILCs producing IL-17 and IL-22.\(^{22}\) During development LTi cells are involved in the formation of secondary lymphoid tissues such as lymph nodes and Peyer’s patches in the intestine.\(^{43}\) Belonging to the family of ILCs, LTi cells are Lin\(^{-}\) CD127\(^{+}\) and produce lymphotixin (LT)\(\alpha\)\(\beta\) and TNF-\(\alpha\) inducing stromal cells to produce adhesion molecules VCAM-1 and ICAM-1, which attract lymphocytes to form lymphoid structures.\(^{43}\) Furthermore during fetal development LTi cells are able to produce IL-17.\(^{44}\) After birth, these Lin\(^{-}\) CD127\(^{+}\) ROR\(\gamma\)t\(^{+}\) cells remain present, and are dedicated to IL-17/IL-22 production, similar to their adaptive Th17/Th22 counterparts.\(^{45}\)
IL-22 production is limited to RORγt+ c-Kit+ ILC that express NCR, like NKp44 or CD56, in humans. NCR+ILC3 can be found at different mucosal sites, such as the intestinal lamina propria, Peyer’s patches, mesenteric lymph nodes and tonsils. IL-22 production is regulated by ligands of the aryl hydrocarbon receptor (AHR). Mucosal epithelial cells respond to IL-22, leading to increased microbial defence, tissue regeneration and protection against tissue damage. IL-22 seems to play a protective role in Citrobacter Rodentium induced colitis. The production of IL-17 is present in ILC3 lacking NCR. IL-17 can recruit neutrophils crucial for defence against microbes, however it has also been shown to be involved in many autoimmune disorders. The majority of ILCs in the intestine belongs to NCR+ ILC3, dedicated to IL-22 production. However, in Crohn’s disease inflamed intestine, IL-22 producing ILC3 are outnumbered by IFN-y producing ILC1.

Lost in translation
Since ILC3 were the first subset of ILCs to be characterized, after NK cells, most is known about these cells. Rag-/- mice, lacking T and B cells, develop an IL-23 dependent colitis after H. Hepaticus infection due to ILCs that produce IL-17, IL-22 and IFN-y. Colitis ameliorated after administration of antibodies against IL-17 and/or IFN-y, and decreased in mice that underwent ILC depletion. Similarly, increased numbers of an IL-17 producing population of Lin-CD45+CD56- ILCs were found to be enriched in the intestine of Crohn’s disease patients. This was also observed in ulcerative colitis colon, however less abundant. Nevertheless, the pathogenic role of IL-17 in mice and the abundance of IL-17 in Crohn’s disease patients, led to the first hypothesis that blocking IL-17 might be a therapeutic strategy. On the contrary, Crohn’s disease patients receiving anti-IL-17 antibodies showed increased disease activity and further trials were not commenced. One could suggest several reasons for these differences between mice and man. First, mice models only mimic IBD but do not reflect the actual triggers for onset and flares of disease. Second, the role of certain cytokines, like IL-17, could differ at different phases of disease. For example, IL-17 might play a pathological role in the development of IBD, but might be essential to restrict inflammation in the chronic phase of the disease and therefore patients with a flare of Crohn’s disease might not benefit from blocking IL-17.
Part II: Therapeutic efficacy in Crohn’s disease patients

Treatment of Inflammatory Bowel Disease
Corticosteroids are effective remission induction drugs suppressing acute inflammation. Most IBD patients respond to a short period of corticosteroids, contrasted by poor maintenance of remission after one year of treatment and serious side effects that have to be taken into account. Immunomodulating drugs, such as thiopurines and methotrexate have been shown to be beneficial for both remission induction and especially maintenance. However, they are limited by a delayed onset of action and adverse events. Despite the improvement of symptoms, no decrease in the need for surgery was found in studies focusing on corticosteroids, thiopurines or methotrexate. Surgical resection of (part of) the intestine is deemed necessary in up to 75% of Crohn’s disease patients and approximately 10–30% of ulcerative colitis patients within the first decade after diagnosis. However, resection of the inflamed intestine does not cure patients, since inflammation often recurs at the site of anastomosis.

In the last decade a revolution in biological drug development has evolved into new possibilities of treatment strategies. Biological drugs target specific inflammatory cytokines. The first biological available for Crohn’s disease was infliximab, a monoclonal chimeric antibody directed against TNF-α. TNF-α is an inflammatory cytokine and plays an important role in the pathogenesis of IBD with elevated levels found in the intestine, serum and stools of patients. Since 1999 infliximab has been available in Europe and efficacy has been shown in several clinical trials. Nonetheless, a substantial number of Crohn’s disease patients do not respond or lose response to infliximab. In 2007 a fully humanized monoclonal antibody antagonizing TNF-α, adalimumab, was approved for treatment of Crohn’s disease patients, broadening treatment armamentarium. However, clinical trials do not reflect the extensive variability in symptoms and endoscopic lesions of Crohn’s disease patients in clinical practice, when decisions are based on global physician’s assessment and not by subjective questionnaires nor mucosal healing.

Markers for therapy response
Assessing therapeutic efficacy in daily practice is a matter of trial and error, changing to another type of therapy when a therapy has turned out to be inadequate. Inadequate therapy leads to severe morbidity among Crohn’s disease patients, typically characterized by chronic bloody diarrhoea, weight loss, abdominal pain and general malaise. Lack of disease control may result in abscess and fistula formation necessitating surgical resection or bowel diversion. Clinical trials have been hampered by the lack of objective markers for therapy response. All the seminal trials made use of subjective disease activity scores to define clinical response. The success of infliximab and adalimumab in the treatment of IBD has boosted research testing other biologicals without much success so far. Only vedolizumab, a humanized immunoglobulin G1 monoclonal antibody to α4β7 integrin that modulates gut lymphocyte trafficking, will be registered in 2014 as a novel biological for the treatment of IBD. Recent studies have shown that vedolizumab is more effective in remission induction and maintenance
of IBD than placebo. Nevertheless, these clinical trials defined clinical response by the subjective disease activity score: CDAI (Crohn’s Disease Activity index). Clinical remission after 6 weeks of induction therapy is defined as a CDAI score of ≤150 points) and clinically meaningful response as CDAI-100 response (≥100-point decrease in the CDAI score). Recent evidence has emerged showing that the CDAI does not accurately predict endoscopic remission and does not correlate with CRP levels. Endoscopic remission is of importance since it has been associated with decreased inflammation after five years and a reduction in the need for steroids. Furthermore, mucosal healing tended to lead to fewer hospitalizations for Crohn’s disease patients on infliximab. However, it is too burdensome for patients and doctors, to perform consecutive colonoscopies to score the Crohn’s disease Endoscopic Index of Severity (CDEIS) as an objective marker of therapy response. Markers that predict therapeutic efficacy in a patient reduce the use of non-efficacious, expensive medical treatments, resulting in a reduction of morbidity, time spent in hospital or sick leave, side effects, as well as costs. Secondly, identification of early markers for therapeutic response can serve as an important tool for drug development. The advantage of such markers over the currently used disease activity indexes is that they are objective and not associated with the high placebo responses. The use of biomarkers for early response to treatment reduces the number of patients needed in trials and will reduce the associated costs of large trials.

**Aim of this thesis**

The main focus of this thesis is to investigate current treatment paradigms for Crohn’s disease patients and unravel part of the innate immune system for potential novel drug targets.
Outline of this thesis

Part I: Innate lymphoid cells in inflammatory bowel disease

Chapter 2
We aimed to identify all known ILC subsets in Crohn’s disease patients’ inflamed intestine. Interestingly, we could identify a novel subset of ILCs dedicated to the production of IFN-y. This subset was distinct from natural killer cells and was dubbed ILC1.

Chapter 3
Here we describe all ILC populations that arise in the intestine during fetal development and the changes that occur after birth when the intestine is colonized with bacteria. During fetal development, the intestine contains ILC3 that differentiate towards gestation.

Part II: Therapeutic efficacy in Crohn’s disease patients

Chapter 4
This chapter reveals potential biomarkers to predict patient’s response to remission induction therapy. Several gene expression profiles were differentially expressed between responders and non-responders early during treatment.

Chapter 5
To asses response to anti-TNF therapeutics in daily clinical practice three cohorts were investigated. First, the clinical response to adalimumab in a large cohort of Crohn’s patients is discussed. Concomitant thiopurine treatment might improve maintenance therapy.

Chapter 6
Second, the clinical response and treatment strategies for infliximab are described. Furthermore, all adverse events that were reported in the past decade are reported.

Chapter 7
Finally, the therapeutic options for highly refractory patients are investigated. Response can be maintained when patients switch from infliximab, to adalimumab and back to infliximab.
References


Introduction


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