Towards therapeutic disease control in inflammatory bowel diseases

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List of abbreviations

3-MA	3-methyladenine
5-ASA	5-aminosalicylic acid
6-MP	6-mercaptopurine
6-TG	6-thioguanine
ADA	adalimumab
ADCC	antibody-dependent cellular toxicity
AIEC	adherent-invasive E. coli
APC	antigen presenting cell
ASCA	anti-Saccharomyces cerevisiae
ATG16L1	autophagy-related 16-like 1
AZA	azathioprine
BMDC	bone marrow derived dendritic cells
CARD15	caspase recruitment domain family, member 15
CCMO	Central Committee on Research involving Human Subject
CD	Crohn's disease
CDAI	Crohn's disease acitivity index
CDEIS	Crohn's disease index of severity
CEACAM6	Carcinoembryonic antigen-related cell adhesion molecule 6
CI	confidence interval
CMV	cytomegalovirus
CRP	C reactive protein
DC	dendritic cell
E. Coli	Escheria Coli
EBV	Epstein-barr virus
ESR	erythrocyte sedimentation rate
FDA	food and drug administration
FSC	forward scatter
GFP	green fluorescent protein
GvHD	Graft versus host disease
GWAS	Genome Wide Association Study
HBV	hepatitis B virus
HL	Hodgkin lymphoma
HSV	herpes simplex virus
HVI	Harvey Bradshaw Index
I2	CD associated bacterial sequence
IBDU	unspecified inflammatory bowel disease
ICAM-1	Inter-Cellular Adhesion Molecule 1
IDO	indoleamine 2,3-dioxygenase
IFNγ	interferon-γ
IFX	infliximab

Ig	immunoglobulin
IL	interleukin
INH	isonicotinylhydrazine
IRGM	immunity-related GTPase family M
IS	immunological synapse
LAMP-2	lysosomal-associated membrane protein 2
LFA-1	lymphocyte function-associated antigen 1
LPMNC	laminia propria mononuclear cell
LPS	lipopolysaccharide
LTA	lymphotoxin-alpha
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MSC	mesenchymal stromal cell
mTNF	membrane bound tumor necrosis factor alpha
Mϕ	macrophage
Mϕ1	type 1 macrophage
Mϕ2	type 2 macrophage
NHL	non-Hodgkin lymphoma
NOD	nucleotide-binding oligomerization domain
OmpC	Escheria coli outer membrane porin C
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PD-L1	programmed cell death 1 ligand 1
Pi	propidium iodide
PML	multifocal leukoencephalopathy
RA	rheumatoid arthritis
RR	relative risk
SES-CD	simple endoscopic score for Crohn's disease
SNP	single nucleotide polymorphism
SSC	side scatter
STAT3	signals transducer and activator of transcription 3
TACE	tumor necrosis factor alpha converting enzyme
TB	tuberculosis
TCR	T cell receptor
TGFβ	tumor growth factor
TIMP	tissue inhibitor of metalloproteinases
TLR	toll-like receptor
TNFSF15	tumor necrosis factor superfamily 15
TNFα	tumor necrosis factor alpha
Tregs	regulatory T cells
UC	ulcerative colitis
VCAM-1	vascular cell adhesion molecule 1

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C The illustrations marked with this icon, can also be found in Chapter 9, 'Full colour illustrations'.

Chapter one

Introduction

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1 The immune system

The immune system protects the human body from disease. To accomplish this, many cell types are involved with different functions and tasks. When bacteria or viruses enter the body, the innate immune system first comes into play. [1] This system is non-specific, and provides an immediate inflammatory response which is characterized by redness, swelling, pain, heat and dysfunction. Once the innate immune system is activated, cells produce several cytokines and chemokines. In response to these secreted factors, other immune cells are recruited to the site of inflammation. The innate immune system then activates the adaptive immune system, consisting of highly specialized cells, B cells and T cells. [2] In the absence of antigen, T cells and B cells are naïve. They are activated by the innate immune system, when an antigen presenting cell (APC) presents an antigen to the B or T cell. Some of these activated B and T cells will become memory cells which provide long-term immune memory as these cells are able to respond to antigens without the help of the innate immune system. This will result in a more rapid and efficient response when the immune system encounters an antigen which has been recognized before.

1.1 The innate immune system

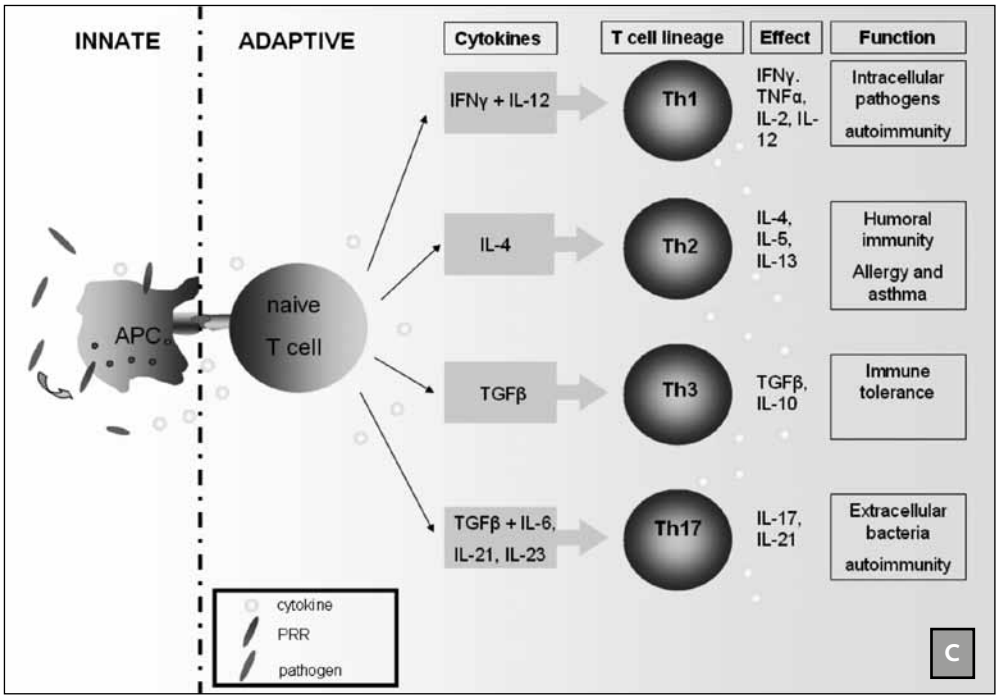
APCs are the key players of the innate immune system: they sense and process antigens, produce cytokines in response to pathogens, and activate the adaptive immune system. APCs express several pattern recognition receptor (PRR) molecules to sense pathogens in the environment. Two important types of PRRs are membrane-associated toll-like receptors (TLR) and cytosolic nucleotide-binding oligomerization domain (NOD proteins). The TLR family includes a family of 10 studied TLRs which all have different specificities for various pathogens. When lipopolyssaccharide (LPS, a cell component of Gram-negative bacteria and responsible for septic shock) binds to TLR4, the cell starts to produce several pro-inflammatory cytokines, among which tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β). [3] Next, other cells are recruited to the site of inflammation and an immune reaction is initiated. NOD2 is located intracellular and recognizes molecules that contain muramyl dipeptide (MDP), which is present in several bacteria. [4]

Dendritic cells (DCs) are the most specialized APC and are usually one of the first cells that come into action once a bacterium or virus enters the body. When a DC senses a pathogen by ligation of a PRR, it phagocytoses ("eats") the potential harmful foreigner, processes it inside the cell, and presents pieces of the protein on the cell membrane loaded on MHCII molecules. [5, 6] After ligation of PRRs, co-stimulatory molecules like CD80, CD83 and CD86 are upregulated. The antigen presented on the MHCII molecule is then recognized by the T cells receptor complex (TCR) on the T cell, and this provides the first signal to initiate an immune response. [7] To achieve a full immune response, additional stimulation is often needed; the interaction of costimulatory molecules on DCs and T cells and the presence of various cytokines provide the second signal and finally determine the outcome of the immune response. [8, 9]

Like DCs, macrophages (Mϕ) are part of the innate immune system, derive from monocytes and function as APCs. They are present in many tissues and contribute to tissue homeostasis. [10, 11] Mϕ are a heterogenous population of cells and have different functions depending on their differentiation status and the type of cytokines present in their environment.

[12-14] Type 1 macrophages are typically induced in the presence of LPS or pro-inflammatory cytokines. In this setting, Mϕ are primed to become effector cells that are highly efficient in killing intracellular bacteria and in the production of pro-inflammatory cytokines. [15] In contrast to Mϕ1, type 2 macrophages (Mϕ2, regulatory macrophage or alternatively activated macrophage) have a more anti-inflammatory phenotype. They are induced by Th2 cytokines, glucocorticoids or immune complexes. Mϕ2 have several characteristics that are functionally different from Mϕ1: they are able to dampen immune responses by inhibiting T cell proliferation, production of anti-inflammatory cytokines and they contribute to wound healing. [16-18] In addition, Mϕ2 inhibit Th1 responses by skewing the immune response towards a Th2 response.

Figure 1 Innate and adaptive immunity.



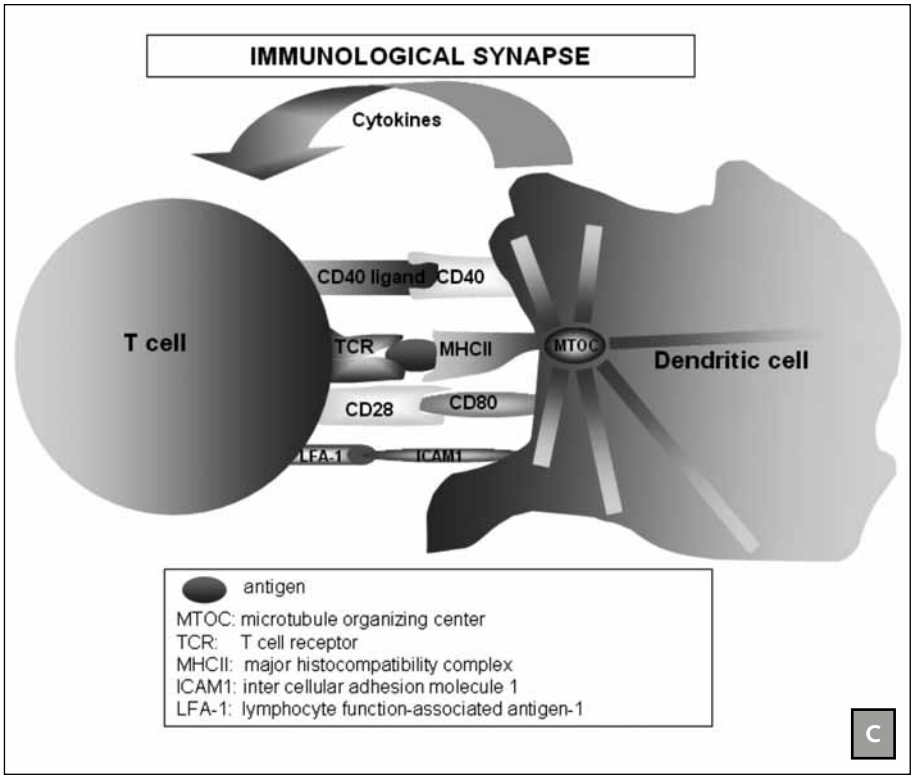
APC antigen presenting cell; IL interleukin; PRR pathogen recognition receptor

1.2 The adaptive immune system

The adaptive immune system consists of lymphocytes, i.e. B cells and T cells. B cells are involved in the production of Immunoglobulins (Ig, i.e. antibodies), whereas T cells differentiate into helper T cells that further support the ongoing immune response. After an APC has presented an antigen to the T cell, the T cell starts to proliferate, and differentiates into one of the known helper T cell lineages: Th1, Th2 or the more recently described Th3 [19] and Th17 (Figure 1). [20, 21] The direction of differentiation is determined by the inflammatory environment and the presence of other factors, like cytokines. A Th1 response is characterized by the production of interferon-γ (IFNγ), TNFα and interleukin-2 (IL-2).

These cytokines stimulate T cell proliferation and activate macrophages. The activated macrophages produce cytokines that further promote Th1 differentiation of T cells. During a Th2 response, IL-4, IL-10 and IL-13 are typically produced, which activate B cells to produce immunoglobulines (Ig) and inhibit Th1 responses. Cytokines produced by Th2 effector cells further augment the differentiation signal towards a Th2 response, thereby maintaining the Th2 response. When the cells differentiate into the Th3 lineage, the T cells start to produce large amounts of transforming growth factor bèta (TGFβ), a factor which is known to be involved in tolerance and for the differentiation of regulatory T cells (Tregs). [22] Tregs are cells with anti-inflammatory properties; they inhibit activation of the immune system and thereby maintain immune homeostasis. [23] A Th17 response is characterized by the massive production of interleukin-17 by T cells, which is induced by IL-23, or IL-6 in combination with TGFβ. [24, 25] IL-17 induces and promotes pro-inflammatory responses, [26] and triggers the production of pro-inflammatory cytokines like TNFα and IL-1β. Th1, Th2, Th3 and Th17 effector T cells all express CD4 on their membrane, making them CD4+ T cells. Another subset of T cells, CD8+ cells, plays an important role in the recognition and elimination of intracellular pathogens. [27] CD8+ T cells recognize antigens presented by MHCI molecules, and are able to kill infected cells by secreting perforin and enzymes. [28]

Figure 2 The immunological synape.



1.3 The immunological synapse

As mentioned before, cytokines present in the inflammatory environment and the interaction between the APC and the T cell together determine the outcome of the immune response. The immunological synapse (IS) is the initial site of interaction between the DC and the T cell. [29, 30]

It is a highly organized structure, with the MHCII-TCR complex in the center, and the co-stimulatory molecules in the periphery (Figure 2). Importantly, a mature synapse is formed only upon recognition of a foreign antigen. When this is the case and an immunological synapse is formed, the actin skeleton polarizes towards the synapse. [31] A proper formation of the IS is important for an efficient T cell response, as destabilization of the IS has been reported to result in decreased T cell signaling. [32] As a consequence, formation of the IS plays a crucial role in the activation of T cells, and thereby the outcome of the immune response.

1.4 The immune system and tolerance in the gastrointestinal tract

The immune system in the gastrointestinal tract has several unique features. In the gut, an enormous amount of microorganisms (more than 500 different bacteria species) [33] is present that constitutes the gut flora (commensals), and intestinal APCs are constantly exposed to these microorganisms and food antigens. An immunologic response to the gut flora or other harmless antigens like food antigens would result in the recruitment of immune cells, production of cytokines, and consequently in inflammation, tissue damage and dysfunction. Therefore it is very important that intestinal APCs do not respond to these antigens, a process called tolerance. [34] The physical barrier separating the commensals from the underlying tissues consists of a single cell layer of epithelial cells and is the first line of defense. The presence of a mucus layer, antibacterial molecules (defensins) and IgA further helps to protect the invasion of antigens. [35] Since APC are then the first cells to respond to an antigen, Mϕ and DCs are the key players of innate immunity and tolerance. [36] To this end, intestinal Mϕ lack several innate response receptors, do not produce pro-inflammatory cytokines in response to various inflammatory signals, [37] and produce large amounts of IL-10 but no IL-12 and IL-23, [38] giving them an Mϕ2-like appearance. [39] Loss of tolerance would lead to an immune reaction, and subsequently result in autoimmunity. In the absence of inflammation but in the presence of commensals, a close balance between effector T cells (i.e. Th1, Th2 or Th17 T cells) and regulatory T cells is maintained by a complex network of cytokines.

2 Inflammatory Bowel Diseases

2.1 Epidemiology, symptoms and diagnosis

Inflammatory bowel disease (IBD) refers to a chronic inflammation affecting the gastrointestinal tract. The highest incidence rates for both Crohn's disease (CD) and ulcerative colitis (UC) are reported in the western world. [40-42] However, the incidence in other parts of the world is increasing. In Europe and the US, incidence rates range from 1.5 to 20.3 per 100.000 person-years for UC, and from 0.7 to 14.6 for CD. The observation that IBD incidence is low in developing countries, suggests that environmental factors and diet play an important role in the pathogenesis.

Crohn's disease (CD) is characterized by deep ulcerations that can occur in the entire gastrointestinal tract, i.e. from the mouth to the anus. [43, 44] The disease may affect only one area in the gut, or several areas with healthy areas in between, so called "skip lesions". An early feature of CD is apthoid ulceration, followed by deep ulcers and fissures in the mucosa at a later stage, which makes up the typical cobblestone pattern. Fistulae and abscesses are often present in a later stage. The inflammation is transmural (affecting all layers of the bowel), and lymphoid hyperplasia, an increase in inflammatory cells and granulomatous lesions are often observed.
Ulcerative colitis (UC) on the other hand, only affects the colon. [43, 44] It can affect the rectum alone (proctitis), it may involve the sigmoid and descending colon (left-sided colitis), or it may involve the whole colon. Typically, the mucosa has a red appearance, bloods easily and is inflamed. The inflammation is restricted to the mucosa, and crypt abscesses and goblet cell depletion are common features.
Typically, a patient presents with abdominal pain, bloody stools, diarrhea and weight loss. Some patients may also have complaints of malaise, fever, nausea and vomiting. Also, CD can be complicated by anal or perianal disease.
The diagnosis IBD can usually be made based on clinical, radiographic and histologic data. [45] The distinct patterns of the two diseases often enable the final diagnosis and differentiation between UC and CD on histologic basis. However, this is not always possible, and sometimes the diagnosis interderminate inflammatory colitis is made. In both UC and CD, anemia is common, and erythrocyte sedimentation rate (ESR) is often raised. CRP has been described as a sensitive marker for CD; elevated CRP levels are detectable in 70 – 100% of the CD patients. On the other hand, only 50 – 60% of UC patients have an elevated CRP at diagnosis. [46, 47]

2.2 Pathogenesis of IBD

CD and UC are diseases of unknown etiology. The fact that higher incidence rates are reported in the Western world compared to developing countries, suggests that environmental factors and nutrition may play a role. However, different incidence rates might also result from differences in access to health care and thus lower incidence rates may be reported in developing countries. Next to environmental factors, genetic factors and defects in innate and adaptive immunity may contribute to inflammatory bowel diseases.

2.2.1 Genetics

IBD has a strong genetic component, since a positive family history for the disease is the largest independent risk factor. It has been reported that 2.2 – 16.2% of the CD patients have a first-degree relative with CD, and in 5.2 – 22% with IBD. For UC, this is 5.7 – 15.5% and 6.6 – 15.8% respectively. [48] Moreover, two studies performed in twins show a pooled estimated concordance in monozygotic twins of 37.3% for CD, and 10% for UC; [49, 50] pooled concordance in dizygotic twins shows 7% for CD and 3% for UC. This suggests that CD might have a stronger genetic component than UC.
However, the disease does not simply result from a single gene defect. Many studies investigating the contribution of genes have been performed, and several genes that are relevant in innate and adaptive immunity have been suggested to be involved in the pathogenesis of IBD. First, the involvement of the *CARD15/NOD2* gene in CD has been shown and con-

firmed in several studies. [51-53] Since CARD15/NOD2 is a known PRR, mutations in this gene might result in altered sensing of bacterial products. Indeed, several mechanisms linking NOD2 dysfunction to CD pathogenesis have been reported, among which enhanced production of IL-1β, [54] abnormalities in TLR2 mediated inflammation in intestinal mϕ [55] and altered NOD2 dependent expression of microbicidal α-defensins. [56, 57] Importantly, NOD2 knockout mice do not spontaneously develop colitis, indicating that defects in NOD2 only are not sufficient to induce inflammation. [58]

SNPs (single nucleotide polymorphisms) in the tumor necrosis factor superfamily 15 (TNFSF15) gene have also been reported in the pathogenesis of IBD. [59, 60] Briefly, TNFSF15 is a strong inducer of IFNγ production in T cells and is upregulated in CD4+/CD8+ T cells and macrophages in the lamina propria of CD patients. [59, 61] Also, a role for SNPs in the IL-23 receptor has been verified in several studies. [62-64] IL-23, together with IL-6 and TGFβ, drives the differentiation of naïve T cells towards a Th17 response, thereby initiating an immune response. In addition, other genes in the IL-23 pathway have been implicated in the pathogenesis of IBD, including IL-12B (encodes the p40 subunit of IL23 and IL12) and signals transducer and activator of transcription 3 (STAT3), further suggesting a prominent role for this pathway. [65-67]

Many other genes have been identified that may play a role in IBD pathogenesis, but a full overview of these genetic defects is beyond the scope of this chapter.

2.2.2 Defects in the immune system in inflammatory bowel diseases

The underlying defect possibly lies in the loss of tolerance towards the mucosal flora, and several defects in innate and adaptive immunity have been reported that may play a role in the development of IBD.

It has been shown that lamina propria mononuclear cells (LPMNCs) from UC and CD patients spontaneously produce large amounts of pro-inflammatory cytokines, thereby triggering an immune response. [68-70] In a mouse model of colitis, increased responsiveness to bacterial stimuli has been reported, [71] resulting in aberrant immunity. This suggests that Mϕ from IBD patients display a more Mϕ1 phenotype, while the ability of intestinal Mϕ to secrete pro-inflammatory cytokines is normally (and preferably) low compared to Mϕ1. Indeed, lower amounts of Mϕ2 were found in mucosal biopsies from active lesions in CD patients compared to non-affected colon of the same patient, and compared to healthy controls. [72] In addition, DCs from both UC and CD patients show higher expression of TLR2 and TLR4 compared to healthy individuals, [73] making them hyperresponsive to bacterial antigens. Colonic macrophages from IBD patients have increased expression of the co-stimulatory molecules CD80 and CD86 [74] resulting in an increased ability to activate T cells. As a consequence of these defects, a Th1 or possibly Th17 response is induced.

Leaks in the epithelial barrier, which is the first line of defense, also have been reported in IBD patients. [75] As a result, pathogens cross the epithelial layer more easily. Interestingly, this defect seems to precede the development of CD in individuals with familial risk, [76] suggesting a causal role for this defect. In addition, overgrowth of mucosa-associated *Escheria coli* has been observed in CD patients; adherent-invasive *E. coli* (AIEC) are found in 36.4% of the CD patients with ileal involvement. Although *E. coli* is considered a commensal, some strains acquire virulence factors. AIEC bind to the CEACAM6 receptor, which is overexpressed in ileal mucosa of CD patients, leading to abnormal colonization. [77] Next, they

invade the intestinal barrier, infect and replicate within mucosal macrophages [78] and induce production of TNFα.
Furthermore, defects in the T cell compartment might contribute to the induction and persistence of IBD. It has been shown that IBD patients have increased numbers of activated T cells in the circulation, and that activated T cells from CD patients are more resistant to apoptosis, [79] a mechanism involved in programmed cell death which takes place after T cell activation and thereby contributes to homeostasis. As a result, the balance between effector T cells and regulatory T cells is disturbed in IBD patients, followed by uncontrolled inflammation.

2.2.3 Autophagy
Another interesting but rather unexpected discovery is the contribution of the *autophagy-related 16-like 1* (ATG16L1) and *immunity-related GTPase family M* (IRGM) genes to CD pathogenesis, [80-82] two genes that are known to be involved in a process called autophagy (referring to the Greek work "autophagos", i.e. "self-eating"). Autophagy was originally described as a cell survival mechanism. When a cell experiences nutrient depletion, autophagy is induced in order to remove damaged organelles. [83] Upon induction of autophagy, a membrane is formed, creating an autophagosome which surrounds the cellular contents and next fuses with lysosomes. [84] More recently, it became clear that autophagy also plays a crucial role in the clearance of intracellular bacteria, [85] and in the delivery of cytoplasmic antigens to MHCII molecules for antigen presentation to T cells. [86] In an experimental ATG16L1 knockdown system, cells showed defective autophagy in response to nutrient depletion and infection, demonstrating the importance of ATG16L1 in the autophagy process. [87] Since ATG16L1 and IRGM have been confirmed in several Genome Wide Association Studies (GWAS), and given the role of autophagy in general and in immunity, it is likely that autophagy plays an important role in the development of CD. Several mechanisms have been suggested that link defective autophagy to CD. In a DSS colitis model, mice lacking ATG16L1 in hematopoetic cells showed increased production of the pro-inflammatory cytokine IL-1β. [88] Furthermore, abnormalities in paneth cells (cells specialized in the secretion of granule contents that contain antimicrobial contents) have been reported in ATG16L1 knockout mice and in CD patients carrying the risk allele. [89]
In summary, environmental, genetic and immunologic defects all contribute to the development of IBD. Likely, the presence of a combination of these factors leads to a loss of response towards the mucosal flora, resulting in inflammation in the gut.

3 Treatment

The main treatment goals in IBD are improving quality of life, reducing hospitalization, surgery and steroid dependency, improving mucosal healing and maintaining clinical remission to control the disease while minimizing side effects. Mucosal healing can lead to significantly higher steroid-free remission rates and less relapses, [90] and is therefore an important goal to achieve in the treatment of CD patients. Induction therapy is concentrated on quickly reducing signs and symptoms of acute inflammation. However, the underlying disease cause remains unchanged and therefore maintenance therapy is often needed to

prevent relapses. Relapsing disease frequently leads to surgical interventions and hospitalization and for that reason maintaining remission is of great importance. Response to treatment is defined as a decrease in Crohn's Disease Activity Index (CDAI) of 70 points (70-points response) or 100 (100-points-response) after four weeks from baseline in non-fistulizing disease. Here, response is defined as a decrease of 70 points unless stated otherwise. In fistulizing disease, response is achieved when a decrease of at least 50% in the number of draining fistulas after ten weeks is observed. Remission is defined as a CDAI score below 150. [91, 92].

The final goal is to understand the course of disease and to finally alter the course towards a less aggressive phenotype. Several therapeutics are available with different effects, side effects and efficacy profiles to achieve the above described goals.

3.1 5-ASA

5-Aminosalicylic acid (5-ASA, mesalazine), a derivate of salicylic acid, is a non-steroidal anti-inflammatory drug (NSAID).

Whereas induction with 5-ASA therapy for patients with mild-to-moderate CD seems effective, [93] it is known that this agent is not effective in inducing remission. [94] In addition, patients with ileal disease do not benefit from 5-ASA and side effects occur in about one-third of the patients. [95] The clinical significance of the CDAI reduction obtained with 5-ASA is controversial, and therefore, 5-ASA has limited value in severe disease and in maintenance in CD patients.

On the other hand, 5-ASA is important in the treatment of UC; the efficacy of 5-ASA in severe UC has been shown in several systematic reviews and meta-analyses. [96, 97]

3.2 Steroids

Glucocorticoids are steroid hormones that bind to the glucocorticoid receptor and activate or suppress certain target genes. This then results in decreased production of pro-inflammatory cytokines and inhibition of T cell proliferation. [98, 99]

Although budesonide is more effective than placebo in inducing remission in acute active CD, [100] it is not effective in maintaining remission. [101] Also, systemic corticosteroids are very effective in inducing remission in the first place, [102] but do not induce long-term remission, [103, 104] mucosal healing [105] and do not reduce the risk for surgery. [106]

Similar to CD, corticosteroids are effective in the induction of remission, [107] and are important in acute severe UC, [108] but are not useful in maintenance therapy. [109] Furthermore, corticosteroids are known to have serious side effects like diabetes mellitus, [110] osteoporosis, depression, hypertension, and as a result these agents are associated with increased morbidity and mortality. Therefore, long-term corticosteroid use is discouraged.

3.3 Thiopurines

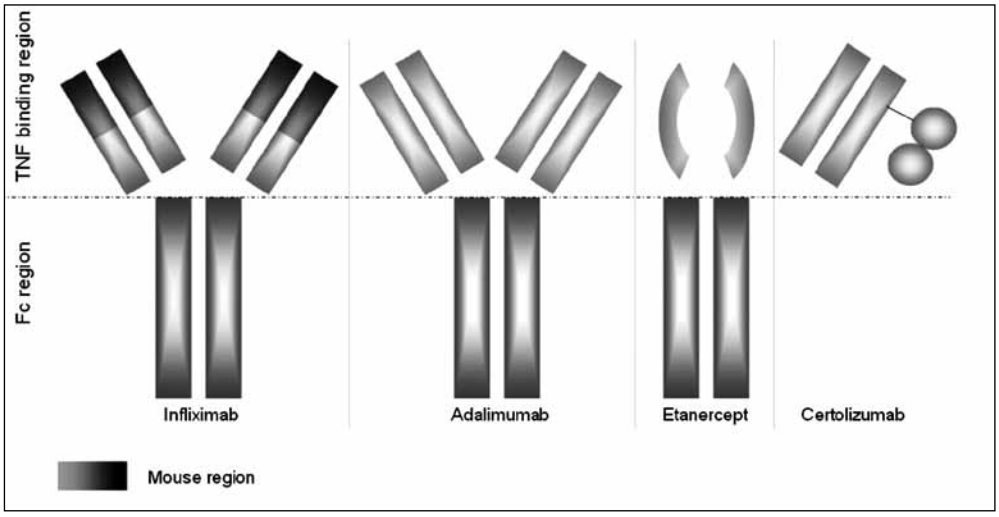
Azahioprine (AZA) and 6-mercaptopurine (6-MP) have been widely used in the treatment of IBD. AZA is a pro-drug which is converted to 6-MP, which is then metabolized to 6-thioguanine (6-TG). This acts as a DNA synthesis inhibitor, and thereby inhibits proliferation of cells, especially lymphocytes. In addition, it has been shown that azathioprine inhibits T cell proliferation by inhibiting APC-T cell conjugation [111] and it induces apoptosis in T cells by modulating Rac1 function. [112]

Thiopurines play an important role in controlling CD and have long term efficacy, [113, 114] but more than half of the patients still depend on corticosteroids. In UC, azathioprine is effective in inducing clinical and endoscopic remission, and is a drug of first choice in patients who are steroid dependent. [115] For maintenance therapy, the evidence for thiopurine in UC is weaker than in CD.
Unfortunately, thiopurine therapy has been associated with myelotoxicity, hepatotoxicity [116] and lymphoma. [117]

3.4 Anti-TNF: structure and mechanism of action

Elevated levels of TNFα are detectable in serum and the intestine of IBD patients, [118, 119] and therefore, blocking this cytokine would potentially alleviate the disease. The introduction of anti-TNF agents during the late '90s has proven to be an effective instrument to achieve the above described treatment goals. Anti-TNF agents induce mucosal healing, reduce steroid dependency, reduce the risk for surgery and hospitalization and improve the patient's quality of life. [91, 120, 121] In addition, healing of endoscopic lesions [121, 122] and reduction of chronic inflammatory infiltrates [122, 123] was achieved.

Figure 3 Different anti-TNF agents.



Anti-TNF agents are designed to neutralize soluble TNF-α, [124] an essential Th_1 cytokine produced by monocytes and T cells. [125] Several anti-TNF agents are available nowadays, and all of them have different structures and properties (Figure 3). Infliximab (Remicade®), is a chimaeric monoclonal antibody, and the first anti-TNF antibody on the market for CD. Adalimumab (Humira®), is a completely humanized anti-TNF antibody, and was designed in the hope to reduce immunogenicity. Adalimumab and infliximab are quite similar in structure; both have an Fc region and a Fab region. Certolizumab (Cimzia®) is different from infliximab and adalimumab, since it does not contain an Fc region and thus can not interact with Fc receptors. Whereas certolizumab was approved by the FDA for the treatment of moderate-to-severe CD, it has not been approved by the EMEA. Etanercept (Enbrel®), a

soluble TNF receptor fusion protein, is an effective drug for the treatment of rheumatoid arthritis and also efficiently neutralizes TNF-α. [124, 126, 127] Surprisingly, etanercept is not beneficial in CD, [128] implicating that neutralizing soluble TNF-α is not the only mechanism of action of anti-TNF agents responsible for their efficacy in CD. One of the differences between infliximab and etanercept is that infliximab induces apoptosis in lamina propria T cells, [129] but etanercept does not. [130] Infliximab, but not etanercept, binds to membrane bound TNF-α (mTNF), which can be cleaved by TNF-α-converting enzyme (TACE) to generate soluble TNF-α. Upon binding to mTNF, infliximab induces antibody dependent cytotoxicity (ADCC) and cell lysis. [131] Mitoma *et al.* described reverse signaling through mTNF induced by infliximab and adalimumab (but not etanercept), leading to cell cycle arrest in Jurkat T cells. [132, 133] Certolizumab does not induce apoptosis, but, like infliximab and adalimumab, inhibits LPS-induced IL-1β production by monocytes. [124] In addition, infliximab induces apoptosis in monocytes from CD patients with active disease. [134] Furthermore, infliximab reduces VCAM-1 (vascular cell adhesion molecule-1) and CD40 expression on mucosal endothelium, thereby disrupting the CD40-CD40L dependent interaction between T cells and endothelium. [135] Infliximab also acts on wound healing: infliximab increases tissue inhibitor of metalloproteinases-1 (TIMP-1) production and reduces matrix metalloproteinase (MMP) activity, and enhances myofibroblast migration *in vitro*. [136]

3.5 Anti-TNF therapy efficacy in clinical trials

The efficacy and safety of anti-TNF therapy has been widely evaluated in clinical studies Targan *et al.* reported response rates at week 4 of overall 65% in patients treated with infliximab vs. 17% in the placebo group. [91] In the ACCENT I trial, 58% responded to infliximab at week 2. [137] In another study, much higher response rates were observed in patients naïve to immunomodulators and biologics with short duration of disease. [138] Response rates of anti-TNF-naïve patients treated with adalimumab were assessed in the CLASSIC-I trial. After 4 weeks, 54% (adalimumab 40/20 mg) to 59% (adalimumab 80/40 and 160/80) showed a clinical response. [92] Clinical response rates of patients receiving certolizumab pegol were evaluated in the PRECISE-1 trial and were 44% at week 4 [139]. The 100-points response at week 6 (primary end point) after full induction therapy was 35%. Both response rates did not reach statistical significance. Patients who received anti-TNF therapy within the previous three months, or had a hypersensitivity or lack of response to a first anti-TNF dose were excluded. Infliximab, adalimumab, as well as certolizumab appeared safe.

Altogether, around one-third of patients treated with infliximab, 45% of patients treated with adalimumab and 56% of patients receiving certolizumab fail to show a clinical response at week 4 or week 6 after full induction therapy. Because there are no head-to-head trials, it is complex to directly compare the results of different studies. It is not known whether these primary non-responders represent a specific group of patients. Patients who do not show a response after a first infusion of infliximab, also fail to show response after subsequent infusions, [91, 140] suggesting that lack of response is stable over time. In general, although many clinical trials have shown the efficacy and safety of anti-TNF therapy, there is a relatively large group of patients displaying lack of response after 4 weeks (primary non-responders). In addition to lack of response, a considerable group of patients lose response following an initial response after several months of treatment. Loss of response is generally defined as a history of initial response and lack of improvement or worsening of symptoms, including:

increased stool frequency, fever, rectal bleeding, daily abdominal pain and recurring drainage from a previously non-draining fistula. [141] Furthermore, a significant number of patients become intolerant to anti-TNF, which is characterized by acute (during or within 24 h post treatment) or delayed (occurring 24 h – 15 days post treatment) infusion reactions.
Lack and loss of response to anti-TNF therapy is an obstacle in the treatment of CD. Predictive factors for lack and loss of response are needed to select patients for a certain approach. This may improve treatment, reduce side-effects and reduce morbidity. Many studies have been done to identify factors for lack and loss of response. Genetic, clinical and demographic factors have been described to play a role in lack of response. FcγRIIIa, [142] TACE [143] and LTA [140] might be possible genetic factors, and young age, [144, 145] luminal CD, [145] short duration of disease [146] and concurrent immusuppression [145, 147-150] possible clinical factors. Patients with lack of response can switch to another anti-TNF, or to a biologic with another mechanism of action. Though controversial, the formation of antibodies against antibodies has been associated with loss of response. The use of concurrent immunosuppressive drugs may reduce the formation of antibodies against antibodies. Dose intensification in patients with low drug through levels or switching to another anti-TNF might be a good option in patients with loss of response.
Also, side effects might complicate anti-TNF treatment. Anti-TNF use is associated with an approximately 21-fold increased risk of tuberculosis (TB) without appropriate safety measures. [151] The TB incidence has been reported to decrease with 78% when suitable safety measures where undertaken. Most cases are presented during the first three months of treatment and have an atypical presentation, which makes the diagnosis more complicating. [152] For that reason, international guidelines advise to assess the risk of TB before starting treatment with an anti-TNF agent, including an X-ray, tuberculin skin testing (depending on national guidelines) and careful evaluation of the TB history. [153] Latent TB may be suspected in case of a positive initial tuberculin skin test and when the patient has recently been exposed to the disease. Physicians should be aware of the possibility of false-negative skin tests, especially when patients are immunocompromized.
Next to reactivation of *mycobacterium tuberculosis*, the use of immunosuppressive agents is associated with opportunistic infections. The risk of opportunistic infections in anti-TNF treated patients is estimated between 0.3 and 0.9%, [154] and an increased risk is observed in patients treated with concommittant immunosuppressives. [155] Indeed, in a large meta-analysis of 21 placebo-controlled trials including 5356 anti-TNF treated patients, the increased risk of opportunistic infections was likely due to disease severity and prednisone use, instead of merely anti-TNF. [156] In line with this observation, no increased risk was found in infections and mortality in 734 anti-TNF treated patients compared to controls, with a median follow-up of 58 months. [149]
Finally, the development of malignanies, and especially lymphomas, is a major concern. Whereas some studies do not show an increased risk, [157-160] other studies do find a moderately elevated risk, especially in patients on thiopurine therapy. [117, 161, 162] Lethal hepatosplenic T cell lymphoma has been reported in young patients on azathioprine/infliximab combination therapy, [163-166] and therefore long-term combination therapy in younger patients is not recommended. Still, the absolute risk appears to be low and should be weighed against the beneficial effects of immunomodulator therapy. In addition, differences in study design and patient recruitment complicate the interpretation of these data. Furthermore, in the

meta-analysis including 21 placebo-controlled trials, [156] no increased risk of malignancy was observed. These data were supported by another study [149] including 734 anti-TNF treated patients. However, long-term safety data are not available yet and therefore awareness of (serious) side effects is warranted.

3.6 Other therapies

There is not much data on the efficacy of methotrexate in UC, but the only randomized placebo-controlled trial did not show any benefit. [167] In contrast, methotrexate efficacy has been shown CD patients. [168] and at the present time, methotrexate therapy is used in patients with active or relapsing CD who are refractory or intolerant to thiopurine therapy or anti-TNF agents. [169] Other immunosuppressives, like ciclosporin or tacrolimus, may be of benefit in patients with severe UC who are intolerant to i.v. corticosteroids. [109] Ciclosporin is of limited value in CD, [45] but data are lacking on the efficacy of tacrolimus in CD.

3.7 Future treatment

Unfortunately, the medication used to control CD is not without risk. Although substantial progression has been made with regard to treatment, there is still no cure for IBD. In addition, side effects like lymphoma, *Mycobacterium tuberculosis* and opportunistic infections further complicate treatment. Moreover, lack and loss of response to anti-TNF therapy are problems in daily practice that are even now unsolved, and surgery is then often the only option left.

Our understanding of the mechanism of action and side effects of several therapies is still incomplete. In an ideal situation, it would be possible to select patients based on genotype or disease phenotype, age or other yet undefined factors for a certain therapeutic strategy. To accomplish this, a better understanding of the pathogenesis of CD and the complex mechanism of action of anti-TNF therapy is warranted in order to tailor therapy. In that way, it is possible to reduce side effects, surgery and chronic use of corticosteroids. In addition, therapy risks need to be re-assessed at any given time point, and if necessary, the therapeutic approach should be re-adjusted. Finally, we have to continue exploring new therapies with less side effects and high efficacy profiles. One interesting development in the treatment of inflammatory disorders, is the administration of mesenchymal stem cells (MSC). MSC are cells with immunosuppressive properties [170, 171] and have been studied in various fields of medicine. Administration of MSCs to patients with severe steroid-refractory graft-versus-host disease (GvDH), including GvDH of the gut, has been shown to be effective. [172, 173] This may be a promising strategy in the treatment of CD.

4 Scope of the thesis

In *chapter 2*, we give an overview of current treatment strategies for CD, especially the top-down approach. Since CD typically progresses from an inflammatory to a fibrotic phenotype, it may be beneficial to interfere in an early disease stage in patients with high risk at developing complicated disease.

In *chapter 3*, we demonstrate the role of autophagy in DCs in regulation of the immunological synapse and CD pathogenesis. We show that decreased levels of autophagy lead to

hyperstabilization of the immunological synapse. This results in increased interaction duration between DCs and T cells and increased T cell activation and IL-17 production. Also, we demonstrate that autophagosomes contain components of the synaps, suggesting that autophagy might be involved in the synaptic breakdown, and thereby plays a role in controlling T cell responses. In addition, we found the same results in patients carrying the ATG16L1 risk allele, indicating a novel role for autophagy in CD pathogenesis by modulating adaptive immune responses.
In *chapter 4*, we further dissect the mechanism of action of anti-TNF agents *in vitro*. We describe the Fc-receptor dependent induction of Mϕ2 upon infliximab therapy and their immunosuppressive phenotype. Since loss of tolerance and hyperresponsiveness contribute to IBD, the induction of Mϕ2 by infliximab might restore the dysbalance.
The induction of Mϕ2 *in vivo* is shown in *chapter 5*, and a significant relation between response to infliximab and induction of Mϕ2 is described. Also, we show the wound healing capacity of infliximab-induced macrophages, further supporting their role in mucosal healing. Furthermore, we show an enhanced induction of Mϕ2 upon infliximab/azathioprine combination treatment, and that Mϕ2 induced by combination treatment have a stronger immunosuppressive phenotype. This might explain the superiority of infliximab/azathioprine combination treatment observed in patients.
In *chapter 6* a Phase I study investigating the safety and feasibility of MSC therapy in steroid-refractory CD patients is described. We show the immunosuppressive properties of MSCs *in vitro* and that MSC therapy in CD patients is safe and feasible. Importantly, no serious side effects were reported during the study period. The efficacy of MSC therapy in CD patients should be further assessed in Phase II/III trials.
In *chapter 7* we further examine the safety profile of common IBD drugs in relation to lymphoma development. In a cohort of approximately 18000 patients, no increased risk was found compared to the general population, but a clear association was observed between thiopurine therapy and EBV positive lymphoma, especially in younger patients. These data give more insight in the risks in specific patient groups.

Reference List

1. Janeway CA, Jr., Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;20:197-216.
2. Medzhitov R, Janeway CA, Jr. Innate immune recognition and control of adaptive immune responses. Semin Immunol 1998;10:351-353.
3. Zhang G, Ghosh S. Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. J Endotoxin Res 2000;6:453-457.
4. Girardin SE, Boneca IG, Viala J et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 2003;278:8869-8872.
5. Clark GJ, Angel N, Kato M et al. The role of dendritic cells in the innate immune system. Microbes Infect 2000;2:257-272.
6. Adema GJ. Dendritic cells from bench to bedside and back. Immunol Lett 2009;122:128-130.
7. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. Science 2010;327:291-295.

8. Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. Nat Rev Immunol 2003;3:984-993.
9. Reis e Sousa. Activation of dendritic cells: translating innate into adaptive immunity. Curr Opin Immunol 2004;16:21-25.
10. Naito M. Macrophage differentiation and function in health and disease. Pathol Int 2008;58:143-155.
11. Naito M, Umeda S, Yamamoto T et al. Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. J Leukoc Biol 1996;59:133-138.
12. Ma J, Chen T, Mandelin J et al. Regulation of macrophage activation. Cell Mol Life Sci 2003;60:2334-2346.
13. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010;32:593-604.
14. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009;27:451-483.
15. Verreck FA, de BT, Langenberg DM et al. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. J Leukoc Biol 2006;79:285-293.
16. Schebesch C, Kodelja V, Muller C et al. Alternatively activated macrophages actively inhibit proliferation of peripheral blood lymphocytes and CD4+ T cells in vitro. Immunology 1997;92:478-486.
17. Kodelja V, Muller C, Tenorio S et al. Differences in angiogenic potential of classically vs alternatively activated macrophages. Immunobiology 1997;197:478-493.
18. Daley JM, Brancato SK, Thomay AA et al. The phenotype of murine wound macrophages. J Leukoc Biol 2010;87:59-67.
19. Carrier Y, Yuan J, Kuchroo VK et al. Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. J Immunol 2007;178:179-185.
20. Murphy KM, Reiner SL. The lineage decisions of helper T cells. Nat Rev Immunol 2002;2:933-944.
21. Crome SQ, Wang AY, Levings MK. Translational mini-review series on Th17 cells: function and regulation of human T helper 17 cells in health and disease. Clin Exp Immunol 2010;159:109-119.
22. Weiner HL. Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. Microbes Infect 2001;3:947-954.
23. Tang Q, Bluestone JA. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol 2008;9:239-244.
24. Harrington LE, Hatton RD et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123-1132.
25. Mangan PR, Harrington LE, O'Quinn DB et al. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature 2006;441:231-234.
26. Liu ZJ, Yadav PK, Su J et al. Potential role of Th17 cells in the pathogenesis of inflammatory bowel disease. World J Gastroenterol 2009;15:5784-5788.
27. Seder RA, Ahmed R. Similarities and differences in CD4+ and CD8+ effector and memory T cell generation. Nat Immunol 2003;4:835-842.

28. Yewdell JW, Norbury CC, Bennink JR. Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines. Adv Immunol 1999;73:1-77.
29. Grakoui A, Bromley SK, Sumen C et al. The immunological synapse: a molecular machine controlling T cell activation. Science 1999;285:221-227.
30. Rodriguez-Fernandez JL, Riol-Blanco L, Delgado-Martin C. What is the function of the dendritic cell side of the immunological synapse? Sci Signal 2010;3:re2.
31. Vicente-Manzanares M, Sanchez-Madrid F. Role of the cytoskeleton during leukocyte responses. Nat Rev Immunol 2004;4:110-122.
32. Al-Alwan MM, Liwski RS, Haeryfar SM et al. Cutting edge: dendritic cell actin cytoskeletal polarization during immunological synapse formation is highly antigen-dependent. J Immunol 2003;171:4479-4483.
33. Hughes JB, Hellmann JJ, Ricketts TH et al. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol 2001;67:4399-4406.
34. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol 2004;4:478-485.
35. Muller CA, Autenrieth IB, Peschel A. Innate defenses of the intestinal epithelial barrier. Cell Mol Life Sci 2005;62:1297-1307.
36. Johansson C, Kelsall BL. Phenotype and function of intestinal dendritic cells. Seminars in Immunology 2005;17:284-294.
37. Smythies LE, Sellers M, Clements RH et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest 2005;115:66-75.
38. Kamada N, Hisamatsu T, Okamoto S et al. Abnormally differentiated subsets of intestinal macrophage play a key role in Th1-dominant chronic colitis through excess production of IL-12 and IL-23 in response to bacteria. J Immunol 2005;175:6900-6908.
39. Smith PD, Ochsenbauer-Jambor C, Smythies LE. Intestinal macrophages: unique effector cells of the innate immune system. Immunol Rev 2005;206:149-159.
40. Russel MG, Dorant E, Volovics A et al. High incidence of inflammatory bowel disease in The Netherlands: results of a prospective study. The South Limburg IBD Study Group. Dis Colon Rectum 1998;41:33-40.
41. Bernstein CN, Blanchard JF, Rawsthorne P et al. Epidemiology of Crohn's disease and ulcerative colitis in a central Canadian province: a population-based study. Am J Epidemiol 1999;149:916-924.
42. Rubin GP, Hungin AP, Kelly PJ et al. Inflammatory bowel disease: epidemiology and management in an English general practice population. Aliment Pharmacol Ther 2000;14:1553-1559.
43. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002;347:417-429.
44. Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. The Lancet 2007;369:1641-1657.
45. Dignass A, Assche GV, Lindsay JO et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. 4 ed. 2010:28-62.
46. Beattie RM, Walker-Smith JA, Murch SH. Indications for investigation of chronic gastrointestinal symptoms. Arch Dis Child 1995;73:354-355.
47. Shine B, Berghouse L, Jones JE et al. C-reactive protein as an aid in the differentiation of functional and inflammatory bowel disorders. Clin Chim Acta 1985;148:105-109.

48. Russell RK, Satsangi J. IBD: a family affair. Best Pract Res Clin Gastroenterol 2004;18:525-539.
49. Thompson NP, Driscoll R, Pounder RE et al. Genetics versus environment in inflammatory bowel disease: results of a British twin study. BMJ 1996;312:95-96.
50. Tysk C, Lindberg E, Jarnerot G et al. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. Gut 1988;29:990-996.
51. Hugot JP, Chamaillard M, Zouali H et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001;411:599-603.
52. Ogura Y, Bonen DK, Inohara N et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001;411:603-606.
53. Hampe J, Cuthbert A, Croucher PJ et al. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. Lancet 2001;357:1925-1928.
54. Maeda S, Hsu LC, Liu H et al. Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. Science 2005;307:734-738.
55. Watanabe T, Kitani A, Murray PJ et al. Nucleotide binding oligomerization domain 2 deficiency leads to dysregulated TLR2 signaling and induction of antigen-specific colitis. Immunity 2006;25:473-485.
56. Kobayashi K, Inohara N, Hernandez LD et al. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. Nature 2002;416:194-199.
57. Wehkamp J, Salzman NH, Porter E et al. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. Proc Natl Acad Sci U S A 2005;102:18129-18134.
58. Pauleau AL, Murray PJ. Role of nod2 in the response of macrophages to toll-like receptor agonists. Mol Cell Biol 2003;23:7531-7539.
59. Thiebaut R, Kotti S, Jung C et al. TNFSF15 polymorphisms are associated with susceptibility to inflammatory bowel disease in a new European cohort. Am J Gastroenterol 2009;104:384-391.
60.Yamazaki K, McGovern D, Ragoussis J et al. Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. Hum Mol Genet 2005;14:3499-3506.
61. Picornell Y, Mei L, Taylor K et al. TNFSF15 is an ethnic-specific IBD gene. Inflamm Bowel Dis 2007;13:1333-1338.
62. Duerr RH, Taylor KD, Brant SR et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 2006;314:1461-1463.
63. Lacher M, Schroepf S, Helmbrecht J et al. Association of the interleukin-23 receptor gene variant rs11209026 with Crohn's disease in German children. Acta Paediatr 2010;99:727-733.
64. Tremelling M, Cummings F, Fisher SA et al. IL23R variation determines susceptibility but not disease phenotype in inflammatory bowel disease. Gastroenterology 2007;132:1657-1664.
65. Lees CW, Barrett JC, Parkes M et al. New IBD genetics: common pathways with other diseases. Gut 2011. Feb 7. [Epub ahead of print]
66. Wang K, Zhang H, Kugathasan S et al. Diverse genome-wide association studies associate the IL12/IL23 pathway with Crohn Disease. Am J Hum Genet 2009;84:399-405.
67. Ferguson LR, Han DY, Fraser AG et al. Genetic factors in chronic inflammation: single nucleotide polymorphisms in the STAT-JAK pathway, susceptibility to DNA damage and Crohn's disease in a New Zealand population. Mutat Res 2010;690:108-115.
68. Reinecker HC, Steffen M, Witthoeft T et al. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin Exp Immunol 1993;94:174-181.

69. Reimund JM, Wittersheim C, Dumont S et al. Mucosal inflammatory cytokine production by intestinal biopsies in patients with ulcerative colitis and Crohn's disease. J Clin Immunol 1996;16:144-150.
70. Zareie M, Singh PK, Irvine EJ et al. Monocyte/macrophage activation by normal bacteria and bacterial products: implications for altered epithelial function in Crohn's disease. Am J Pathol 2001;158:1101-1109.
71. Takakura R, Kiyohara T, Murayama Y et al. Enhanced macrophage responsiveness to lipopolysaccharide and CD40 stimulation in a murine model of inflammatory bowel disease: IL-10-deficient mice. Inflamm Res 2002;51:409-415.
72. Hunter MM, Wang A, Parhar KS et al. In Vitro-Derived Alternatively Activated Macrophages Reduce Colonic Inflammation in Mice. Gastroenterology 2010;138(4):1395-40.
73. Hart AL, Al-Hassi HO, Rigby RJ et al. Characteristics of intestinal dendritic cells in inflammatory bowel diseases. Gastroenterology 2005;129:50-65.
74. Rogler G, Hausmann M, Spottl T et al. T-cell co-stimulatory molecules are upregulated on intestinal macrophages from inflammatory bowel disease mucosa. Eur J Gastroenterol Hepatol 1999;11:1105-1111.
75. Soderholm JD, Olaison G, Peterson KH et al. Augmented increase in tight junction permeability by luminal stimuli in the non-inflamed ileum of Crohn's disease. Gut 2002;50:307-313.
76. Irvine EJ, Marshall JK. Increased intestinal permeability precedes the onset of Crohn's disease in a subject with familial risk. Gastroenterology 2000;119:1740-1744.
77. Barnich N, Carvalho FA, Glasser AL et al. CEACAM6 acts as a receptor for adherent-invasive E. coli, supporting ileal mucosa colonization in Crohn disease. J Clin Invest 2007;117:1566-1574.
78. Glasser AL, Boudeau J, Barnich N et al. Adherent invasive Escherichia coli strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. Infect Immun 2001;69:5529-5537.
79. Sturm A, de Souza HS, Fiocchi C. Mucosal T cell proliferation and apoptosis in inflammatory bowel disease. Curr Drug Targets 2008;9:381-387.
80. Hampe J, Franke A, Rosenstiel P et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet 2007;39:207-211.
81. Kuballa P, Huett A, Rioux JD et al. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. PLoS One 2008;3:e3391.
82. Parkes M, Barrett JC, Prescott NJ et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet 2007;39:830-832.
83. Mizushima N, Klionsky DJ. Protein turnover via autophagy: implications for metabolism. Annu Rev Nutr 2007;27:19-40.
84. Yorimitsu T, Klionsky DJ. Autophagy: molecular machinery for self-eating. Cell Death Differ 2005;12 Suppl 2:1542-1552.
85. Birmingham CL, Smith AC, Bakowski MA et al. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J Biol Chem 2006;281:11374-11383.
86. Schmid D, Pypaert M, Munz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity 2007;26:79-92.

87. Rioux JD, Xavier RJ, Taylor KD et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet 2007;39:596-604.
88. Saitoh T, Fujita N, Jang MH et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 2008;456:264-268.
89. Cadwell K, Liu JY, Brown SL et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 2008;456:259-263.
90. Baert F, Moortgat L, van AG et al. Mucosal healing predicts sustained clinical remission in patients with early-stage Crohn's disease. Gastroenterology 2010;138:463-468.
91. Targan SR, Hanauer SB, van Deventer SJH et al. A Short-Term Study of Chimeric Monoclonal Antibody cA2 to Tumor Necrosis Factor {alpha} for Crohn's Disease. N Engl J Med 1997;337:1029-1036.
92. Hanauer SB, Sandborn WJ, Rutgeerts P et al. Human Anti-Tumor Necrosis Factor Monoclonal Antibody (Adalimumab) in Crohn's Disease: the CLASSIC-I Trial. Gastroenterology 2006;130:323-333.
93. Sandborn WJ, Feagan BG, Lichtenstein GR. Medical management of mild to moderate Crohn's disease: evidence-based treatment algorithms for induction and maintenance of remission. Aliment Pharmacol Ther 2007;26:987-1003.
94. Hanauer SB, Stromberg U. Oral Pentasa in the treatment of active Crohn's disease: A meta-analysis of double-blind, placebo-controlled trials. Clin Gastroenterol Hepatol 2004;2:379-388.
95. Taffet SL, Das KM. Sulfasalazine. Adverse effects and desensitization. Dig Dis Sci 1983;28:833-842.
96. Bebb JR, Scott BB. How effective are the usual treatments for ulcerative colitis? Aliment Pharmacol Ther 2004;20:143-149.
97. Sutherland L, Macdonald JK. Oral 5-aminosalicylic acid for induction of remission in ulcerative colitis. Cochrane Database Syst Rev 2006;CD000543.
98. Barnes PJ, Adcock I. Anti-inflammatory actions of steroids: molecular mechanisms. Trends Pharmacol Sci 1993;14:436-441.
99. Boumpas DT, Paliogianni F, Anastassiou ED et al. Glucocorticosteroid action on the immune system: molecular and cellular aspects. Clin Exp Rheumatol 1991;9:413-423.
100. Otley A, Steinhart AH. Budesonide for induction of remission in Crohn's disease. Cochrane Database Syst Rev 2005;CD000296.
101. Benchimol EI, Seow CH, Otley AR et al. Budesonide for maintenance of remission in Crohn's disease. Cochrane Database Syst Rev 2009;CD002913.
102. Benchimol EI, Seow CH, Steinhart AH et al. Traditional corticosteroids for induction of remission in Crohn's disease. Cochrane Database Syst Rev 2008;CD006792.
103. Faubion WA, Jr., Loftus EV, Jr., Harmsen WS et al. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. Gastroenterology 2001;121:255-260.
104. Steinhart AH, Ewe K, Griffiths AM et al. Corticosteroids for maintenance of remission in Crohn's disease. Cochrane Database Syst Rev 2003;CD000301.
105. Modigliani R, Mary JY, Simon JF et al. Clinical, biological, and endoscopic picture of attacks of Crohn's disease. Evolution on prednisolone. Groupe d'Etude Therapeutique des Affections Inflammatoires Digestives. Gastroenterology 1990;98:811-818.
106. Cosnes J, Nion-Larmurier I, Beaugerie L et al. Impact of the increasing use of immunosuppressants in Crohn's disease on the need for intestinal surgery. Gut 2005;54:237-241.

107. Lennard-Jones JE, Longmore AJ, Newell AC et al. An assessment of prednisone, salazopyrin, and topical hydrocortisone hemisuccinate used as out-patient treatment for ulcerative colitis. Gut 1960;1:217-222.

108. Campieri M, Adamo S, Valpiani D et al. Oral beclometasone dipropionate in the treatment of extensive and left-sided active ulcerative colitis: a multicentre randomised study. Aliment Pharmacol Ther 2003;17:1471-1480.

109. Travis SP, Stange EF, Lemann M et al. European evidence-based Consensus on the management of ulcerative colitis: Current management. J Crohns Colitis 2008;2:24-62.

110. Donihi AC, Raval D, Saul M et al. Prevalence and predictors of corticosteroid-related hyperglycemia in hospitalized patients. Endocr Pract 2006;12:358-362.

111. Poppe D, Tiede I, Fritz G et al. Azathioprine suppresses ezrin-radixin-moesin-dependent T cell-APC conjugation through inhibition of Vav guanosine exchange activity on Rac proteins. J Immunol 2006;176:640-651.

112. Tiede I, Fritz G, Strand S et al. CD28-dependent Rac1 activation is the molecular target of azathioprine in primary human CD4+ T lymphocytes. J Clin Invest 2003;111:1133-1145.

113. Bouhnik Y, Lemann M, Mary JY et al. Long-term follow-up of patients with Crohn's disease treated with azathioprine or 6-mercaptopurine. Lancet 1996;347:215-219.

114. Prefontaine E, Macdonald JK, Sutherland LR. Azathioprine or 6-mercaptopurine for induction of remission in Crohn's disease. Cochrane Database Syst Rev 2010;6:CD000545.

115. Ardizzone S, Maconi G, Russo A et al. Randomised controlled trial of azathioprine and 5-aminosalicylic acid for treatment of steroid dependent ulcerative colitis. Gut 2006;55:47-53.

116. de Boer NK, van Bodegraven AA, Jharap B et al. Drug Insight: pharmacology and toxicity of thiopurine therapy in patients with IBD. Nat Clin Pract Gastroenterol Hepatol 2007;4:686-694.

117. Beaugerie L, Brousse N, Bouvier AM et al. Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. The Lancet 2009;374:1617-1625.

118. Plevy SE, Landers CJ, Prehn J et al. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. J Immunol 1997;159:6276-6282.

119. van Deventer SJH. Review article: targeting TNF as a key cytokine in the inflammatory processes of Crohns disease the mechanisms of action of infliximab. Alimentary Pharmacology & Therapeutics 1999;13:3-8.

120. Rutgeerts P, D'Haens G, Targan S et al. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. Gastroenterology 1999;117:761-769.

121. van Dullemen HM, van Deventer SJ, Hommes DW et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 1995;109:129-135.

122. D'Haens G, Van Deventer S, Van Hogezand R et al. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. Gastroenterology 1999;116:1029-1034.

123. Baert FJ, D'Haens GR, Peeters M et al. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. Gastroenterology 1999;116:22-28.

124. Nesbitt A, Fossati G, Bergin M et al. Mechanism of action of certolizumab pegol (CDP870): in vitro comparison with other anti-tumor necrosis factor alpha agents. Inflamm Bowel Dis 2007;13:1323-1332.
125. Schreiber S, Nikolaus S, Hampe J et al. Tumour necrosis factor [alpha] and interleukin 1[beta] in relapse of Crohn's disease. The Lancet 1999;353:459-461.
126. Moreland LW, Schiff MH, Baumgartner SW et al. Etanercept Therapy in Rheumatoid Arthritis: A Randomized, Controlled Trial. Ann Intern Med 1999;130:478-486.
127. Moreland LW, Baumgartner SW, Schiff MH et al. Treatment of Rheumatoid Arthritis with a Recombinant Human Tumor Necrosis Factor Receptor (p75)-Fc Fusion Protein. N Engl J Med 1997;337:141-147.
128. Sandborn WJ, Hanauer SB, Katz S et al. Etanercept for Active Crohn's Disease: A Randomized, Double-Blind, Placebo-Controlled Trial. Gastroenterology 2001;121:1088-1094.
129. ten Hove T, van Montfrans C, Peppelenbosch MP et al. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. Gut 2002;50:206-211.
130. Van den Brande JMH, Braat H, van den Brink G et al. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology 2003;124:1774-1785.
131. Scallon BJ, Moore MA, Trinh H et al. Chimeric anti-TNF-[alpha] monoclonal antibody cA2 binds recombinant transmembrane TNF-[alpha] and activates immune effector functions. Cytokine 1995;7:251-259.
132. Mitoma H, Horiuchi T, Hatta N et al. Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-[alpha]. Gastroenterology 2005;128:376-392.
133. Mitoma H, Horiuchi T, Tsukamoto H et al. Mechanisms for cytotoxic effects of anti-tumor necrosis factor agents on transmembrane tumor necrosis factor alpha-expressing cells: comparison among infliximab, etanercept, and adalimumab. Arthritis Rheum 2008;58:1248-1257.
134. Lügering A, Schmidt M, Lügering N et al. Infliximab Induces Apoptosis in Monocytes From Patients With Chronic Active Crohn's Disease by Using a Caspase-Dependent Pathway. Gastroenterology 2001;121:1145-1157.
135. Danese S, Sans M, Scaldaferri F et al. TNF-{alpha} Blockade Down-Regulates the CD40/CD40L Pathway in the Mucosal Microcirculation: A Novel Anti-Inflammatory Mechanism of Infliximab in Crohn's Disease. J Immunol 2006;176:2617-2624.
136. Di Sabatino A, Pender SLF, Jackson C et al. Functional Modulation of Crohn's Disease Myofibroblasts by Anti-Tumor Necrosis Factor Antibodies. Gastroenterology 2007;133:137-149.
137. Hanauer SB, Feagan BG, Lichtenstein GR et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. The Lancet 2002;359:1541-1549.
138. Colombel JF, Sandborn WJ, Reinisch W et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med 2010;362:1383-1395.
139. Sandborn WJ, Feagan BG, Stoinov S et al. Certolizumab Pegol for the Treatment of Crohn's Disease. N Engl J Med 2007;357:228-238.
140. Taylor KD, Plevy SE, Yang H et al. ANCA Pattern and LTA haplotype relationship to clinical responses to anti-TNF antibody treatment in crohn's disease. Gastroenterology 2001;120:1347-1355.

141. Sandborn WJ, Rutgeerts P, Enns R et al. Adalimumab Induction Therapy for Crohn Disease Previously Treated with Infliximab: A Randomized Trial. Ann Intern Med 2007;146:829-838.
142. Louis E, El GZ, Vermeire S et al. Association between polymorphism in IgG Fc receptor IIIa coding gene and biological response to infliximab in Crohn's disease. Aliment Pharmacol Ther 2004;19:511-519.
143. Dideberg V, Théâtre E, Farnir Fdr et al. The TNF/ADAM 17 system: implication of an ADAM 17 haplotype in the clinical response to infliximab in Crohn's disease. Pharmacogenetics and Genomics 2006 Oct;16(10):727-34
144. Kugathasan S, Werlin SL, Martinez A et al. Prolonged duration of response to infliximab in early but not late pediatric Crohn's disease. Am J Gastroenterol 2000;95:3189-3194.
145. Vermeire S, Louis E, Carbonez A et al. Demographic and clinical parameters influencing the short-term outcome of anti-tumor necrosis factor (infliximab) treatment in Crohn's disease. Am J Gastroenterol 2002;97:2357-2363.
146. Lionetti P, Bronzini F, Salvestrini C et al. Response to infliximab is related to disease duration in paediatric Crohn's disease. Aliment Pharmacol Ther 2003;18:425-431.
147. Arnott ID, McNeill G, Satsangi J. An analysis of factors influencing short-term and sustained response to infliximab treatment for Crohn's disease. Aliment Pharmacol Ther 2003;17:1451-1457.
148. Parsi MA, Achkar JP, Richardson S et al. Predictors of response to infliximab in patients with Crohn's disease. Gastroenterology 2002;123:707-713.
149. Schnitzler F, Fidder H, Ferrante M et al. Long-term outcome of treatment with infliximab in 614 patients with Crohn's disease: results from a single-centre cohort. Gut 2009;58:492-500.
150. Lemann M, Mary JY, Duclos B et al. Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. Gastroenterology 2006;130:1054-1061.
151. Carmona L, Gomez-Reino JJ, Rodriguez-Valverde V et al. Effectiveness of recommendations to prevent reactivation of latent tuberculosis infection in patients treated with tumor necrosis factor antagonists. Arthritis Rheum 2005;52:1766-1772.
152. Gardam MA, Keystone EC, Menzies R et al. Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. Lancet Infect Dis 2003;3:148-155.
153. Rahier JF, Ben-Horin S, Chowers Y. European evidence-based Consensus on the prevention, diagnosis and management of opportunistic infections in inflammatory bowel disease. J Crohn's and colitis (2009), doi:10.1016/j.crohns.2009.02.010
154. Sandborn WJ, Loftus EV. Balancing the risks and benefits of infliximab in the treatment of inflammatory bowel disease. Gut 2004;53:780-782.
155. Toruner M, Loftus EV, Jr., Harmsen WS et al. Risk factors for opportunistic infections in patients with inflammatory bowel disease. Gastroenterology 2008;134:929-936.
156. Lichtenstein GR, Feagan BG, Cohen RD et al. Serious Infections and Mortality in Association With Therapies for Crohn's Disease: TREAT Registry. Clinical Gastroenterology and Hepatology 2006;4:621-630.
157. Lewis JD, Bilker WB, Brensinger C et al. Inflammatory bowel disease is not associated with an increased risk of lymphoma. Gastroenterology 2001;121:1080-1087.
158. Askling J, Brandt L, Lapidus A et al. Risk of haematopoietic cancer in patients with inflammatory bowel disease. Gut 2005;54:617-622.
159. Persson PG, Karlen P, Bernell O et al. Crohn's disease and cancer: a population-based cohort study. Gastroenterology 1994;107:1675-1679.

160. Loftus EV, Jr., Tremaine WJ, Habermann TM et al. Risk of lymphoma in inflammatory bowel disease. Am J Gastroenterol 2000;95:2308-2312.

161. Kandiel A, Fraser AG, Korelitz BI et al. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. Gut 2005;54:1121-1125.

162. Farrell RJ, Ang Y, Kileen P et al. Increased incidence of non-Hodgkin's lymphoma in inflammatory bowel disease patients on immunosuppressive therapy but overall risk is low. Gut 2000;47:514-519.

163. Burger DC, Florin TH. Hepatosplenic T-cell lymphoma following infliximab therapy for Crohn's disease. Med J Aust 2009;190:341-342.

164. Mackey ACR, Green LP, Leptak CM et al. Hepatosplenic T Cell Lymphoma Associated with Infliximab Use in Young Patients Treated for Inflammatory Bowel Disease: Update. [Letter]. Journal of Pediatric Gastroenterology & Nutrition 2009;48:386-388.

165. Ochenrider MG, Patterson DJ, Aboulafia DM. Hepatosplenic T-cell lymphoma in a young man with Crohn's disease: case report and literature review. Clin Lymphoma Myeloma Leuk 2010;10:144-148.

166. Shale M, Kanfer E, Panaccione R et al. Hepatosplenic T cell lymphoma in inflammatory bowel disease. Gut 2008;57:1639-1641.

167. Oren R, Arber N, Odes S et al. Methotrexate in chronic active ulcerative colitis: a double-blind, randomized, Israeli multicenter trial. Gastroenterology 1996;110:1416-1421.

168. Feagan BG, Rochon J, Fedorak RN et al. Methotrexate for the treatment of Crohn's disease. The North American Crohn's Study Group Investigators. N Engl J Med 1995;332:292-297.

169. Fraser AG. Methotrexate: first-line or second-line immunomodulator? Eur J Gastroenterol Hepatol 2003;15:225-231.

170. Krampera M, Glennie S, Dyson J et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003;101:3722-3729.

171. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-1822.

172. Le Blanc K, Frassoni F, Ball L et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-1586.

173. Le Blanc K, Rasmusson I, Sundberg B et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439-1441.

Chapter two

Early intensive treatment for Crohn's disease

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IBD Springer Text Book
Under review

Abstract

During recent years, the treatment for patients with Crohn's disease has changed substantially. With the introduction of anti-TNFα antibodies, the treatment goals have been more ambitious. Today, upon diagnosing Crohn's disease, clinicians are able to use predictors of a complicated disease course and act accordingly when designing an individual medical strategy.

Crohn's disease typically progresses from an inflammatory disease phenotype in the early stage of the disease to a more severe complicated disease phenotype, characterized by fibrosis and stenoses. At this point, the disease is difficult to treat and fibrotic lesions are often irreversible. Therefore, intensive therapy in an early disease stage, within the so-called 'Window of Opportunity', is favorable in patients who are likely to develop a complicated disease phenotype. This disease-modifying strategy is aimed at avoiding late stage disease-related complications and at inducing steroid-free remission as soon as possible. Indeed, with this approach, higher response rates, higher rates of mucosal healing, and a higher percentage of patients achieving steroid-free remission is observed. However, not all patients will need this early intensive approach, and long-term safety is one of the concerns.

In this chapter, we discuss which patients can be selected for an early intervention approach, which safety measures should be undertaken, how patients should be monitored, and how therapy might be de-escalated once remission has been achieved.

Introduction

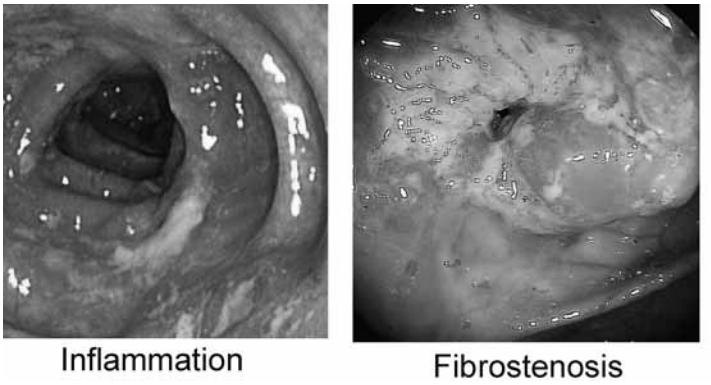
In recent years, the medical care for patients suffering from Crohn's disease (CD) has changed considerably. Nowadays, clinicians are equipped with a robust set of tools, supported by a substantial body of literature, on how to act upon diagnosing Crohn's disease for the first time. There seems general consensus on the diagnostic process, using a full endoscopic work-up in combination with radiologic and histopathologic assessments. [1] Once diagnosed, it is up to the team of IBD health professionals to fully inform and educate the patient and his/her relatives, and to install and monitor a vigorous medical regime.
The main treatment goals are 1) to achieve clinical remission of steroids as soon as possible 2) to achieve endoscopic remission 3) to avoid hospitalization and surgery 4) to improve the quality of life. Indeed, today we are able to monitor the success of each of the treatment goals very effectively: ad 1) monitoring the rate of clinical remission in daily practice can be performed on a week to week basis by, for example, a specialized IBD nurse, using well defined and validated parameters of general well being, abdominal pain, stool frequency, extra intestinal manifestations and general symptoms like fever and weight. More and more, IBD centers are establishing *remote care* delivery systems for monitoring disease activity indices on a regular basis and thus partly allowing self-management. [2] Next to clinical parameters, a defined set of laboratory values like CRP is recommended nowadays for monitoring. In addition, most steroid tapering schedules can be completed within 8 weeks. Intervention is required much sooner than 8 weeks should patients continue to have active disease. Ad 2) the paradigm of mucosal healing has been well accepted upon publication of a large number of studies showing the relevance of endoscopic assessments for the prediction of clinical remission. [3-5] Alternative biomarkers for mucosal healing, for instance fecal calprotectin and fecal lactoferrin, are under development since endoscopy remains an invasive procedure. [6-8] Again, these fecal tests could also be very well for home monitoring of mucosal healing. Ad 3) it has also been shown that induction of clinical and endoscopic remission decreases the number of hospitalizations and surgeries in CD patients. [9] Ad 4) Similar to clinical remission, the quality of life of CD patients dramatically improves upon reaching the previously discussed treatment goals. [4, 10, 11]
In summary, today's physicians are able to act immediately upon diagnosing CD with effective and safe drugs. This chapter clarifies i) why there is this '*Window of Opportunity*' the first years after the diagnosis of CD ii) why intensive treatment (also referred to as Top Down) is superior in selected cases iii) which safety measures should be taken to prevent (serious) adverse events during intense therapy iv) how to optimally monitor patients and lastly v) the possibilities for de-escalating therapy once deep remission is achieved.

Window of opportunity in Crohn's disease

When a patient is diagnosed with CD, it is an important and challenging issue how to optimally treat the patient and at which point a certain therapy should be initiated. In established CD, early disease should be distinguished from late disease, since CD is characterized by progression of inflammatory disease (early disease) to a more complicated stricturing, penetrating and fibrotic disease (late disease). [12] Fibrostenosis and perforations are

not *per se* associated with early CD, usually patients with recently diagnosed CD have pure inflammatory lesions (Figure 1). However, when the disease evolves, the number of complications increases and many patients will develop strictures or fistulae formation. In this stage, complications caused by tissue remodeling and fibrosis following long-standing disease are irreversible and difficult to treat with the anti-inflammatory agents. Often, surgery can't be avoided at this stage. The progression from early to late disease is accompanied by a change in mucosal cytokine profiles. Early CD is characterized by a pronounced Th1 response, whereas in late disease Th2 cytokines are predominating. [13] The concept that mucosal T cell regulation is different in early and late disease, suggests that patients with late disease respond different to therapies. Indeed, superior therapy efficacy is observed in patients with newly diagnosed CD compared to patients with a longer disease duration. [14-17] The progression from early to complicated late disease course has also been reported in rheumatoid arthritis (RA). [18, 19] In RA patients, therapy is aimed at preventing late disease when complications and bone destruction are irreversible. In this light, intervening in an early stage of the disease has proven to be very effective and superior to treatment in a late disease stage. [19-21]

Figure 1 Inflammation (left panel) and fibrostenosis (right panel).



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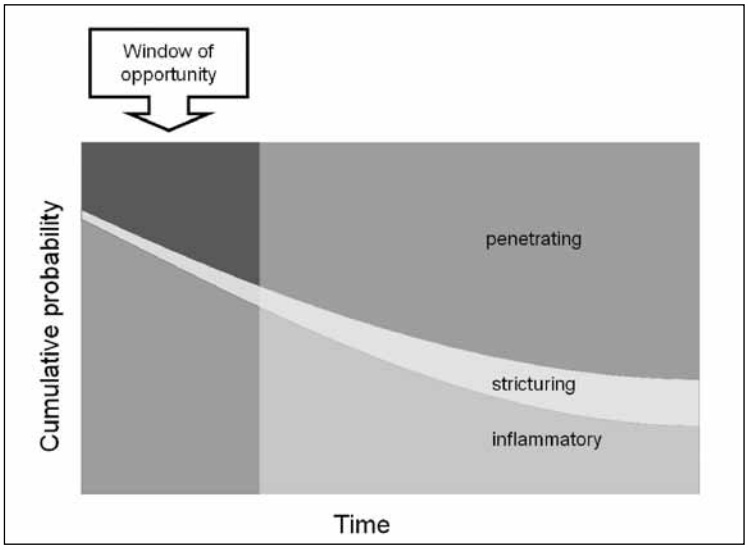
The fact that disease progresses to complicated disease suggests that there is a particular time window at which therapy is most effective and favorable. In order to prevent progression to a complicated phenotype, it is of great importance to intervene in an early stage of the disease, the so-called "Window of Opportunity" (Figure 2). At this point, it is most likely still possible to change the course of the disease towards a less aggressive phenotype, to control symptoms, induce mucosal healing and to induce and maintain clinical and endoscopic remission.

Figure 2 Window of opportunity.



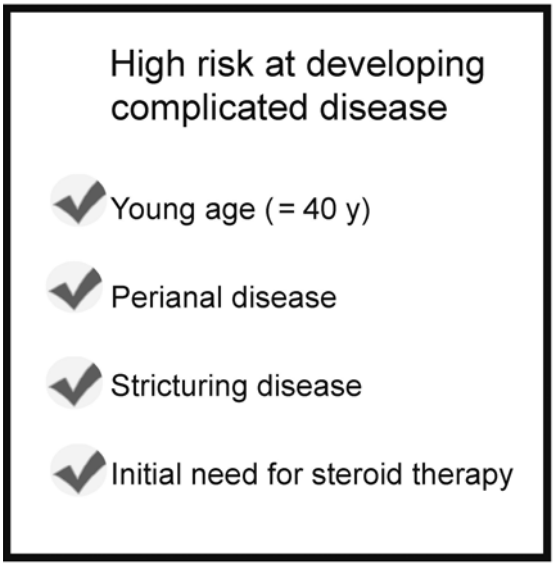
Adapted with permission from Cosnes *et al.* Inflamm Bowel Dis. 2002

Early intensive treatment ("Top-Down")

Selecting patients for early intensive treatment

The window of opportunity is especially important for patients who are likely to develop a disabling disease course. Risk factors that predict a disabling disease course have now been identified and confirmed in several studies (Figure 3). Although "disabling disease" has various definitions in different studies, clinical factors predicting the course of disease have been independently verified. In a retrospective study involving 1188 patients, disabling disease was defined as more than 2 steroid courses, hospitalization, immunomodulators or surgery within the first 5 years of diagnosis. [22] *Young age* at diagnosis, presence of *perianal disease* at diagnosis and initial *need for corticosteroids* were found to be predictive factors for disabling disease. In addition, when 2 of these factors were present at diagnosis, 84% of the patients developed disabling disease within 5 years; when 3 factors were present, 91% developed a disabling disease course. These factors were all independently confirmed in another study. In this study, disabling disease was defined as complex perianal disease, colonic resection, ≥ 2 small bowel resections or a definitive stoma, also within the first 5 years of diagnosis. [23] Using one definition or the other, several factors that are present at diagnosis have been identified and confirmed that predict a disabling disease outcome, including a young age (≤ 40 y), presence of perianal disease, stricturing disease and the initial need for steroid therapy. [22-24] Patients with these symptoms at diagnosis are at high risk for a complicated disease course. Therefore, early intensive treatment in selected patients could prevent the development of complicated disease, and thereby prevent hospitalization and surgery.

Figure 3 Predictive factors at diagnosis for selection of high-risk patient.



Biomarkers predicting disease outcome in CD are currently lacking. Serological- genetic- and immunological factors are being studied and would be helpful to further adequately select patients for a certain therapy strategy. Elevated levels of CRP correlate with more severe clinical, endoscopic and histologic disease. [25] However, CRP did not very well predict milder disease, and some patients with severe disease have normal CRP levels. Consequently, additional markers are needed. Positive anti-Saccharomyces cerevisiae antibody (ASCA) status has been associated with early surgery, [26] ileal disease [27] and poor outcome [28] and might therefore be a useful marker in the selection of patients. In another study, a well defined correlation was found between triple positive status for ASCA, anti-OmpC as well as anti-I2 and small bowel surgery. [29] Furthermore, mutations in NOD2 [30] and CARD15 [31] increase the risk for surgery in patients with pediatric-onset CD.
Large trials should further confirm the value of these markers in predicting disease course. When combining these factors, a profile can be established including phenotypic, genetic and serologic markers to facilitate the selection of patients.

Early intensive strategy in Crohn's disease

The benefit of the disease-modifying strategy has been shown in several studies. The first clues pointing towards the benefit of so-called early intervention strategies in CD, result from retrospective analyses from large randomized, placebo-controlled trials. In a study evaluating the maintenance of response and remission of adalimumab, a clear difference was observed in patients with different disease durations. [32] The rate of patients with remission maintenance at week 26 was 56% in patients with a disease duration less than 2 years, 35% in patients with a disease duration of 2 – 5 years and 37% in patients with a disease duration more than 5 years.

In a prospective, randomized controlled trial, including 130 patients with newly diagnosed CD naïve to corticosteroids, immunomodulators or anti-TNF agents, efficacy of step-up versus top-down therapy was evaluated. [33] The top-down approach involved a *treatment algorithm* starting with infliximab 5 mg/kg at weeks 0, 2 and 6 and azathioprine 2.5 mg/kg/d. Patients randomized to the step-up treatment algorithm were initially treated with corticosteroids (current standard around 1999), followed by azathioprine in case the corticosteroids were not able to control disease activity. Should this combination fail to avoid relapse, infliximab treatment was initiated. The co-primary endpoint in this study was clinical remission off steroids and without surgery at weeks 26 and 52. At both time points, a significant greater proportion of patients in the early combined intervention group met this endpoint compared to the conventional therapy group. At week 26, 39 (60.0%) of 65 patients in the top-down group were in remission without corticosteroids and without surgical resection, compared with 23 (35.9%) of 64 step-up patients, with an absolute difference of 24.1% (95% CI 7.3 - 40.8, $p = 0.0062$). Corresponding rates at week 52 were 40/65 (61.5%) and 27/64 (42.2%) (absolute difference 19.3%, 95% CI 2.4 - 36.3, $p = 0.0278$). Furthermore, after two years, mucosal healing was observed in 73% of the patients in the early intervention group, whereas mucosal healing was seen in only 30% of the patients in the conventional treatment group. Importantly, 19% of the patients in the step-up group were still on steroids at this time point, whereas 0% of the patients in the early intervention group were receiving steroids. Also, it has been shown that complete mucosal healing in patients with early-stage Crohn's disease is associated with significantly higher steroid-free remission rates 4 years after therapy began. [3] Complete mucosal healing, defined as a Simple Endoscopic Score of 0 after 2 years of therapy, was the only factor that predicted sustained, steroid-free remission 3 and 4 years after therapy was initiated; it was observed in 17 of 24 patients (70.8%) vs 6 of 22 patients with lesions detected by endoscopy (27.3%, Simple Endoscopic Score >0) ($p = 0.036$; odds ratio = 4.352; 95% confidence interval, 1.10-17.220). Fifteen of 17 patients with mucosal healing at year 2 maintained in remission without further infliximab infusions during years 3 and 4 ($p = 0.032$; odds ratio = 4.883; 95% confidence interval, 1.144 - 20.844). Finally, in this step-up versus top-down study, the number of side effects did not differ significantly between the two groups. Obviously, remission rates in the two groups did not differ significantly after 1 year since the two treatment algorithms were both designed to control disease, and allowed intensification when disease activity persisted.

Effective early intervention has also been reported in children. Twenty-nine patients receiving infliximab in either step-up (11 patients) or top-down (18 patients) regimen where evaluated 8 weeks after initiation of treatment. [34] Remission was achieved in 3/11 patients in the step-up group and in 15/18 in the top-down group. In the top-down group, significant improvement was observed in PCDAI score and perianal fistula status. Furthermore, in a retrospective study of 36 pediatric CD patients, [35] relapse rate after 24 months was significantly lower in the early intervention group than in the conventional therapy group.

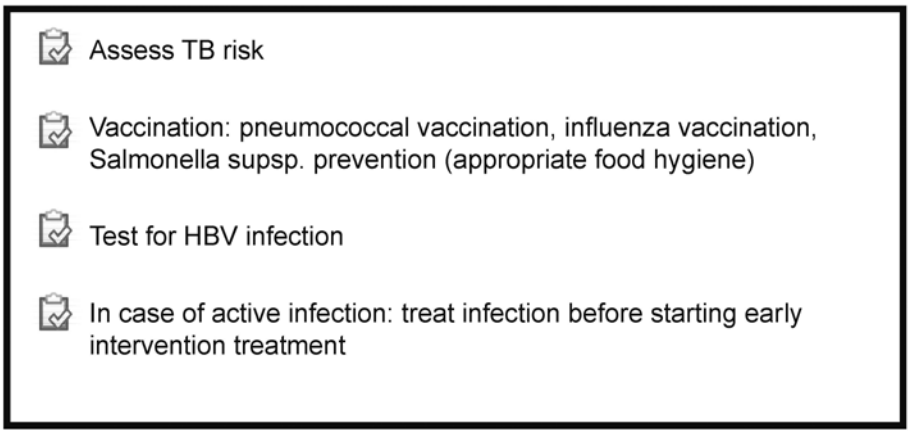
Finally, the SONIC trial evaluated the efficacy of infliximab monotherapy, azathioprine monotherapy, and the two drugs combined in 508 adults with moderate-to-severe Crohn's disease who had not undergone previous immunosuppressive or biologic therapy. [15] Patients were randomly assigned to receive an intravenous infusion of 5 mg of infliximab per kilogram of body weight at weeks 0, 2, and 6 and then every 8 weeks plus daily oral placebo capsules; 2.5 mg of oral azathioprine per kilogram daily plus a placebo infusion on the standard

schedule; or combination therapy with the two drugs. Of the 169 patients receiving combination therapy, 96 (56.8%) were in corticosteroid-free clinical remission at week 26 (the primary end point), as compared with 75 of 169 patients (44.4%) receiving infliximab alone ($p = 0.02$) and 51 of 170 patients (30.0%) receiving azathioprine alone ($p < 0.001$ for the comparison with combination therapy and $p = 0.006$ for the comparison with infliximab). Similar numerical trends were found at week 50. At week 26, mucosal healing had occurred in 47 of 107 patients (43.9%) receiving combination therapy, as compared with 28 of 93 patients (30.1%) receiving infliximab ($p = 0.06$) and 18 of 109 patients (16.5%) receiving azathioprine ($p < 0.001$ for the comparison with combination therapy and $p = 0.02$ for the comparison with infliximab). Thus, infliximab plus azathioprine was shown to be the superior combination for inducing remission.
In conclusion, early intensive treatment, consisting of the combination of a thiopurine with an anti-TNF, should be considered in *all* patients who have a high risk for a complicated disease course, i.e. young age at diagnosis, presentation with peri-anal or stricturing disease, or initial needs of steroids.

Safety measures for early intensive treatment

An important issue related to early intensive treatment is the safety of long-term use of biologics and immunomodulators. Patients are exposed to potentially toxic agents, and therefore appropriate safety measures should be undertaken before immunomodulators and biologicals are initiated (Figure 4). Safety measures are aimed at prevention of infections and awareness of several rare complications.
It appears that patients treated with immunomodulators have an increased risk for influenza infections, pneumococcal infections, and *Salmonella* supp. infections. [36] Therefore, influenza vaccination, pneumococcal vaccination and appropriate food hygiene (avoiding raw eggs, unpasteurized milk, raw meet) is recommended. Also, reactivation of latent HBV is considered a serious risk, and therefore all IBD patients should be tested to exclude HBV. [36] Patients with active chronic infection should be treated according to standard antiviral therapy. Nucleoside/nucleotide analogues are preferred since IFN therapy might exacerbate the colitis. Seronegative patients should receive HBV vaccination, patients might need a higher dose of immunizing antigen since vaccination efficacy is affected by the number of immunomodulators.
Screening for cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Herpes simplex virus (HSV) before starting immunomodulator therapy is not recommended. In case of severe EBV infection, immunomodulator therapy should be discontinued. Latent subclinical CMV infection is also no contra-indication for starting immunomodulators. However, CMV colitis should be excluded in refractory IBD cases and in case of systemic CMV, immunomodulator therapy should be discontinued. [36]

Figure 4 Safety measures.



Anti-TNF use is associated with an approximately 21-fold increased risk of tuberculosis (TB) without appropriate safety measures. [37] The TB incidence has been reported to decrease with 78% when suitable safety measures where undertaken. Most cases are presented during the first three months of treatment and have an atypical presentation, which makes the diagnosis more complicating. [38] For that reason, international guidelines advise to assess the risk of TB before starting treatment with an anti-TNF agent, including an X-ray, tuberculin skin testing (depending on national guidelines) and careful evaluation of the TB history. [36] Latent TB may be suspected in case of a positive initial tuberculin skin test and when the patient has recently been exposed to the disease.

Physicians should be aware of the possibility of false-negative skin tests, especially when patients are immunocompromised. When the patient is diagnosed with latent TB, treatment with the full therapeutic antituberculous regimen should be initiated and it is advised to delay anti-TNF treatment with at least 3 months. The full anti-TB regimen consists of isoniazid (INH) for 6 to 9 months. An association between isoniazid related hepatoxicity and methotrexate and sulphasalazine has been reported in the rheumatology setting, but this is not clear for IBD. When active TB is diagnosed, anti-TNF treatment is ideally delayed until anti-TB treatment is completed. However, solid data on the ideal timing during anti-TB treatment are lacking and in case of medical urgency, physicians may consider to start anti-TNF therapy despite positive TB diagnosis. Advice from a TB expert is in that case recommended. When TB is diagnosed during anti-TNF treatment, the anti-TNF agent should be discontinued and TB therapy should be started. Anti-TNF therapy can be resumed if needed after 2 months. In any event, supervision of a thoracic physician or infectious disease specialist is advised. 5-ASA, azathioprine, methotrexate and steroids can be continued during anti-TB therapy. All patients should be monitored carefully for signs of cough, fever and weight loss and treating physicians should be aware of uncommon extrapulmonary TB as well as the more common lung disease.

How to optimally monitor patients

1 Disease activity

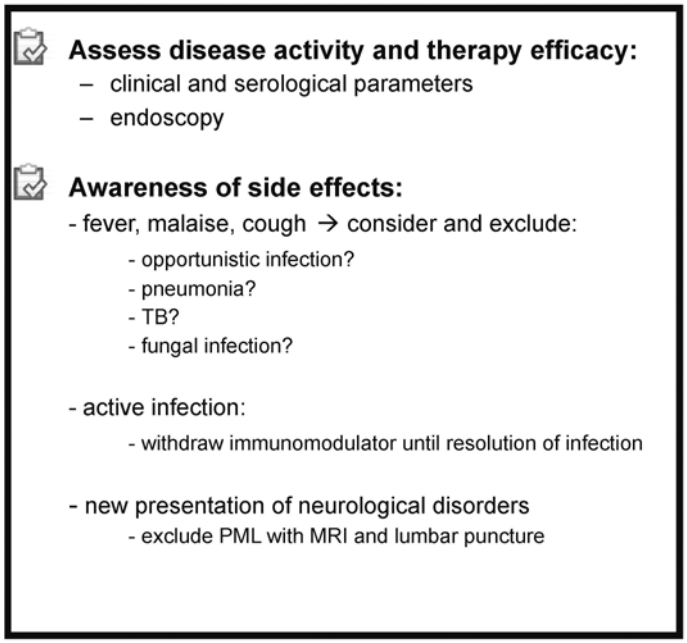
Prior to installing a medical strategy, it is essential to discuss the monitoring scenario with each patient. Monitoring of both disease activity and drug safety is mandatory, irrespective of early- or late stage disease. Although guidelines discuss monitoring practices, the evidence in the literature is currently lacking. Optimally, the clinical monitoring of Crohn's disease activity should be done in a standardized manner using for instance the Crohn's Disease Activity Index (CDAI), the Harvey Bradshaw Index (HBI) or other validated indices. However, for routine clinical practice this is usually not accepted. Using simple Physician Global Assessments is very simple and easy to do, and a reasonable alternative. These clinical assessments are required on a weekly basis once remission induction is initiated, and less often once remission is achieved. In addition to monitoring clinical disease activity, clinicians should also monitor CRP each time a clinical assessment is made. Finally, the correct monitoring strategy of early intensive treatment also involves assessment of mucosal healing. It has been shown that, following surgical remission, Crohn's ulceration can be detected within 3 to 6 months following surgery preceding clinical symptoms. [39] Mucosal healing predicts long-term outcome and is associated with less surgery and hospitalization.[4, 40] In a retrospective study, it has been shown that patients with severe ulcerations at colonoscopy are more likely to have a complicated disease course, [41] and in an ongoing study, a strong association was found between mucosal healing and decreased risk of relapse. [42] Therefore, assessing mucosal healing is a useful tool to predict the course of disease, to monitor disease activity and to further optimize treatment.

Non-invasive fecal markers such as fecal lactoferrin and calprotectin are currently being evaluated for their usefulness to measure disease activity. Increased levels of calprotectin and lactoferrin have been shown to correlate with CDEIS scores [7, 43] and therefore these markers might be useful tools to assess disease activity. In addition, it has been shown that lactoferrin and calprotectin are valuable markers to predict relapse. [44] In patients with high (>150 microgr/gr) calprotectin levels or positive lactoferrin, a higher risk for relapse was observed. The value of lactoferrin and calprotectin as markers for disease activity has also been demonstrated in paediatric disease. [45] However, it has been suggested that these markers may be less valuable in patients with ileal CD, [46] since the disease location might interfere with accurate fecal detection. Future studies are needed to further confirm the value of these markers in daily practice, define clear cut-off values and to finally identify a combination of markers with higher sensitivity and specificity.

2 Side effects

In order to evaluate the risk benefit of the early intensive treatment, a careful monitoring of potential, and sometimes avoidable adverse events is mandatory (Figure 5). Patients on immunosuppressive therapy and with malnutrition are at risk for opportunistic infections. [47] For that reason, patients with fever, cough and systemic illness should be carefully examined. In case of an active infection, it is advised to withdraw immunomodulators until the resolution of infection. In case of persistent fever and when the patient is treated with an anti-TNF agent, *mycobacterium tuberculosis* (TB) should be excluded. Patients diagnosed with pneumonia should be treated with an antibiotic covering *S. pneunomiae*. [36]

Figure 5 Monitoring strategies for patients with early intensive therapy.



Furthermore, about 60 – 80% of the European population has latent JC virus, which can cause progressive multifocal leukoencephalopathy (PML) upon reactivation. Since reactivation of the JC virus is associated with systemic immunosuppression, treating physicians ought to be aware of this rare situation, especially in patients presenting with new onset neurological symptoms, in which case the patient should receive an MRI scan and lumbar puncture.

Studies show divergent data on the occurrence of malignant lymphoma in IBD patients receiving immunomodulators. Whereas some studies do not show an increased risk, other studies do find a moderately elevated risk, especially in patients on thiopurine therapy. [48-50] Still, the absolute risk appears to be low and should be weighed against the beneficial effects of immunomodulator therapy. On the other hand, awareness is advised. Lethal hepatosplenic T cell lymphoma has been reported in young patients on azathioprine/infliximab combination therapy, [51-54] and therefore long-term combination therapy in younger patients is not recommended.

After all, when taking appropriate safety measures, anti-TNF and immunomodulator therapy appears to be relatively safe. Safety data from referral centers and randomized controlled trials do not show an increased risk of malignancies or infections in anti-TNF treated patients. In a large meta-analysis of 21 placebo-controlled trials including 5356 patients, no increased risk of death, serious infection or malignancy compared to controls was reported. [55] In line with this observation, no increased risk was found in infections, mortality or malignancy in 734 anti-TNF treated patients compared to controls, with a median follow-up of 58 months. [11] In addition, no increased risk of malignancy was observed in patients treated

with anti-TNF in a large cohort of CD patients. [56] However, long-term safety data are not available yet and therefore awareness of (serious) side effects is warranted.

Possibilities for de-escalating therapy once remission is achieved

Once remission is achieved, it is important to know if, when and how to de-escalate therapy. Although severe adverse events are rare, especially when appropriate safety measures are undertaken and patients are correctly monitored, they can occur and therefore establishing an individualized risk-benefit ratio is encouraged. The disadvantages of discontinuation of therapy should be taken into account, including relapse, possibly lower response to re-induction therapy, infusion reactions and surgery. Particularly important when considering de-escalation is the earlier pattern of the disease and response to therapies. In addition, several factors may predict relapse, including smoking, previous steroid use and elevated fecal calprotectin and CRP.

Anti-TNF and anti-TNF/azathioprine combination therapy

The question how and when to de-escalate infliximab therapy is of great interest. Trials investigating this matter are currently ongoing, and therefore there is not much data and consensus on this topic yet.

The proportion of patients with infliximab-induced remission that relapsed after discontinuing infliximab was assessed in a prospective single-center study. [57] In this study, infliximab was stopped in patients who were treated with infliximab for at least one year, and who were in steroid-free remission for 6 months. After the median follow up time of 12 months, about 50% of the patients relapsed. On the other hand, 35% of the patients were still doing well up to the end of the follow-up time (nearly 7 years). Therefore, it seems that the patients that are still doing well after 7 years represent a specific group of patients. Age, gender, number of infliximab infusions and disease location were all excluded as predictors for relapse in this study. These results were supported by data from an observational study evaluating the long-term effects of infliximab. [11] In this study, 20% of the patients who experienced a sustained clinical response to infliximab, maintained remission after infliximab discontinuation.

Interim results of the STORI (infliximab discontinuation in Crohn's disease patients in stable remission on combined therapy with immunosuppressors) showed similar results. [58] In this prospective study, relapse was assessed in patients on combination therapy for more than 1 year and in stable remission for ≥ 6 months after stopping infliximab therapy. About 50% of the patients relapsed within 1 year of discontinuation; patients retreated with infliximab after relapse responded well. In addition, risk factors that predict relapse were detected. A univariate analysis revealed current smoking, previous steroid treatment, lower haemoglobin, higher CDAI, ultrasensitive C reactive protein and faecal calprotectin as predictive markers. In a multivariate analysis, CDEIS ≥ 2, usCRP > 5 mg/dl, haemoglobin ≤ 14.5 g/dl and infliximab trough levels ≥ 2 μg/ml were found to predict relapse. Furthermore, the risk of relapse increased as the number of risk factors increased. [42]

In a study investigating the ability to stop immunosuppressives in patients treated with combination therapy, an enduring response was observed after withdrawal of immunosup-

pressives. [59] Importantly, they reported low infliximab trough levels before immunosuppressive withdrawal as a predictor for surgery. Risk factors for relapse after azathioprine withdrawal in patients treated with infliximab/azathioprine combination therapy were identified in another study. [60] Infliximab/azathioprine exposure duration ≤ 811 days, CRP ≥ 5 mg/L and platelet count ≥ 298 x 10^9 were found to predict infliximab failure, which was defined as disease flare, hypersensitivity reactions leading to infliximab discontinuation or surgery. After 12 and 24 months, resp. 85% and 41% of the patients were infliximab-failure free.
In summary, it appears from these preliminary data that there is a distinct group of patients that does well after de-escalation therapy. It is of great interest to identify the factors that discriminate patients with sustained remission from the patients that relapse, in order to further tailor therapy and to prevent unnecessary side effects. Studies are currently ongoing, and the preliminary data need to be confirmed in large cohorts.

Thiopurines

Furthermore, there are conflicting data with regard to discontinuation of thiopurine therapy once a patient is in remission. In a large randomized, placebo-controlled double blind trial, azathioprine discontinuation was associated with a 50% relapse rate after 54,5 months, regardless of the duration of remission. [61]
In a study evaluating disease recurrence after azathioprine withdrawal, a lower rate of relapse was seen in patients who received azathioprine ≥ 4 years than in patients treated with azathioprine ≤ 4 years. [62]

Concluding remarks

Early intervention therapy in CD is beneficial in selected patients who are likely to develop a severe disease course. Young age at diagnosis, the presence of perianal disease, stricturing disease and the initial need for corticosteroids are factors predicting a complicated disease course. When taking appropriate safety measures, this approach appears to be relatively safe. However, treating physicians should be aware of opportunistic infections and other rare complications, and patients should be monitored carefully on a regular basis. The value of serological, immunologic and genetic markers in monitoring and predicting disease are currently under investigation, and could be helpful to further optimize therapy.

Competing interests: none

Reference List

1. Dignass A, Assche GV, Lindsay JO et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. 4 ed. 2010:28-62.
2. Elkjaer M, Shuhaibar M, Burisch J et al. E-health empowers patients with ulcerative colitis: a randomised controlled trial of the web-guided 'Constant-care' approach. Gut 2010;59:1652-1661.
3. Baert F, Moortgat L, Van Assche G et al. Mucosal Healing Predicts Sustained Clinical Remission in Patients with Early-Stage Crohn's Disease. Gastroenterology 2010;138(2):463-8.
4. Schnitzler F, Fidder H, Ferrante M et al. Mucosal healing predicts long-term outcome of maintenance therapy with infliximab in Crohn's disease. Inflamm Bowel Dis 2009;15(9):1295-301.
5. Geboes K, Rutgeerts P, Opdenakker G et al. Endoscopic and histologic evidence of persistent mucosal healing and correlation with clinical improvement following sustained infliximab treatment for Crohn's disease. Curr Med Res Opin 2005;21:1741-1754.
6. Vieira A, Fang CB, Rolim EG et al. Inflammatory bowel disease activity assessed by fecal calprotectin and lactoferrin: correlation with laboratory parameters, clinical, endoscopic and histological indexes. BMC Res Notes 2009;2:221.
7. Sipponen T, Karkkainen P, Savilahti E et al. Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings. Aliment Pharmacol Ther 2008;28:1221-1229.
8. Langhorst J, Elsenbruch S, Koelzer J et al. Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. Am J Gastroenterol 2008;103:162-169.
9. Feagan BG, Panaccione R, Sandborn WJ et al. Effects of Adalimumab Therapy on Incidence of Hospitalization and Surgery in Crohn's Disease: Results From the CHARM Study. Gastroenterology 2008;135:1493-1499.
10. Bastida G, Nos P, Aguas M et al. The effects of thiopurine therapy on health-related quality of life in Inflammatory Bowel Disease patients. BMC Gastroenterol 2010;10:26.
11. Schnitzler F, Fidder H, Ferrante M et al. Long-term outcome of treatment with infliximab in 614 patients with Crohn's disease: results from a single-centre cohort. Gut 2009;58:492-500.
12. Cosnes J, Cattan S, Blain A et al. Long-term evolution of disease behavior of Crohn's disease. Inflamm Bowel Dis 2002;8:244-250.
13. Kugathasan S, Saubermann LJ, Smith L et al. Mucosal T-cell immunoregulation varies in early and late inflammatory bowel disease. Gut 2007;56:1696-1705.
14. Breedveld FC. The PREMIER study: A multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. Arthirits Rheum 2006;54(1):26-37.
15. Colombel JF. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med. 2010 Apr 15;362(15):1383-95
16. Kugathasan S, Werlin SL, Martinez A et al. Prolonged duration of response to infliximab in early but not late pediatric Crohn's disease. Am J Gastroenterol 2000;95:3189-3194.
17. Lionetti P, Bronzini F, Salvestrini C et al. Response to infliximab is related to disease duration in paediatric Crohn's disease. Aliment Pharmacol Ther 2003;18:425-431.
18. Quinn MA, Emery P. Window of opportunity in early rheumatoid arthritis: possibility of altering the disease process with early intervention. Clin Exp Rheumatol 2003;21:S154-S157.

19. Roberts LJ, Cleland LG, Thomas R et al. Early combination disease modifying antirheumatic drug treatment for rheumatoid arthritis. Med J Aust 2006;184:122-125.
20. Breedveld FC, Emery P, Keystone E et al. Infliximab in active early rheumatoid arthritis. Ann Rheum Dis 2004;63:149-155.
21. Durez P, Malghem J, Nzeusseu TA et al. Treatment of early rheumatoid arthritis: a randomized magnetic resonance imaging study comparing the effects of methotrexate alone, methotrexate in combination with infliximab, and methotrexate in combination with intravenous pulse methylprednisolone. Arthritis Rheum 2007;56:3919-3927.
22. Beaugerie L, Seksik P, Nion-Larmurier I et al. Predictors of Crohn's disease. Gastroenterology 2006;130:650-656.
23. Loly C, Belaiche J, Louis E. Predictors of severe Crohn's disease. Scand J Gastroenterol 2008;43:948-954.
24. Lichtenstein GR, Olson A, Travers S et al. Factors associated with the development of intestinal strictures or obstructions in patients with Crohn's disease. Am J Gastroenterol 2006;101:1030-1038.
25. Solem CA, Loftus EV, Jr., Tremaine WJ et al. Correlation of C-reactive protein with clinical, endoscopic, histologic, and radiographic activity in inflammatory bowel disease. Inflamm Bowel Dis 2005;11:707-712.
26. Forcione DG, Rosen MJ, Kisiel JB et al. Anti-Saccharomyces cerevisiae antibody (ASCA) positivity is associated with increased risk for early surgery in Crohn's disease. Gut 2004;53:1117-1122.
27. Linskens RK, Mallant-Hent RC, Murillo LS et al. Genetic and serological markers to identify phenotypic subgroups in a Dutch Crohn' s disease population. Dig Liver Dis 2004;36:29-34.
28. Kim BC, Park S, Han J et al. Clinical significance of anti-Saccharomyces cerevisiae antibody (ASCA) in Korean patients with Crohn's disease and its relationship to the disease clinical course. Dig Liver Dis 2007;39:610-616.
29. Mow WS, Vasiliauskas EA, Lin YC et al. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. Gastroenterology 2004;126:414-424.
30. Lacher M, Helmbrecht J, Schroepf S et al. NOD2 mutations predict the risk for surgery in pediatric-onset Crohn's disease. J Pediatr Surg 2010;45:1591-1597.
31. Kugathasan S, Collins N, Maresso K et al. CARD15 gene mutations and risk for early surgery in pediatric-onset Crohn's disease. Clin Gastroenterol Hepatol 2004;2:1003-1009.
32. Colombel JF, Sandborn WJ, Rutgeerts P et al. Adalimumab for Maintenance of Clinical Response and Remission in Patients With Crohn's Disease: The CHARM Trial. Gastroenterology 2007;132:52-65.
33. D'Haens G, Baert F, van AG et al. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. Lancet 2008;371:660-667.
34. Kim MJ, Lee JS, Lee JH et al. Infliximab therapy in children with Crohn's disease: A one-year evaluation of efficacy comparing 'top-down' and 'step-up' strategies. Acta Paediatr 2010.
35. Lee JS, Lee JH, Lee JH et al. Efficacy of early treatment with infliximab in pediatric Crohn's disease. World J Gastroenterol 2010;16:1776-1781.
36. Rahier JF, Ben-Horin S, Chowers Y. European evidence-based Consensus on the prevention, diagnosis and management of opportunistic infections in inflammatory bowel disease. J. Crohn's and colitis (2009), doi:10.1016/j.chrons.2009.02.010

37. Carmona L, Gomez-Reino JJ, Rodriguez-Valverde Vet al. Effectiveness of recommendations to prevent reactivation of latent tuberculosis infection in patients treated with tumor necrosis factor antagonists. Arthritis Rheum 2005;52:1766-1772.
38. Gardam MA, Keystone EC, Menzies R et al. Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. Lancet Infect Dis 2003;3:148-155.
39. Rutgeerts P, Geboes K, Vantrappen G et al. Predictability of the postoperative course of Crohn's disease. Gastroenterology 1990;99:956-963.
40. Froslie KF, Jahnsen J, Moum BA et al. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. Gastroenterology 2007;133:412-422.
41. Allez M, Lemann M, Bonnet J et al. Long term outcome of patients with active Crohn's disease exhibiting extensive and deep ulcerations at colonoscopy. Am J Gastroenterol 2002;97:947-953.
42. Louis E, Vernier-Massouille G, Grimaud J. Infliximab discontinuation in Crohn's disease patients in stable remission on combined therapy with immunosuppressors: a prosepctive ongoing cohort study. Gastroenterology 2009;136:A-146
43. Sipponen T, Savilahti E, Kolho KL et al. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. Inflamm Bowel Dis 2008;14:40-46.
44. Gisbert JP, Bermejo F, Perez-Calle JL et al. Fecal calprotectin and lactoferrin for the prediction of inflammatory bowel disease relapse. Inflamm Bowel Dis 2009;15:1190-1198.
45. Joishy M, Davies I, Ahmed M et al. Fecal calprotectin and lactoferrin as noninvasive markers of pediatric inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2009;48:48-54.
46. D'Inca R, Dal PE, Di L et al. Can calprotectin predict relapse risk in inflammatory bowel disease? Am J Gastroenterol 2008;103:2007-2014.
47. Toruner M, Loftus EV, Jr., Harmsen WS et al. Risk factors for opportunistic infections in patients with inflammatory bowel disease. Gastroenterology 2008;134:929-936.
48. Kandiel A, Fraser AG, Korelitz BI et al. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. Gut 2005;54:1121-1125.
49. Beaugerie L, Brousse N, Bouvier AM et al. Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. The Lancet 2009;374:1617-1625.
50. Farrell RJ, Ang Y, Kileen P et al. Increased incidence of non-Hodgkin's lymphoma in inflammatory bowel disease patients on immunosuppressive therapy but overall risk is low. Gut 2000;47:514-519.
51. Burger DC, Florin TH. Hepatosplenic T-cell lymphoma following infliximab therapy for Crohn's disease. Med J Aust 2009;190:341-342.
52. Mackey ACR, Green LP, Leptak CM et al. Hepatosplenic T Cell Lymphoma Associated with Infliximab Use in Young Patients Treated for Inflammatory Bowel Disease: Update. [Letter]. Journal of Pediatric Gastroenterology & Nutrition 2009;48:386-388.
53. Ochenrider MG, Patterson DJ, Aboulafia DM. Hepatosplenic T-cell lymphoma in a young man with Crohn's disease: case report and literature review. Clin Lymphoma Myeloma Leuk 2010;10:144-148.
54. Shale M, Kanfer E, Panaccione R et al. Hepatosplenic T cell lymphoma in inflammatory bowel disease. Gut 2008;57:1639-1641.

55. Lichtenstein GR, Feagan BG, Cohen RD et al. Serious Infections and Mortality in Association With Therapies for Crohn's Disease: TREAT Registry. Clinical Gastroenterology and Hepatology 2006;4:621-630.
56. Biancone L, Orlando A, Kohn A et al. Infliximab and newly diagnosed neoplasia in Crohn's disease: a multicentre matched pair study. Gut 2006;55:228-233.
57. Waugh AW, Garg S, Matic K, Gramlich L et al. Maintenance of clinical benefit in Crohn's disease patients after discontinuation of infliximab: long-term follow-up of a single centre cohort. Aliment Pharmacol Ther 2010;32:1129-1134.
58. Louis E, Vernier-Massouille G et a;. Infliximab discontinuation in Crohn's disease patients in stable remission on combined therapy with immunosuppressors: interim analysis of a prospective cohort study. Gut 2008;57:A66
59. Bossuyt PJJ, Juergens M, Ballet V. Discontinuation of immunosuppressives in patients with Crohn's disease treated with scheduled maintenance infliximab: prospective long-term follow-up and influence on infliximab trough levels.Gut 2010;59 (Supll III) A79
60. Oussalah A, Chevaux JB, Fay R. Predictors of infliximab failure after azathioprine withdrawal in Crohn's disease treated with combination therapy. Am J Gastroenterol 2010;105(5):1142-9.
61. Treton X, Bouhnik Y, Mary JY et al. Azathioprine withdrawal in patients with Crohn's disease maintained on prolonged remission: a high risk of relapse. Clin Gastroenterol Hepatol 2009;7:80-85.
62. Pica R, Avallone EV, Cassieri C. Optimal duration of maintenance treatment with azathioprine in patients with inflammatory bowel disease. Gut 2010;59(Suppl III): A38.

Chapter three

Dendritic cell autophagy attenuates adaptive immune responses by destabilization of the immunological synapse

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Manuscript in preparation

Abstract

Autophagy-related gene variants are associated with the development of Crohn's disease, an aberrant inflammatory response to the intestinal flora. How autophagy is linked to the loss of immune tolerance has not been established. We demonstrate that autophagy regulates the stability of the immunological synapse in dendritic cells (DC). Autophagy is induced upon formation of the immunological synapse, and autophagosomes preferentially localize to the site of interaction. Autophagosomes engulf synaptic components such as MHCII and ICAM-1, targeting them for degradation. Knockdown of the autophagy-related genes *ATG16L1* and *IRGM* in DC results in hyperstable DC-T cell interactions, leading to increased T cell signaling and Th17 differentiation. Similar aberrations were observed in DC obtained from patients with Crohn's disease carrying the disease associated polymorphisms in *ATG16L1*. Our findings show that autophagy is induced upon formation of the immunological synapse and acts as a negative regulator of T cell activation and Th17 differentiation.

Introduction

A number of genome-wide association studies have associated autophagy-related genes *ATG16L1* and *IRGM* with the development of Crohn's disease, an inflammatory bowel disease. [1-3] During autophagy, the cell envelops damaged or excess proteins with a lipid bilayer, thus creating an autophagosome. The autophagosome then fuses with a lysosome, resulting in the degradation of the protein and the release of free amino acids. Although the pathway was initially described as a cell survival mechanism during nutrient depletion, [4] more recent studies show that the process is also involved in the proper function of various immune cells, including pathogen degradation and antigen processing by phagocytes. [5-7] The Crohn's disease risk alleles result in decreased autophagic function, suggesting that autophagy is required to limit the intestinal immune response. Mice expressing a hypomorphic variant of *ATG16L1* display aberrant Paneth cell morphology and decreased secretion of anti-microbial proteins into the intestinal lumen. [8] Additionally, autophagy is involved in the killing of intracellular pathogens by phagocytes, including *Salmonella enterica* and *Mycoplasma tuberculosis*. This suggested that the effects of autophagy on intestinal inflammation are secondary, as impaired autophagy leads to hampered innate immune responses and microbial overgrowth, which in turn results in hyperstimulation of the intestinal immune system. However, we hypothesized that autophagy also has a direct effect in the regulation of immunity.

Dendritic cells (DC) are key players in the maintenance of immune homeostasis, as they link the innate and the adaptive immune system and can drive both immunogenic and tolerogenic responses. The mechanisms by which DC can dampen immune responses include secretion of cytokines which induce Foxp3+ regulatory T cells and skew the T cell response to a less damaging subtype. [9, 10] Additionally, a lack of costimulatory molecules or expression of inhibitory proteins such as IDO and PD-L1 can lead to T cell anergy and thus also limit immunogenic responses. [11, 12] In contrast to the volume of data on these topics, the involvement of the immunological synapse in the regulation of immune responses by DC has been studied to a limited degree.

The immunological synapse is the site of contact between DC and T cells and is characterized by a highly organized structure containing molecules involved in antigen recognition such as MHC class II and the T cell receptor and adhesion molecules ICAM-1 and LFA-1. [13] The proper formation is important for the efficacy and outcome of the ensuing immune response, and synaptic destabilization has been shown to result in decreased T cell signaling. [14] Although the formation of the immunological synapse as well as the signaling pathways involved have been described in some detail from the T cell side, the regulation of synapse formation in DC has remained largely unexplored.

Given the pivotal role of DC and DC-T cell interactions in immunity and the role of autophagy in the control of intestinal inflammation, we studied the role of autophagy in DC, in particular during cell-cell interactions. We report here that autophagy is induced in DC upon formation of an immunological synapse. Autophagosomes localize preferentially towards the site of interaction and engulf components of the synapse. DC in which expression of the autophagy-related genes *ATG16L1* or *IRGM* is decreased show increased stability of the immunological synapse, enhanced stimulation of T cell proliferation and increased Th17 activation. Our data suggest that autophagy acts as a negative feedback mechanism in

DC-T cell interactions and that a loss of this negative feedback mechanism may be a contributing factor in the pathogenesis of Crohn's disease.

Material and methods

Mice

C57BL/6-Tg(TcraTcrb)425Cbn/J (OT-II) mice carrying the $OVA_{323-339}$ specific T cell receptor were obtained from Jackson Laboratories (Bar Harbor, ME) and supplied with water and standard chow ad libitum. Experimental procedures were approved by the local Animal Ethics Committee in accordance with national guidelines.

Antibodies, immunofluorescence and flow cytometry

Anti-IFNγ, anti-IL-17 and anti-IL-4 were obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-CD40, CD80, CD83, CD86, CD209, anti-HLA-DR-FITC and isotype controls were obtained from BD Biosciences (San Jose, CA). Anti-HLR-DRbeta and anti-LAMP2 were obtained from Abcam (Cambridge, MA), anti-GFP, anti-mouse AlexaFluor 546, anti-rat AlexaFluor 568 and anti-rabbit-AlexaFluor 488 from Invitrogen (Carlsbad, CA). For immunofluorescence, cells were adhered to poly-L-lysine (Sigma Aldrich, Deisenhofen, Germany) coated coverslips, fixed in 4% paraformaldehyde, stained in permeabilisation buffer (PBS containing 0.05% Triton X-100) and embedded in SlowFade Gold (Invitrogen). Images were obtained on a Leica TCS SP2 confocal system equipped with 488 nm argon and 543 HeNe lasers (Leica, Mannheim, Germany) and processed using ImageJ software.
For flow cytometry of surface markers, cells were washed and stained in PBS/0.5% BSA, washed again and fixed in 2% PFA. For cytokine staining, cells were washed in PBS, fixed in 2% PFA, permeabilized and stained in 0.5% saponin and washed in PBS/0.5% BSA. All samples were analyzed using a FACSCalibur (BD Bioscience) and FlowJo Software (Treestar, Ashland, OR).
For immunoblotting the following antibodies were used: anti-human p62 lck ligand (BD), anti-GFP serum (Invitrogen), anti-pLAT Tyr191 (Cell Signaling, Beverly, MA).

Cell isolation and generation of dendritic cells

Monocytes and lymphocytes were isolated from buffy coats using Ficoll and Percoll density gradients according to a previously described protocol. [15] For generation of human DC, monocytes were then cultured for 6-8 days in AIM-V culture medium (Invitrogen) in the presence of recombinant human GM-CSF and IL-4 (both 100 ng/ml, RnD Systems, Inc. Minneapolis, MN).
For patient studies, Crohn's disease patients were genotyped for rs_2241880 (ATG16L1) and rs_5743293 (NOD2) using polymerase chain reaction restriction fragment length polymorphisms. Inclusion criteria were homozygosity for either the risk or wild type allele of ATG16L1 and homozygosity for the wild type allele of NOD2. Venous heparinized blood was obtained and monocytes were isolated using Ficoll density gradient followed by magnetic bead isolation (Miltenyi). Monocytes were then used for further analysis and culture. All patients gave informed consent and the study was approved by the Ethical Review Committee.

For generation of mouse dendritic cells (BMDC), bone marrow was isolated from femur and cultured in RMPI 1640 culture medium (Invitrogen), in the presence of 10% FCS and 20 ng/ml recombinant mouse GM-CSF (RnD Systems Inc.) for 7-9 days.

Transfection of dendritic cells

ON-TargetPlus ATG16L1, IRGM and NOD2 specific siRNA pools were obtained from Dharmacon (Epsom, United Kingdom), and control non-specific siRNA from Ambion (Austin, Texas). All were transfected using Dharmafect 4 (Dharmacon) reagent according to the manufacturer's protocol. Plasmid encoding eGFP-LC3 fusion protein was described previously (Addgene plasmid 11546 [16]). To generate the plasmid encoding the SNAP-ICAM-1 fusion protein, human ICAM-1 was amplified by PCR from Addgene plasmid 8632 [17] using targeted primers (forward CAGGCGCGCCAGCTCCCAGCAGCCCCCGG, reverse CAGGATCCTCAGGGAGGCGTGGCTTG) containing BamHI and ASCI restriction sites and cloned into the pSNAP-Tag vector (New England Biolabs, Ipswich, MA). All plasmids were transfected using Lipofectamine 2000 reagent (Invitrogen). Surface expression of SNAP-ICAM was visualized by staining of transfected DC with SNAP-Surface 549 cell impermeant fluorescent dye (New England Biolabs) according to the manufacturer's suggestions.

RNA isolation and quantitative pcr

RNA was isolated using the RNAeasy mini kit (Qiagen, Hilden, Germany) and cDNA was generated using RevertAid reverse transcriptase (Fermentas, St Leon-Rot, Germany) and random hexamer primers (Promega, Leiden, The Netherlands). Quantitative pcr reactions were carried out using Quantitekt primer assays and SybrGreen PCR Kit (both Qiagen). For relative expression, all data was normalized against expression of the household gene GAPDH.

DC-T cell cluster formation and analysis

DC-T cell clusters were induced as described previously. [14] Briefly, DC and T cells were centrifuged together at 50x*g* in a 15 ml conical tube and incubated at 37°C for times indicated. Where indicated DC were pre-treated with 10 mM 3-methyladenine (Sigma Aldrich, Deisenhofen, Germany) for 2 hours at 37 °C after which cells were washed carefully. For antigen specific interactions, $OVA_{323\text{-}339}$ and control $OVA_{257\text{-}264}$ peptide (2 μg/ml, both AnaSpec Inc, San Jose, CA) were added. For Western Blot analysis cells were then lysed in lysisbuffer (Cell Signaling Technology, Beverly, MA) containing Protease Inhibitor Cocktail (MP Biomedicals Inc., Solon, OH), and homogenized by ultrasound sonication. Samples were run on SDS-PAGE gels under reducing conditions and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Membranes were blocked by incubation in 5% blocking powder (Bio-Rad, Hercules, CA) and incubated with primary and secondary antibodies in 1% blocking powder (Biorad). Expression was detected by Lumilight Plus (Roche, Woerden, The Netherlands) and image analysis was performed using ImageJ software.

For analysis of cytoskeletal polarization, clusters were plated on Poly-L-lysine (Sigma) coated slides and fixed in 4% paraformaldehyde. Polarization of the cytoskeleton was visualized using phalloidin-AlexaFluor 488 (Invitrogen) and scored on a three point scale by two independent observers.

Mixed lymphocyte reaction and Th subset induction

For mixed lymphocyte reactions, DC were co-cultured with allogeneic lymphocytes for 72 hours. For OVA specific reactions, BMDC were cocultured with isolated OTII splenocytes in the presence of $OVA_{323-339}$ peptide for 72 hours. Proliferation was measured by ^{3}H-thymidine incorporation assay.

For analysis of Th subset induction, CD45RO+ T cells were isolated from peripheral blood using magnetic beads (Miltenyi Biotec) and co-cultured with autologous DC in the presence or absence of anti-IFNγ (10 μg/mL, RnD Systems) for 4 days and restimulated during 4-6 hours using Phorbol Myristate Acetate and ionomycin (both Sigma) in the presence of Golgistop (BD Biosciences).

Time lapse analysis

DC were plated on glass bottom microwell dishes (MatTek Corp. Ashland, MA) and allowed to adhere for 2 hours at 37°C. When appropriate, OVA peptide was added during adhesion. Supernatant was removed and lymphocytes (human: peripheral blood lymphocytes, mouse: OT-II spleen cells) were added to the plates and allowed to settle for 1 minute. Sequential images were then taken every 20 seconds for 30 minutes using a Zeiss Axiovert 200M inverted microscope and processed using OpenLab 3.09 software (Perkin Elmer, Waltham, MA). The resulting image sequences were analysed by two independent observers.

Statistics

Statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL) or GraphPad (Graphpad software Inc., La Jolla, CA). Analyses included Mann-Whitney U test (non paired data), Wilcoxon square rank test (paired data), Kruskall Wallis test followed by Dunn's multiple comparisons (multiple comparisons) and Spearman's Rho (correlations). Data was considered significant if $p<0.05$.

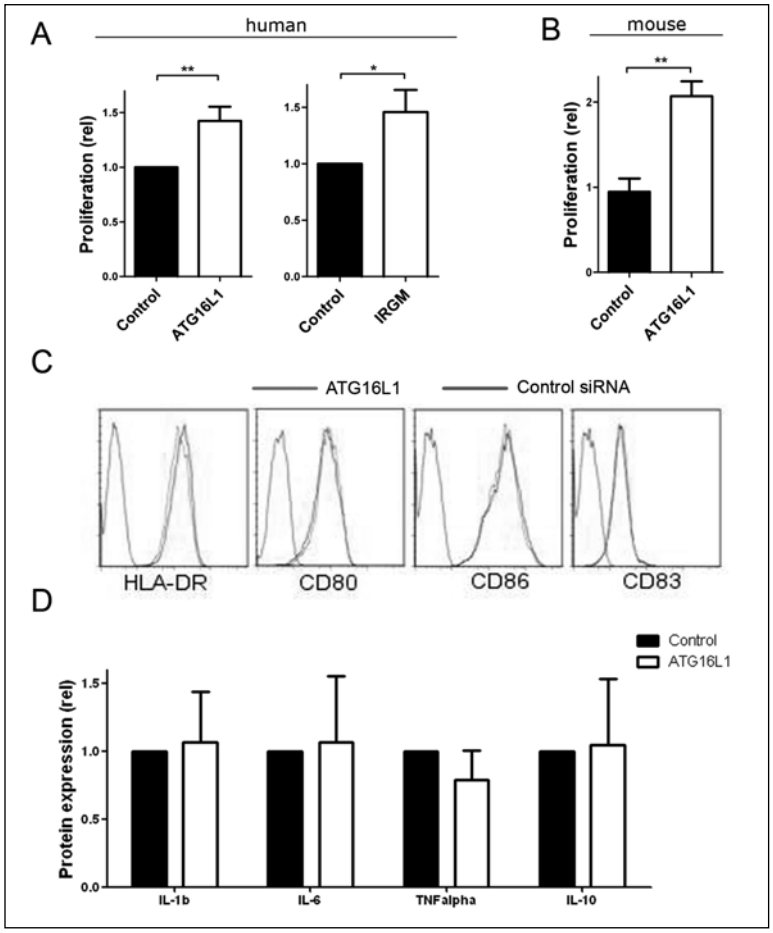
Results

Decreased DC autophagy results in increased T cell stimulatory capacity

Expression of ATG16L1 was decreased in human DC using siRNA technology and confirmed by quantitative PCR and immunoblotting for the autophagy target p62 (Supplementary Figure 1*A/B*). Although autophagy has been described as a cell death pathway, under our experimental (nutrient sufficient) conditions cell viability was not altered in ATG16L1^{low} DC (Supplementary Figure 1*C*).

ATG16L1^{low} DC induced significantly more proliferation in allogeneic lymphocytes than control DC (Figure 1*A*). To verify that the increased stimulatory capacity of ATG16L1^{low} cells was due to diminished autophagy rather than an unknown function of ATG16L1, results were confirmed by inhibition of a second autophagy related protein, IRGM (Figure 1*A*). Additionally, autophagy knockdown in murine BMDC resulted in increased proliferation of ovalbumin-specific T cells in the presence of their cognate antigen (Figure 1*B*), suggesting the mechanism also operates in antigen specific responses and is conserved across species. Expression of activation markers including HLA-DR, CD80, CD86 and CD83 was not altered after knockdown, neither before nor after co-culture with lymphocytes (Figure 1*C* and data

Figure 1 Decreased DC autophagy results in increased T cell proliferation without phenotypic maturation.



(a) Inhibition of autophagy genes ATG16L1 or IRGM in DC results in increased allogeneic T cell stimulation in an MLR culture. Cells were co-cultured for 72-96 hours, data normalized against proliferation in the control sample of the same donor, n=10 and n=9 respectively. (b) Decreased autophagy in BMDC pulsed with OVA results in increased OTII proliferation. Cells were co-cultured for 96-120 hours, data normalized against control sample, mean and s.e.m. of three independent experiments shown. (c) Inhibition of ATG16L1 expression does not alter DC maturation. DC were treated with ATG16L1 (green line) or control (red line) siRNA for 48 hours and analyzed by flow cytometry, blue lines indicate control staining. Data is representative of four independent experiments. (d) Inhibition of ATG16L1 expression does not alter DC cytokine secretion. Cyokine secretion of DC co-cultured with allogeneic lymphocytes (ratio 1:5) was determined in supernatants by cytometric bead array. Data normalized against control of the individual donors. Mean and s.e.m. of three individual experiments shown. (a,b) * indicates $p<0.05$, ** $p<0.01$

not shown). Furthermore, decreased expression of ATG16L1 did not affect the secretion of proinflammatory cytokines IL-1β, IL-6 and TNF-α, nor of the regulatory cytokine IL-10 (Figure 1*D*), suggesting that the immunogenic phenotype was not due to increased DC maturation.

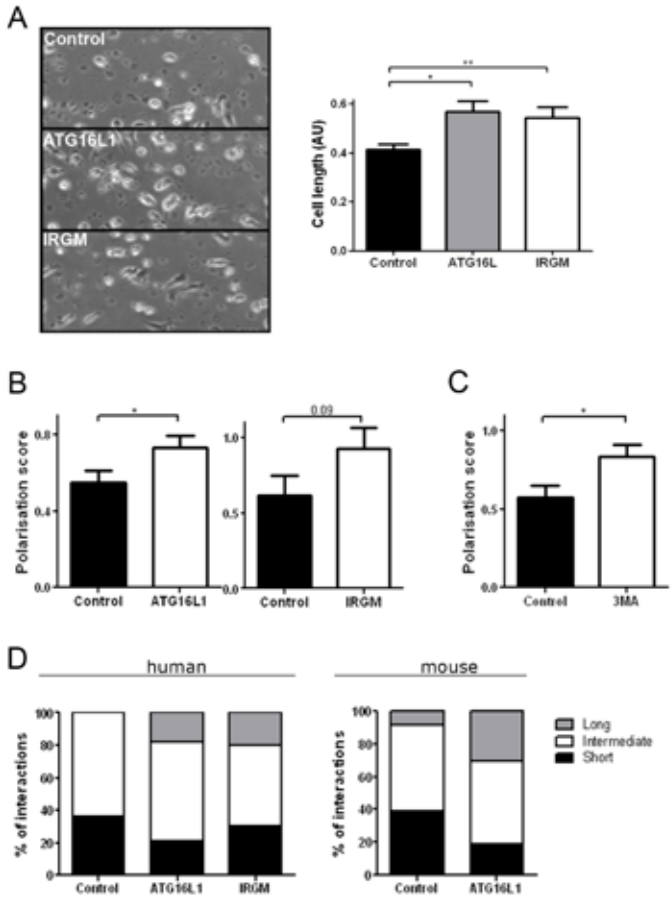
Decreased autophagy increases interaction strength in DC – T cell interactions

Interestingly, knockdown of ATG16L1 and IRGM gene expression resulted in a change in the gross morphology of DC, in that cells showed a more elongated phenotype (Figure 2*A*). This suggests that autophagy affects the cytoskeletal organization of DC. As polarization of the actin cytoskeleton is a key feature of DC-T cell interactions, we reasoned that such cytoskeletal effects may also affect DC mediated immune responses. Upon antigen recognition, DC and T cells form an immunological synapse, a highly organized molecular structure at the site of contact between the two cells. [13] One characteristic of the immunological synapse is the polarization of the actin skeleton towards the site of interaction. The relevance of this polarization is shown by the fact that inhibition of cytoskeletal rearrangements results in decreased T cell activation. [18] We hypothesized that autophagy knockdown would alter polarization of the DC actin skeleton. Therefore, allogeneic DC-T cell interactions were induced *in vitro* and the level of polarization was measured. Significantly stronger actin polarization was seen in DC in which autophagy was inhibited either by ATG16L1 or IRGM knock-down (Figure 2*B*). Again, this finding was consistent in OVA-specific murine BMDC-T cell interactions (Supplementary fig. 2*A*).

We further confirmed the role of the autophagy pathway as a whole using the autophagy inhibitor 3-methyladenine (3-MA). Since it has been shown previously that T cells require some level of autophagy to prevent activation induced cell death, [19] it was not possible to add 3-MA directly to the cultures. Instead, DC were pre-incubated with 3-MA for two hours. Cells were than washed carefully, and used in subsequent polarization experiments. Indeed, pharmacological inhibition of autophagy also resulted in increased cytoskeletal polarization (Figure 2*C*), suggesting a role for the pathway as a whole.

The outcome of immune responses is not only determined by the polarization and recruitment of molecules, but also by the duration of the interaction. [20, 21] As has been described previously, inhibition of proper immunological synapse formation by the myosin II inhibitor blebbistatin resulted in decreased duration of DC-T cell interactions and decreased T cell activation (data not shown). To measure the effect of decreased autophagy on the duration of DC-T cell interactions, we performed time lapse experiments. Both ATG16L1^{low} and IRGM-^{low} DC engaged in more prolonged interactions with allogeneic T cells (Figure 2*D*, left). In accordance with a conserved role for autophagy in immune regulation, similar results were obtained for the interaction between murine BMDC loaded with cognate antigen and OTII splenocytes (Figure 2*D*, right). Together this data suggests that in DC, autophagy is a regulatory mechanism, controlling the strength and duration of DC-T cell interactions and thereby the ensuing immune response.

Figure 2 Decreased autophagy leads to increased cytoskeletal rearrangements in DC and prolonged DC-T cell interactions.

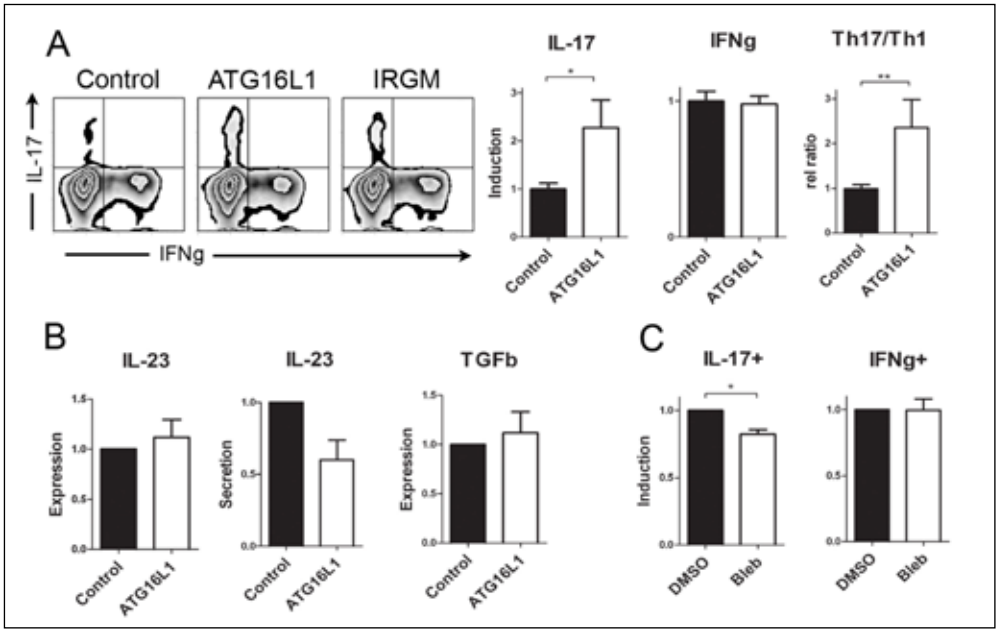


(a) DC generated from siRNA treated monocytes show an elongated phenotype. Representative light microscopy image of each condition (magnification 200x, left). Cell length was measured by image analysis in arbitrary units (right). Mean and s.e.m. of >50 cells per condition is shown. (b,c) DC-T cell clusters were generated by centrifugation at 50g and maintained for 30 min. Actin skeleton was visualized by phalloidin staining and scored for actin polarization on a three point scale (0-2). Bars represent mean and s.e.m., representative figure of three independent experiments. (c) DC were pre-treated with 3MA for 2 hours and washed carefully prior to clustering with T cells. (d) Time lapse analysis of DC-T cell interactions. Allogeneic T cells were added to adherent siRNA treated human DC (ratio 5:1, left panel) and OTII cells were added to adherent siRNA treated BMDC in the presence of cognate antigen (OVA323-339 peptide, 1 μg/ml, ratio 1:5, right panel). All samples were analyzed for 30 minutes by time lapse microscopy and total duration of individual interactions were calculated in duplicate samples. Interactions were categorized as short (< 3 minutes, black)), intermediate (3-15 minutes, white) or long >15 minutes, grey). Results are representative of five independent experiments. * indicates $p<0.05$, ** $p<0.01$

Decreased DC autophagy results in Th17 polarization

One important function of DC in the regulation of immune responses is the skewing of T cells. Although the sharp distinction between Th1, Th2 and Th17 cells has proven less obvious in humans than in rodents, human T cell subsets also produce varying degrees of the effector cytokines IFNγ, IL-4 and IL-17. The stability of DC-T cell interactions has been shown to influence the Th1/Th2 balance, [22] but data regarding the effect on Th17 has not been available thus far. Given the potential role of the Th17 axis in inflammatory bowel disease, [23, 24] we tested the effect of decreased autophagy on the polarization of Th17 cells. To this end, DC were co-cultured with CD45RO+ T cells without the addition of exogenous cytokines. Both ATG16L1^{low} and IRGM^{low} DC induced significantly more IL-17 producing cells in an array of donors (Figure 3*A*). In contrast, the level of IFNγ producing cells was not altered (Figure 3*A*).

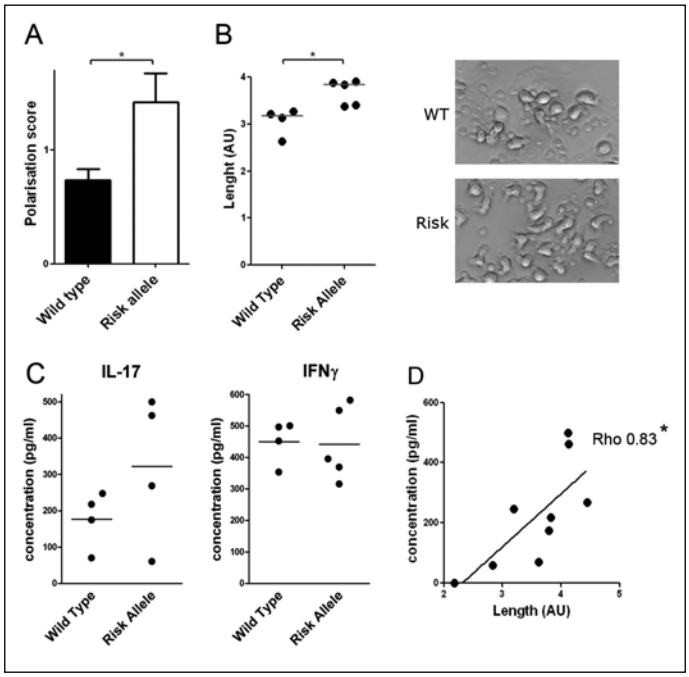
Figure 3 Immunological synapse hyperstability results in increased Th17 polarization.



(a,b) Isolated CD45RO+ T cells were co-cultured with autologous siRNA treated DC for 4 days, restimulated by PMA/ionomycin and analyzed by flow cytometry (a), quantitative pcr or ELISA (b). (a) Left panel shows representative data, bars represent mean (normalized against control sample in each donor) and s.e.m. of duplicate experiments in five different donors. (b) Bars represent mean relative mRNA expression and s.e.m. (left and right panel, n=3 and n=4 respectively) normalized against GAPDH and CD11c expression in each individual donor. Middle panel represents mean protein level and s.e.m. of four individual donors. (c) Isolated CD45RO+ T cells were co-cultured with autologous siRNA treated DC for 4 days in the presence of blebbistatin (5 μM) or vehicle control (DMSO), restimulated by PMA/ionomycin and analyzed by flow cytometry. Data are normalized against vehicle control for each donor and represent mean and s.e.m. (n=5). * indicates $p<0.05$, ** $p<0.01$

Various cytokines have been implicated in the development of Th17 cells *in vitro*, including IL-23, IL-6 and TGF-β. However, IL-23 mRNA levels were unaltered and protein levels were decreased rather than increased in ATG16L1 siRNA treated DC (Figure 3*B*). Furthermore, DC production of TGFβ1 and IL-6 was also comparable in autophagy^{low} and control cells (Figure 3*B* and Figure 1*D*), suggesting that increased secretion of Th17 inducing cytokines is not the mechanism underlying the T cell skewing observed. To further analyze the role of synaptic stability in the activation of Th17 responses, we used the myosin II inhibitor blebbistatin. Complete myosin inhibition would quench all cellular interactions and be toxic in long term cultures, such as those required for induction of Th17 responses. Therefore, we used a relatively low dose in order to destabilize but not completely abolish immunological synapse formation. Interestingly, we found that in the presence of blebbistatin polarization towards the Th17 phenotype was decreased, whereas the induction of Th1 was not significantly affected (Figure 3*C*). This results in a decreased Th17/Th1 balance, suggesting that

Figure 4 ATG16L1 Crohn's disease susceptibility allele induces DC hyperpolarisation and tilted Th17/Th1 balance.



(a) Monocyte-T cell clusters were induced by centrifugation at 50*g*, actin was stained using phalloidin and actin polarization was scored on a three point scale (0-2, n=9). (b) Monocytes isolated from peripheral blood were cultured in the presence of GM-CSF and IL-4 for six days, two images were taken from each well at random locations and processed by image analysis (n=9), right panel shows representative images from 2 donors . (c) DC were co-cultured with allogeneic T-cells for 96 hours. Supernatant was then collected for cytokine measurements by ELISA (d) Cell length as measured in section (a) was correlated to IL-17 concentration in supernatants using Spearman's Rho. * indicates $p<0.05$

the stability of the immunological synapse itself may be involved in determining the polarization of the immune response.

ATG16L1 risk allele carrier DC display hyperpolarization and increased Th17 induction

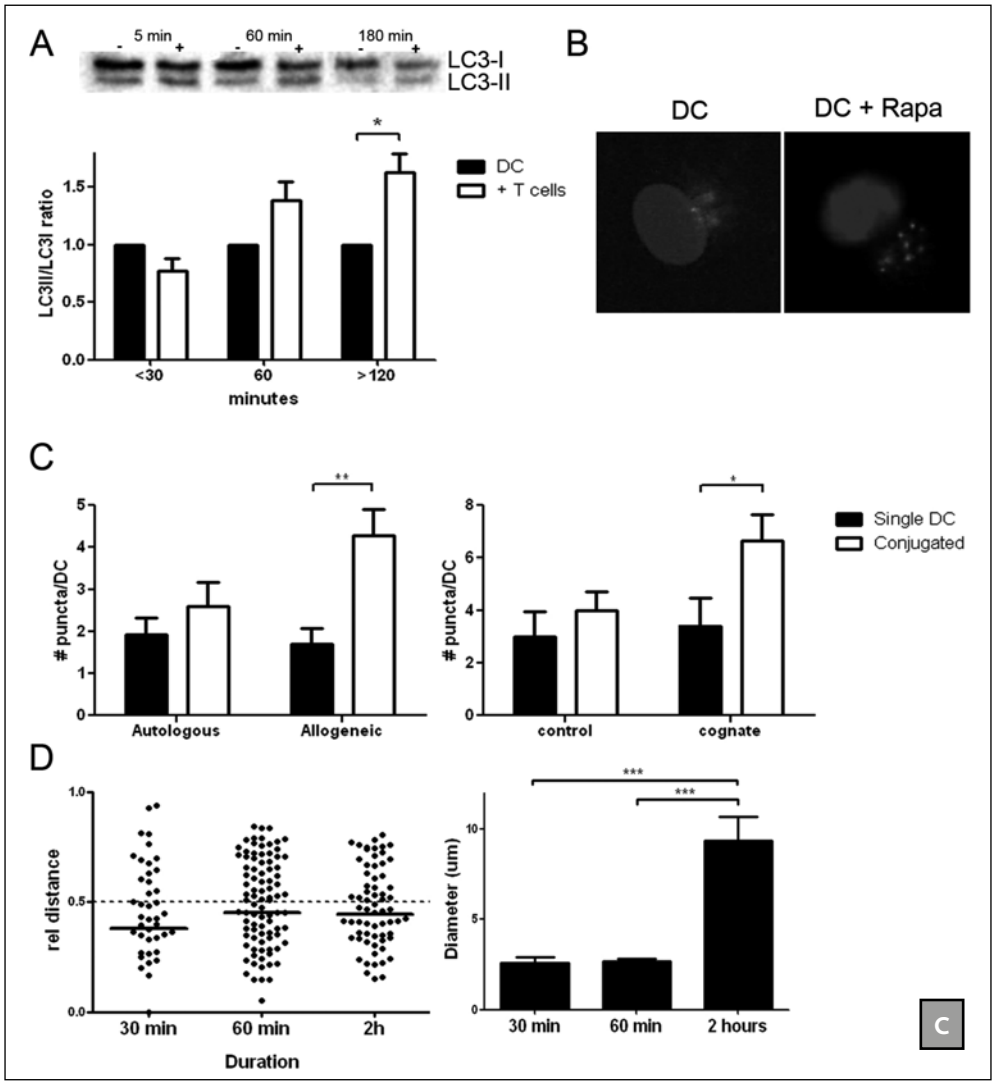
To confirm the physiological relevance of autophagy-based immune regulation, we recruited Crohn's disease patients homozygously carrying either the wild type or the T300A risk allele for ATG16L1. [1] Since protein interactions between ATG16L1 and NOD2 have been described previously, we excluded carriers of the NOD2 3020insC risk allele to avoid confounding by this polymorphism. In accordance with our previous data, monocytes obtained from ATG16L1 risk allele carriers exhibited significant hyperpolarization of the actin cytoskeleton, indicating more stable synapses (Figure 4*A*). Furthermore, DC generated from patients carrying the risk allele were significantly longer than DC from patients expressing the wild type allele, similar to the data obtained using gene knock-down (Figure 4*A*). Furthermore, co-culture of CD45RO+ T cells and T300A expressing DC resulted in a trend towards increased levels of IL-17 but not IFNγ secretion compared to wild type DC (Figure 4*B*). Strikingly, the level of IL-17 secretion was strongly correlated to DC length, but not to activation marker expression (Figure 4*C* and data not shown), further supporting the link between the cytoskeletal aberrancies and the increased presence of Th17 cells.

Immunological synapse formation induces autophagy in DC

To further decipher the mechanism by which autophagy regulates stability of the immunological synapse, we evaluated whether autophagy is upregulated by synapse formation. During autophagy, the autophagosomal protein LC3 is converted from 18 kD (LC3 I) to 16 kD (LC3 II), leading to an increased LC3 II/ LC3 I ratio. [25] This ratio can therefore be used as a measure of the level of autophagy. GFP-LC3 transfected DC were incubated in the presence or absence of allogeneic T cells, and the LC3 II/I ratio was determined by immunoblotting against GFP. Indeed, DC co-incubated with allogeneic T cells for more than 30 minutes showed autophagic processing compared to DC alone (Figure 5*A*).

This finding was further confirmed by quantification of GFP-LC3 puncta (which are formed upon induction of autophagy, Figure 5*B*) in single and clustered DC in the same image field. The number of LC3 puncta was increased significantly in clustered DC compared to single DC, suggesting the induction of autophagy (Figure 5*C*). To verify that autophagy is induced by the actual formation of the immunological synapse rather than the mere proximity of a lymphocyte, we compared allogeneic and autologous DC-T cell clusters. In allogeneic clusters, a real synapse is formed, including polarization of the actin cytoskeleton, while in an autologous setting, clusters are formed, but these do not lead to the formation of a full synapse. [14] Again, in the allogeneic setting, a significantly higher level of autophagy was present in clustered cells compared to single DC. However, in autologous interactions the number of puncta in clustered DC was not increased compared to the number of LC3 puncta in single DC, indicating that under these conditions, autophagy was not induced (Figure 5*C*). Similarly, a significant increase in autophagy occurred in BMDC-OTII splenocyte clusters when DC presented the cognate $OVA_{323\text{-}339}$ peptide resulting in a full synapse. However, when DC were loaded with the control $OVA_{257\text{-}264}$ peptide (Figure 5c) autophagy remained unaltered, emphasizing the fact that it is not the mere clustering of DC and T cells that leads to autophagy, but the actual cognate interaction.

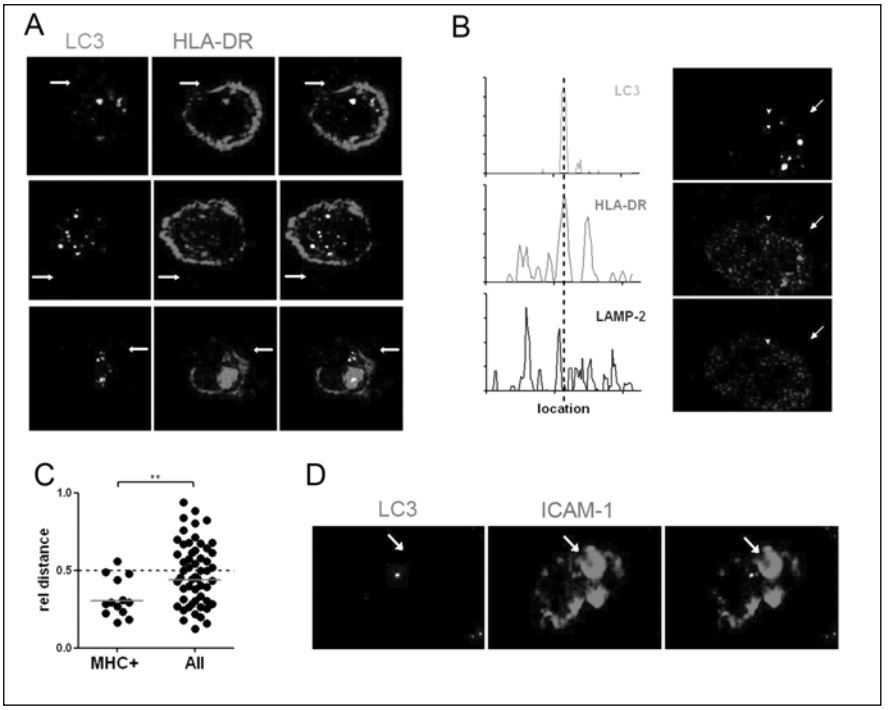
Figure 5 Immunological synapse formation induces autophagy in DC.



(a) GFP-LC3 transfected DC were incubated in the absence (black bars) or presence (white bars) of allogeneic lymphocytes (times indicated above lanes), samples were then blotted for GFP and LC3II/LC3I ratio was calculated by image analysis. Western blot representative of three independent experiments. Data normalized against control samples, bars represent mean and s.e.m. of duplicate experiments. (b) GFP-LC3 transfected DC were treated with rapamycin (right, 20 μg/ml) and analyzed by immunofluorescence. (c) Clusters were induced between GFP-LC3 transfected DC and autologous or allogeneic T cells (left), or between BMDC and OTII cells in the presence of control or cognate antigen (right). Number of LC3 puncta/DC were then scored in unconjugated DC (black bars) and DC conjugated to T cells (white bars) in the same image fields. Data representative of three individual experiments, bars represent mean and s.e.m. (d) Clusters were induced between GFP-LC3 transfected DC and allogeneic T cells and maintained for indicated periods. Cells were then imaged by confocal microscopy and relative distance was calculated as (distance of LC3+ spot to contact site / maximum width of the cell at site of synapse). Spot size was measured by image analysis. * indicates $p<0.05$, ** $p<0.01$

To further establish whether this autophagy induction was related to stability of the immunological synapse, we measured the relative distance between the autophagosome and the synapse (that is, the distance between the LC3+ spot and the site of cell-cell contact relative to the maximum width of the cell). If the autophagosomes are distributed randomly throughout the cell, this relative distance would have an average of 0.5. However, when measuring the relative distance over a large number of clusters, the average distance is significantly below 0.5, suggesting a preferential localization towards the immunological synapse (Figure 5*D*).

Figure 6 Autophagosomes are involved in degradation of synaptic components.



(a) Clusters of GFP-LC3 transfected DC and allogeneic T cells were induced for 60 minutes and stained for expression of HLA-DR (red). Subcellular localization of positive staining was determined by confocal imaging. Three individual clusters are shown, arrows indicate position of T cell in cluster. (b) Position of LC3+/MHC+ and LC3+/MHC- spots were calculated as relative to maximum width of the cell. Data representative of three independent experiments in four donors. (c) Clusters of GFP-LC3 transfected DC and allogeneic T cells were induced for 60 minutes and stained for expression of HLA-DR (red) and LAMP2 (blue). Arrows indicate location of T cell in cluster. Arrowheads indicate line of analysis shown in line graphs. Graphs show relative signal intensity along the same line for all three stainings. Data representative of three individual experiments in different donors. (d) GFP-LC3 and SNAP-ICAM transfected DC were surface stained for ICAM (red). ICAM localized around phagocytic cups (indicated by arrows) and co-localized with LC3. Picture representative of two independent experiments. (a,c,d) All images magnification 630x and taken at a single Z-stack level in each cell

Autophagosomes close to the immunological synapse contain synaptic components

When we analyzed the formation of autophagosomes during formation of the immunological synapse, we not only observed a preferential localization towards the contact site, but also an increase in the size of autophagosomes between 30 minutes and 2 hours of DC-T cell interactions (Figure 5*D*). Given that autophagy is mainly involved in the degradation of cellular components, we hypothesized that these growing structures are sites of degradation of synaptic components, thereby destabilizing the synapse. To test this, clusters of GFP-LC3 transfected DC and allogeneic T lymphocytes were induced as described before, and localization of the central immunological synapse component MHC II was determined by confocal imaging. In line with our hypothesis, clustered DC showed clear colocalization of autophagosomes and MHC II (Figure 6a). Furthermore, distance measurements revealed that while autophagosomes in general are localized somewhat preferentially near the synapse, LC3+ spots containing MHC II molecules are localized significantly closer to the synapse (Figure 6*B*). To exclude the possibility that the MHC II/LC3 colocalization was caused by fusion of autophagosomes and late endosomes/lysosomes, [6] samples were also stained for the endo/lysosomal marker LAMP2. Although the majority of spots stained positive for all three markers, puncta positive for LC3 and MHCII but negative for LAMP2 were also observed, showing these spots are in fact autophagosomes containing non-lysosomal derived MHCII (Figure 6*C*). To further confirm this data, colocalization of the immunological synapse component ICAM-1 was determined using SNAP-tag technology. After expression in the cell of interest, the SNAP-tag can be labeled fluorescently using a cell non-permeable dye, resulting in the selective staining of proteins present on the outside of the cell membrane. We created an SNAP-ICAM-1 fusion construct, and labeled membrane ICAM-1 during formation of the immunological synapse. Similar to MHC II, ICAM-1 colocalized with LC3 puncta close to the synapse (Figure 6*D*). Since ICAM-1 staining was only possible at the cell surface these ICAM-1 molecules have to be membrane derived. The presence of immunological synapse derived molecules in autophagosomes close to the synapse strongly suggests that these autophagosomes play an active role in degradation and thereby regulation of the synapse.

Discussion

We demonstrate that cognate interactions between DC and T cells induce autophagy which in turn negatively regulates the interaction. Decreased autophagy in DC resulted in increased formation of the immunological synapse, prolonged DC-T cell interactions and increased T cell activation and Th17 polarization.

Autophagy was first described over three decades ago, but was initially only considered a cell survival mechanism. The identification of several autophagy genes as susceptibility genes for Crohn's disease has lead to renewed attention for the field, in particular its role in the immune system. Autophagy contributes to innate immunity through degradation of intracellular pathogens, including *Mycoplasma Tuberculosis*, *Listeria Monocytogenes* and *Salmonella Enterica*. [26, 27, 28] Diminished autophagy may therefore result in defective innate immunity, excessive microbial expansion and unbalanced adaptive immune activation. This mechanism, known as the theory of innate immunodeficiency, has been proposed to be

responsible for the elevated immune activation seen in Crohn's disease patients carrying these SNP. However, although impaired clearance of intracellular pathogens may contribute to the excessive immune responses seen in Crohn's disease, our study shows that a deficient autophagy can also directly hyperactivate T cell responses.

Previous studies into formation of the immunological synapse mainly focused on the T cell. The 'other side' of the synapse (ie, the DC) has received relatively little attention, leading to a void in the knowledge on this topic. However, inhibition of actin polarization specifically in DC hampers the formation of a proper synapse and significantly decreases T cell activation and proliferation. [14] Additionally, a recent study showed recruitment of the signaling molecule phosphatidylinositol-4,5-biphosphate to the synapse as well as local activation of Akt1 in DC suggesting active signaling in DC. [29] Our study further confirms the active participation of DC in immunological synapse formation, as autophagy knockdown specifically in DC results in altered synapses. This effect appears to be part of a negative feedback mechanism, as autophagic activity increases upon formation of a mature immunological synapse. Dendritic cells have a basal level of constitutive autophagy, which is increased upon synapse induction. Most likely only part of these autophagosomes are involved in regulation of the immunological synapse, whereas others play a role in cellular homeostasis. This may explain why localization of autophagosomes is somewhat polarized to the synapse, but still displays a relative big spread. Synaptic components such as MHCII and ICAM-1 are engulfed by autophagosomes, targeting them for degradation. Although no data is available on the relationship between the number of molecules present and the strength of the immunological synapse, it is not hard to imagine that a decrease in the number of interacting molecules leads to a relative destabilization of the synapse. Previously, it has been shown that membrane expression of MHCII is regulated through ubiquitination of the molecule and that ubiquitination can target proteins to the autophagosomes. [30-32] Therefore, ubiquitination of synaptic components may be the mechanism behind the trafficking observed. Interestingly, ubiquitination-related polymorphisms have recently been associated with the development of Crohn's disease. [33, 34] Whether these polymorphisms have similar consequences for the regulation of cell-cell interactions as autophagy related defects would be an interesting topic of investigation.

Interestingly, a recent study shows that the protein encoded by another Crohn's disease susceptibility gene, NOD2, is necessary for proper localization of ATG16L1 at the cell membrane. [35] The susceptibility variant of NOD2 lacks this function, resulting in abnormal cytoplasmic ATG16L1 distribution. Given the important role of the membrane in cell-cell interactions, it is tempting to speculate that membrane localization of ATG16L1 is also necessary for proper regulation of DC-T cell interactions. Indeed, in our experiments, decreased expression of NOD2 resulted in a DC hyperpolarization similar to that observed in autophagy^{low} DC (Supplementary Figure 2*B*). This suggests that NOD2 and ATG16L1 risk alleles can independently lead to decreased pathogen degradation as well as increased adaptive immune activation, thus explaining why the risks carried by these alleles appear to be independent rather than cumulative.

Several studies have highlighted the importance of autophagy in the antigen presentation by MHC class II molecules. When influenza matrix antigen was targeted to the autophagosome, increased presentation and T cell activation was observed. [6] More recently, *Atg5^{lox}CD11c^{cre}* mice lacking autophagy specifically in DC show decreased MHC II antigen presenta-

tion due to decreased autophagosomal-lysosomal fusion. [36] Consequently, these DC were less capable of inducing T cell activation and mice showed impaired immune responses. The different results in this study compared to ours may be explained by the fact that the previous study used a complete knockout model, whereas in our model DC display decreased autophagy levels, but not a complete deficiency. Differential levels of autophagy may be required for regulation of antigen processing versus regulation of immunological synapse stability. Whereas the complete knockout is a relevant model to study if a specific gene plays any role in a given process, our model may be more reflective of the effect of hypomorphic alleles as seen in Crohn's disease patients. Indeed, several of the effects seen in our autophagy^{low} DC are mirrored by results in DC generated from Crohn's disease patients carrying the ATG16L1 risk allele, confirming the physiological relevance of our findings.

The role of the immunological synapse in the polarization of T helper responses has been investigated previously in the context of Th1/Th2 skewing. For example, synapses resulting in Th1 polarization contain a clear ICAM-1 ring structure and exclusion of CD45 from the central synapse, while synapses resulting in Th2 skewing contain more phosphotyrosines.[37] To the best of our knowledge, no data are available on the role of the synapse in the induction or activation of Th17 cells. In our experiments, secretion of the Th17 inducing factors IL-23, IL-6 and TGF-β [38] was not increased in autophagy^{low} DC, indicating that the Th17 polarization observed is not due to altered cytokine secretion. Although we can not formally exclude that the increase in Th17 is the result of alterations in DC other than increased immunological synapse formation, we have not observed any such alterations. Furthermore, destabilization of the synapse using blebbistatin resulted in a decreased Th17 skewing, further supporting our hypothesis that hyperstability of the immunological synapse positively correlates with the induction of Th17 responses.

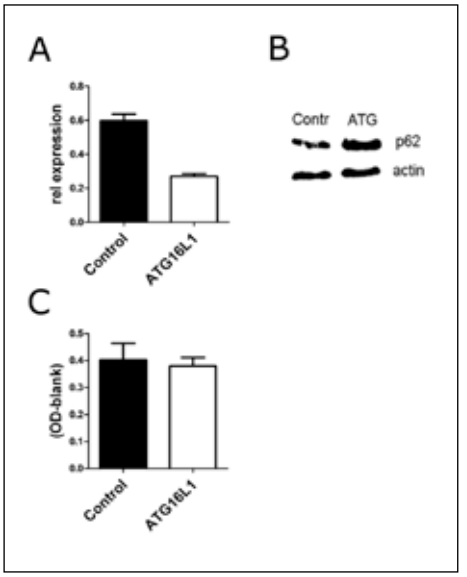
Our results demonstrate a novel role for autophagy in the regulation of immunological synapse stability and subsequent strength and polarization of the adaptive immune response. The clinical implications of these findings are shown by the fact that Crohn's disease patients carrying polymorphisms in ATG16L1 display similar alterations in their DC, including hyperpolarization and a tendency towards increased induction of IL-17 producing T cells. Interestingly, azathioprine, a frequently used therapy for Crohn's disease, has been shown to destabilize DC-T cell interactions through inhibition of the Rac pathway. [39] Our data suggest that this may in fact be a therapeutic mechanism for this drug in Crohn's disease. Additionally, other medications interfering with immunological synapse formation may be new candidates for Crohn's disease treatment.

In summary, this study shows that upon formation of the immunological synapse, autophagy is induced, which then acts as a negative feedback regulator of DC-T cell interactions by destabilizing the synapse. In Crohn's disease patients carrying the ATG16L1 risk allele, autophagic activity is decreased, leading to hyperstable DC-T cell interactions and increased Th17 activation.

Competing interests: none

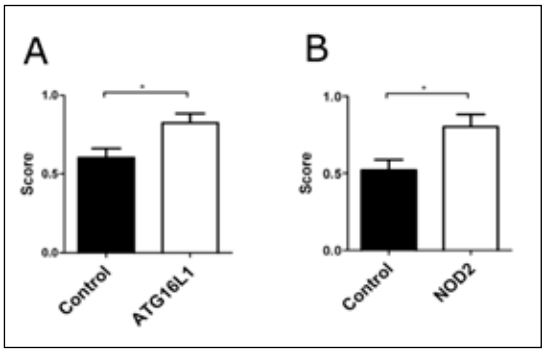
Supplementary figures

Supplementary figure 1.



(a) DC were treated with specific or control siRNA for 48 hours and analyzed by quantitative PCR. All data was normalized against GAPDH. Bars indicate mean expression and s.e.m., representative of >10 experiments. (b) siRNA treated DC were stimulated with rapamycin for 12 hours (20 μg/ml) and immunoblotted for expression of p62. Data representative of three donors. (c) siRNA treated DC were cultured for three days in RPMI culture medium containing 10% FCS and tested for viability by MTS assay. Bars represent mean and s.e.m. of three individual donors

Supplementary figure 2.



Clusters were formed between T cells and DC (a) pre-treated with 3MA (1 mM, 2 hours) or (b) transfected using NOD2 siRNA. Data representative of three individual experiments. Actin skeleton was visualized by phalloidin staining and scored for actin polarization on a three point scale (0-2). Bars represent mean and s.e.m. of >100 cells/experiment, ** $p<0.01$

Reference List

1. Barrett JC, Hansoul S, Nicolae DL et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 2008;40:955-962.
2. Libioulle C, Louis E, Hansoul S et al. Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. PLoS Genet 2007;3:e58.
3. Rioux JD, Xavier RJ, Taylor KD et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet 2007;39:596-604.
4. Lum JJ, Bauer DE, Kong M et al. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell 2005;120:237-248.
5. Birmingham CL, Smith AC, Bakowski MA et al. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J Biol Chem 2006;281:11374-11383.
6. Schmid D, Pypaert M, Munz C. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity 2007;26:79-92.
7. Singh SB, Davis AS, Taylor GA et al. Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science 2006;313:1438-1441.
8. Cadwell K, Liu JY, Brown SL et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 2008;456:259-263.
9. Iwasaki A, Kelsall BL. Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. J Exp Med 1999;190:229-239.
10. Sun CM, Hall JA, Blank RB et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 2007;204:1775-1785.
11. Lutz MB, Suri RM, Niimi M et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. Eur J Immunol 2000;30:1813-1822.
12. Munn DH, Sharma MD, Lee JR et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science 2002;297:1867-1870.
13. Dustin ML, Tseng SY, Varma R, et al. T cell-dendritic cell immunological synapses. Curr Opin Immunol 2006;18:512-516.
14. Al-Alwan MM, Liwski RS, Haeryfar SM et al. Cutting edge: dendritic cell actin cytoskeletal polarization during immunological synapse formation is highly antigen-dependent. J Immunol 2003;171:4479-4483.
15. Repnik U, Knezevic M, Jeras M. Simple and cost-effective isolation of monocytes from buffy coats. J Immunol Methods 2003;278:283-292.
16. Jackson WT, Giddings TH, Jr., Taylor MP et al. Subversion of cellular autophagosomal machinery by RNA viruses. PLoS Biol 2005;3:e156.
17. Staunton DE, Marlin SD, Stratowa C et al. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell 1988;52:925-933.
18. Ilani T, Vasiliver-Shamis G, Vardhana S et al. T cell antigen receptor signaling and immunological synapse stability require myosin IIA. Nat Immunol 2009;10:531-539.

19. Feng CG, Zheng L, Jankovic D et al. The immunity-related GTPase Irgm1 promotes the expansion of activated CD4+ T cell populations by preventing interferon-gamma-induced cell death. Nat Immunol 2008;9:1279-1287.
20. Celli S, Lemaitre F, Bousso P. Real-time manipulation of T cell-dendritic cell interactions in vivo reveals the importance of prolonged contacts for CD4+ T cell activation. Immunity 2007;27:625-634.
21. Katzman SD, O'Gorman WE, Villarino AV et al. Duration of antigen receptor signaling determines T-cell tolerance or activation. Proc Natl Acad Sci U S A 2010;107:18085-18090.
22. Azar GA, Lemaitre F, Robey EA et al. Subcellular dynamics of T cell immunological synapses and kinapses in lymph nodes. Proc Natl Acad Sci U S A 2010.
23. Fujino S, Andoh A, Bamba S et al. Increased expression of interleukin 17 in inflammatory bowel disease. Gut 2003;52:65-70.
24. Yen D, Cheung J, Scheerens HD et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J Clin Invest 2006;116:1310-1316.
25. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. Int J Biochem Cell Biol 2004;36:2503-2518.
26. Vergne I, Singh S, Roberts E et al. Autophagy in immune defense against Mycobacterium tuberculosis. Autophagy 2006;2:175-178.
27. Birmingham CL, Smith AC, Bakowski MA et al. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J Biol Chem 2006;281:11374-11383.
28. Gutierrez MG, Master SS, Singh SB et al. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 2004;119:753-766.
29. Fooksman DR, Shaikh SR, Boyle S et al. Cutting edge: phosphatidylinositol 4,5-bisphosphate concentration at the APC side of the immunological synapse is required for effector T cell function. J Immunol 2009;182:5179-5182.
30. de GA, Camosseto V, Thibodeau J et al. MHC class II stabilization at the surface of human dendritic cells is the result of maturation-dependent MARCH I down-regulation. Proc Natl Acad Sci U S A 2008;105:3491-3496.
31. Kim PK, Hailey DW, Mullen RT et al. Ubiquitin signals autophagic degradation of cytosolic proteins and peroxisomes. Proc Natl Acad Sci U S A 2008;105:20567-20574.
32. Walseng E, Furuta K, Goldszmid RS et al. Dendritic Cell Activation Prevents MHC Class II Ubiquitination and Promotes MHC Class II Survival Regardless of the Activation Stimulus. J Biol Chem 2010;285:41749-41754.
33. Franke A, McGovern DP, Barrett JC et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010;42:1118-1125.
34. Fransen K, Visschedijk MC, van SS et al. Analysis of SNPs with an effect on gene expression identifies UBE2L3 and BCL3 as potential new risk genes for Crohn's disease. Hum Mol Genet 2010;19:3482-3488.
35. Travassos LH, Carneiro LA, Ramjeet M et al. Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol 2010;11:55-62.
36. Lee HK, Mattei LM, Steinberg BE et al. In Vivo Requirement for Atg5 in Antigen Presentation by Dendritic Cells. Immunity 2010;32:227-239.
37. Thauland TJ, Koguchi Y, Wetzel SA et al. J Immunol 2008;181:393-399.

38. Manel N, Unutmaz D, Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol 2008;9:641-649.
39. Poppe D, Tiede I, Fritz G et al. Azathioprine suppresses ezrin-radixin-moesin-dependent T cell-APC conjugation through inhibition of Vav guanosine exchange activity on Rac proteins. J Immunol 2006;176:640-651.

Chapter four

Anti-TNFα antibodies induce regulatory macrophages in an Fc region dependent manner

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Abstract

Anti-TNFα antibodies are effective in Crohn's disease whereas soluble TNFα receptors have failed to show clinical efficacy. The molecular mechanism that underlies the differences between these compounds has not been elucidated. Here we aimed to examine the mechanism of action of the immunosuppressive effect of anti-TNFα antibodies on activated T cells.

We studied the effect of anti-TNFα antibodies infliximab and adalimumab, the soluble TNFα receptor etanercept, pegylated F(ab') fragment certolizumab and certolizumab-IgG on primary activated T cells. T cells were grown in isolation or in a mixed lymphocyte reaction (MLR). Proliferation was measured by ^{3}H thymidine incorporation and apoptosis was examined using Annexin V labeling and a colorimetric assay for activated caspase-3. Macrophage phenotype was assayed by flow cytometry and cytokine secretion.

Infliximab and adalimumab reduced proliferation in an MLR, whereas etanercept and certolizumab did not. This effect was completely abolished after blocking Fc receptors. Infliximab F(ab')2 fragment failed to inhibit proliferation whereas certolizumab-IgG gained the ability to inhibit proliferation. In the MLR anti-TNFs induced a new population of macrophages in an Fc region dependent manner. These macrophages were found to have an immunosuppressive phenotype, in terms of their capacity to inhibit proliferation of activated T cells, production of anti-inflammatory cytokines and the expression of the regulatory macrophage marker CD206.

Regulatory macrophages have immunosuppressive properties and play an important role in wound healing. Our data show that anti-TNFs induce regulatory macrophages in an Fc region dependent manner. This mechanism of action of anti-TNFs may contribute to the resolution of inflammation.

Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease that results from a dysregulated immune response of unknown aetiology. [1-3] Tumour necrosis factor alpha (TNFα), a cytokine produced by activated macrophages, monocytes and T cells, [4] is a key mediator in immune responses and is increased in serum and intestine in CD and synovium in rheumatoid arthritis (RA). [5-7]

Since their introduction in the '90s, anti-TNFα antibodies are commonly used for the treatment of Crohn's disease. Various classes of anti-TNFs have been introduced, and although all classes efficiently neutralize TNFα, they show different efficacy profiles. The antibodies infliximab [8, 9] and adalimumab [10, 11] were shown to be effective in both inducing and maintaining remission in CD patients. On the other hand, two soluble receptors; etanercept and onercept were ineffective in inducing remission in CD patients. [12, 13] Also, the humanized anti-TNFα antibody CDP571 which was designed as an IgG_4 to reduce interaction with Fc receptors in the hope to reduce side effects also failed to show effectiveness in CD. [14] These clinical data strongly suggest that neutralizing TNFα may not be the sole mechanism of action of anti-TNFα treatment in Crohn's disease.

Several effector mechanisms of anti-TNFα treatment have been proposed that are independent of TNFα neutralizing activity. Examples are the induction of apoptosis in T cells and monocytes via binding of membrane bound TNFα (mTNFα), [15-17] antibody-dependent-cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity. [18, 19] Furthermore, a role for Fc receptors has been proposed based on the association between a polymorphism in the Fc gamma receptor IIIa and the biological response to infliximab. [20] Fc receptors bind the Fc region of an antibody or antibody-complex, resulting in myeloid cell activation, phagocytosis and cytokine secretion. The importance of Fc receptors in the mechanism of action of other antibody therapies such as anti-CD20 and anti-Human Epidermal growth factor Receptor 2 (Her2) has been described before. [21, 22] In $Fc\gamma^{-/-}$ mice which are unable to bind Fc regions, both anti-CD20 and anti-Her2 lost their efficacy in reducing tumor size, demonstrating a key role for the Fc receptor in the mechanism of action of these antibodies. Although a number of mechanisms have been suggested, it is still unclear why certain anti-TNFs are effective in Crohn's disease and other anti-TNFs are not. Many studies on the mechanism of action of anti-TNFα focus on binding to mTNFα and reverse signaling in T cells, [18, 23] whereas the effect and importance of binding to Fc receptors has not been established thus far.

In this study, we found that in order for an anti-TNFα to inhibit T cell proliferation *in vitro*, the compound needs to bind to mTNFα on activated T cells and posses an Fc region to interact with the Fc receptor on antigen presenting cells. Upon this binding, a distinct macrophage subset is induced with immunosuppressive capacities, including the production of anti-inflammatory cytokines and inhibition of T cell proliferation.

Material and methods

Antibodies and Reagents

Infliximab, certolizumab, adalimumab and etanercept were prepared according to manufacturers' recommendations. Certolizumab-IgG was obtained from UCB (UCB, Belgium), and IgG_{1k} was obtained from Sigma.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated by Ficoll Paque density-gradient centrifugation. After washing, monocytes were isolated by Percoll density-gradient centrifugation. CD3 positive T cells were isolated from PBMCs using negative magnetic bead separation (Invitrogen). For T cell activation, cells were activated with αCD3/αCD28 antibodies (Sanquin) at the indicated concentration or αCD3/αCD28 beads (Invitrogen) (1 bead/5 cells). PBMCs and T cells were cultured in RPMI 1640 containing 10% heat-inactivated FCS.

Dendritic cells (DCs) were obtained by culturing monocytes with GM-CSF (50 ng/mL, R&D), IL-4 (50 ng/mL, R&D) for 7 days in AIM-V medium. To generate macrophages, monocytes were cultured in 6 wells plates for 5 days.

Infliximab induced macrophages ($M\phi_{ind)}$ were isolated from MLR cultures using CD14 microbeads according to manufacturer's protocol (Miltenyi). Next, cells were cultured in RPMI 1640, containing 10% heat-inactivated FCS. For light microscopy, cells were adhered to poly-L-lysine coated coverslips and stained using DiffQuick.

Binding to membrane TNFα

For mTNFα binding assays, infliximab, certolizumab, adalimumab, etanercept and control IgG were labeled with a fluorescent dye using a commercially available kit according to the manufacturers' instructions (Alexa Fluor 647 protein labeling kit, Pierce). CD3 positive T cells were activated with αCD3/CD28 antibodies for 48 hours. Cells were collected, washed three times in FACS buffer (PBS containing 1% BSA) and incubated with various amounts of labeled anti-TNFα compound or IgG control for 30 minutes on ice. Binding of infliximab F(ab')2 fragment was assessed in a competition assay; activated T cells were incubated with labeled infliximab (10 μg/mL) and increasing concentrations of unlabeled infliximab F(ab')2 fragment at the same time. After washing, binding to mTNFα was analyzed by flow cytometry.

Allogeneic mixed lymphocyte reaction (MLR)

PBMCs from two healthy donors were cultured in a 1:1 ratio in RPMI 1640 culture medium. After 48 hours of activation, cells were treated with the indicated compound (infliximab, adalimumab, certolizumab, etanercept, Certolizumab-IgG or IgG control, all at 10 μg/mL) for up to 7 days where indicated. When appropriate, Fc receptors were saturated by treating MLRs with IgG (10 μg/mL, Sigma) for 6 – 16 hours and next treated with anti-TNF compound or IgG control for 2 days. Finally, proliferation was measured using a ^{3}H-thymidine incorporation assay.

Assays for apoptosis

PBMCs or isolated T cells were activated in an MLR or with CD3/CD28 antibodies for 48 hr, and treated with anti-TNF compound or control (10 μg/mL). Cells were collected, washed three times, and stained with Annexin V and Pi and analyzed by flowcytometry.
For measurement of caspase-3 enzymatic activity, a colorimetric assay was used as described before. [24] Cell lysates were generated from MLR cultures and protein concentration was determined by BCA analysis (Pierce). Lysates were incubated with a saturating concentration of 25μM specific enzyme substrate Ac-Aps-Glu-Val-Asp-AMC (Ac-DEVD-AMC, Bachem, Germany) in 100mM HEPES buffer with 10% sucrose, 10mM dithiothreitol and 0.1% Nonidet-P40. Samples were incubated at 37°C and fluorescent AMC release was monitored (Fluostar Optima plate reader).

FACS analysis

Human monocytes and DCs were plated in 6 well plates (2 x 10^6 cells/well) and cultured with or without LPS (Sigma, 100 ng/mL) for 16 hours, and treated with anti-TNFα compound or IgG control (Sigma, 10 μg/mL) for 25 – 48 hours. Cells were harvested, washed, and stained for αCD14-FITC, αCD40-FITC, αCD80-Pe, αCD83-APC, αCD86-APC, αHLA-DR-FITC and appropriate controls (all BD) for 30 minutes on ice. For analysis of marker expression on monocytes, macrophages and $M\phi_{ind}$, cells were cultured in 6 wells plates and stained for αCD14-FITC, αCD16-FITC, αCD32-APC, αCD40-FITC, αCD80-Pe, αCD83-APC, αCD86-APC, αCD206-APC, αCD209-Pe or αHLA-DR-FITC (all BD). Finally, expression was analyzed by flow cytometry using a FACS Calibur (BD) and FlowJo software (Treestar Inc, Ashland, OR). Expression was calculated as MFI specific staining – MFI control.

Generation of Infliximab F(ab')2 fragment

Infliximab F(ab')2 fragments were generated using a Fab Preparation Kit according to the manufacturers protocol (Pierce). Purity of the resulting fraction was analyzed by SDS-PAGE followed by Coomassie Blue staining and showed no remaining intact antibodies.

Cytokine detection by cytokine beads array (CBA)

DCs, Mϕ1 or $M\phi_{ind}$ were treated or untreated with LPS for 24 hours, supernatants were collected and stored at -20 until use. A CBA was performed according to manufacturer's protocol. Data were analyzed with FlowJo (Treestar).

Statistical analysis

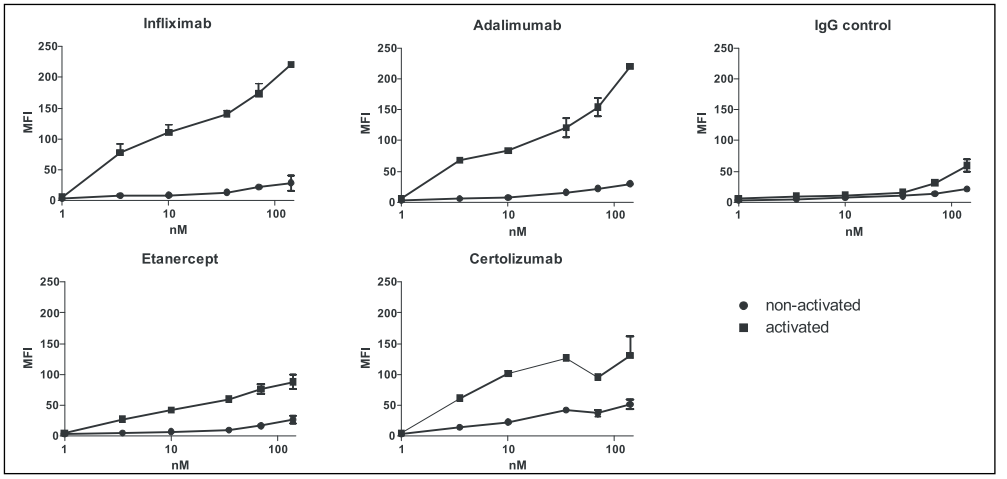
Results are representative for at least three independent experiments and show means ± SEM unless otherwise indicated. For statistical analysis, one way ANOVA was used followed by Bonferroni post test. Results were considered significant when $p < 0.05$.

Results

Anti-TNFα compounds bind TNFα on activated T cells to varying degrees

To assess binding of compounds to mTNFα, anti-TNFα compounds and IgG control were labeled with a fluorescent dye, and binding of fluorescent labeled compounds to activated T-cells was compared to binding to non-activated cells. Binding of infliximab and adalimumab to mTNFα was highly efficient, binding of certolizumab was intermediate and binding of etanercept was low compared to the IgG control (Figure 1).

Figure 1 Anti-TNF compounds bind to mTNF in varying degrees.



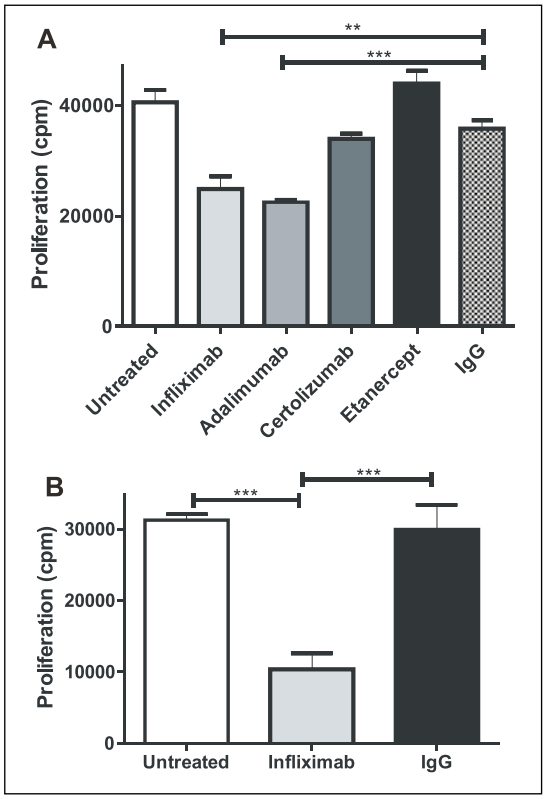
Naïve or CD3/CD28 activated T cells were labeled with a fluorescent dye (Alexa Fluor). Next, cells were incubated with different labeled anti-TNF compounds or IgG control for 30 minutes and binding to activated or non-activated T cells was analyzed by flow cytometry. Data are shown as means ± SD from 3 independent experiments

Anti-TNFα compounds with an Fc region suppress T cell activation but only in an MLR

We examined the effect of various anti-TNFα compounds on the proliferation of activated T cells and found that all anti-TNFα agents slightly inhibited T cell proliferation although this did not reach statistical significance (Supplementary Figure 1*A*). This minor effect was similar for all anti-TNFα compounds and irrespective of their capacity to bind mTNFα, suggesting it may be due to the neutralizing effect on soluble TNFα.

As it has previously been described that anti-TNFs can induce apoptosis in T cells, we examined effects on apoptosis of T cells cultured in isolation or in an MLR. No apoptosis was induced in CD4+ cells treated with anti-TNFs as detected by Annexin V/Pi staining (Supplementary Figure 1*B*). Also, no apoptosis was observed at increasing concentrations, or when we used other cell types or different detection methods (Jurkat cells, lymphocytes, monocytes, Annexin V staining, Caspase 3 activity assay; data not shown). As a result, we hypothesized that anti-TNFs do not have a direct effect on T cells grown in isolation and we established a mixed lymphocyte reaction as a model to further elucidate their anti-inflammatory properties. Again, in this model, no apoptosis was observed (Supplementary Figure

Figure 2 Anti-TNFs differ in their capacity to inhibit proliferation in an MLR.



(A) MLR cultures were treated with anti-TNF compound or IgG control (all 10 μg/mL) for 36 - 48 hours. Proliferation was measured by thymidine incorporation. Only infliximab and adalimumab significantly inhibited proliferation (** $P < 0.01$ and *** $P < 0.001$). (B) CD14+ cells and CD4+ cells from two healthy donors were cocultured for 48 hours. Next, cultures were treated with infliximab or IgG control (10 μg/mL) for 72 hours. Proliferation was measured by thymidine incorporation. Data show means ± SD from 2 independent experiments

1*C*). In addition, no differences were observed in caspase 3 activity whether we used heat inactivated serum or serum without heat inactivation (Supplementary Figure 1*D*). However, a reproducible effect on T cell proliferation was observed with some of the anti-TNFα compounds in the MLR. We found that infliximab and adalimumab inhibit proliferation in this model, whereas etanercept and certolizumab do not (Figure 2*A*). The suppressive effect was much stronger than the effect observed in T cells alone (up to 50% inhibition in an MLR vs 15% inhibition in T cells alone (Supplementary fig 1*A*). These findings suggest that the various anti-TNFα compounds have distinct properties that may result in different pharmacological behavior. To further elucidate the cell types involved in this observed effect, we established an MLR containing purified CD14+ cells and CD4+ T cells. In this assay, infliximab strongly inhibited proliferation compared to the untreated or IgG treated condition (Figure 2*B*). These data show that CD4+ T cells and CD14+ cells are sufficient to induce inhibition of proliferation.

The main difference between the two assays we used is that an MLR contains not only T cells, but also antigen presenting cells (APCs). As these cells may also express low levels of mTNFα, binding of the anti-TNF compounds may affect the activation of the APC and thus decrease its T cell stimulatory capacity. To test this hypothesis, we incubated monocytes and dendritic cells with anti-TNFα compounds in the presence or absence of LPS and analyzed the expression of costimulatory molecules. No effect was found for any of the anti-TNFα compounds tested, either on the expression of costimulatory molecules or on the expression of HLA-DR (Supplementary Figure 2), indicating that infliximab does not affect the activation of APCs.

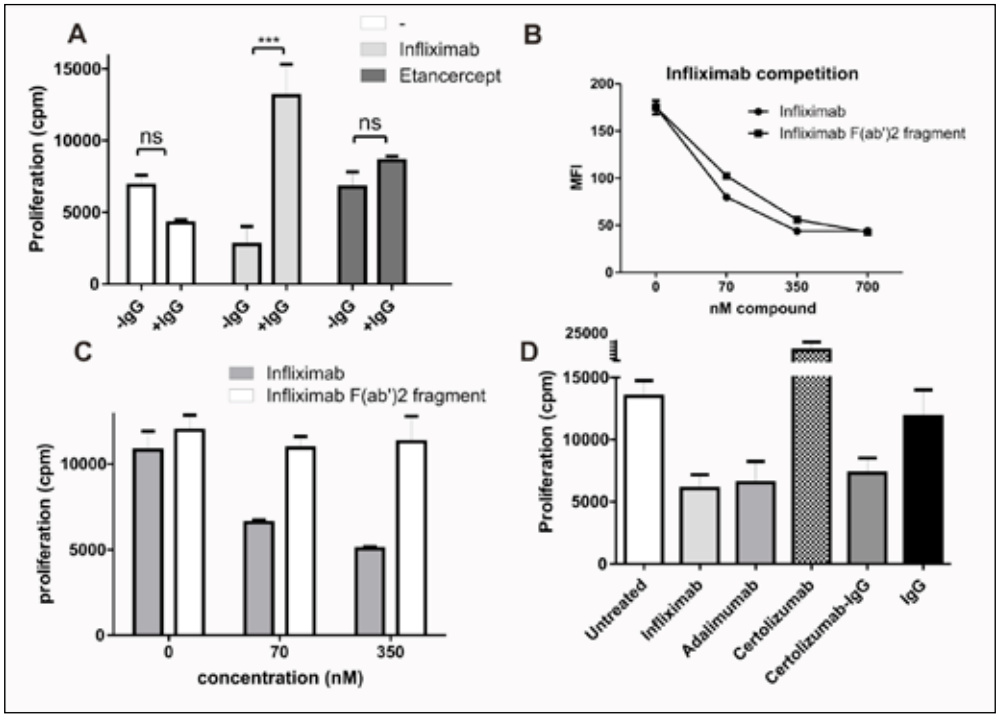
Inhibition of proliferation is abolished when binding to Fc receptors is inhibited

We found that only infliximab and adalimumab (Figure 2) were effective in the MLR, and both of these agents are characterized by efficient binding to mTNFα on the T cells and the presence of an Fc region. In contrast, certolizumab does not contain an Fc region whereas etanercept does, but does not bind efficiently to membrane bound TNFα. We hypothesized that the combination of efficient binding to T cells and the ability to bind and activate an Fc receptor plays an important role in the immunosuppressive function of these compounds in our system. To examine the contribution of the Fc receptor, the ability to inhibit MLR responses was tested after blocking Fc receptors with IgG. Indeed, we found that the inhibition of proliferation by infliximab was completely abolished after saturation of Fc receptors (Figure 3*A*).

To further examine the role of the Fc receptor, infliximab F(ab')2 fragments were generated from infliximab. This compound is identical to the structure of infliximab, except for the fact that it does not contain an Fc region. Successful digestion was confirmed by Coomassie Blue staining (data not shown). To confirm that infliximab F(ab')2 fragment remained capable of binding to mTNF, a competition assay was performed. In this assay, infliximab was labeled with a fluorescent dye, and activated T cells were incubated with a constant concentration of labeled infliximab and increasing concentrations of either infliximab F(ab')2 fragment or infliximab at the same time. Binding of fluorescent infliximab to mTNFα decreases in the presence of a competitor, in this assay infliximab itself or infliximab F(ab')2 fragment. The experiment showed that infliximab F(ab')2 fragment competes with fluorescent infliximab to the same extent as infliximab itself (Figure 3*B* and Supplementary Figure 3), demonstrating that infliximab F(ab')2 fragment binds to mTNF to the same degree as infliximab. In contrast, infliximab F(ab')2 fragment did not inhibit proliferation in an MLR, (Figure 3*C*). This effect was also absent at higher concentrations. These data further support a crucial role for the Fc region in the effects of infliximab and adalimumab on T cells in the MLR.

The previous results indicated that binding to the Fc receptor is necessary for the immunosuppressive effect of anti-TNFα; however, Fc receptor binding alone is not sufficient since IgG and etanercept do not inhibit proliferation although these compounds do contain an Fc region. Therefore, we hypothesized that both binding the Fc receptor and binding mTNFα is required to inhibit T cell proliferation. As shown in figure 1, certolizumab binds to mTNF, albeit at an intermediate level. However, certolizumab does not contain an Fc region and does not inhibit proliferation in an MLR (Figure 2). Strikingly, certolizumab-IgG, a compound containing the mTNFα binding region of certolizumab as well as an Fc region does inhibit proliferation to the same extent as infliximab and adalimumab (Figure 3*D*), fur-

Figure 3 Infliximab induced T cell proliferation is completely abolished when binding to Fc receptors is inhibited.



(A) Fc receptors were saturated by treating MLRs with IgG (10 μg/mL) for 6 – 16 hours following activation. Next, cells were treated with the indicated compound. Proliferation was measured using a 3H-thymidine incorporation assay. *** $p \leq 0.001$ (B) T cells were activated for 48 hours with CD3/CD28 beads. Infliximab was labeled with a fluorescent dye (Alexa Fluor 647). Cells were incubated with infliximab Alexa Fluor (70 nM) and different concentrations of infliximab and infliximab F(ab')2 fragment. Fluorescence was measured with Flow Cytometry. (C, D) Cells in an MLR were treated with the indicated compound for 48 hours. Next, proliferation was measured by thymidine incorporation

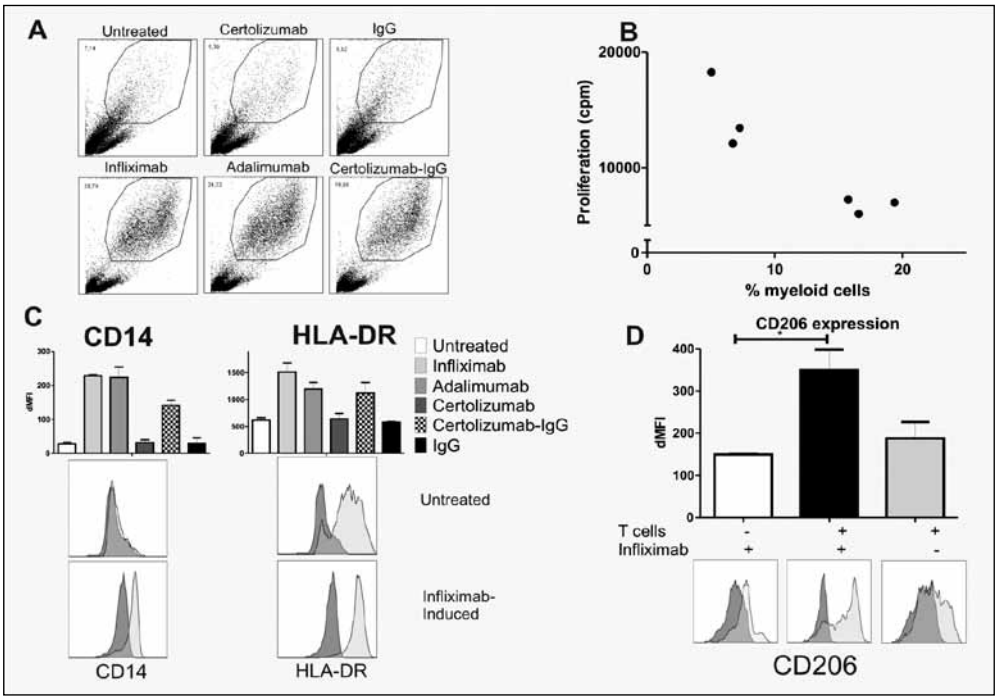
ther confirming that both binding Fc receptor and binding to mTNF is responsible for the immunosuppressive effects of anti-TNF.

Only infliximab, adalimumab and certolizumab-IgG induce a distinct CD14+ and HLA-DR+ cell population in an MLR which correlates with inhibition of T cell activation

To further investigate the mechanisms involved in the inhibition of T cell activation in an MLR, we evaluated the cells by flowcytometry after one week of treatment. We noticed a distinct cell population characterized by high forward scatter and intermediate side scatter upon treatment with infliximab, adalimumab or certolizumab-IgG, which was absent when cells were untreated or treated with certolizumab or IgG (Figure 4*A*). Importantly, the presence of these cells correlates with the degree of inhibition of proliferation induced by the

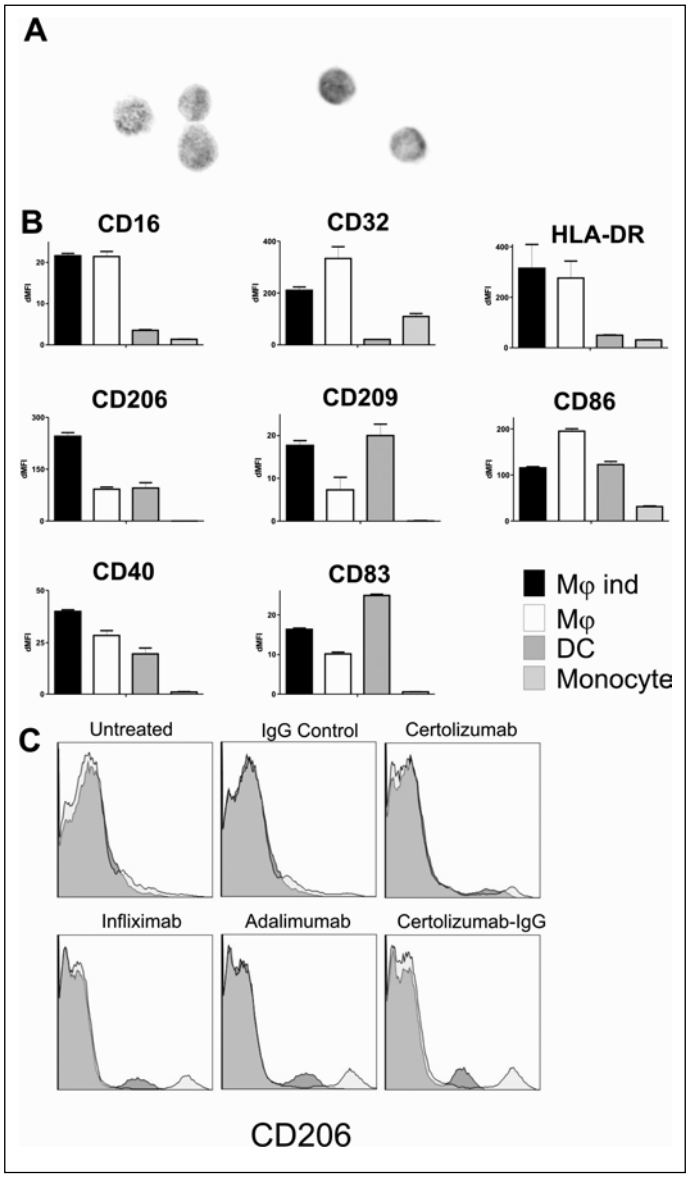
compounds in the MLR, indicating their involvement in the inhibition of T cell activation (Figure 4*B*). Based on the cell size and the position of the cells in the FSC/SSC plot, we hypothesized that these cells might be macrophages. This idea was further supported by the fact that the population expressed the macrophage marker CD14 as well as HLA-DR (Figure 4*C*). This finding also facilitated the isolation of the cell population based on the expression of CD14. Since this induced cell population (further referred to as $M\phi_{ind}$) was absent when cells were treated with certolizumab or IgG, and present upon treatment with infliximab, adalimumab and certolizumab-IgG, this effect seems to be mediated by the Fc region. The characteristics of the different anti-TNFα agents are summarized in Table 1.

Figure 4 Only infliximab, adalimumab and certolizumab-IgG induce a distinct CD14+ and HLA-DR+ population in an MLR.



(A) Cells in an MLR were treated with anti-TNF compound or IgG control (all 10 μg/mL) for 7 days. Cells were analyzed on the FSC/SSC. (B) Correlation between the presence of myeloid cells analyzed on the FSC/SSC and proliferation. (C) Cells in an MLR were treated with anti-TNF compound or IgG control (all 10 μg/mL) for 7 days, stained for HLA-DR and CD14 and analyzed on the FACS. Cells were gated as in 4A. Graphs show MFI ± SD and representative histograms of CD14 and HLA-DR expression of an untreated or infliximab-treated MLR are shown. Grey = isotype, white = expression. (D) T cells are required for the differentiation of $M\phi_{ind}$. CD14+ cells were treated with infliximab in the presence or absence of CD4+ T cells, and CD206 expression was analyzed by flowcytometry. CD206 expression was upregulated only when CD14+ cells were cocultured with CD4+ cells. A representative graph is shown from 2 independent experiments. Upper panel: MFI ± SD. Lower panel: histogram plots. Black = isotype control, grey = CD206 expression

Figure 5 Characterization of Mϕind.



(A) $M\phi_{ind}$ have a macrophage like appearance (40x magnification) Macrophages ($M\phi_{ind}$: left panel, Mϕ1: right panel) were stained using DiffQuick (B) Mϕ1, monocytes, DCs or $M\phi_{ind}$ were stained for different markers and expression was measured on the FACS. (C) Cells in an MLR were treated with anti-TNF compound or IgG control (all 10 μg/mL) for 7 days and stained for CD206 or isotype control

Finally, we examined whether the presence of T cells was required for the differentiation of $M\phi_{ind.}$ To address this question, we isolated CD14+ cells and cultured these cells in the presence or absence of CD4+ T cells, treated the cells with infliximab and analyzed CD206 expression by flowcytometry. We found an upregulation of CD206 when CD14+ cells were cocultured with CD4+ cells. This upregulation was absent when CD14+ cells were cultured without T cells (Figure 4*D*). These data show that T cells are required for the infliximab-induced differentiation of $M\phi_{ind.}$

$M\phi_{ind}$ express costimulatory molecules, Fc receptors and the regulatory macrophage marker CD206

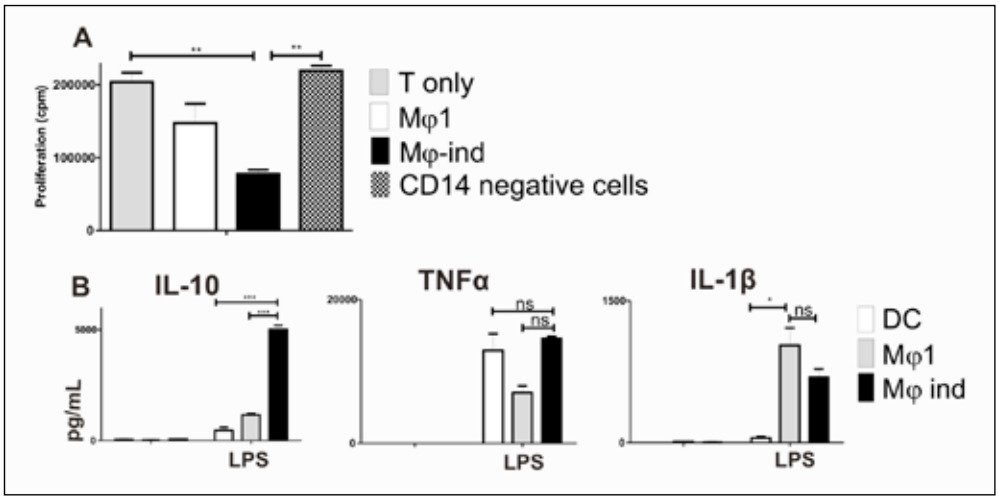
Since the cell population induced by some anti-TNFs expresses CD14 and HLA-DR and looks like macrophages (Figure 5*A*), we sought to further characterize the phenotype of these cells. Therefore we isolated $M\phi_{ind}$ based on CD14 expression and compared the expression level of different markers with the levels on monocytes, DCs and macrophages. Dendritic cells were cultured in the presence of IL-4 and GM-CSF, macrophages in the presence of IFN-γ (further referred to as Mϕ1) and monocytes were freshly isolated from blood. $M\phi_{ind}$ express CD40, CD80, CD83 and CD86 comparable to the expression of these markers on Mϕ1, as well as CD16 (FcγRIII, activating Fc receptor) and CD32 (FcγRIIb, inhibitory Fc receptor) (Figure 5*B*). Furthermore, $M\phi_{ind}$ express CD206 (mannose receptor), a commonly used marker for regulatory macrophages (alternatively activated macrophages, type 2 macrophages or Mϕ2), which was absent on Mϕ1. We confirmed the presence of this marker in the primary MLR and found CD206 expression only in the infliximab, adalimumab or certolizumab-IgG treated conditions (Figure 5*C*). Therefore, we hypothesized that $M\phi_{ind}$ are macrophages with regulatory properties.

$M\phi_{ind}$ have anti-inflammatory properties

Because $M\phi_{ind}$ are only present in the conditions where inhibition of T cell proliferation was observed, we hypothesized that $M\phi_{ind}$ inhibit T cell responses. To test this hypothesis, we isolated the $M\phi_{ind}$ from an MLR and co-cultured these cells with pre-activated isolated T cells from a third donor (secondary MLR). Indeed, we found a strong immunosuppressive effect that was induced by $M\phi_{ind}$ and not by Mϕ1. In addition, this effect was absent when activated T cells were co-cultured with the CD14 negative fraction of the MLR, indicating that $M\phi_{ind}$ mediate the immunosuppressive effect (Figure 6*A*).

To further characterize $M\phi_{ind}$, we evaluated the cytokine profile of these cells in the presence and absence of LPS, and compared this to the cytokine production by DCs and Mϕ1. In accordance with their regulatory properties, $M\phi_{ind}$ produce large amounts of IL-10 in response to LPS (figure 6*B*). Also, $M\phi_{ind}$ secrete less IL-1β in response to LPS compared to Mϕ1. The secretion of TNFα was comparable to that produced by DCs. Taken together, these data indicate that $M\phi_{ind}$ induced by infliximab, adalimumab or certolizumab-IgG have anti-inflammatory properties.

Figure 6 Mϕind have anti-inflammatory properties.



(A) Isolated CD3/CD28 activated T cells from a third donor were co-cultured with $M\phi_{ind}$, Mϕ1, or the CD14 negative fraction of the MLR. Proliferation was measured by thymidine incorporation. (B) $M\phi_{ind}$, Mϕ1 and DCs were cultured in the presence or absence of LPS. Supernatants were collected after 24 hours and cytokine production was measured by CBA. Data are shown as means ± SD from 3 independent experiments. * $p \leq 0.05$, *** $p \leq 0.001$, ns = not significant

Discussion

In the present study, we find that drugs used to target TNFα have different functional properties. The anti-TNFα antibodies bind to mTNFα on activated T cells and inhibit their proliferation but only in the presence of antigen presenting cells. In contrast, a soluble TNFα receptor or F(ab')2 fragments of the anti-TNFα antibodies do not affect T cell proliferation under the same circumstances. Our experiments show that the immunosuppressive properties of the anti-TNFα antibodies are dependent on their Fc region. We find that the antibodies differentiate blood derived monocytes to a regulatory macrophage phenotype ($M\phi_{ind}$). $M\phi_{ind}$ have anti-inflammatory properties since they inhibit proliferation of activated T cells, produce IL-10 and express the regulatory macrophage marker CD206.

It was previously described that infliximab may induce low levels of apoptosis in T cells as determined by TUNEL staining of cytospins of infliximab treated activated Jurkat cells, a lymphoblastoid T cell line. [15] Also, others found that anti-TNFα antibodies induce apoptosis in Jurkat cells that stably overexpress a non-cleavable form of mTNFα using Annexin V-PI staining as a read out. [18, 23] Additionally, apoptosis was described in primary lymphocytes isolated from the lamina propria of CD patients. [16] These data are in contrast to our current results, where none of the anti-TNFα compounds affected either apoptosis or proliferation of Jurkat cells or primary T cells grown in isolation despite extensive attempts. The difference between the previous studies and ours most likely lies in the use of endogenous levels of mTNFα expression in contrast to overexpression and the use of highly viable peripheral

blood lymphocytes rather than lamina propria lymphocytes which had undergone extensive isolation procedures. In line with this is the study by Bedini *et al,* [25] who described that anti-TNFα antibodies suppress activation of T cells in the presence of either immature dendritic cells or monocytes. Indeed, we find similar properties of the anti-TNFα antibodies but not of the other anti- TNFα compounds in the presence of monocytes in an MLR. The results we obtained in this assay are robust and reproducible.

In contrast to some earlier studies, we did not detect binding of etanercept to mTNFα. Importantly, binding of etanercept to mTNFα has only been described in experiments in which a non-cleavable mTNFα was stably overexpressed. [18, 26] In untransfected activated primary lymphocytes it was found that etanercept has weak mTNFα binding activity [16] similar to the results of our current experiments. Thus strong binding of etanercept to mTNFα may only occur in situations in which a non cleavable mTNFα is artificially overexpressed.

An important role for Fc receptor binding has been shown for the mechanism of action of other antibody therapies. Fc receptor binding of rituximab (a chimaeric monoclonal anti-CD20 antibody) was found to be indispensable for the clearance of B cells. [27] In addition, the FcγRIIIA-158V allotype has a higher affinity for binding to IgG_1 than the FcγRIIIA-158F allotype and an association between the *FCGR3A* genotype and clinical response to rituximab was observed in several studies. [27, 28] The *FCGRIIIA* genotype has also been associated with outcome in other antibody therapies, such as cetuximab (chimaeric monoclonal anti-EGFR antibody). [29] In line with this, antibodies with higher Fc receptor affinity have been developed to augment antibody efficacy in animal models. [22, 30] The same polymorphism has been associated with response to infliximab, however with conflicting results. [20, 31] In addition, CDP571, an IgG_4 antibody against TNFα, was found to be ineffective for steroid-sparing in CD. [14] Interestingly, IgG_4 has very low affinity for binding Fc receptors [32] and our data suggest that this could play a causal role in the disappointing performance of CDP571 in the treatment of Crohn's disease.

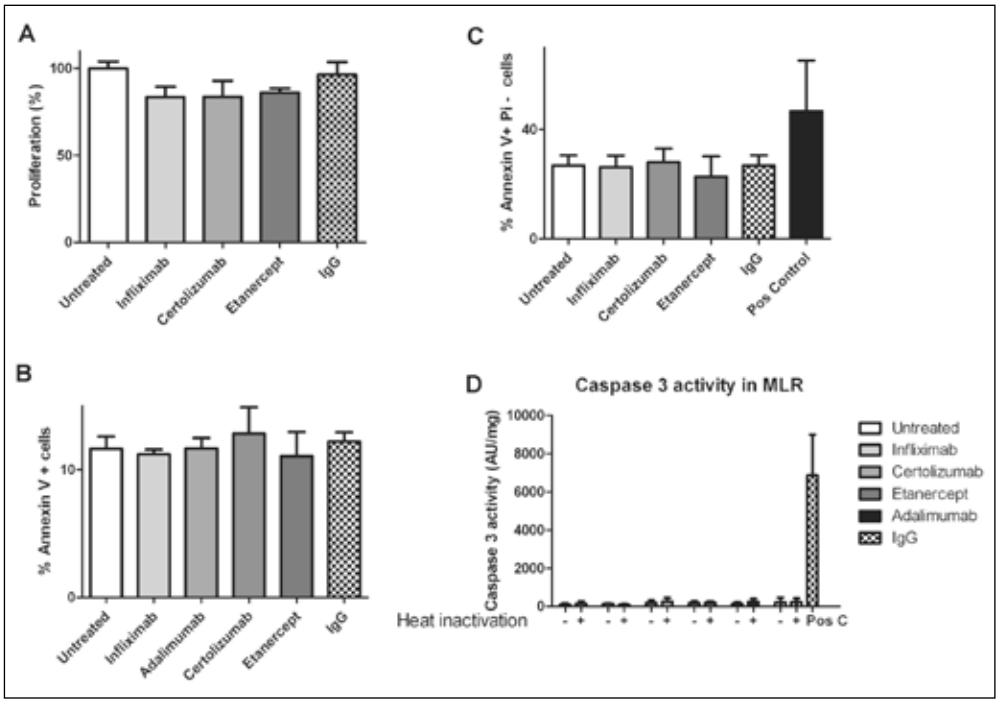
The effects observed with various anti-TNFs in the MLR may have implications for our understanding of the clinical activity of the compounds in patients with Crohn's disease. It is tempting to speculate that Fc receptor dependent differentiation of alternatively activated macrophages plays a role in mucosal healing in patients with Crohn's disease, since alternatively activated macrophages play a major role in wound healing. [33] Recently, it has been shown that regulatory macrophages cultured *in vitro* reduce colonic inflammation in mice and that patients with active CD have reduced numbers of these cells compared to patients with inactive CD. [34] Also, lamina propria macrophages produce large amounts of IL-10 and have anti-inflammatory properties, indicating that these cells are involved in gut homeostasis. [35] Thus far, our data has been limited to *in vitro* experiments, and it would be highly informative to verify the generation of regulatory macrophages *in vivo* in patients receiving anti-TNF therapy. Furthermore, although we have shown *in vitro* that an Fc region is required for inhibition of proliferation and the induction of regulatory macrophages, the *in vivo* clinical benefit of certolizumab has been shown in several studies. [36, 37] Certolizumab was found to be more effective than placebo to induce and maintain remission in CD patients and anti-TNFs are likely to exert their effects through multiple mechanisms.

The anti-TNFα antibodies and soluble receptors not only differ in their clinical efficacy in Crohn's disease but it has also been reported that the risk of tuberculosis with infliximab or adalimumab was 13 to 17 times higher than with etanercept. [38] Interestingly, mycobacteria such as *M. Tuberculosis* bind stronger to Mϕ2 than to Mϕ1, and have a prolonged survival in Mϕ2. [39] In this light it is interesting to note that Mϕ2 are strongly induced by infliximab and adalimumab in our experiments but significantly less by etanercept (data not shown). In addition, the mannose receptor plays an important role in the phagocytosis of mycobacteria [40] and we found that the mannose receptor CD206 is upregulated on $M\phi_{ind}$. Therefore it is tempting to speculate that the increased risk of tuberculosis in patients treated with infliximab or adalimumab is at least partly mediated by the induction of $M\phi_{ind}$.
In conclusion, our data show that the anti-TNFα antibodies have immunosuppressive properties that are distinct from those of the soluble TNFα receptor etanercept and F(ab')2 fragments of anti-TNFα. These distinct properties may have implications for both the effects and side effects of anti-TNFα compounds in patients *in vivo*. This finding sheds more light on the complex mechanism of action of infliximab and anti-TNFα therapies in general and gives new directions for the future development of new antibody based therapy for CD.

Competing interests: These authors disclose the following: Gijs van den Brink receives grants/research support from MSD, Ferring, and Giuliani; Daniel Hommes receives grants/research support and is a consultant for Abbott, MSD, UCB, Giuliani, Ferring, Serono, AstraZeneca, Falk, and Tramedico. The remaining authors disclose no conflicts.

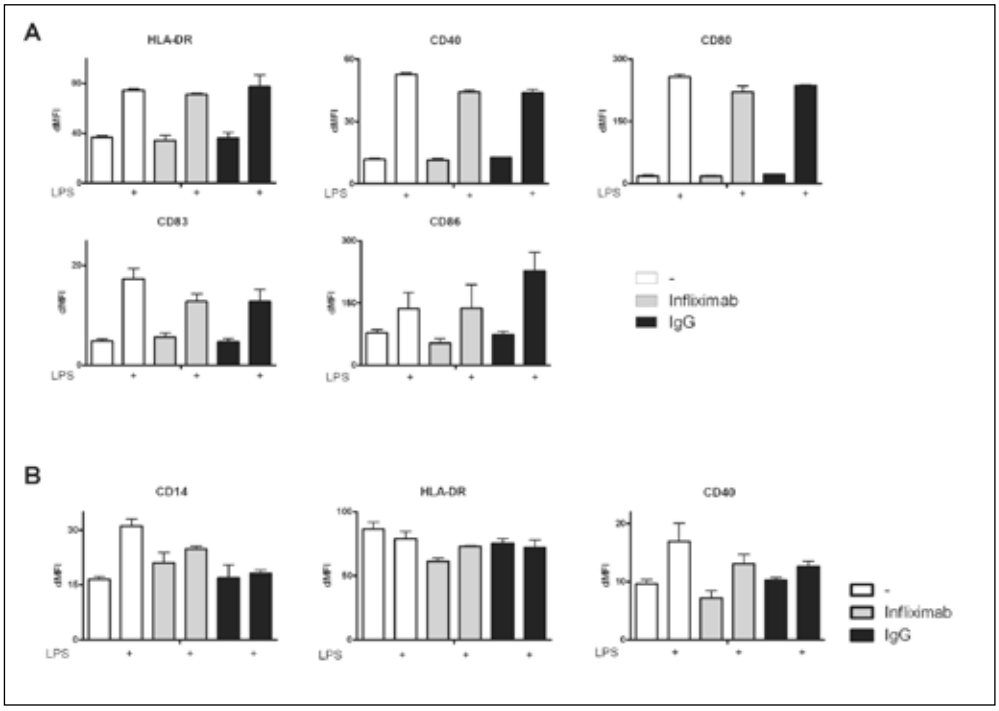
Supplementary Figures

Supplementary Figure 1 Anti-TNFs do not induce apoptosis in activated T cells or an MLR, and do not inhibit proliferation in isolated T cells.



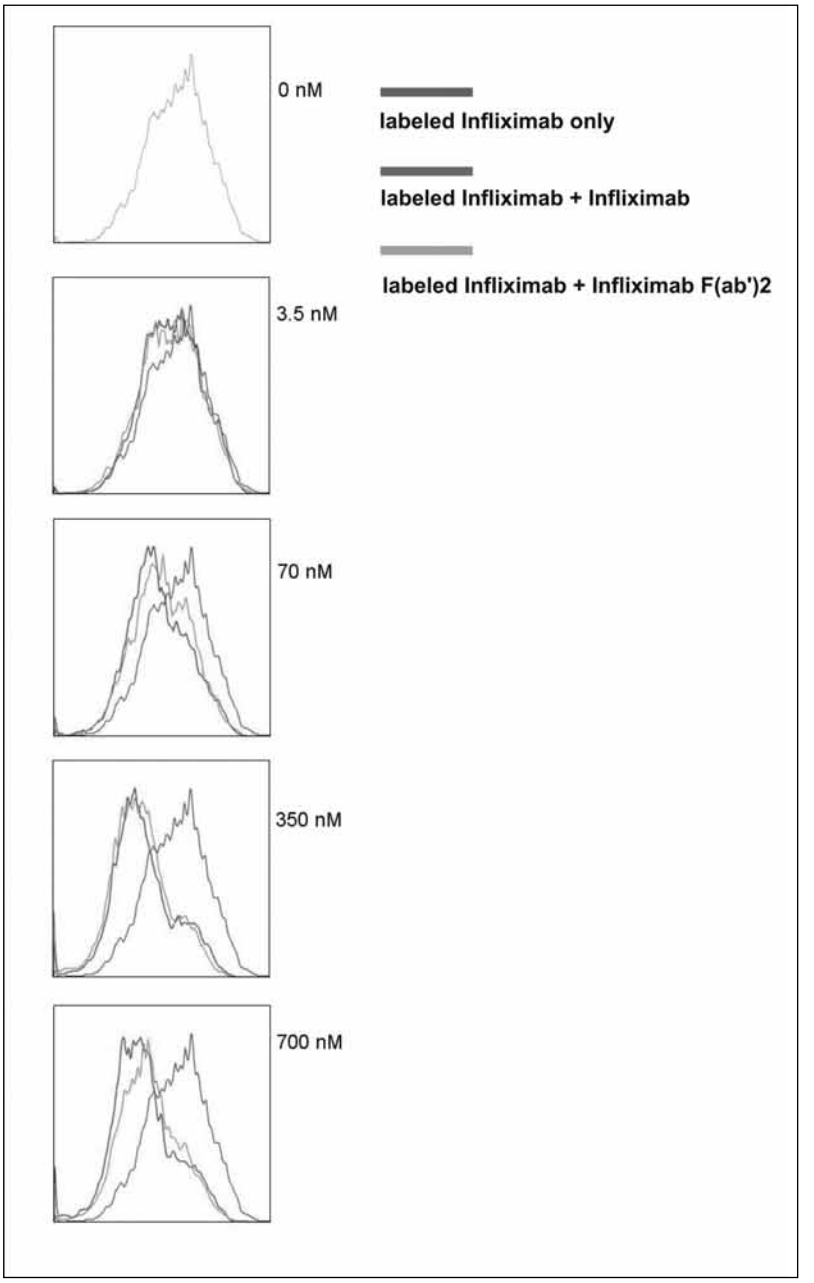
(A) T cells were isolated from PBMCs using negative magnetic bead separation (Invitrogen) and activated with CD3/CD28 antibodies for 48 hr. Next, cells were treated with anti-TNF or IgG control (10ug/mL) for 48 hrs. Finally proliferation was measured by thymidine incorporation. (B) T cells were stained with annexin V and Pi and fluorescence was measured on the FACS. (C) MLR cultures were stained with annexin V and Pi and fluorescence was measured on the FACS. (D) Apoptosis in an MLR with heat inactivated serum and serum without heat inactivation was measured in a caspase 3 activity assay. All data are shown as means ± SD

Supplementary Figure 2 Infliximab has no effect on costimulatory molecules on DCs and monocytes.



MoDCs (A) or monocytes (B) were activated with 100 ng/mL LPS for 16hr, and treated with anti-TNF for 24 hr. Cells were stained with αHLA-DR, αCD40, vCD80, αCD83, αCD86 and isotype control and analyzed by flowcytometry. All data are shown as means ± SD

Supplementary Figure 3 Infliximab F(ab')2 fragment binds to activated T cells.



T cells were activated for 48 hours with CD3/CD28 beads. Infliximab was labeled with a fluorescent dye (Alexa Fluor 647). Cells were incubated with infliximab Alexa Fluor (70 nM) and different concentrations of infliximab and infliximab F(ab')2 fragment. Fluorescence was measured with Flow Cytometry

Reference List

1. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002;347:417-429.
2. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. Gastroenterology 1998;115:182-205.
3. Swidsinski A, Ladhoff A, Pernthaler A, et al. Mucosal flora in inflammatory bowel disease. Gastroenterology 2002;122:44-54.
4. Schreiber S, Nikolaus S, Hampe J et al. Tumour necrosis factor [alpha] and interleukin 1[beta] in relapse of Crohn's disease. The Lancet 1999;353:459-461.
5. Plevy SE, Landers CJ, Prehn J et al. A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. J Immunol 1997;159:6276-6282.
6. van Deventer SJH. Review article: targeting TNF as a key cytokine in the inflammatory processes of Crohns disease the mechanisms of action of infliximab. Alimentary Pharmacology & Therapeutics 1999;13:3-8.
7. Kontoyiannis D, Pasparakis M, Pizarro TT et al. Impaired On/Off Regulation of TNF Biosynthesis in Mice Lacking TNF AU-Rich Elements: Implications for Joint and Gut-Associated Immunopathologies. Immunity 1999;10:387-398.
8. Targan SR, Hanauer SB, van Deventer SJH et al. The Crohn's Dc. A Short-Term Study of Chimeric Monoclonal Antibody cA2 to Tumor Necrosis Factor {alpha} for Crohn's Disease. N Engl J Med 1997;337:1029-1036.
9. Hanauer SB, Feagan BG, Lichtenstein GR et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. The Lancet 2002;359:1541-1549.
10. Hanauer SB, Sandborn WJ, Rutgeerts P et al. Human Anti-Tumor Necrosis Factor Monoclonal Antibody (Adalimumab) in Crohn's Disease: the CLASSIC-I Trial. Gastroenterology 2006;130:323-333.
11. Sandborn WJ, Hanauer SB, Rutgeerts P et al. Adalimumab for maintenance treatment of Crohn's disease: results of the CLASSIC II trial. Gut 2007;56:1232-1239.
12. Sandborn WJ, Hanauer SB, Katz S et al. Etanercept for Active Crohn's Disease: A Randomized, Double-Blind, Placebo-Controlled Trial. Gastroenterology 2001;121:1088-1094.
13. Rutgeerts P, Sandborn WJ, Fedorak RN et al. Onercept for moderate-to-severe Crohn's disease: a randomized, double-blind, placebo-controlled trial. Clin Gastroenterol Hepatol 2006;4:888-893.
14. Feagan BG, Sandborn WJ, Lichtenstein G et al. CDP571, a humanized monoclonal antibody to tumour necrosis factor-alpha, for steroid-dependent Crohn's disease: a randomized, double-blind, placebo-controlled trial. Aliment Pharmacol Ther 2006;23:617-628.
15. ten Hove T, van Montfrans C, Peppelenbosch MP et al. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. Gut 2002;50:206-211.
16. Van den Brande JMH, Braat H, van den Brink GR et al. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. Gastroenterology 2003;124:1774-1785.
17. Lügering A, Schmidt M, Lügering N et al. Infliximab Induces Apoptosis in Monocytes From Patients With Chronic Active Crohn's Disease by Using a Caspase-Dependent Pathway. Gastroenterology 2001;121:1145-1157.
18. Mitoma H, Horiuchi T, Tsukamoto H et al. Mechanisms for cytotoxic effects of anti-tumor necrosis factor agents on transmembrane tumor necrosis factor alpha-expressing cells: comparison among infliximab, etanercept, and adalimumab. Arthritis Rheum 2008;58:1248-1257.

19. Arora T, Padaki R, Liu L et al. Differences in binding and effector functions between classes of TNF antagonists. Cytokine 2009;45:124-131.
20. Louis E, El GZ, Vermeire S et al. Association between polymorphism in IgG Fc receptor IIIa coding gene and biological response to infliximab in Crohn's disease. Aliment Pharmacol Ther 2004;19:511-519.
21. Clynes RA, Towers TL, Presta LG et al. Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. Nat Med 2000;6:443-446.
22. Zalevsky J, Leung IW, Karki S et al. The impact of Fc engineering on an anti-CD19 antibody: increased Fcgamma receptor affinity enhances B-cell clearing in nonhuman primates. Blood 2009;113:3735-3743.
23. Mitoma H, Horiuchi T, Hatta N et al. Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-[alpha]. Gastroenterology 2005;128:376-392.
24. de HP, de Bruin EC, Klein-Kranenbarg E et al. Caspase-3 activity predicts local recurrence in rectal cancer. Clin Cancer Res 2007;13:5810-5815.
25. Bedini C, Nasorri F, Girolomoni G et al. Antitumour necrosis factor-alpha chimeric antibody (infliximab) inhibits activation of skin-homing CD4+ and CD8+ T lymphocytes and impairs dendritic cell function. Br J Dermatol 2007;157:249-258.
26. Kaymakcalan Z, Sakorafas P, Bose S et al. Comparisons of affinities, avidities, and complement activation of adalimumab, infliximab, and etanercept in binding
to soluble and membrane tumor necrosis factor. Clin Immunol 2009;131:308-316.
27. Cartron G, Dacheux L, Salles G et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 2002;99:754-758.
28. Dall'Ozzo S, Tartas S, Paintaud G et al. Rituximab-Dependent Cytotoxicity by Natural Killer Cells: Influence of FCGR3A Polymorphism on the Concentration-Effect Relationship. Cancer Res 2004;64:4664-4669.
29. Bibeau F, Lopez-Crapez E, Di FF et al. Impact of Fc{gamma}RIIa-Fc{gamma}RIIIa polymorphisms and KRAS mutations on the clinical outcome of patients with metastatic colorectal cancer treated with cetuximab plus irinotecan. J Clin Oncol 2009;27:1122-1129.
30. Horton HM, Bernett MJ, Pong E et al. Potent in vitro and in vivo activity of an Fc-engineered anti-CD19 monoclonal antibody against lymphoma and leukemia. Cancer Res 2008;68:8049-8057.
31. Louis EJ, Watier HE, Schreiber S et al. Polymorphism in IgG Fc receptor gene FCGR3A and response to infliximab in Crohn's disease: a subanalysis of the ACCENT I study. Pharmacogenet Genomics 2006;16:911-914.
32. Woof JM, Burton DR. Human antibody-Fc receptor interactions illuminated by crystal structures. Nat Rev Immunol 2004;4:89-99.
33. Kodelja V, Muller C, Tenorio S, Schebesch C, Orfanos CE, Goerdt S. Differences in angiogenic potential of classically vs alternatively activated macrophages. Immunobiology 1997;197:478-493.
34. Hunter MM, Wang A, Parhar KS et al. In Vitro-Derived Alternatively Activated Macrophages Reduce Colonic Inflammation in Mice. Gastroenterology 2010;138(4):1395-40.
35. Takada Y, Hisamatsu T, Kamada N et al. Monocyte Chemoattractant Protein-1 Contributes to Gut Homeostasis and Intestinal Inflammation by Composition of IL-10-Producing Regulatory Macrophage Subset. J Immunol 2010;184:2671-2676.

36. Sandborn WJ, Feagan BG, Stoinov S et al. Certolizumab Pegol for the Treatment of Crohn's Disease. N Engl J Med 2007;357:228-238.
37. Schreiber S, Khaliq-Kareemi M, Lawrance IC et al. Maintenance therapy with certolizumab pegol for Crohn's disease. N Engl J Med 2007;357:239-250.
38. Tubach F, Salmon D, Ravaud P et al. Risk of tuberculosis is higher with anti-tumor necrosis factor monoclonal antibody therapy than with soluble tumor necrosis factor receptor therapy: The three-year prospective French Research Axed on Tolerance of Biotherapies registry. Arthritis Rheum 2009;60:1884-1894.
39. Verreck FA, de BT, Langenberg DM et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. Proc Natl Acad Sci U S A 2004;101:4560-4565.
40. Kang PB, Azad AK, Torrelles JB et al. The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. J Exp Med 2005;202:987-999.

Chapter five

Regulatory macrophages induced by infliximab are involved in healing *in vivo* and *in vitro*

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Abstract

Regulatory macrophages play an important role in wound healing and gut homeostasis and have anti-inflammatory properties. Induction of this cell type ($M\phi_{ind}$) by the anti-TNF antibodies, infliximab and adalimumab, has recently been shown *in vitro*. Also, the superiority of infliximab/azathioprine combination therapy over infliximab or azathioprine monotherapy has recently been established, but the mechanism behind this remains unclear. The aim of this study is to examine the induction of regulatory macrophages in patients with and without mucosal healing in response to infliximab. In addition, we studied the effect of infliximab/azathioprine combination treatment on the differentiation and function of regulatory macrophages.

IBD patients (n=10) underwent endoscopy before and after first infliximab treatment. Immunohistochemical staining of CD68 and CD206 was performed in all patients. Mixed lymphocyte reactions (MLR) were treated with infliximab, azathioprine or both. Macrophage phenotype was evaluated by flow cytometry and inhibition of T cell proliferation was measured in a secondary MLR containing macrophages and third party lymphocytes.

A significant induction of regulatory macrophages was observed in patients with mucosal healing after treatment with infliximab, this induction was absent in patients without mucosal healing. In addition, $M\phi_{ind}$ have the ability to induce wound healing in an *in vitro* model, further suggesting a key role for infliximab induced macrophages in mucosal healing. Upon infliximab/azathioprine combination treatment, an increased number of regulatory macrophages was observed. These macrophages also displayed stronger immunosuppressive properties than macrophages induced by infliximab monotherapy.

These data show that regulatory macrophages may be involved in mucosal healing, and provide a rationale for the superiority of infliximab/azathioprine combination treatment observed in the clinic.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) of unknown etiology resulting in loss of tolerance towards the mucosal flora. Traditionally, therapy to control IBD was aimed at reducing symptoms, but at the present time treatment is more and more focused on inducing mucosal healing and altering the disease course. The introduction of anti-TNF agents in the '90's has been an important breakthrough to accomplish this. Anti-TNF agents have been shown to induce mucosal healing, reduce steroid dependency, reduce the risk for surgery and hospitalization, and to improve the patient's quality of life. [1-5] However, not all anti-TNF agents that have been introduced in the clinic appeared effective. For instance, the TNF receptor fusion protein etanercept [6] and the IgG_4 anti-TNF antibody CDP571 [7] lacked clinical efficacy although these agents have the ability to neutralize soluble TNF. This observation raised the question why some anti-TNF agents are effective and others are not. We have recently shown that in order for an anti-TNF to inhibit T cell proliferation *in vitro*, the compound needs to bind to membrane bound TNF on activated T cells and posses an Fc region to interact with Fc receptors. [8] Upon this binding, a distinct macrophage subset is induced ($M\phi_{ind}$) with immunosuppressive capacities, including the production of anti-inflammatory cytokines and inhibition of T cell proliferation. Furthermore, it has been shown that in vitro derived regulatory macrophages reduce colonic inflammation in mice, [9] and inhibit proliferation of activated T cells. [10] On top of this, regulatory macrophages play a crucial role in wound healing, [11] and therefore the induction of this cell type may be of particular interest in the treatment of IBD and induction of mucosal healing.

Recently, a large randomized, placebo-controlled trial demonstrated the superiority of infliximab/azathioprine combination treatment over infliximab or azathioprine monotherapy in inducing and maintaining remission and mucosal healing in patients with CD. [12] The exact mechanism underlying this effect is unknown, but it likely results from the additional effect of a second immunosuppressive. Also, it is possible that azathioprine suppresses infliximab induced immunogenicity, thereby increasing infliximab efficacy. However, the specific mechanisms involved in this superiority and in mucosal healing in general are so far not completely understood.

In this study, we aimed to investigate the induction of regulatory macrophages in patients responding to infliximab therapy versus those who did not respond. Also, we examined the wound healing capacity of infliximab-induced regulatory macrophages *in vitro*. In addition, we examined the effect of infliximab/azathioprine combination treatment on the induction and function of regulatory macrophages *in vitro*.

Materials and methods

Patients

The patient material used in this study was obtained from University Hospital Gasthuisberg in Leuven (ClinicalTrials.gov number NCT00639821). All patients gave written informed consent. Ten patients with active IBD (4 UC, 6 CD), refractory to corticosteroids and/or immunosuppression, were examined. The patients underwent endoscopy with biopsies

from affected bowel (colon for UC and colonic CD, and ileum for ileal CD) within a week prior to the first infliximab infusion of 5 mg /kg body weight. Patients received a second endoscopy with biopsies at week 4 - 6. The biopsies were taken at sites of active inflammation but at a distance of ulcerations. When healing was observed at the second endoscopy, biopsies were obtained in the areas where lesions were present before therapy. The endoscopist was not blinded to treatment.
Next, biopsies were fixed in Carnoy's fixative for up to 5 hours and then dehydrated, cleared and paraffin-embedded for histological examination and/or immunohistochemistry. Haematoxylin-eosin stained slides from the paraffin block of each patient were used to score chronic intestinal inflammation using a previously reported scoring system for UC [13] and for CD. [14] The pathologists were blinded to treatment. Response to infliximab was assessed 4-6 weeks after the first infliximab treatment and classified as has been described before. [15] Briefly, for colonic CD, a response was defined as complete mucosal healing with a decrease of at least 3 points on the histological score. [14] For UC, a response consisted of a decrease to a Mayo endoscopic subscore of 0 or 1 with a decrease to grade 0 or 1 on the histological score. [13] For ileal CD (3 patients), patients with a clear improvement of the ulcerations and a decrease on the histological score [14] were considered responders. Patients who did not achieve this healing were considered non-responders although some of them showed an improvement on the histologic or endoscopic score.

Immunohistochemistry

For immunohistochemistry, Mϕ2 cells were defined as CD206+/CD68+ cells. Mϕ2 were detected using an anti-human CD206 goat polyclonal antibody (AF2534, R&D systems, Abingdon, UK) and anti-human CD68 mouse monoclonal antibody (clone KP1, M0814, Dako Belgium NV, Heverlee, Belgium). Briefly, 5 μm-thick sections were cut from the paraffin blocks of Carnoy-fixed endoscopic biopsies from the patients. After drying, deparaffinization and rehydration, epitope retrieval was performed at high pH (Dako PT Link machine, Dako Belgium NV, Heverlee, Belgium). Sections were then washed 3 times 5 min (Envision Flex wash buffer, Dako) and Envision Flex Peroxidase-Blocking Reagent (Dako) was applied for 10 min at room temperature. After a second wash step, sections were incubated with the anti-human CD206 antibody (R&D Systems, dilution 1:40) or with the anti-human CD68 antibody (Dako, dilution: 1:2000) for 30 min at room temperature. Following a third wash step, bound primary antibody was visualized by incubating the slides for 30 min with Envision Flex/HRP (Dako) and application of the Envision DAB+ Chromogen (Dako) for 10 min at room temperature. After rinsing, the slides were counterstained with haematoxylin, dehydrated, cleared and mounted. Negative controls (no application of primary antibody) were run together with the test samples. All the stains were evaluated by an experienced pathologist (GDH). Finally, the stains were analyzed using ImageJ software.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were isolated by Ficoll Paque density-gradient centrifugation. Mφ1 macrophages were generated as described previously. [10] Briefly, monocytes were isolated by Percoll density-gradient centrifugation and cultured in RPMI 1640 containing 10% heat-inactivated FCS for 5 days. CD3 positive T cells were isolated from PBMCs using negative magnetic bead separation (Invitrogen, Paisley,

UK). Where appropriate, T cells were activated with αCD3/αCD28 beads (Invitrogen) (1 bead/5 cells).

Allogeneic mixed lymphocyte reaction (MLR)

PBMCs from two healthy donors were cultured in a 1:1 ratio in RPMI 1640 culture medium to establish a mixed lymphocyte reaction (MLR). After 48 hours of activation, cells were treated with infliximab (10 μg/ml), azathioprine (1 μM) or a combination, for 5 days. Were indicated, cells were treated with 10 μg/ml certolizumab. Finally, proliferation was measured using a ^{3}H-thymidine incorporation assay.

Isolation of infliximab-induced macrophages ($M\phi_{ind}$) and infliximab/azathioprine-induced macrophages ($M\phi_{ind/aza}$)

Infliximab induced macrophages ($M\phi_{ind}$) and infliximab/azathioprine induced macrophages ($M\phi_{ind/AZA}$) were isolated from MLR cultures from healthy volunteers. We have previously shown that upon infliximab treatment, a distinct cell population occurs on the FCS/SSC, which is strongly positive for several markers, among which CD206 (a commonly used regulatory macrophage marker) and CD14, and that these cells have immunosuppressive properties. The induction, phenotype and isolation of this cell subset have been extensively described before. [8] In brief, $M\phi_{ind}$ and $M\phi_{ind/aza}$ were isolated from the MLR based on the expression of CD14 using CD14 microbeads according to manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany). Next, cells were counted and cultured in RPMI 1640, containing 10% heat-inactivated FCS.

In vitro wound healing assay

The wound healing scratch assay was performed as previously described with a few modifications. [16] HCT116 colon epithelial cells were cultured to approximately 70% confluence in 6 wells plates under standard culture conditions. The plates were marked in order to create a reference point to identify the wound at the same place at different time points. Next, a wound was created using a plastic tip, and pictures were taken at a phase-contrast microscope. $M\phi_{ind}$ and pro-inflammatory type 1 macrophages from healthy volunteers were added to the cell culture (100.000 cells/well). To quantify the degree of wound healing, pictures were taken again after 24 hrs, and the percentage closure was calculated using Image J software.

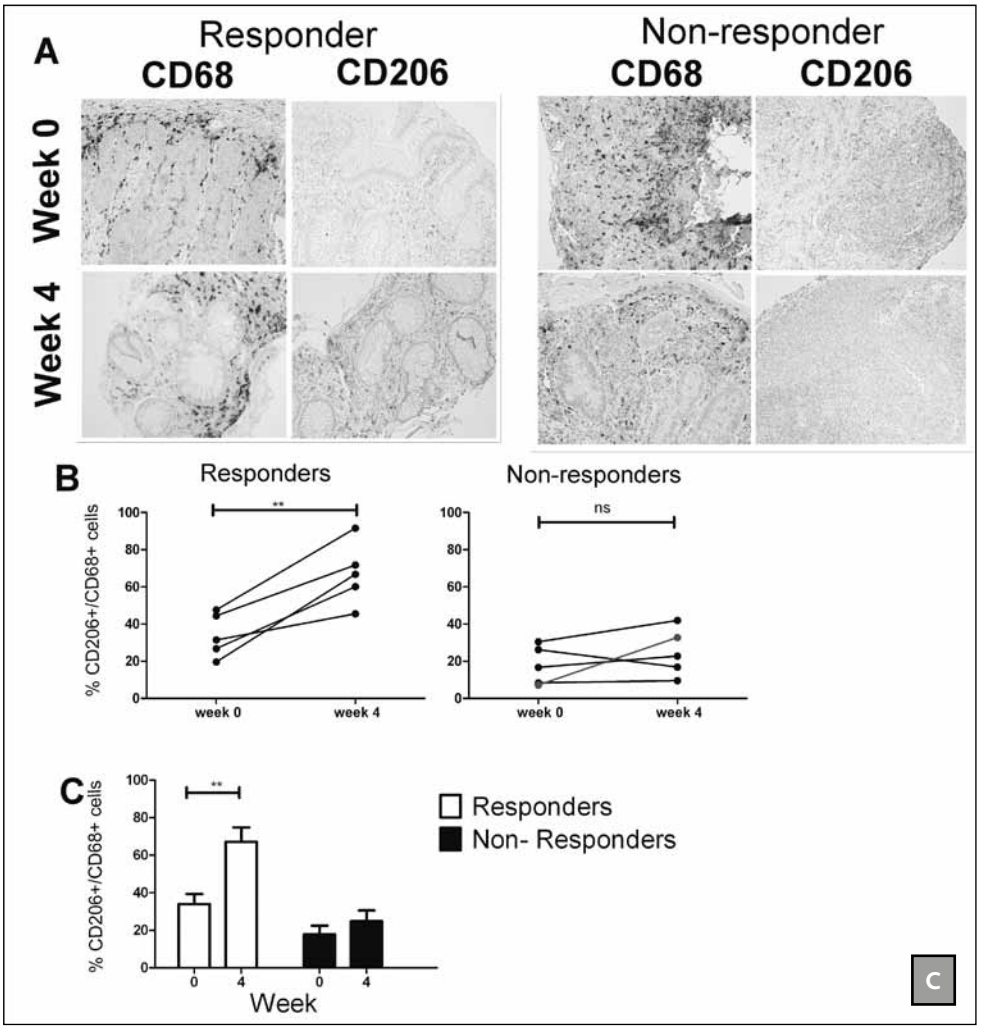
FACS analysis of regulatory macrophages

MLR were treated with infliximab, azathioprine, or a combination. After 5 days, cells were harvested, washed with FACS buffer, and stained for CD206 or isotype control. Finally, expression was analyzed by flow cytometry using a FACS Calibur (BD) and FlowJo software (Treestar Inc, Ashland, OR)

Statistical analysis

Results are representative for at least three independent experiments and show means ± SEM unless otherwise indicated. For statistical analysis, one way ANOVA was used followed by Bonferroni post test. Results were considered significant when $p < 0.05$. Paired *t* test was used to calculate statistical significance in patients before and after infliximab therapy. Results were considered significant when $p < 0.05$

Figure 1 Infliximab induces regulatory macrophages in responders but not in non-responders.



A) Representative pictures of CD206/CD68 stainings in a patient responding to infliximab (left panel) and a non-responder (right panel). Pictures of stained biopsies before and after infliximab induction therapy are shown. B). Induction of CD206/CD68 regulatory macrophages per patient. A significant induction of CD206/CD68+ cells is observed in patients responding to infliximab (left panel), whereas this induction is absent in non-responders (right panel). The red line represents a patient with a partial response observed during endoscopy. C). Non-responders have lower amounts of CD206/CD68+ cells at baseline. ** $p \leq 0.01$ Data were analyzed using paired *t* test

Results

Infliximab induces regulatory macrophages in responders but not in non-responders

We have shown previously that anti-TNF antibodies induce regulatory macrophages *in vitro* [8] and this cell type plays an important role in wound healing. [11] Now, we aimed to investigate whether the induction of regulatory macrophages is also observed *in vivo* in patients responding to infliximab therapy. Biopsies were obtained from six CD patients (3 responders, 3 non-responders) and 4 UC patients (2 responders, 2 non-responders) before (week 0) and after (week 4 - 6) first infliximab treatment. Patient characteristics are shown in table 1. Slides from each patient before and after therapy were stained for CD68 and CD206 (also called MRC1 or MMR). CD206 is a commonly used marker for regulatory macrophages, and CD68 is a macrophage marker. Since patients with a response to infliximab usually have a decrease in the number of CD68+ cells, we used the ratio CD206+/CD68+ to identify the induction of regulatory macrophages.

A clear and significant increase was observed in the percentage of CD206+ macrophages in patients responding to infliximab induction therapy, indicating that regulatory macrophages were induced (Figure 1*A* and 1*B*). This induction was observed in each individual responding to infliximab therapy Figure 1*B*, left panel). Importantly, induction of CD206+/CD68+ cells was absent in patients not responding to infliximab therapy (Figure 1*B*, right panel). One patient (Figure 1b, right panel, red line) was considered a non-responder on

Table 1 Patient characteristics at week 0 and week 4-6.

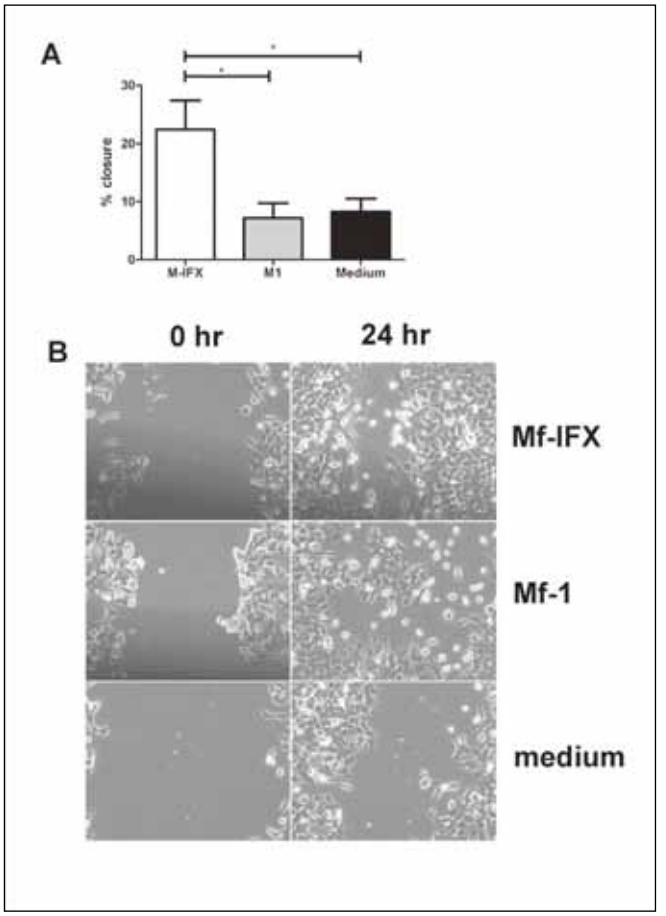
Pt nr	*UC/ CDi/ CDc*	*R/NR*	*Histological score Week 0*	*Histological score Week 4*	*Endoscopic score Week 0*	*Endoscopic score Week 4*	*%CD206/ CD68+ cells Week 0*	*% CD206/ CD68+ cells Week 4*
1	UC	R	5.4	0.3	3	1	19,7	66,8
2	UC	R	5.2	0.1	2	0	47,6	91,6
3	UC	NR	5.4	5.3	3	3	16,7	22,8
4	UC	NR	5.1	5.3	2	3	30,5	42
5	CDc	R	14/16	6/16	Active colitis	healing	26,8	60,1
6	CDc	NR	16/16	16/16	Active colitis	Active colitis	8,4	9,6
7	CDc	NR	10/16	10/16	Active colitis	Incomplete healing	7,2	32,8
8	CDi	R	10/16	3/16	Active colitis	healing	31,5	45,5
9	CDi	R	12/16	5/16	Active ileitis	Incomplete healing	44,5	71,8
10	CDi	NR	7/16	8/16	Active ileitis	Active ileitis	26,2	16,9

Biopsies were obtained before (week 0) and after (week 4 - 6) infliximab therapy. Histologic and endoscopic scores and the percentage of CD206/CD68+ cells at 0 and 4 weeks are shown. Pathologists who scored histology were blinded to treatment, endoscopists were not blinded to treatment, and the percentage CD206/CD68+ was calculated using Image J software.

UC Ulcerative colitis, CDc colonic Crohn's disease, CDi ileal Crohn's disease, R responder, NR non-responder

histological basis, but did show a partial response on endoscopy. An increase of CD206+/CD68+ cells was observed in this patient.
Taken together, these data show that regulatory macrophages similar to those induced by infliximab *in vitro*, are induced *in vivo* in patients responding to infliximab therapy.

Figure 2 $M\phi_{ind}$ induce wound healing in vitro.



A) $M\phi_{ind}$ induce wound healing *in vitro* B) representative pictures of the wound healing assay. Data are representative of at least three independent experiments. Data were analyzed using one-way ANOVA and bonferroni post-test. * $p \leq 0.05$

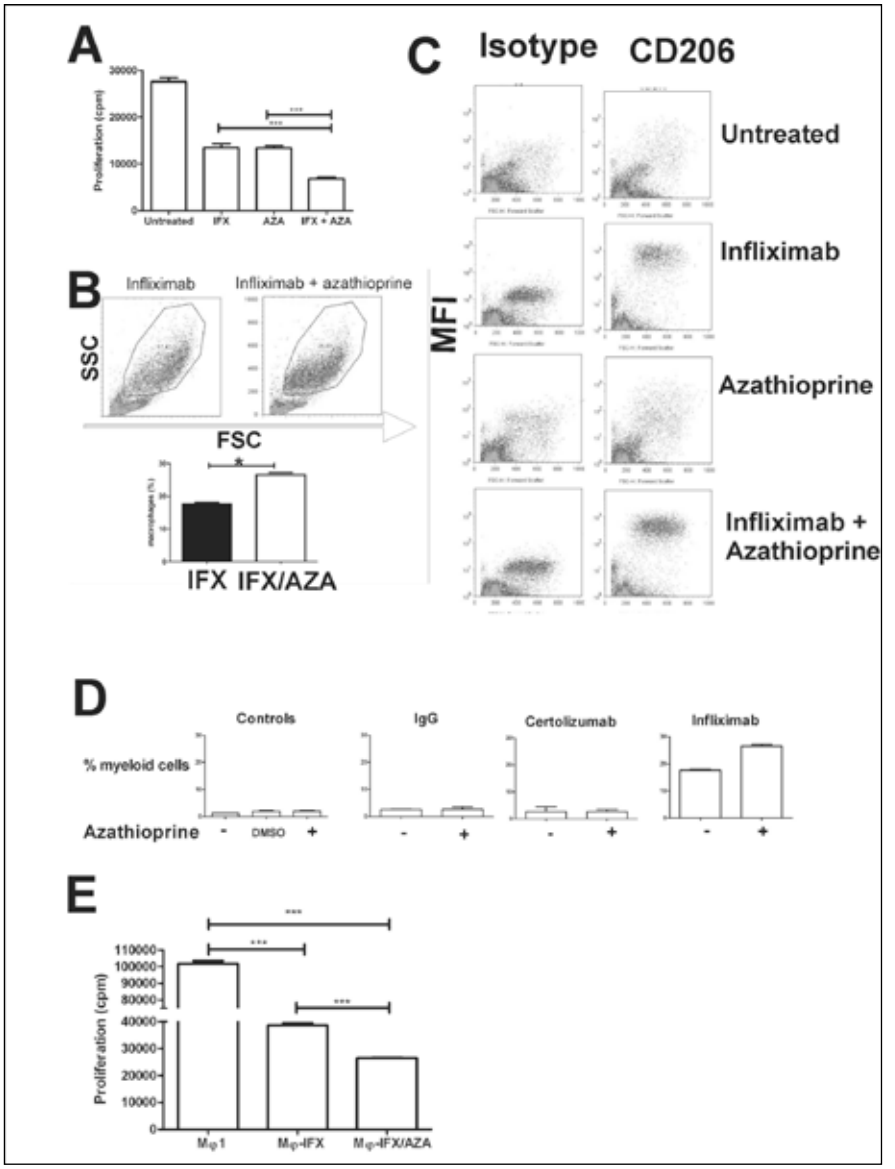
Infliximab-induced regulatory macrophages induce wound healing *in vitro*
It has been shown previously that regulatory macrophages generated *in vitro* by addition of IL-4 and IL-13 exhibit wound healing capacities. The data described above show an increased presence of macrophages displaying a regulatory phenotype after infliximab therapy. However, the presence of CD206+/CD68+ cells in patients presenting with mucosal healing does not discriminate between infliximab-mediated effects and wound healing effects induced by

another mechanism such as immunosuppression in general. Therefore, we aimed to assess specifically the wound healing capacity of macrophages induced by infliximab treatment.
To this end, macrophages were isolated from MLR cultures treated with infliximab (regulatory $M\phi_{ind}$) or generated from monocyte cultures (inflammatory Mϕ1). HCT116 colonic epithelial cells were cultured and a wound was created in the monolayer. Mϕ1, $M\phi_{ind}$ or control medium was added to the wells and images were taken at t=0 and t=24 hours. As expected, Mϕ1 did not induce wound healing above the level of control (Figure 2). In contrast, $M\phi_{ind}$ showed a clear capacity to enhance wound healing up to two-fold compared to Mϕ1 or medium alone. These data are in line with the results observed in patients responding to infliximab therapy, and further suggest that regulatory macrophages induced by infliximab induce wound healing.

Enhanced induction and function of regulatory macrophages upon infliximab/azathioprine combination treatment *in vitro*

Since the induction of $M\phi_{ind}$ correlates with response to infliximab (Figure 1), and higher response rates are observed in patients receiving infliximab/azathioprine combination treatment compared to either monotherapy, [12] we hypothesized that infliximab/azathioprine combination treatment might enhance the induction of $M\phi_{ind}$ *in vitro*. To answer this question, we treated mixed lymphocyte cultures with infliximab, azathioprine or a combination. Azathioprine and infliximab monotherapy inhibited T cell proliferation in an MLR to the same extent (Figure 3*A*). As expected, when cells in an MLR were treated with a combination of infliximab and azathioprine, T cell proliferation was further inhibited. To investigate whether this observation was a cumulative effect of a second immunosuppressive or whether another mechanism was involved, we studied the effect of combination treatment on the induction of regulatory macrophages. As shown previously, [8] anti-TNF antibodies induce a distinct subset of cells, characterized by high forward and high side scatters and expression of CD14 and CD206 when analyzed by flowcytometry. When cells were treated with azathioprine monotherapy, no induction of regulatory macrophages was observed. Strikingly, we found a significantly increased induction of this CD206+ subset upon combination treatment (Figure 3*B/C*).
We have previously shown that anti-TNF agents induce regulatory macrophages in an Fc region dependent manner, [8] and that agents lacking the Fc fragment, do not induce this cell type. Therefore, we also evaluated the combination azathioprine/certolizumab (lacking the Fc fragment) to examine whether the observed effect of enhanced $M\phi_{ind/AZA}$ was induced by infliximab in particular or a general effect of TNFα neutralization. Indeed, the effect was specific for the combination treatment of azathioprine/infliximab, since the induction was absent upon azathioprine/certolizumab or azathioprine/IgG treatment (Figure 3*D*).
To further examine the properties of regulatory macrophages induced by infliximab/azathioprine combination treatment ($M\phi_{ind/AZA}$), we aimed to investigate their immunosuppressive function. Therefore, we isolated regulatory macrophages based on CD14 expression, and co-cultured equal numbers of these cells with activated T cells from a third donor. Strikingly, $M\phi_{ind/AZA}$ displayed a stronger immunosuppressive phenotype, since $M\phi_{ind/AZA}$ showed enhanced ability to inhibit T cell proliferation compared to $M\phi_{ind}$ (Figure 3*E*).
These data show that combination treatment is superior to monotherapy *in vitro* with respect to the induction of regulatory macrophages. Not only the number of regulatory mac-

Figure 3 Enhanced induction of regulatory macrophages upon infliximab / azathioprine combination therapy.



A). Infliximab and azathioprine inhibit T cell proliferation in an MLR to the same extent, but stronger inhibition is observed upon infliximab/azathioprine combination therapy. B) Enhanced induction of regulatory macrophages in an MLR. C) Enhanced number of CD206+ cells upon infliximab/azathioprine combination therapy. This induction is absent when cells are treated with azathioprine monotreatment. D) The enhanced induction of regulatory macrophages is specific for infliximab, and is absent when co-treated with certolizumab or control IgG. E) $M\phi_{ind/AZA}$ are superior to $M\phi_{ind}$ in inhibiting T cell proliferation. 20.000 macrophages were plated and cocultured with 100.000 T cells. All figures are representative figures of at least three independent experiments. Data were analyzed using one-way ANOVA and bonferroni post-test *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

rophages is increased, but the macrophages also display a stronger immunosuppressive phenotype. These findings may provide a partial explanation for the superiority of infliximab/azathioprine combination therapy observed in patients with CD.

Discussion

The data presented here show the induction of regulatory macrophages after infliximab treatment in IBD patients. Patients responding to infliximab showed a significant induction of CD206+/CD68+ cells, whereas this induction was absent in non-responders. Since we used endoscopic and histologic healing as a definition of response, these data suggest that the induction of regulatory macrophages may be involved in the process of mucosal healing. The fact that we observe increased numbers of CD206+/CD68+ cells in patients responding to infliximab therapy does not prove directly that this effect is mediated by infliximab. However, we have previously shown induction of CD206+ macrophages by infliximab *in vitro*. [8] Additionally, we now show here that CD206+ macrophages induced by infliximab *in vitro* have wound healing capacity, similar to what has been described for IL-4/IL-13 induced regulatory macrophages previously. [17] Together these data suggests that the induction of regulatory macrophages by infliximab may contribute to mucosal healing in patients clinically responding to the treatment.

A recent clinical trial has shown that combination treatment of azathioprine and infliximab is superior to monotreatment in the induction of remission and mucosal healing in CD patients. [12] In our in vitro system, azathioprine did not induce significant numbers of macrophages. However, in combination with infliximab, azathioprine potentiated both the number and immunosuppressive capacity of CD206+ macrophages. Although the superiority of combination treatment *in vivo* probably results at least in part from the additive effects of two immunosuppresives, our data suggest an additional layer of actual synergism which may also contribute to the increased mucosal healing observed in patients.

In order to maintain tolerance, lamina propria macrophages normally display a type 2 macrophage (Mϕ2) phenotype. Mϕ2 are functionally different from type 1 macrophages (Mϕ1), since they have minor ability to respond to bacterial stimuli, and produce anti-inflammatory cytokines rather than pro-inflammatory cytokines. [18, 19] In IBD patients, lamina propria macrophages have a more Mϕ1 phenotype, as lamina propria mononuclear cells (LPMNCs) from IBD patients spontaneously produce large amounts of pro-inflammatory cytokines and are hyperresponsive to bacterial stimuli. [20-22] In line with this, lower amounts of Mϕ2 were found in mucosal biopsies from active lesions in CD patients compared to non-affected colon of the same patient, and compared to healthy controls. [9] This suggests that lamina propria macrophages from CD patients are skewed towards an Mϕ1 phenotype, thus contributing to the defect in tolerance. Since regulatory macrophages contribute to tolerance towards the mucosal flora and CD patients have decreased numbers of this cell type, the induction of Mϕ2 might be an interesting target in restoring the disturbed balance.

In conclusion, our data demonstrate the induction of regulatory macrophages in IBD patients responding to infliximab therapy. In addition, we show that infliximab induced macrophages indeed have wound healing capacity, suggesting a potential role in mucosal healing. Finally, we show that infliximab/azathioprine combination treatment potentiates

the induction of regulatory macrophages, thus providing a new rationale for the superiority of infliximab/azathioprine combination treatment observed in the clinic.

Reference List

1. Targan SR, Hanauer SB, van Deventer SJH et al. The Crohn's Dc. A Short-Term Study of Chimeric Monoclonal Antibody cA2 to Tumor Necrosis Factor for Crohn's Disease. N Engl J Med 1997;337:1029-1036.
2. Rutgeerts P, D'Haens G, Targan S et al. Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. Gastroenterology 1999;117:761-769.
3. van Dullemen HM, van Deventer SJ, Hommes DW et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 1995;109:129-135.
4. D'Haens G, Van Deventer S, Van Hogezand R et al. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. Gastroenterology 1999;116:1029-1034.
5. Baert FJ, D'Haens GR, Peeters M et al. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. Gastroenterology 1999;116:22-28.
6. D'Haens G, Swijsen C, Noman M et al. Etanercept in the treatment of active refractory Crohn's disease: a single-center pilot trial. Am J Gastroenterol 2001;96:2564-2568.
7. Feagan BG, Sandborn WJ, Lichtenstein G et al. CDP571, a humanized monoclonal antibody to tumour necrosis factor-alpha, for steroid-dependent Crohn's disease: a randomized, double-blind, placebo-controlled trial. Aliment Pharmacol Ther 2006;23:617-628.
8. Vos AC, Wildenberg ME, Duijvestein M et al. Anti-tumor necrosis factor-alpha antibodies induce regulatory macrophages in an Fc region-dependent manner. Gastroenterology 2011;140:221-230.
9. Hunter MM, Wang A, Parhar KS et al. In Vitro-Derived Alternatively Activated Macrophages Reduce Colonic Inflammation in Mice. Gastroenterology 2010;138(4):1395-40.
10. Verreck FA, de BT, Langenberg DM et al. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-gamma- and CD40L-mediated costimulation. J Leukoc Biol 2006;79:285-293.
11. Daley JM, Brancato SK, Thomay AA et al. The phenotype of murine wound macrophages. J Leukoc Biol 2010;87:59-67.
12. Colombel JF, Sandborn WJ, Reinisch W et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med 2010;362:1383-1395.
13. Geboes K, Riddell R, Ost A et al. A reproducible grading scale for histological assessment of inflammation in ulcerative colitis. Gut 2000;47:404-409.
14. D'Haens GR, Geboes K, Peeters M et al. Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. Gastroenterology 1998;114:262-267.
15. Arijs I, De HG, Lemaire K et al. Mucosal gene expression of antimicrobial peptides in inflammatory bowel disease before and after first infliximab treatment. PLoS One 2009;4:e7984.
16. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc 2007;2:329-333.
17. Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003;3:23-35.

18. Smythies LE, Sellers M, Clements RH et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest 2005;115:66-75.
19. Kamada N, Hisamatsu T, Okamoto S et al. Abnormally differentiated subsets of intestinal macrophage play a key role in Th1-dominant chronic colitis through excess production of IL-12 and IL-23 in response to bacteria. J Immunol 2005;175:6900-6908.
20. Reinecker HC, Steffen M, Witthoeft T et al. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin Exp Immunol 1993;94:174-181.
21. Reimund JM, Wittersheim C, Dumont S et al. Mucosal inflammatory cytokine production by intestinal biopsies in patients with ulcerative colitis and Crohn's disease. J Clin Immunol 1996;16:144-150.
22. Zareie M, Singh PK, Irvine EJ et al. Monocyte/macrophage activation by normal bacteria and bacterial products: implications for altered epithelial function in Crohn's disease. Am J Pathol 2001;158:1101-1109.

Chapter six

Autologous bone marrow derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study

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Abstract

Background and aim: Mesenchymal stromal cells (MSCs) are pluripotent cells that have immunosuppressive effects both *in vitro* and in experimental colitis. Promising results of MSC therapy have been obtained in patients with severe graft versus host disease of the gut. Our objective was to determine the safety and feasibility of autologous bone marrow derived MSC therapy in patients with refractory Crohn's disease (CD).
Patients and intervention: Ten adult patients with refractory CD (8 females/2 males) underwent bone marrow aspiration under local anesthesia. Bone marrow MSCs were isolated and expanded *ex vivo*. MSCs were tested for phenotype and functionality *in vitro*. Nine patients received 2 doses of 1-2x10^6 cells/kg bodyweight, intravenously, 7 days apart. During follow up, possible side effects and changes in patients' Crohn's disease activity index (CDAI) scores were monitored. Colonoscopies were performed at week 0 and 6, and mucosal inflammation was assessed by using the Crohn's disease endoscopic index of severity (CDEIS).
Results: MSC isolated from CD patients showed similar morphology, phenotype and growth potential compared to MSCs from healthy donors. Importantly, immunomodulatory capacity was intact, as CD MSCs significantly reduced peripheral blood mononuclear cell proliferation *in vitro*. MSC infusion was without side effects, besides a mild allergic reaction probably due to the cryopreservant DMSO in one patient. Baseline median CDAI was 326 (224-378). Three patients showed clinical response (CDAI decrease ≥70 from baseline) 6 weeks post treatment, conversely three patients required surgery due to disease worsening.
Conclusions: Administration of autologous bone marrow derived MSCs appears safe and feasible in the treatment of refractory CD. No serious adverse events were detected during bone marrow harvesting and administration.

Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastro-intestinal tract, including Crohn's disease (CD) and ulcerative colitis (UC). Despite the improvements in IBD management with the introduction of anti-TNF compounds, remission often remains difficult to maintain. Many patients suffer from a poor quality of life due to disease relapse, repeated surgeries, extra intestinal manifestations and drug side-effects. Therefore, novel therapeutic approaches need to be explored.
Mesenchymal Stromal Cells (MSCs) are nonhematopoietic stromal cells exhibiting multilineage differentiation capacity and the ability to mediate immunosuppressive and anti-inflammatory effects. [1-3] MSCs are easily isolated from various tissues, [4-6] including the bone marrow, and are capable of *ex vivo* expansion. Moreover, MSCs can be cryopreserved without loss of phenotype or differentiation potential. [7] Systemic infusion of MSCs ameliorated the clinical and histopathologic severity of experimental colitis, abrogating body weight loss, diarrhea, and inflammation and increasing survival. [8, 9] Furthermore in humans, transplantation of bone marrow (bm) derived MSCs has led to improvement of corticoid refractory graft versus host disease (GvHD), including GvHD of the gut [10, 11] and MSCs obtained from adipose tissue induced healing in complex perianal fistulas in patients with CD. [12] Although the mechanisms underlying these effects are not fully elucidated, it has been shown that both cell-cell contact and the secretion of growth factors and cytokines are involved. [13, 14] The potential role of MSCs in the modulation of immune responses and tissue regeneration aroused interest to use MSCs as a novel cellular therapy to treat Crohn's disease. [15]
In this clinical phase I study, we evaluated the safety and feasibility of intravenous infusion of autologous bmMSC in refractory CD patients. Additionally the functionality of the bmMSCs of the CD patients was studied focusing on culture potential, morphology, cell surface marker profiling, differentiation potential and immunosuppressive properties. Finally, the effect of bmMSCs on various drugs used to treat Crohn's disease was assessed.

Methods

Patient selection

On January 14th 2008, the Medical Ethical Committee of the Leiden University Medical Center (LUMC) and the Central Committee on Research involving Human Subject (CCMO, The Hague, the Netherlands) approved this phase I study on autologous bmMSCs in the treatment of refractory CD (registered in the Netherlands National Trial Register under study number NTR1360 www.trialregister.nl). All patients gave written informed consent. Criteria for patient inclusion were that patients were at least 18 years of age and had moderate to severe CD, as defined by a baseline Crohn's Disease Activity Index (CDAI) score between 220 and 450. Furthermore, patients had to be refractory to the standard treatment options for CD. We defined refractory patients as patients that, at some time during the course of the disease, must have received steroids, immunosuppressive agents (for example, azathioprine, 6-mercaptopurine or methotrexate) or anti-TNF therapy which did not result in an adequate response to treatment. The following medications were allowed: 5-aminosalicylates and corticosteroids (at a stable dosage regimen for at least 4 weeks) and

methotrexate, azathioprine, or 6-mercaptopurine (at least 12 weeks, with stable dosage regimen for at least 8 weeks). Infliximab was discontinued at least 8 weeks prior to enrolment. All patients continued current treatment at the time of infusion. Before bone marrow harvest for MSC isolation and expansion, patients were thoroughly screened including medical history, physical examination, standard laboratory investigations and chest x-ray to rule out tuberculosis. Each patient was also screened for human immunodeficiency virus (HIV), syphilus, hepatitis B and C virus. Patients were excluded if they had a history of lymphoproliferative disease or malignancy within the past five years, when they exhibited serious infections or when in need of immediate surgery. Colonoscopy was performed at baseline to confirm disease activity.

Laboratory methods for clinical expansion of MSCs

MSC isolation and expansion

MSCs were expanded according to a common protocol devised by the European Group for Blood and Bone Marrow Transplantation developmental committee, as previously described. [10] Bone marrow was harvested by aspiration from the iliac crest from patients under local anesthesia in the outpatient clinic. Bone marrow mononuclear cells (MNC) were isolated by Ficoll density gradient (density 1.077 g/cm^3) centrifugation. Washed cells were resuspended in Dulbecco's modified Eagle's-low glucose medium (Invitrogen, Paisley, UK) supplemented with penicillin and streptomycin (Lonza, Verviers, Belgium) and 10% fetal bovine serum (FBS, HyClone, Logan, UT), without any additional growth factors. MNCs were plated at a density of 160.000 cells per cm^2. Cultures were maintained at 37fflC in a humidified atmosphere containing 5% CO2 in 175 cm^2 flasks (Greiner Bio-One, Frickenhausen, Germany). When the cultures reached near confluence (>80%), the cells were detached by treatment with trypsin/ EDTA (Lonza, Verviers, Belgium) and replated at a density of 4000 cells per cm^2. MSCs were passaged up to a maximum of three times. When sufficient MSCs were expanded, cells were harvested and cryopreserved in isotonic buffered salt solution supplemented with 10% dimethyl sulphoxide (LUMC Pharmacy, the Netherlands). Data on MSCs obtained from healthy donors matched for age and gender, was obtained from previous studies. [10, 16, 17] MSCs for these studies were sourced either from a family or non-related (third party) donor. Donors were informed about and consented to the possibility of the use of their MSCs for preclinical studies/analysis. All donors underwent routine donor control examination and screening tests, according to the standard procedures required for bone marrow donors. Following eligibility, donors donated 50-100cc of bone marrow under local anesthesia as described above.

Characterization of MSC products

Morphology was monitored twice a week throughout the culture period by light microscopy. *Cell viability* was determined at each passage and harvest procedure by trypan blue staining in a Bürker chamber. *Immunophenotyping* of cultured MSCs was performed using flow cytometry. The following markers were analyzed: HLA II (DR), CD73, CD90, CD31, CD34, CD45, CD80 (Becton Dickinson, Franklin Lakes, NJ, USA) and CD105 (Ancell, Bayport, MN, USA). The samples were analyzed on a FACSCalibur™ using CellQuest Pro software (Becton Dickinson). *Absence of contamination by pathogens* was tested at culture initiation and harvest of the MSC product by aerobic and anaerobic cultures (Becton Dickinson, Bac-

tec plus aerobe/F and Bactec plus anaerobe/F). *Genetic stability* of the expanded MSCs was tested by karyotype analysis using a standard G-banding procedure.

Clinical application of MSC products

Release criteria for clinical use of MSCs included product sterility, absence of visible cell clumps, spindle-shape morphology, expression of CD73, CD90, and CD105 surface molecules (>90%) and a normal karyotype in at least 20 observed metaphases.

Laboratory methods for supportive research

In vitro differentiation

MSCs were plated at 5x10^4 cells/cm^2 in 24-well culture plates and kept in complete medium until 80-90% confluency was reached. For osteogenic differentiation cells were stimulated for 21 days in standard medium supplemented with 50 μg/mL ascorbic acid, 10 mM β-Glycerolphosphate and 10^{-8}M dexamethasone and were stained with fast blue for alkaline phosphatase. For adipogenic differentiation, cultures were stimulated for 21 days with complete medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 100 μM indomethacin, 5 μg/ml insulin and 10^{-6} M dexamethasone. Lipid droplets were revealed by staining with Oil Red O. Control MSCs were grown in non-conditioned medium. All chemicals were from Sigma-Aldrich (St Louis, Missouri, USA)

MSC/peripheral blood mononuclear cell (PBMC) proliferation assay

Cultured MSCs from Crohn's disease patients were plated in flat bottom 96 well plates (Costar, Zwijndrecht, the Netherlands) and allowed to attach overnight. PBMCs were isolated from whole blood of CD patients before MSC infusion. PBMCs were stimulated anti-CD28/anti-CD3 coated Dynabeads (1 bead/5 cells, Invitrogen) per 1x10^6 cells and were seeded in IMDM with 5% human serum (Sanquin, Amsterdam, the Netherlands), 5% FBS and 100 IU/mL IL-2 (LUMC Pharmacy, Leiden, the Netherlands) per well. Proliferation was measured by ^{3}H-thymidine incorporation. In order to test the effect of Crohn's disease medication on the immunosuppressive properties of MSCs, the proliferation assay was also performed in the presence of indicated medications: 10 μg/mL infliximab, 10ug/mL adalimumab, 10 μM dexamethasone (Sigma-Aldrich), 1μM azathioprine (Sigma-Aldrich), 4um 6-mercaptopurine (Sigma-Aldrich), and 100 μg/ml methotrexate (Pharmachemie BV Haarlem, the Netherlands) performed as described above.

Cell bead array cytometric assay

Production of TNF-α, IL-1b, IL-10, and IL-6 in MSC/PBMC supernatants, colon biopsy homogenates and serum was determined using a CBA kit according to the manufacturer's instructions (BD Biosciences, San Jose, CA).

Study design

Patients received 2 doses of MSCs, 7 days apart, at week 0 and 1. Just before clinical application, cryopreserved cells were thawed and cells were infused intravenously at a target dose of 1-2x10^6 cells/kg bodyweight. Patients were clinically assessed at weeks 0, 1, 2, 4, 6, and 14. At each visit, adverse events were ascertained, concomitant medications were recorded and samples for clinical laboratory evaluations and the patients' CDAI score were

Table 1 Baseline characteristics of included patients.

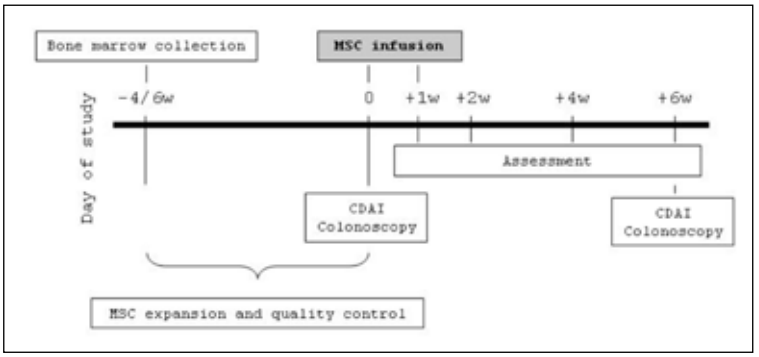
	Patient number									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Age (y)	24	36	33	34	34	42	29	32	19	30
Sex	F	F	F	F	F	M	M	F	F	F
Disease duration (y)	10	5	15	3	11	4	7	12	3	10
CDAI at screening	442	346	283	316	337	237	316	280	255	277
Baseline CDAI	332	341	254	326	350	266	378	224	NA	304
Disease localization	ileo-colonic	colonic, ileum ND	colonic, ileum ND	ileum	ileum	colonic	colonic, ileum ND	colonic, ileum ND	no active disease	colonic
Perianal disease	y	n	y, perianal abscess (inactive)	n	y (inactive)	no	y (inactive)	no	no	y (inactive)
Extra-intestinal manifestations	arthralgias	arthralgias	No	arthralgias	No	no	cheilititis granulomatosa	no	no	no
Current medical therapies	CS 50mg, AZA	MTX, ADA	CS 10mg	CS 5mg	CS 40mg	MTX	CS 30mg, 6-MP	CS 5mg, MTX	NA	CS 5mg, MTX
Height (cm)	175	167	174	172	158	160	187,5	173	157	161
Weight (kg)	59,3	68,6	74	99,9	46	78	107,5	113,2	50,5	53,1
Current smoker / Smoking history	no / no	no / no	no / no	no / yes	no / no	no / yes	yes / yes	no / no	no / yes	no / yes

Prior medical therapies	CS, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, ADA	5-ASA, CS, ATB, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, 6MP, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA, TAC	CS, AZA, IFX, MTX, ADA	5-ASA, CS, AZA, IFX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, ADA	5-ASA, CS, ATB, AZA, IFX, MTX, CZP, ADA	5-ASA, CS, AZA, IFX, MTX, CZP, ADA, HSCT
Prior surgeries	ileocoecal resection	no	ileocoecal resection	ileocoecal resection	ileocoecal resection, colostoma on colon transversum	no	no	no	no	no

Abbreviations: F female, M male, n no, y yes, NA not applicable, ND not determined, 5-ASA mesalamine, CS corticosteroids, AZA azathioprine, 6-MP 6-mercaptopurine, ATB antibiotics, MTX methotrexate, IFX infliximab, CZP certoluzimab pegol, ADA adalimumab, TAC tacrolimus, HSCT mobilisation phase of hematopoietic stem cell transplantation

obtained. Colonoscopies were performed at week 0 and 6, and mucosal inflammation was assessed using the Crohn's disease endoscopic index of severity (CDEIS). The study flow chart is depicted in Figure 1.

Figure 1



Statistical analysis

Data were analyzed using SPSS (SPSS Inc., Chicago, IL) or GraphPad (Graphpad software Inc., La Jolla, CA). Analyses included the Kruskall Wallis test followed by Dunn's multiple comparisons (multiple comparisons), two-sided *t*-test and Wilcoxon signed-rank test for paired data. P-values <0.05 were considered significant.

Results

Patients

In total ten patients (eight females/two males, median age 32,5 and range 19-42 years) with moderate to severe Crohn's disease (median CDAI score at screening of 299,5 and range 255-442) were included in the study and underwent bone marrow aspiration under local anesthesia. Besides some local pain at the puncture site afterwards, bone marrow aspiration was well tolerated by all patients. Baseline characteristics are presented in table 1. The bone marrow aspiration procedure resulted in sufficient bone marrow to expand MSC up to the required therapeutic doses (supplementary Table 1).

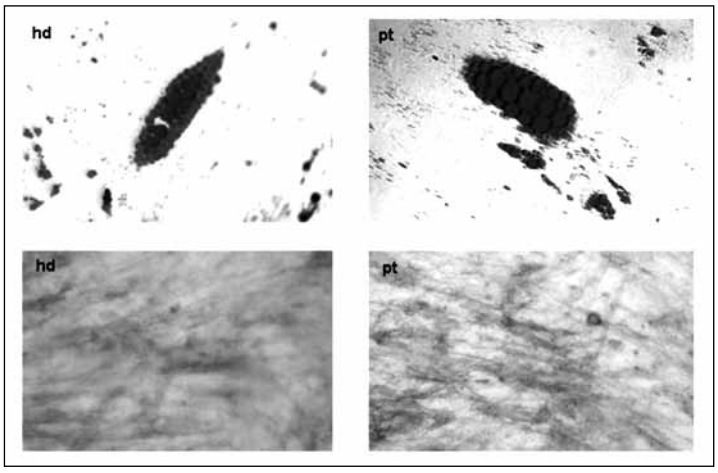
One patient with a CDAI score of 255 at screening, showed no active disease on colonoscopy and was therefore excluded for further MSC administration (patient 9). The baseline median CDAI score of the nine treated patients was 326 (range 224-378). During MSC infusion, patients were closely monitored. MSC infusion was successful and without relevant side effects. In one patient a transient mild allergic reaction occurred which was probably due to the cryopreservant DMSO. Moreover, all patients noticed the typical smell and taste due to the DMSO up to 48 hours after infusion. Other adverse events in the first 6 weeks of the protocol were ruled unlikely to be associated with MSC treatment and included AEs such as common cold and headache (Table 2).

Table 2 Adverse events in MSC treated patients (week 0-6).

	n	*Patient number*
Likely related to MSC infusion		
Allergic reaction	1	4
Typical taste and smell	9	all
Headache	3	1,4,7
Unlikely related		
Worsening CD*	2	1,7
Dizziness	1	1
Nausea	2	1,2
Vomiting	1	1
Bloating	1	3
Abdominal pain	3	3,5,8
Hemorrhoid	1	4
Fever	1	4
Lack of appetite	2	1,4
Fatigue	2	5,8
Diarrhea	1	8
Common cold	1	10
Otitis media acuta	1	2

Two serious adverse events (*) were reported due to worsening of disease requiring hospitalization

Figure 2 Mesenchymal stromal cells (MSCs) from Crohn's disease patients differentiate into mesenchymal lineages.



(A) Adipocyte differentiation was demonstrated in MSCs cultured from a healthy donor (hd) and from a patient with Crohn's disease (pt) after which cells were stained with Oil Red O to show lipid droplets in the cytoplasm of the cell.

(B) For osteoblast differentation MSCs were stained with fast blue to show alkaline phosphatase activity. Non-conditioned MSCs did not stain for Oil Red O, whereas fast blue gave slight background staining (not shown)

Table 3 Flow cytometric analysis of cultured bmMSCs from CD patients and healthy donors matched for gender and age (mean/median age CD patients 31,3/32,5 and hd 32,1/32,5).

	Patient number										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Average*
HLA-DR	5,1	5,7	7,9	5,7	1,0	5,4	0,8	0,9	5,0	5,8	4,3
CD31	1,9	2,1	1,7	2,1	1,7	1,1	2,2	1,6	2,0	2,0	1,8
CD73	99,9	99,7	99,9	99,6	99,3	99,7	99,9	99,2	90,4	98,1	98,6
CD45	1,3	1,6	1,9	2,1	3,6	1,3	0,9	1,2	1,4	1,3	1,7
CD105	100,0	99,9	100,0	100,0	100,0	100,0	100,0	99,9	99,9	100,0	100,0
CD80	3,1	6,2	10,3	18,0	4,2	3,3	1,0	0,2	0,3	29,4	7,6
CD90	100,0	100,0	99,9	99,9	99,3	99,6	99,8	100,0	99,8	99,9	99,8
CD34	6,0	4,0	4,6	9,5	2,8	5,6	6,5	0,8	10,1	4,3	5,4
	Healthy donor number										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*Average*
HLA-DR	10,5	3,7	0,6	4,2	12,8	10,1	1,4	9,1	5,3	3,1	6,1
CD31	2,6	1,7	2,5	1,7	2,8	2,9	1,7	3,1	0,4	1,8	2,1
CD73	99,4	97,4	99,7	99,2	99,7	96,7	94,0	96,4	99,4	99,4	98,1
CD45	2,6	0,7	1,4	1,9	1,2	0,6	1,8	1,2	0,6	1,9	1,4
CD105	99,9	99,9	99,8	100,0	100,0	99,9	99,9	100,0	100,0	99,9	99,9
CD80	1,5	6,6	3,6	6,2	2,2	19,6	19,8	2,2	2,3	3,8	6,8
CD90	99,9	98,9	99,9	99,8	99,9	99,9	99,0	99,8	100,0	99,9	99,7
CD34	4,2	1,7	2,9	4,0	7,1	19,2	6,6	16,5	5,1	4,0	7,1

bmMSC from refractory Crohn's disease patients are comparable to MSCs from healthy donors

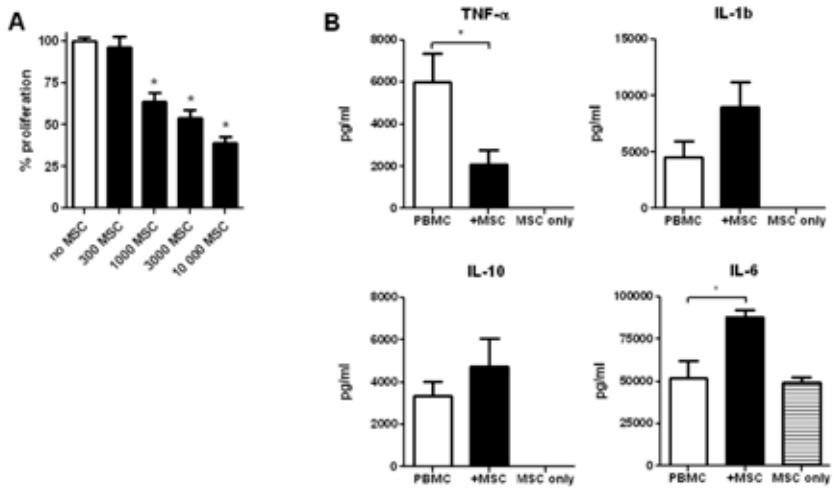
Approximately hundred milliliters of bone marrow was aspirated from each patient and bmMSCs were isolated and cultured. MSCs from Crohn's disease patients showed the typical spindle-shaped morphology and similar growth potential and yield compared to MSCs from healthy donors (Supplementary Figure 1). Depending on the number of cells needed and the yield of cells, MSCs were harvested in the first, second or third passage (Supplementary Table 1). All MSC cultures showed normal karyotyping. Immunophenotypical characterization was performed by FACS analysis, and showed similar phenotyping as described for healthy controls (Table 3).

Furthermore, CD MSCs were able to differentiate along the osteogenic and adipogenic lineages when cultured in appropriate culture medium (Figure 2).

bmMSCs from refractory Crohn's disease patients suppress immune responses *in vitro*

In the presence of autologous bmMSCs proliferation of PBMCs was reduced in a cell dose-dependent fashion (Figure 3*A*) and a decreased TNF-α production was observed. An increase of IL-1b, IL-6 was seen, as well as an increase in the regulatory cytokine IL-10 (Figure 3*B*). MSC proliferation was not affected by co-medication (infliximab, adalimumab, dex-

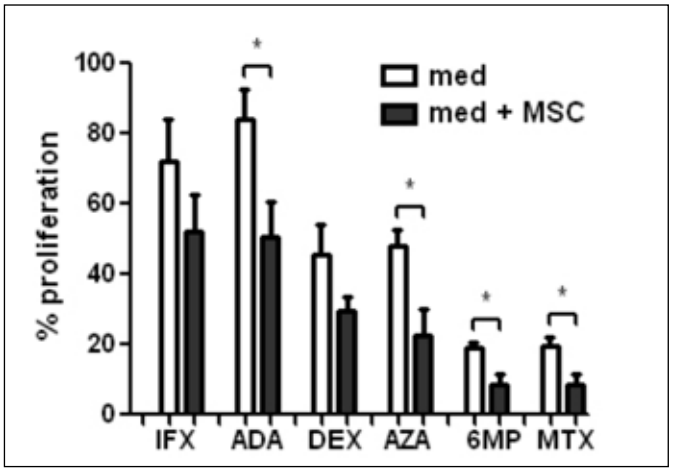
Figure 3 Mesenchymal stromal cells (MSCs) from Crohn's disease patients significantly inhibit the proliferation of peripheral blood mononuclear cells (PBMCs) and this inhibition is dose-dependent.



(A) 100 000 PBMCs cells were stimulated with anti-CD3/CD28 beads in the absence (white column) or presence (black columns) of indicated numbers of autologous MSCs. Proliferation measured by ^{3}H-thymidine uptake in counts per minute was expressed as a percentage of PBMCs proliferation without MSCs for each individual patient. (B) Cytocine production in the supernatants of PBMC cultures and 10 000MSC/100 000 PBMC cocultures. MSCS alone produce IL-6 (lower right). In the presence of MSCs, TNF-α production is lowered, whereas IL-1b and IL-10 production is increased. Bars represent the mean and SEM of data from 10 patients in triplo. *p< 0.05 for significant differences

amethasone, azathioprine, 6-mercaptopurine and methotrexate) *in vitro* (data not shown). However, MSCs do have an additive effect on inhibition of PBMC proliferation by various Crohn's disease medication *in vitro* (Figure 4).

Figure 4 Mesenchymal strom cells (MSCs) have an additive effect on the inhibition of PBMC proliferation by Crohn's disease medication.



In the presence of indicated Crohn's disease medication proliferation of 100 000 stimulated PBMCs added to 1000 MSC (black bars) compared to PBMC without MSCs (white bars) was measured. Proliferation measured by ^{3}H-thymidine uptake in counts per minute was expressed as a percentage relative to PBMC proliferation in control medium (set to 100%). Bars represent mean and SEM. *p< 0.05 for significant differences between test conditions based (two-sided *t*-test)

Clinical response

Clinical assessment was performed on all patients using CDAI scoring. Two patients were excluded before the primary endpoint was met. The first patient was a chronic severe steroid refractory patient on the waiting list for surgery. Although an initial drop of CDAI score was seen, this patient was excluded when presented with a CDAI >450 due to poor general condition and persistent rectal blood loss. Patient 7 was withdrawn from the study four days after the first MSC infusion because of continuing abdominal aches and bloody diarrhea. In this case infliximab treatment was resumed. CDAI scores improved in 5 patients, clinical response (defined as a drop in CDAI>70) was seen in 3 patients at week 6 (Table 4).

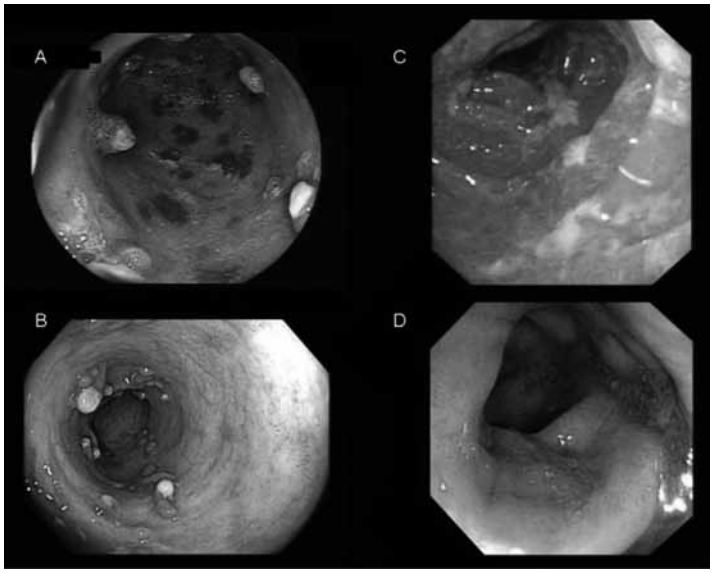
Table 4 Clinical scores of patients at week 0, 1, and 6.

		Patient number								
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*10*
CDAI (0-600)										
wk		332	341							
0*	First infusion			254	326	350	266	378	224	304
wk 1	Second infusion	305	281	182	318	306	247	#452	167	ND
wk 6	Primary endpoint	#473	185	179	267	314	160	ND	340	354
Surgical resection										
	In week number	7	n	n	n	12	n	n	14	n

In the 6 month follow up period, three patients underwent surgery in indicated week after MSC infusion. *baseline, #withdrawn from study, ND not determined, n no

Remission (CDAI <150) was not achieved in any of the patients. Three patients had a reduction of 70 points in CDAI score, this decrease could in most cases be ascribed to solid stools and a decrease in soft stool frequency. In a period of 14 weeks, three patients required surgery due to disease worsening (Table 4). No significant differences in C-reactive protein (CRP) levels were seen.

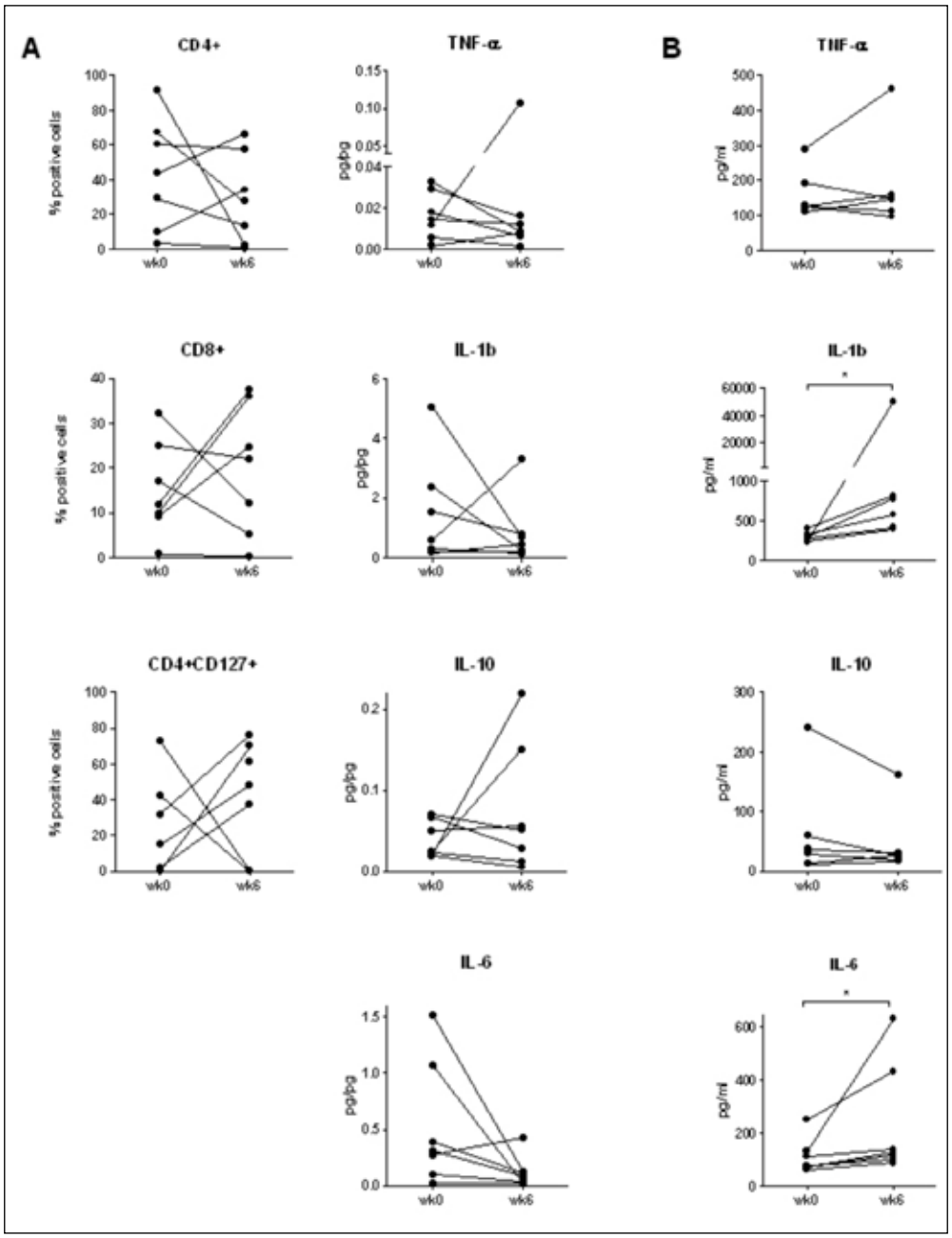
Figure 5 Endoscopy 0 (upper panels) and at 6 weeks (lower panels) after mesenchymal stromal cell (MSC) treatment (two administrations of 2x10^6 autologous bmMSCs) shows clear mucosal healing.



C

Pictures A and B are from patient 2, pictures C and D from patient 3

Figure 6



CD4+, CD8+ and CD4+CD127+ populations in biopsies of inflamed mucosa at week 0 and 6 (A, left panels). Cytokine levels of TNF-α, IL-1b, IL-10 and IL-6 were determined in mucosal biopsies (A, right panels) and serum (B) at week 0 and 6. Bars represent mean and SEM. *p< 0.05 for significant differences

Endoscopy

Endoscopic improvement, observed by a drop in CDEIS of 10,0 and 24,7 points, was seen in two patients with extensive CD localized in the colon (Figure 5).

In the other five patients no significant endoscopic improvement was seen between baseline and six weeks post-infusion. At week 0 and 6, levels of CD4+, CD8+ and CD4+CD127+ populations were determined in biopsies of inflamed mucosa (fig 6A left panels). Lower CD4+ T cells and higher CD4+CD127+ regulatory T cells were observed at week 6 when compared to week 0. Cytokine levels of TNF-α, IL-1b, IL-10 and IL-6 were determined in mucosal biopsies and serum at week 0 and 6. In general, cytokine levels went down in the mucosa (Figure 6*A* right panels), whereas an increase of cytokine levels in the serum was seen (Figure 6*B*).

Discussion

This phase I study shows that bone marrow harvesting and expansion of bmMSCs from refractory Crohn's disease patients is feasible and that MSCs cultured from Crohn's disease patients are similar to MSCs from healthy donors, in for example plastic adherence, spindle-shaped morphology, growth potential (Supplementary Figure 1), surface marker expression, lack of hematopoietic markers (Table 3) and differentiation capability (Figure 2). In addition, MSCs from refractory Crohn's disease patients are able to inhibit autologous PBMC proliferation and inhibit TNF-α production *in vitro* (Figure 3). Furthermore, autologous bmMSC infusion appears to be safe as intravenous MSC infusions were clinically well tolerated. Reported adverse events directly related to MSC infusion were a mild and transient allergic reaction in one patient and the typical taste and smell of the cryopreservant DMSO noticed by all patients (Table 2).

Although the design of this study does not allow conclusions on efficacy, after two infusions with autologous bmMSCs, endoscopic improvement was seen in two patients (Figure 5), while three patients required surgery due to worsening of disease (Table 4). Patients included were chronic active patients refractory to all currently available medical therapeutic options. One could speculate that the immunomodulatory effect of MSCs might not be sufficient to induce clinical remission in this category of patients. Further (randomized) trials in also less refractory patients are therefore warranted

In order to study the biologic effects of systemic MSC infusion in refractory CD patients we analyzed CD4+CD127+ expression on T cells obtained from colonic biopsies and determined cytokine production in both colon homogenates and serum. We observed a trend of lower CD4+ T cells and higher CD4+CD127+ regulatory T cells at week 6 when compared to week 0, although the number of patients in this study was not enough to reach statistical significance. In addition, cytokine levels went down in mucosal biopsies, indicating a decrease in intestinal inflammation (Figure 6A right panels). The apparent reciprocal increase in serum cytokine levels (Figure 6B) may be the result of altered distribution of inflammatory cells. Due to the decreased local inflammation, leukocytes are no longer recruited to the intestine, but remain in the circulation, thus increasing the systemic cytokine levels. Similar findings have been observed for regulatory T cells in CD [18] and plasmacytoid dendritic cells in dermal inflammation. [19]

The intravenous route of administration and target dose of 1-2x10^6 cells/kg bodyweight were based on experience with other protocols in GvHD. Intravenous infusion of cells is an easy, minimal invasive and routinely performed procedure with proven safety so far. Although it has been suggested that MSCs home to sites of inflammation, it is unknown how many cells will eventually reach the intestine in CD patients. In a case report, Dinesen *et al.* [20] showed that MSCs administration via selective mesenteric artery cannulation was safe and feasible. This approach may possibly increase the number of cells reaching the affected organ.

In the case of autologous MSCs, an ongoing discussion is whether MSCs are affected by or may contribute to the underlying disease. For instance, MSCs from patients with systemic lupus erythematosus are difficult to expand in culture and yield low cell numbers [21] and those from patients with multiple myeloma have been shown to be impaired and possibly contribute to the pathogenesis of the disease. [22] In this study we demonstrate that MSCs obtained from refractory Crohn's disease patients show similar growth potential, yield and properties when studied *in vitro* in comparison to MSCs from healthy donors. Our data supports work published recently [23] and suggests that bmMSCs from patients with refractory Crohn's disease are not affected by the disease. Unfortunately, there is no golden standard test to assess the functionality of MSCs and it has not been demonstrated that *in vitro* effectiveness of MSCs can be translated to clinical effectiveness, making true extrapolation of this topic difficult.

Another important issue is the effect of MSCs on concomitant medication. [24] To address this matter, we studied the effect of MSCs on various routinely used CD medications *in vitro*. We found that adding various CD medications to MSC cultures did not affect cell proliferation. Furthermore, adding MSCs to proliferating PBMCs in the presence of tested medications, gave a cumulative effect on the inhibition of PBMC proliferation (Figure 4), suggesting that MSCs can be used in addition to concomitant CD medication.

A concern in cell based therapies with *ex vivo* expanded cells is the formation of tumors. Previous work indicated that in mice, MSCs stimulate the growth of cancers [25, 26] and promote metastasis. [27] Additionally, extensive *in vitro* expansion of cells may induce genetic instability. Although an increased risk on tumor formation has never been confirmed in humans, patients with a history of malignancy were excluded from this study. To minimize the risk of transformation of cells we have expanded MSCs in the absence of growth factors, plated MSCs in moderate cell concentrations and used only low passage numbers. Furthermore, we karyotype the MSC product before clinical release to confirm normal karyotyping and did not observe any aberrancies.

In conclusion, our data suggests that intravenous application of autologous bmMSCs is feasible and well tolerated. Furthermore, bmMSC administration may produce clinical benefits in severe refractory Crohn's disease. Therefore, further studies should be designed to examine MSCs as a potential treatment for Crohn's disease.

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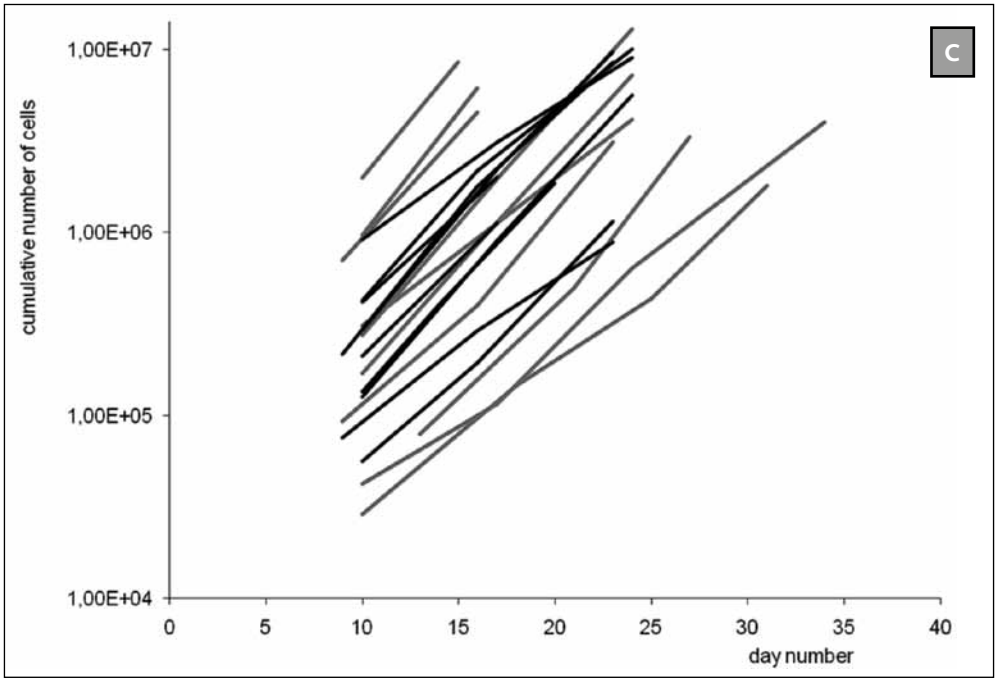
Ethics approval: The study was approved by the Medical Ethical Committee of the LUMC and the Central Committee on Research involving Human Subject (CCMO, The Hague, the Netherlands). www.trialregister.nl identifier: NTR1360.

Competing interests: None declared

Supplementary files

Supplementary figure 1.



MSC expansion of CD patients (red) and healthy volunteers (black) expressed as theoretical cumulative cell number per ml harvested bone marrow

Supplementary table 1.

Patient number	*ml bone marrow collected*	*Days of culture*	*Passage number*	*Number of cells (x10^6)/kg/ infusion*	*Total number of cells infused*
1	120	15	1	1,9	220
2	97	24	2	1,9	260
3	106	24	2	2,0	300
4	114	16	1	2,0	400
5	106	24	2	1,6	150
6	111	31	3	0,9	146
7	96	23	2	1,1	240
8	109	34	3	1,5	346
9	100	16	1	NA	NA
10	102	27	2	2,1	220

Overview of bone marrow collection, time needed for MSC culture and final MSC product infused. NA not applicable

Reference List

1. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-1822.
2. Di Nicola M, Carlo-Stella C, Magni M et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002;99:3838-3843.
3. Krampera M, Glennie S, Dyson J et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003;101:3722-3729.
4. Zuk PA, Zhu M, Ashjian P et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13:4279-4295.
5. Tondreau T, Meuleman N, Delforge A, et al. Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. Stem Cells 2005;23:1105-1112.
6. Erices A, Conget P, Minguell JJ. Mesenchymal progenitor cells in human umbilical cord blood. Br J Haematol 2000;109:235-242.
7. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 1997;64:278-294.
8. Gonzalez-Rey E, Anderson P, Gonzalez MA et al. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 2009;58:929-939.
9. Gonzalez MA, Gonzalez-Rey E, Rico L et al. Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. Gastroenterology 2009;136:978-989.
10. Le Blanc K, Frassoni F, Ball L et al Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-1586.
11. Le Blanc K., Rasmusson I, Sundberg B et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004;363:1439-1441.
12. Garcia-Olmo D, Garcia-Arranz M, Herreros D. Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease. Expert Opin Biol Ther 2008;8:1417-1423.
13. Ren G, Zhang L, Zhao X et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. Cell Stem Cell 2008;2:141-150.
14. Parekkadan B, van PD, Suganuma K et al. Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PLoS One 2007;2:e941.
15. Duijvestein M, van den Brink GR, Hommes DW. Stem cells as potential novel therapeutic strategy for inflammatory bowel disease. Journal of Crohn's and Colitis 2008;2:99-106.
16. Bernardo ME, Ball LM, Cometa AM et al. Co-infusion of ex vivo-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. Bone Marrow Transplant 2011;46(2):200-7
17. Ball LM, Bernardo ME, Roelofs H et al. Co-transplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem cell transplantation. Blood 2007;110(7):2764-7.

18. Li Z, Arijs I, De HG et al. Reciprocal changes of Foxp3 expression in blood and intestinal mucosa in IBD patients responding to infliximab. Inflamm Bowel Dis 2010;16(8):1299-310.
19. Nestle FO, Conrad C, Tun-Kyi A et al. Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. J Exp Med 2005;202:135-143.
20. Dinesen L, Wang A, Vianello F et al. Mesenchymal stem cells administered via novel selective mesenteric artery cannulation for the treatment of severe refractory Crohn's disease. Journal of Crohn's and Colitis 2010;3:Pages S51-S52.
21. Sun LY, Zhang HY, Feng XB et al. Abnormality of bone marrow-derived mesenchymal stem cells in patients with systemic lupus erythematosus. Lupus 2007;16:121-128.
22. Arnulf B, Lecourt S, Soulier J et al. Phenotypic and functional characterization of bone marrow mesenchymal stem cells derived from patients with multiple myeloma. Leukemia 2007;21:158-163.
23. Bocelli-Tyndall C, Bracci L, Spagnoli G et al. Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes in vitro. Rheumatology (Oxford) 2007;46:403-408.
24. Hoogduijn MJ, Crop MJ, Korevaar SS et al. Susceptibility of human mesenchymal stem cells to tacrolimus, mycophenolic acid, and rapamycin. Transplantation 2008;86:1283-1291.
25. Studeny M, Marini FC, Champlin RE et al. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 2002;62:3603-3608.
26. Djouad F, Plence P, Bony C et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003;102:3837-3844.
27. Karnoub AE, Dash AB, Vo AP et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature 2007;449:557-563.

Chapter Seven

Risk of Malignant Lymphoma in Patients with Inflammatory Bowel Diseases, a Dutch Nationwide Study

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Abstract

Immune suppressant medications such as thiopurines and anti-tumor necrosis factor agents are important for maintaining disease control in most patients with inflammatory bowel diseases (IBD), however, their use have been associated with the development of malignant lymphoma. The purpose of this Dutch nationwide study was to estimate the relative risk of malignant lymphoma in IBD patients.

IBD patients who developed a lymphoma between 1997 and 2004 were identified using the Dutch National Database of PALGA. Data from confirmed cases were collected from individual hospitals, including data on Epstein Barr virus. The age-adjusted 8-years incidence of malignant lymphoma in the Netherlands was retrieved from the Central Bureau of Statistics.

Forty-two hospitals were visited and 285 matches evaluated in the total cohort of 17834 IBD patients. Forty-four lymphomas were observed resulting in a relative risk of 1.27 (95% CI: 0.92 – 1.68). Only 19 of 44 patients (43%) were exposed to AZA/6-MP. Remarkably, 92% of patients (11/12) with EBV positive lymphoma used AZA/6-MP, in contrast to only 19% patients (4/21) with EBV negative lymphoma, suggesting a strong relation between EBV positive lymphoma and thiopurine use.

This nationwide study does not suggest a significant overall increased risk for lymphoma in IBD patients. A distinct correlation between EBV positive lymphoma and AZA/6MP use was observed.

Introduction

An approximate fourfold increased risk of a malignant lymphoma in inflammatory bowel disease (IBD) patients treated with azathioprine/6-mercaptopurine (AZA/6MP) was observed in a meta-analysis several years ago. [1] Since the absolute risk was considered low and not affecting the risk-benefit ratio, global IBD guidelines continued to endorse the use of AZA/6MP as maintenance therapy for IBD. Two recent developments have occurred that could potentially challenge this. First, reports on cases with hepatosplenic T-cell lymphoma suggested a role for thiopurines possibly in combination with anti-TNF therapy. [2] Second, a recent French prospective nationwide study from the Cesame Study Group involving almost twenty thousand IBD patients, demonstrated a significant increased risk for lymphoma development in patients receiving thiopurine therapy. [3]

IBD lymphoma cases have typically involved post-transplant lymphoproliferative disorder-like B-cell disorders associated with Epstein-Barr virus suggesting a causal role for immunosuppression. However, inflammation itself might contribute to the excess lymphoma risk. Indeed, a longer duration of IBD was an independent risk factor for developing a lymphoma in the Cesame study. Also, in rheumatoid arthritis it has been evident that severe disease can be associated with an increased risk for lymphoproliferative disease irrespective of immunomodulatory therapy. [4, 5]

Although the cumulative absolute risk for developing lymphoproliferative disorders in patients receiving thiopurine therapy appears to be low, we investigated the lymphoma incidence in a Dutch nationwide study. Specifically, we were interested in the association between the use of thiopurines and the development of lymphoproliferative disorder-like B-cell disorders in IBD patients associated with EBV.

Methods

Cases

To identify all IBD patients in the Netherlands who developed a malignant lymphoma, the PALGA database was used. PALGA is the Dutch nationwide network and registry of histo- and cytopathology, which contains standardized abstracts of all 16 million inhabitants of the Netherlands since 1990. [6] Patients in this registry are identified by date of birth, gender and the first 4 characters of their family name. The abstracts contain encrypted patient information, demographic data and a summary of the pathology report coded in accord with Systematized Nomenclature of Medicine (SNOMED) terminology issued by the College of American Pathologists. We queried the PALGA database according to a standardized procedure, after which an independent review committee consented to this search and subsequent identification of cases. The PALGA case-numbers were generated in an anonymous way. Subsequently, local pathologists were contacted to identify the associated medical files. Local gastroenterologists or treating physicians assisted to retrieve the required datasets. Finally, participating hospitals were visited and anonymized datasets of case reports matched by PALGA were entered in the case report forms (CRFs) using unique study numbers.

Dutch lymphoma incidence and expected number

Age-adjusted incidence numbers of Hodgkin Lymphoma (HL) and Non-Hodgkin Lymphoma (NHL) in the general population in the Netherlands were obtained from the Central Bureau for Statistics (2000 – 2004) and used to calculate the 8-years incidence and subsequently the expected number in our IBD-cohort. The total number of the cohort was calculated using the estimated sum of patients per participating hospital and was estimated at 23216. Because 23% of the case histories was not available for full analysis, the final cohort size was estimated at 17834 IBD patients (77% * 23216). Forty-two medical centers were participating in this study, both academic and peripheral, and we used the Dutch age and gender specific incidence numbers from the Central for Statistics to calculate the number of IBD patients in each age and gender group.
Subsequently, the expected number of lymphomas in IBD patients was calculated using the 8-years age-adjusted incidence numbers.

Design of the study

From the PALGA registry all patients with both IBD and lymphoma were selected between 1997 and 2004. This time window was chosen since thiopurine therapy management became more prominent and more generally applied around 1995. Criteria adopted for the data base search were based on the association of the term "ulcerative colitis", "Crohn's disease", "inflammatory bowel disease", "inflammation", with "malignancy" and "malignant lymphoma" coupled to "oesophagus", "stomach", "small intestine" or "colon". The search disclosed only cases recorded between 1st January 1997 and 31st December 2004. Lesions histologically diagnosed as "multiple myeloma", or "adenomatous polyposis coli" as well as "colorectal carcinoma" were excluded from the study. When it wasn't clear whether the diagnosis was UC or CD, the diagnosis of the latest pathology report and/or medical file was recorded as definite diagnosis. When the diagnosis was "nonspecific colitis", and the patient used immunosuppressive agents, it was assumed that the patient had either CD or UC, and was recorded in the CRF as "IBD" and was subsequently verified in the individual medical file. The final selection of the cases was done after an analysis of the medical records in the participating hospitals. Cases were excluded if the objective diagnosis of lymphoma or IBD was lacking.

Data management and statistical analysis

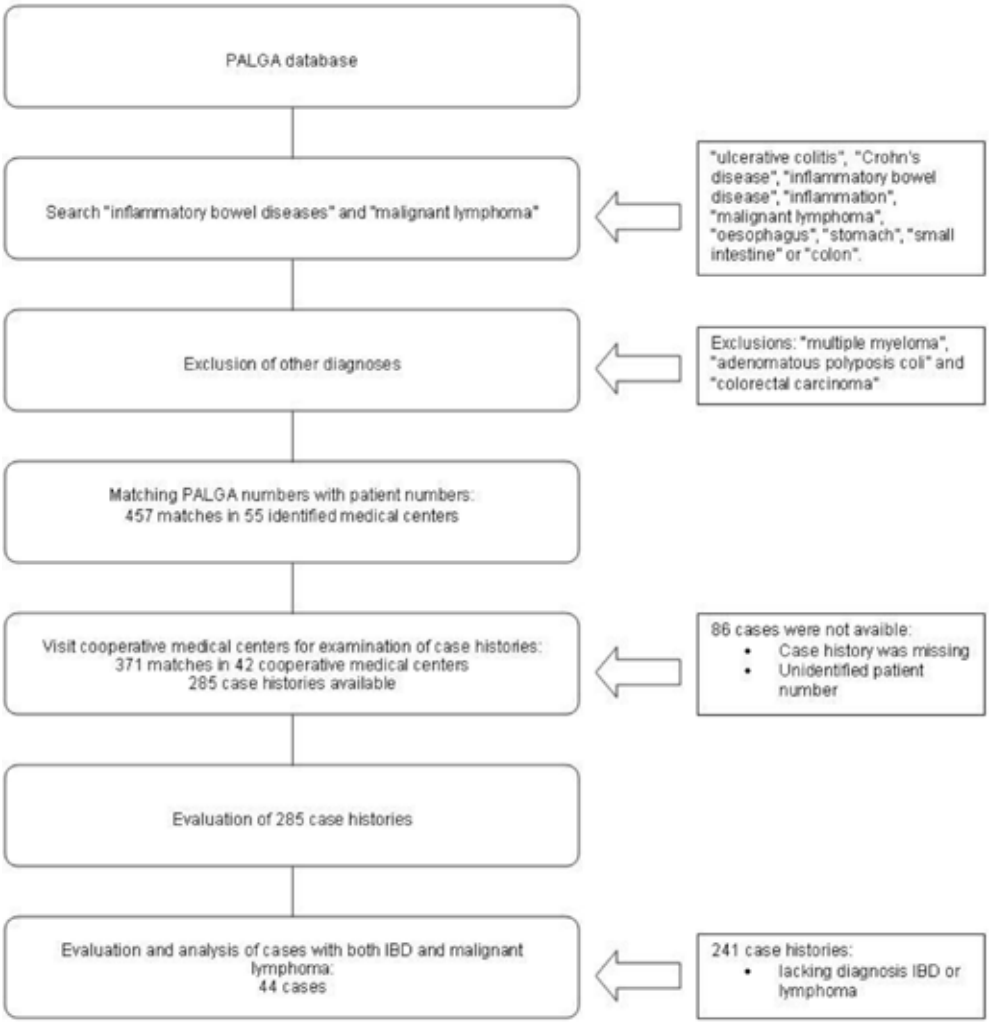
From all cases an *a priori* defined dataset was retrieved and entered in a dedicated Excel database. The dataset included both general information such as gender, age, height and weight, as well as details on disease duration, medication, onset of disease, and phenotypic characteristics such as stenoses, fistulas, surgery, complications and family history of IBD. In addition, the year of diagnosis of malignant lymphoma, location and type of lymphoma, treatment, patient survival and the presence of EBV were retrieved. Descriptive and analytical statistics were performed using Microsoft Excel. The relation between EBV positive lymphoma and azathioprine was calculated using Fisher exact test.

Results

Cases

The PALGA database search identified a total of 371 cases with both IBD and lymphoma between 1997 and 2004. Figure 1 depicts the search and selection algorithm. Eighty-six case histories (86/371, 23%) proofed not to be present for full analysis and could therefore not be analyzed. Subsequently, 42 Dutch medical centers were visited to analyze the medical files of in total 285 matches. In 241 cases, an objective diagnosis of IBD or malignant lymphoma could not be confirmed. Thus, 44 cases of malignant lymphoma in a cohort of 17834 IBD patients were included for final analysis. Table 1 shows the number of cases and patient characteristics.

Figure 1 Search and selection strategy.



Lymphoma risk

Since 44 cases were observed in a total IBD population of 17834, and 34.63 cases were expected (Table 2), the relative risk (RR) of developing a malignant lymphoma was 1.27 (95% CI 0.92 – 1.68). Hence, no overall increased risk was observed. However, a statistically significant increased risk was observed in the age categories 35 – 39 (RR 9.32, 95% CI: 3.89 – 17.07) and 45 – 49 (RR 3.99, 95% CI: 1.22 – 8.36). The increased RR in these two groups suggests an association between lymphoma and a diagnosis of IBD for patients of these ages.

Also, a gender specific analysis was performed, since the lymphoma incidence numbers in males are higher than in females and therefore, the expected number of lymphoma in males would be higher. In line with this, more lymphomas were observed in males (27/44, 61%) than in females (17/44, 39%). In all age groups taken together, the risk in males and females was similar, and there was no significant increased risk for either males or females for lymphoma in IBD (males: RR: 1.21 95% CI 0.79 – 1.72; females: RR: 1.23 95% CI: 0,70 – 1,89). However, in both males and females, a statistically significant risk was found in the age group 35 – 39 y and this increased relative risk was particularly outspoken in males (males: RR: 10.25 95% CI: 2.56 – 23.05; females: RR: 6.74 95% CI: 1.20 – 16.77). In all other

Table 1 Characteristics of cases.

	EBV positive	*EBV negative*	*EBV status unknown*	*Total*
number of cases	12/44 (27%)	21/44 (48%)	11/44 (25%)	44
Age				
mean ± SD (y)	46.75 ± 17.56	53.62 ± 14.31	61.64 ± 13.74	53.75 ± 15.74
median (range, y)	37.5 (24 – 78)	58 (21 – 74)	63 (36 – 79)	56.5 (21 – 79)
Male (%)	5/12 (42%)	15/21 (71%)	7/11 (64%)	27/44 (61%)
IBD				
CD	6/12 (50%)	6/21 (29%)	3/11 (27%)	15/44 (34%)
UC	6/12 (50%)	12/21 (57%)	6/11 (55%)	24/44 (55%)
IBDU		3/21 (14%)	2/11 (18%)	5/44 (11%)
Duration between IBD and lymphoma				
mean ± SD (y)	12.05 ± 6.75*	13.76 ± 10.97	12.73 ± 8.21	13.06 ± 9.21
median (range, y)	13 (2.5 – 25)	12 (0.5 – 36)	11 (1 – 31)	12 (0.5 – 36)
AZA/6MP use (%)	11/12 (92%)**	4/21 (19%)**	4/11 (36%)	19/44 (43%)
Duration of AZA/6MP use mean ± SD (y)	4.32 ± 4.08	3.63 ± 1.7	1.3 ± 0.71***	3.58 ± 3.3
median (range, y)	3 (0.25 – 12)	3.25 (2 – 6)	1.33 (0.58 – 2)	2.5 (0.25 – 12)

EBV = Epstein Barr virus, CD = Crohn's disease, UC = ulcerative colitis, IBDU = unspecified IBD, AZA = azathioprine, 6MP = 6-mercaptopurine

* of 1 patient in this group the time between IBD diagnosis and lymphoma was unknown; ** medication use of 1 patient in both groups was unknown; *** of 1 patient in this group the duration of azathioprine use was unknown

age groups, the relative risk for both males and females was similar and not significantly increased. In conclusion, males initially have a higher risk of developing lymphoma, and the fact that we observed more male than female cases, is therefore probably not related to IBD or treatment.

EBV associated lymphomas

Of the 44 lymphoma specimens, 12 were EBV positive and 21 negative. In 11 cases the EBV status could not be ascertained. Table 3 depicts the 12 individual IBD cases with an EBV-related lymphoma in detail. Of these, 11 patients were treated with thiopurines in combination with 5-ASA and prednisone. One case is supposed to be related to immunosuppression after liver transplantation. Six patients were diagnosed with CD (4 female and 2 male), 6 with UC (3 female, 3 male). None of the patients used anti-TNF compounds. Nine out of twelve lymphomas were localized in the gastrointestinal tract and all EBV+ lymphomas but one were of the diffuse large B cell type. A considerable variation was found in the dose and duration of the thiopurine use (duration ranging from months to 12 years, dose ranging from 50 – 350 mg/day), and in time between onset of IBD and lymphoma diagnosis (2.5 – 25 years).

Table 2 Expected and observed numbers of lymphoma and IBD in the cohort (1997 – 2004).

Age	*Expected number of cases*	*Observed number of cases (EBV positive lymphoma)*	*RR (95% CI)*
0 – 4	0.002	0	
5 – 9	0.013	0	
10 – 14	0.044	0	
15 – 19	0.318	0	
20 – 24	0.748	2 (1)	2.67 (0.23 – 7.79)
25 – 29	0.975	0	
30 – 34	0.783	2 (1)	2.56 (0.22 – 7.45)
35 – 39	0.858	8 (5)	9.32 (3.89 – 17.07)*
40 – 44	1.188	1	0.84 (0 – 3.37)
45 – 49	1.252	5 (1)	3.99 (1.22 – 8.36)*
50 – 54	1.834	3	1.64 (0.29 – 4.07)
55 – 59	1.924	5 (1)	2.60 (0.79 – 5.44)
60 – 64	2.136	6 (1)	2.81 (0.98 – 5.57)
65 – 69	3.370	4	1.19 (0.30 – 2.67)
70 – 74	4.913	4	0.81 (0.20 – 1.83)
75 – 79	5.729	4 (2)	0.70 (0.17 – 1.57)
80 – 84	5.022	0	
85 – 89	2.833	0	
> 90	0.788	0	
Total	34.63	44	1.27 (0.92 – 1.68)

Table 3 Characteristics of EBV positive lymphomas.

	Year of lymphoma diagnosis	*Age at lymphoma diagnosis*	*Sex*	*Aza / 6MP*	*Dose and duration*	*Lymphoma site*	*Lymphoma type*	*IBD*	*Time between IBD and lymphoma*	*Outcome*
1	2004	37	M	Y	Aza 200 mg/day 8 years	ileum	Diffuse large B	CD	15 years	RTX + surgery, survival
2	2001	35	M	Y	Aza 150 mg/day Months	sigmoid	Diffuse large B	UC	6 years	RTX + surgery, survival
3	2004	37	M	Y	Aza, 100 mg/day 8 years	lymph nodes, bone marrow, lungs, spleen, liver, stomach	Diffuse large B	CD	17 years	RTX, died 2004
4	2004	34	F	Y	Aza, 100-350 Mg/day 3 years	lungs, dermis, liver, kidney	Diffuse large B	UC	13 years	CHOP, survival
5	2001	36	F	Y	Aza, 100 mg/day 12 years	liver, perianal	Diffuse large B	CD	15 years	CAVmP, survival
6	2001	62	F	Y	Aza, 50 mg/day 3 months (1 y MTX for RA)	spleen, rectum	?	UC	2,5 years	CHOP, survival
7	2000	38	F	Y	Aza, 150 mg/day 2 y	dermis, lungs	?	CD	25 years	Remission and survival
8	2001	58	F	Y	Aza, 50-150 mg/day 4 years	perirectal	Diffuse large	CD	6 years	RTX, died 2001
9	2000	24	F	Y	6-MP 50 mg/day 1 year	stomach	?	UC	13 years	RTX, died of sepsis 2000
10	1999 (post mortem)	78	F	Y	Aza, 50-150 Mg, 1,5 y	brain	Follicular B cell	CD	4 years	Unknown, no survival
11	1999	46	M	Y	Aza, 100mg/day 1 year	Descending colon	Diffuse large	UC	16 years	Surgery, no survival
*12**	2003	76	M	N		Unknown	unknown	UC	unknown	Chemotherapy, survival

M = male, F = female, aza = azathioprine, 6-MP = 6-mercaptopurine, CD = Crohn's disease, UC = ulcerative colitis, RTX = rituximab

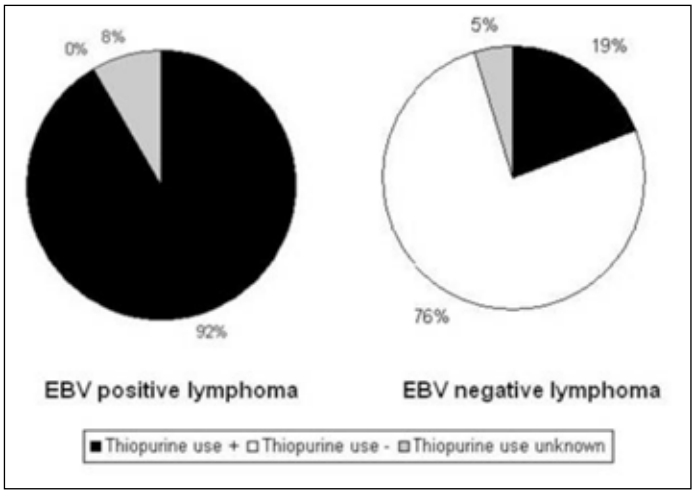
* In this case the EBV-positive lymphoma was thought to be related to immunosuppression after liver transplantation

Twenty-one EBV negative lymphomas were observed (Table 4). Of these, four patients used azathioprine. Nineteen patients used mesalazine, prednisone or a combination, one patient didn't receive any therapy at all and from one patient the prescribed medication was unknown. Of these 21 lymphomas, 10 lymphomas were diffuse large B cells lymphomas, so there was more variation in the type of lymphoma. Twelve out of 21 lymphomas were localized in the gastrointestinal tract. Twelve patients were diagnosed with UC (8 male, 4 female), six with CD (4 male, 2 female) and three with unspecified IBD (IBDU). The time between IBD and lymphoma diagnosis ranged from 6 months to 36 years.

Association between azathioprine/6-MP use and EBV positive lymphomas

Of the 44 cases, 19 patients were exposed to AZA/6MP (range: 3 months –12 yrs), 23 patients never used AZA/6MP and from two patients it was unknown whether they used AZA/6-MP. Of the 19 lymphomas in thiopurine users, 15 were tested for EBV. Remarkably, 11 lymphomas were EBV positive (73%) and 4 were EBV negative (27%), and there was a significant association between thiopurine use and EBV positive lymphoma ($p<0{,}001$). Twenty-three patients didn't use AZA/6MP, and in this group 16 lymphomas were tested for EBV status. No EBV positive lymphomas were found. From 2 patients it was unknown whether they used AZA/6-MP, one tested negative for EBV and one was positive for EBV. The latter case, however, was thought to be related to immunosuppression following liver transplantation (Figure 2 and table 5). Most EBV positive lymphomas were diffuse large B cell lymphomas. Ten out of 21 EBV negative lymphomas were diffuse large B cell lymphomas. Whether these lymphomas arose as a result of the disease itself or because of the use of immune suppressive agents has not been elucidated yet. Since EBV positive lymphomas arise especially in the setting of immunosuppression, the occurrence of these lymphomas suggested a strong relation with the use of immunosuppressive drugs, especially thiopurines, in IBD patients.

Figure 2 Thiopurine use in EBV positive and EBV negative lymphomas.



A significant association between EBV+ lymphoma and thiopurine use was found ($p \leq 0.001$)

Table 4 Characteristics of EBV negative lymphomas.

Pt no	*Year of lymphoma diagnosis*	*Age at lymphoma diagnosis*	*Sex*	*Aza / 6MP*	*Dose and duration*	*Other medication*	*Lymphoma site*	*Lymphoma type*	*IBD*	*Time between IBD and lymphoma*	*Therapy & Outcome*
1	2000	21	M	Y	3,5 y 100 – 200mg	pred	bone marrow, spleen, liver	T cell	CD	4 years	Chemo, no survival
2	2004	58	M	N	-	-	stomach	Diffuse large B cell, stage IV B	UC	29 years	Chop, survival
3	2000	53	M	N	-	5-ASA pred	liver	Diffuse large B cell	UC	29 years	Rituximab, Chop, no survival
4	2001	63	M	N	-	5-ASA	colon	Marginal zone B cell	UC	28 years	No therapy, survival
5	2004	40	F	Y	3 y, 50mg	5-ASA pred	dermis	T cell	UC	12 years	MTX, complete remission
6	2000	31	M	N	-	5-ASA pred	retroperitoneal	Diffuse large B cell	IBDU	6 months	Surgery, no survival
7	1999	47	F	N	-	5-ASA pred	retroperitoneal	Diffuse large B cell st IV	UC	22 years	CHOP, Survival (complete remission)
8	2001	59	M	N	-	5-ASA	mandibula, bone marrow	Diffuse large B cell	IBDU	5 years	Radiotherapy, chemo, no survival
9	2002	66	M	N	-	5-ASA	stomach	Follicular B cell	IBDU	19 years	Surgery and CHOP, no survival
10	2003	66	F	N	-	5-ASA	submandibular	Diffuse large B cell	CD	10 years	CHOP, no survival
11	2004	38	M	N	-	5-ASA	duodenum	B cell	UC	2,5 years	Autologous stem cell transplantation, survival

12	2002	48	M	N	-	5-ASA pred	retroperitoneal	follicular	CD	16 years	Radiotherapy, survival
13	1997	72	F	N	-	5-ASA	spleen	B cell	UC	6 months	Surgery, survival
14	2002	55	M	N	-	5-ASA	bone marrow, lymph nodes, spleen, liver, kidney	Follicular B cell	CD	5 years	CVP, no survival
15	2002	74	M	N	-	5-ASA pred	Submandibular	Follicular B cell	UC	17 years	CVP / CHOP survival
16	2000	69	M	N	-	5-ASA pred	supraclavicular	Mantel cell	UC	36 years	CHOP, no survival
17	1999	35	F	Y	2 y, 75 – 125mg	5-ASA pred	liver, spleen, bone marrow	Diffuse large B cell, st IV	UC	4 years	CVP / CHOP, no survival
18	2001	64	M	Y	6 y 50 – 50 mg	5-ASA pred	Rectum	Diffuse large B cell	UC	17 years	CHOP, survival
19	1998	59	M	?		?	Ileum	Diffuse large B cell	CD	Months?	No survival
20	1997	47	M	N		5-ASA	Liver	Diffuse large B cell	UC	23 years	CHOP, survival
21	1997	61	F	N		Pred 5-ASA	Coecum		CD	9 years	survival

Thio = thiopurine, pred = prednisone, EBV Pos = EBV positive

Table 5 Thiopurine use and EBV status.

EBV status	*Thiopurine use +*	*Thiopurine use -*	*Thiopurine use unknown*	*Total*
Positive	11**	0	1*	12
Negative	4	16	1	21
Unknown	4	7	0	11
Total	19	23	2	44

* In this case the lymphoma could also be related to immunosuppression after liver transplantation
** A significant association between EBV+ lymphoma and thiopurine use was found ($p \leq 0.001$)

Discussion

This nationwide study in the Netherlands suggests that there is no overall statistically significant increased risk of malignant lymphoma in IBD patients (RR 1.27, 95% CI: 0.92 – 1.68). Two relevant additional observations were made: 1) almost all patients with an EBV positive lymphoma used AZA/6-MP, and 2) these EBV post-transplant like lymphomas were particularly prevalent in the younger aged adult patients (< 50 years). This was a remarkable finding since the expected number of lymphomas increases with age and is quite low in younger age categories of the normal population. Therefore, these data suggest that younger aged adult patients may have an increased risk. Although more lymphomas were observed in males (61%), more lymphomas were expected since the lymphoma incidence in males is higher than in females. Both in males and females no significant increased risk for lymphoma was found, except in the age group 35 – 39. Risks were similar and not significantly increased for males and females in other age groups. Lymphomas of different origin were found in the study population, which included both CD and UC patients in different age categories. Since the study population is a mixed population and not all patients received immunomodulators, it is possible that the calculated risk underestimates the actual risk in patients receiving immunomodulators, and overestimates the risk in patients that do not.
The current literature does not offer clear and conclusive answers on the risk of predisposing factors for lymphoma development in IBD. It seems that our results are comparable to those of reported population based studies. Two large population based studies including 16996 IBD patients in the UK [7] and 47679 IBD patients in Sweden [8] failed to show a significant increased risk of lymphoma (RR for CD 1.4 and 1.3, RR for UC 1.2 and 1.0 resp.). In addition, other population based studies did not show a significant increased risk. [9-11]
In contrast, one population-based, retrospective Canadian IBD study did observe an increased risk of 2.4 in CD patients but not in UC. [12] In 2000, Farrell *et al.* observed a small increased risk in a cohort of 782 IBD patients. [13] In 2002, Dayharsh *et al.* observed in 10,000 IBD patients from a tertiary center a two-fold risk, also including EBV positive lymphomas. [14] Also, two other studies found a significant increased risk. [1, 3] Reasons for the conflicting results of the different studies could be the design of the study, selection of patients, referral bias, or a different incidence of NHL and HL in the general population. For instance, it is possible that single center studies experience referral bias, and meta-analyses performed on the cases reported in single center studies might overestimate the actual risk. In addition,

the prospective study performed by Beaugerie *et al*. showed a significant increased risk but was also designed differently; the investigators aimed to evaluate the lymphoma risk in thiopurine users versus non users. Because of differences in design and patient recruitment, it is complicated to compare the outcomes of different studies.

Furthermore, we found that especially younger aged adult patients are at risk. This observation is in line with another study [15] and rather interesting, since lymphoma incidence in the general population increases with age, and therefore the increased risk in the younger aged adult patients suggests that the lymphoma is more likely related to the disease or the treatment. However, it is not known why this age group might have an increased risk. Future studies are needed to elucidate why this particular group has an increased risk, and how this vulnerable group can be protected from an increased risk. Also, we confirmed a significant association between EBV positive lymphoma and the use of thiopurines. This was observed previously in other studies and case reports. [1, 16-18]

There has been a longstanding concern regarding anti-TNF therapy and the risk of lymphoma. Anti-TNFs were introduced in the Netherlands in the late '90s so anti-TNF was introduced during our study period (1997 – 2004). None of our cases used anti-TNF therapy, and the issue of anti-TNF therapy and the risk of lymphoma was not addressed in this study.

It is unclear to what extent disease severity plays a role in the development of malignant lymphoma in IBD. In rheumatoid arthritis (RA), a strong relation between disease activity and lymphoma risk has been reported. [4, 19] One particular problem with such an assumption is the selection bias of patients because association evidently does not imply causation. It is likely that use of thiopurine derivatives also reflects severity of the disease, which in itself could well be part of the pathogenesis of lymphoma development. This concern has also been voiced by others. [20]

A few limitations of this study should be noted. First of all, most medical centers in the Netherlands do not register their IBD patients in research dedicated databases. Consequently, the size of the total IBD patient cohort in our study is an estimation drawn from hospital information systems. Second, a considerable number of case histories could not be retrieved or were incomplete. As a result, we were unable to fully evaluate 23% of the possible cases. Although this a large percentage, the missing case reports were both in tertiary centers as well as peripheral centers and distributed over 21 medical centers, arguing against the idea of selection bias or over- or under presentation of a certain patient group and suggesting that the missing case reports over 21 medical centers are caused by random inaccuracies. In addition, given the number of medical centers included in our study (42 in total), the representativeness of our cohort and nationwide design of the study, this study provides a valid estimation of the relative risk of malignant lymphoma among IBD patients. We were unable to identify specific risk factors other than age in this study, since the design of this study is not eligible for the identification of other risk factors. Future studies are needed to identify other risk factors, such as disease severity, and to find out which patient groups are at risk.

In conclusion, we report no statistically significant overall increased relative risk for malignant lymphoma development in IBD patients, and a significant association of thiopurine derivative use and development of EBV-positive lymphoma. Furthermore, a significantly increased risk was found in younger aged adult patients. Should we now be reluctant to

prescribe thiopurines in our IBD population? Probably not, since thiopurine use has been so successful in maintenance therapy and in avoidance of corticosteroids, its benefit is well appreciated. In risk-benefit models, it has been suggested that a 10-fold risk is necessary for the overall effect of thiopurines in IBD to be detrimental. [21]

Competing interests: none to declare

Reference List

1. Kandiel A, Fraser AG, Korelitz BI et al. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. Gut 2005;54:1121-1125.
2. Shale M, Kanfer E, Panaccione R et al. Hepatosplenic T cell lymphoma in inflammatory bowel disease. Gut 2008;57:1639-1641.
3. Beaugerie L, Brousse N, Bouvier AM et al. Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. The Lancet 2009;374:1617-1625.
4. Baecklund E, Ekbom A, Sparen P et al. Disease activity and risk of lymphoma in patients with rheumatoid arthritis: nested case-control study/ BMJ 1998;317:180-181.
5. Jones M, Symmons D, Finn J et al. Does exposure to immunosuppressive therapy increase the 10 year malignancy and mortality risks in rheumatoid arthritis? A matched cohort study. Br J Rheumatol 1996;35:738-745.
6. Casparie M, Tiebosch AT, Burger G et al. Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. Cell Oncol 2007;29:19-24.
7. Lewis JD, Bilker WB, Brensinger C et al. Inflammatory bowel disease is not associated with an increased risk of lymphoma. Gastroenterology 2001;121:1080-1087.
8. Askling J, Brandt L, Lapidus A et al. Risk of haematopoietic cancer in patients with inflammatory bowel disease. Gut 2005;54:617-622.
9. Persson PG, Karlen P, Bernell O et al Crohn's disease and cancer: a population-based cohort study. Gastroenterology 1994;107:1675-1679.
10. Palli D, Trallori G, Bagnoli S et al. Hodgkin's disease risk is increased in patients with ulcerative colitis. Gastroenterology 2000;119:647-653.
11. Loftus EV, Jr., Tremaine WJ, Habermann TM et al. Risk of lymphoma in inflammatory bowel disease. Am J Gastroenterol 2000;95:2308-2312.
12. Bernstein CN, Blanchard JF, Kliewer E et al. Cancer risk in patients with inflammatory bowel disease: a population-based study. Cancer 2001;91:854-862.
13. Farrell RJ, Ang Y, Kileen P et al. Increased incidence of non-Hodgkin's lymphoma in inflammatory bowel disease patients on immunosuppressive therapy but overall risk is low. Gut 2000;47:514-519.
14. Dayharsh GA, Loftus EV, Jr., Sandborn WJ et al. Epstein-Barr virus-positive lymphoma in patients with inflammatory bowel disease treated with azathioprine or 6-mercaptopurine. Gastroenterology 2002;122:72-77.

15. Siegel CA, Marden SM, Persing SM et al. Risk of Lymphoma Associated With Combination Anti-Tumor Necrosis Factor and Immunomodulator Therapy for the Treatment of Crohn's Disease: A Meta-Analysis. Clinical Gastroenterology and Hepatology 2009;7:874-881.
16. Crispino P, Pica R, Angelucci E et al. Hematological malignancies in chronic inflammatory bowel diseases: report of five cases and review of the literature. Int J Colorectal Dis 2007;22:553-558.
17. Schwartz LK, Kim MK, Coleman M et al. Case report: lymphoma arising in an ileal pouch anal anastomosis after immunomodulatory therapy for inflammatory bowel disease. Clin Gastroenterol Hepatol 2006;4:1030-1034.
18. Kumar S, Fend F, Quintanilla-Martinez L et al. Epstein-Barr virus-positive primary gastrointestinal Hodgkin's disease: association with inflammatory bowel disease and immunosuppression. Am J Surg Pathol 2000;24:66-73.
19. Baecklund E, Iliadou A, Askling J et al. Association of chronic inflammation, not its treatment, with increased lymphoma risk in rheumatoid arthritis. Arthritis Rheum 2006;54:692-701.
20. McGovern DP, Jewell DP. Risks and benefits of azathioprine therapy. Gut 2005;54:1055-1059.
21. Lewis JD, Schwartz JS, Lichtenstein GR. Azathioprine for maintenance of remission in Crohn's disease: benefits outweigh the risk of lymphoma. Gastroenterology 2000;118:1018-1024.

Chapter eight

Summarizing discussion

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This thesis focuses on various strategies towards disease control in IBD; new treatment targets, working mechanism of established therapies, side effects and future therapy. A better understanding of disease pathology and the working mechanism of IBD therapy, could help to find new treatment targets, disease markers and novel therapies. Also, this would allow the selection of patients for a certain therapy based on genotype or phenotype.
An overview of a selection strategy based on disease phenotype is provided in **chapter two**. It has been shown that patients with young age at onset of disease, presence of perianal disease, stricturing disease and initial need for corticosteroids have a high risk at developing a complicated disease course. In addition, CD typically evolves from an inflammatory disease to a fibrotic disease. At this stage, the disease is difficult to treat and response rates to various therapies, among which anti-TNF, are lower than in patients with recently diagnosed disease. It is suggested that patients with a high risk of developing complicated disease would particularly benefit from a "top-down" approach. Importantly, side effects need to be weighed against the benefits, and strong genetic or phenotypic factors in order to predict disease course, therapeutic response and risk of side effects are still lacking but would be highly valuable.

A new role for autophagy in CD pathogenesis: clinical implications

Since autophagy has been described as a degradation mechanism, [1, 2] many studies on the role of autophagy in CD pathogenesis focused on its role in the clearance of pathogens. [3-5] A defect in the autophagy pathway could lead to impaired clearance of intracellular organisms, and in that way contribute to uncontrolled microbial expansion and excessive immunity. Likely, these mechanisms contribute to disease pathogenesis, but the results described in **chapter three** provide new insight into the role of autophagy in the absence of pathogens. We found that autophagy is important in the breakdown of the immunological synapse, and in that way identified a role of autophagy in tolerance and immunity in general. Importantly, we also confirmed these findings in patients carrying the autophagy SNP, and these data may explain the aberrant immune response observed in these patients. The identification of a new role for autophagy in the pathogenesis of CD may provide new therapeutic targets in patients carrying this SNP. Interestingly, azathioprine has been described to suppress APC-T cell interactions, [6] and therefore patients carrying the autophagy risk allele may benefit from azathioprine therapy in particular. In addition, other autophagy inducing drugs may be effective in this specific patient group, such as rapamycin, which inhibits mammalian target of rapamycin (mTOR) and thereby induces autophagy. Indeed, two case reports show an impressive effect of this drug, [7, 8] but this has not been tested in patients with the risk allele specifically. In addition, the efficacy of this drug has not been evaluated in clinical trials. Yet, selecting patients based on genotype for a certain therapy might be a promising future strategy.

Relevance of Mϕ2 induction and future implications

In **chapter four** we describe the Fc region dependent induction of regulatory macrophages *in vitro* by anti-TNF antibodies and we further show their immunosuppressive features. Both the presence of monocytes and T cells was required in order to induce Mϕ2. Interestingly, this induction was only observed when mixed lymphocyte reaction cultures were treated with agents that have the ability to bind to mTNF and have an Fc region to interact with Fc receptors. Anti-TNF agents that do not bind to mTNF (etanercept, onercept) and anti-TNF agents that do not interact with Fc receptors (certolizumab, CDP571) did not induce Mϕ2 *in vitro*. These agents are also the ones that failed to show efficacy in clinical practice, and it is tempting to speculate that the induction of Mϕ2 is crucial in this process.
Mucosal healing is nowadays an important treatment goal, since it has been shown that mucosal healing is associated with less surgery, less hospitalization, less corticosteroid use and improved quality of life. [9-11] The factors that contribute to mucosal healing are not completely understood, but the role of regulatory macrophages (Mϕ2) in wound healing in general has been extensively studied before. [12, 13] Next to wound healing properties, Mϕ2 have immunosuppressive effects. They produce mainly anti-inflammatory cytokines, [14] have minor ability to respond to bacterial stimuli, [15] and inhibit proliferation of activated T cells. [16] Since IBD is thought to result from loss of tolerance towards the mucosal environment, the induction of this cell type might be of particular interest in restoring the balance and inducing mucosal healing. Indeed, decreased numbers of this cell type are found in the mucosa of IBD patients. [17] Since the successful introduction of anti-TNF agents during the late '90s in terms of mucosal healing and improved quality of life, [18-21] much research has focused on the mechanisms of action to elucidate factors involved in mucosal healing. In addition, the inefficacy of several anti-TNF agents in the clinic suggested that neutralization of TNFα was not the most important aspect.
In **chapter five**, we confirmed the induction of Mϕ2 *in vivo* and showed that patients responding to infliximab therapy have increased numbers of CD206+/CD68+ cells after 4 weeks of induction therapy compared to baseline. Since we used endoscopic and histologic healing as a definition of response, these data suggest that the induction of Mϕ2 may be important in the process of mucosal healing. Also, we demonstrated the wound healing capacity *in vitro*, which further suggests a critical role of this cell type in mucosal healing.
The induction of Mϕ2 by anti-TNFα antibodies provides a model which helps to elucidate several other features related to anti-TNF treatment. Not only may this hypothesis help to explain why certain anti-TNF agents have not succeeded in clinical practice, also the superiority of infliximab/azathioprine combination treatment observed in the clinic in terms of remission induction and maintenance and mucosal healing [22] may be explained. The results obtained with combination treatment in the clinic correlate properly with the results we observed *in vitro*. We found increased numbers of Mϕ2 upon combination treatment, and interestingly, these macrophages also displayed stronger immunosuppressive properties. In addition, this hypothesis may help to resolve several side effect issues, for instance the increased risk of tuberculosis which is observed with anti-TNF antibodies. [23, 24] Remarkably, this increased risk is only observed with the anti-TNF antibodies, and not with the soluble TNF receptor etanercept, both in human [25] and in mice. [26] It has been shown that mycobacteria like *M. tuberculosis* bind stronger to Mϕ2 than Mϕ1, [27] and that the mannose receptor

CD206 plays a role in the phagocytosis of *M. tuberculosis*. [28] Interestingly, we only found an induction of Mϕ2 only in the conditions treated with anti-TNF antibodies, and in addition we observed an upregulation of CD206 on these induced cells. The results we obtained *in vitro* fit properly with the observations *in vivo*, and insight into these mechanisms provides new opportunities to challenge this problem in the future.

The immunosuppressive effect of Mϕ2 is probably mediated by a soluble factor, as we observe strong dose-dependent inhibition of T cell proliferation when we culture T cells with Mϕ2-conditioned medium (data not shown). However, we did not find wound-healing effects of Mϕ2-conditioned medium (data not shown) and this might suggest that the immunosuppressive properties and wound healing properties of Mϕ2 are mediated by distinct factors. Therefore, the exact mechanisms that underlie Mϕ2-induced effects are yet to be defined, but likely both soluble factors and cell-cell interactions are involved in the various processes. IL-10 has been proposed as a responsible factor in Mϕ2-induced amelioration of colonic disease in mice, [17] but it is less likely that IL-10 is the key factor leading to the results we observed *in vitro*. In our hands, Mϕ2 mainly produce large amounts of IL-10 in response to LPS, but we observed strong immunosuppressive and wound healing properties in the absence this stimulus. Therefore, we think that IL-10 may contribute to the effects, but is unlikely to be the key mediator. Several other factors have been described in the literature, including the induction of Treg, [29] but this mechanism was not involved in our *in vitro* experiments.

We found that patients that do not respond to infliximab therapy have lower amounts of Mϕ2 at baseline, and it is possible that these patients have a defect in the differentiation or the recruitment of Mϕ2. Identifying factors involved in this defect and Mϕ2 differentiation in general would allow the selection of patients for a certain therapeutic strategy. A recent paper showed that UC patients with high IL13Rα2 expression at baseline, show poor response to infliximab therapy. [30] IL13Rα2 has been described to function as a decoy receptor, and thereby prevents proper IL-13 signaling. [31] Interestingly, IL-13 in combination with IL-4 is a known inducer of regulatory macrophages, [32, 33] and disturbed IL-13 signaling might therefore result in reduced induction of regulatory macrophages.

It would be of great value to identify the factors that are responsible for the induction and function of Mϕ2, since this would facilitate the identification of a marker that reflects disease severity and therapy efficacy. In addition, the identification of responsible factors would possibly provide new treatment targets.

The findings described in chapter 4 and 5 may have further implications. The idea that binding to Fc receptors is a critical step in the induction of Mϕ2 suggests that the generation of an anti-TNF antibody with stronger affinity to the Fc receptor might have enhanced efficacy. Indeed, antibodies with higher affinity to Fc receptors have been developed in other fields of medicine in order to augment efficacy, [34, 35] and this approach may be promising in the development of new therapies with greater efficacy for the treatment of IBD as well.

Although the introduction of anti-TNF agents was an important development in the treatment of IBD, about 30% do not respond to this therapy, and about 30% lose response after a certain period of time. Because side effects and occasionally limited efficacy are still obstacles in the treatment of IBD, there is need for new therapies with less toxicity and higher efficacy. Mesenchymals stem cells (MSCs) are multipotential nonhematopoetic progenitor cells that can be isolated from various tissues, including the bone marrow. Like Mϕ2, MSCs

have immunomodulatory [36, 37] and wound healing [38-40] properties. Several mechanisms have been suggested that may be involved in these processes, including cell-cell dependent mechanisms as well as soluble factors. Interestingly, it has been described that MSCs are able to induce Mϕ2. [41-43] Because of their wound healing and immunomodulatory properties, these cells are of particular interest in various fields of medicine, including in the treatment of IBD. In **chapter six** we described the treatment of CD patients with autologous MSC in a Phase I trial. Importantly, MSCs from CD patients showed the same characteristics and immunomodulatory properties as MSCs from healthy controls. No serious side effects were reported during the study period, and a clinical response was observed in 3 patients; 3 patients needed surgery. Since all patients had severe refractory disease, it is complicated to speculate on efficacy. MSCs seem to have high potential to serve as an efficacious cell based therapy in IBD, and currently numerous studies are underway to further evaluate this.

Side effects and risk profiles: current therapy and future implications

As mentioned before, the drugs used for the treatment of IBD are not without risk. Therefore, a better understanding of the mechanism of action of IBD drugs enables the identification of factors leading to side effects. In addition, a careful evaluation of side effects and defining specific risk groups is important to limit side effects as much as possible. Cancer, and especially lymphoma, is a long-standing concern in patients treated with immunosuppressive agents.

In **chapter seven** we aimed to assess the risk of lymphoma in IBD patients and to find specific patient groups at risk. Also, we investigated whether we could establish an association between azathioprine use and lymphoma. In this retrospective, nation-wide study, we observed 44 lymphomas in a cohort of approximately 18000 IBD patients. Compared to lymphoma risk in the general population, the risk of lymphoma in IBD patients was not increased in this study. However, a clear increased risk was observed in patients in the age groups 35 – 39 and 45 – 49 and a significant correlation was found between thiopurine use and the development of EBV^+ lymphoma. None of the 44 cases used anti-TNF agents, but it is risky to draw any conclusions from that observation since this study was not designed to specifically study lymphoma risk in anti-TNF treated patients. Our data are in line with other population-based studies, [44-47] but in contrast to data from a meta-analysis [48] and a recent prospective study. [49] Discrepancies in outcome may result from differences in patient recruitment, study design and a different lymphoma incidence in the general population. The latter determines the calculated expected number and in that way the relative risk. Nevertheless, it has been estimated that only a ten-fold increased risk would outweigh the benefit of thiopurine treatment, [50] so although a slightly increased risk is found in some studies, this would still have limited clinical implications. On the other hand, since it appears that particularly patients ≤ 50 years have an increased risk, and patients with young onset of disease more often require intensive therapy, watchfulness is warranted in this vulnerable group. It is yet unknown why younger patients might have an increased risk, and which factors may be involved. In addition, it remains to be determined to what extent disease severity and disease duration contribute to lymphoma development, or whether this simply results from immunosuppression in general, or azathioprine in particular. Other

aspects like medication dose, therapy duration, and disease-related factors like disease location and age at onset of disease also may play a role. Although our study involved approximately 18000 IBD patients in the Netherlands, the relative small number of 44 lymphomas does not provide the power which is needed to calculate the contribution of these specific factors. This would further allow the stratification of patients for a certain therapy, especially with regard to side effects.
In summary, the studies described in this thesis provide new insight into our current knowledge of IBD therapy. Increasing knowledge of the working mechanism of anti-TNF agents, safety profiles, cell-based therapy and the identification of new possible drug targets, could eventually lead to personalized treatment with higher efficacy and less toxicity.

Reference List

1. Berry DL, Baehrecke EH. Growth arrest and autophagy are required for salivary gland cell degradation in Drosophila. Cell 2007;131:1137-1148.
2. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science 2000;290:1717-1721.
3. Birmingham CL, Smith AC, Bakowski MA et al. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J Biol Chem 2006;281:11374-11383.
4. Perrin AJ, Jiang X, Birmingham CL et al. Recognition of bacteria in the cytosol of Mammalian cells by the ubiquitin system. Curr Biol 2004;14:806-811.
5. Gutierrez MG, Master SS, Singh SB et al. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 2004;119:753-766.
6. Poppe D, Tiede I, Fritz G et al. Azathioprine suppresses ezrin-radixin-moesin-dependent T cell-APC conjugation through inhibition of Vav guanosine exchange activity on Rac proteins. J Immunol 2006;176:640-651.
7. Massey DC, Bredin F, Parkes M. Use of sirolimus (rapamycin) to treat refractory Crohn's disease. Gut 2008;57:1294-1296.
8. Dumortier J, Lapalus MG, Guillaud O et al. Everolimus for refractory Crohn's disease: a case report. Inflamm Bowel Dis 2008;14:874-877.
9. Schnitzler F, Fidder H, Ferrante M et al. Mucosal healing predicts long-term outcome of maintenance therapy with infliximab in Crohn's disease. Inflamm Bowel Dis 2009;15(9):1295-301.
10. Baert F, Moortgat L, Van Assche G et al. Mucosal Healing Predicts Sustained Clinical Remission in Patients with Early-Stage Crohn's Disease. Gastroenterology 2010;138(2):463-8.
11. Froslie KF, Jahnsen J, Moum BA et al. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. Gastroenterology 2007;133:412-422.
12. Daley JM, Brancato SK, Thomay AA et al. The phenotype of murine wound macrophages. J Leukoc Biol 2010;87:59-67.
13. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008;8:958-969.
14. Kamada N, Hisamatsu T, Okamoto S et al. Abnormally differentiated subsets of intestinal macrophage play a key role in Th1-dominant chronic colitis through excess production of IL-12 and IL-23 in response to bacteria. J Immunol 2005;175:6900-6908.

15. Smythies LE, Sellers M, Clements RH et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. J Clin Invest 2005;115:66-75.
16. Schebesch C, Kodelja V, Muller C et al. Alternatively activated macrophages actively inhibit proliferation of peripheral blood lymphocytes and CD4+ T cells in vitro. Immunology 1997;92:478-486.
17. Hunter MM, Wang A, Parhar KS et al. In Vitro-Derived Alternatively Activated Macrophages Reduce Colonic Inflammation in Mice. Gastroenterology 2010;138(4):1395-405.
18. Targan SR, Hanauer SB, van Deventer SJH et al. The Crohn's Dc. A Short-Term Study of Chimeric Monoclonal Antibody cA2 to Tumor Necrosis Factor {alpha} for Crohn's Disease. N Engl J Med 1997;337:1029-1036.
19. van Dullemen HM, van Deventer SJ, Hommes DW et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 1995;109:129-135.
20. D'Haens G, Van Deventer S, Van Hogezand R et al. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. Gastroenterology 1999;116:1029-1034.
21. Baert FJ, D'Haens GR, Peeters M et al. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. Gastroenterology 1999;116:22-28.
22. Colombel JF, Sandborn WJ, Reinisch W et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. N Engl J Med 2010;362:1383-1395.
23. Keane J, Gershon S, Wise RP et al. Tuberculosis Associated with Infliximab, a Tumor Necrosis Factor {alpha}-Neutralizing Agent. N Engl J Med 2001;345:1098-1104.
24. Denis B, Lefort A, Flipo RM, et al. Long-term follow-up of patients with tuberculosis as a complication of tumour necrosis factor (TNF)-alpha antagonist therapy: safe re-initiation of TNF-alpha blockers after appropriate anti-tuberculous treatment. Clin Microbiol Infect 2008;14:183-186.
25. Tubach F, Salmon D, Ravaud P et al. Risk of tuberculosis is higher with anti-tumor necrosis factor monoclonal antibody therapy than with soluble tumor necrosis factor receptor therapy: The three-year prospective French Research Axed on Tolerance of Biotherapies registry. Arthritis Rheum 2009;60:1884-1894.
26. Plessner HL, Lin PL, Kohno T et al. Neutralization of tumor necrosis factor (TNF) by antibody but not TNF receptor fusion molecule exacerbates chronic murine tuberculosis. J Infect Dis 2007;195:1643-1650.
27. Verreck FA, de BT, Langenberg DM et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. Proc Natl Acad Sci U S A 2004;101:4560-4565.
28. Kang PB, Azad AK, Torrelles JB et al. The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. J Exp Med 2005;202:987-999.
29. Savage ND, de BT, Walburg KV et al. Human anti-inflammatory macrophages induce Foxp3+ GITR+ CD25+ regulatory T cells, which suppress via membrane-bound TGFbeta-1. J Immunol 2008;181:2220-2226.
30. Arijs I, Li K, Toedter G et al. Mucosal gene signatures to predict response to infliximab in patients with ulcerative colitis. Gut 2009;58:1612-1619.

31. Rahaman SO, Sharma P, Harbor P et al. IL-13R(alpha)2, a decoy receptor for IL-13 acts as an inhibitor of IL-4-dependent signal transduction in glioblastoma cells. Cancer Res 2002;62:1103-1109.
32. Hart PH, Bonder CS, Balogh J et al. Differential responses of human monocytes and macrophages to IL-4 and IL-13. J Leukoc Biol 1999;66:575-578.
33. Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003;3:23-35.
34. Zalevsky J, Leung IW, Karki S et al. The impact of Fc engineering on an anti-CD19 antibody: increased Fcgamma receptor affinity enhances B-cell clearing in nonhuman primates. Blood 2009;113:3735-3743.
35. Horton HM, Bernett MJ, Pong E et al. Potent in vitro and in vivo activity of an Fc-engineered anti-CD19 monoclonal antibody against lymphoma and leukemia. Cancer Res 2008;68:8049-8057.
36. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-1822.
37. Krampera M, Glennie S, Dyson J et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 2003;101:3722-3729.
38. Stappenbeck TS, Miyoshi H. The role of stromal stem cells in tissue regeneration and wound repair. Science 2009;324:1666-1669.
39. Chen L, Tredget EE, Liu C et al. Analysis of allogenicity of mesenchymal stem cells in engraftment and wound healing in mice. PLoS One 2009;4:e7119.
40. Chen L, Tredget EE, Wu PY et al. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS One 2008;3:e1886.
41. Kim J, Hematti P. Mesenchymal stem cell-educated macrophages: a novel type of alternatively activated macrophages. Exp Hematol 2009;37:1445-1453.
42. Maggini J, Mirkin G, Bognanni I et al. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. PLoS One 2010;5:e9252.
43. Zhang QZ, Su WR, Shi SH et al. Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing. Stem Cells 2010;28:1856-1868.
44. Lewis JD, Bilker WB, Brensinger C et al. Inflammatory bowel disease is not associated with an increased risk of lymphoma. Gastroenterology 2001;121:1080-1087.
45. Askling J, Brandt L, Lapidus A et al. Risk of haematopoietic cancer in patients with inflammatory bowel disease. Gut 2005;54:617-622.
46. Persson PG, Karlen P, Bernell O et al. Crohn's disease and cancer: a population-based cohort study. Gastroenterology 1994;107:1675-1679.
47. Loftus EV, Jr., Tremaine WJ, Habermann TM et al. Risk of lymphoma in inflammatory bowel disease. Am J Gastroenterol 2000;95:2308-2312.
48. Kandiel A, Fraser AG, Korelitz BI et al. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. Gut 2005;54:1121-1125.
49. Beaugerie L, Brousse N, Bouvier AM et al. Lymphoproliferative disorders in patients receiving thiopurines for inflammatory bowel disease: a prospective observational cohort study. The Lancet 2009;374:1617-1625.
50. Lewis JD, Schwartz JS, Lichtenstein GR. Azathioprine for maintenance of remission in Crohn's disease: benefits outweigh the risk of lymphoma. Gastroenterology 2000;118:1018-1024.

Chapter nine

Appendices

Nederlandse samenvatting voor niet-ingewijden

Inflammatoire darmziekten

Crohn's disease (CD, ook wel ziekte van Crohn genoemd) en ulcerative colitis (UC, colitis ulcerosa) zijn chronische inflammatoire darmziekten (IBD, inflammatory bowel diseases). Patiënten hebben doorgaans klachten als buikpijn, diarree, bloed bij de ontlasting, gewichtsverlies en algehele malaise. De ziekte is chronisch, en is op dit moment helaas niet te genezen. De ziekte heeft een grote invloed op de kwaliteit van leven van de patiënt, en veel patiënten gebruiken chronisch medicatie die het immuunsysteem onderdrukken (immuunsuppressiva) en ondergaan een of meerdere operaties. Bij CD kan het hele spijsverteringsstelsel zijn aangedaan (dat wil zeggen van mond tot anus); UC betreft alleen de dikke darm. Zowel UC als CD kunnen op elke leeftijd voorkomen, maar de meeste nieuwe gevallen worden vastgesteld tussen het 20e en 40e levensjaar.

In de darm leven miljoenen bacteriën (de darmflora) die belangrijk zijn voor verschillende processen, en die ook een rol spelen in het afweersysteem. In de normale situatie reageert het afweersysteem niet op de darmflora, omdat dit zou leiden tot een ontsteking (een proces dat tolerantie wordt genoemd). In het geval van IBD reageert het afweersysteem toch op de darmflora, waardoor een afweerreactie in gang wordt gezet welke resulteert in een chronische ontsteking. Hoewel er veel onderzoek wordt verricht naar het ontstaan van beide ziekten, is het niet geheel duidelijk waarom de tolerantie wordt doorbroken. Het is bekend dat zowel CD als UC een sterke genetische component heeft, aangezien mensen die een familielid hebben met de ziekte, een grotere kans hebben om de ziekte ook te krijgen dan mensen die geen familielid hebben met de ziekte. Verschillende grote studies (Genome Wide Association Studies, GWAS) zijn uitgevoerd om defecten in genen op te sporen die mogelijk geassocieerd zijn met CD en/of UC. Er zijn defecten gevonden in genen die een rol spelen in verschillende componenten van het immuunsysteem, maar ook defecten in genen waarvan minder goed bekend is hoe dit geassocieerd is met de ziekte. Hoewel deze GWAS studies weliswaar aanwijzingen verschaffen over welke genen mogelijk betrokken zijn, blijken daarnaast ook omgevingsfactoren een belangrijke rol te spelen. Zowel UC als CD zijn dus ziekten die multifactorieel van aard zijn; dat wil zeggen dat er op dit moment niet duidelijk een verantwoordelijke factor aangewezen kan worden die leidt tot de ziekte.

De behandeling is voornamelijk gericht op het verbeteren van de kwaliteit van leven, het verminderen van hospitalisatie, operaties en steroïde-gebruik, en het behouden van klinische remissie. Vaak gebeurt dit met immuunsuppressiva, zodat de afweerreactie onderdrukt wordt. De medicatie die gebruikt wordt om de ziekte onder controle te krijgen is echter niet zonder bijwerkingen. De medicamenten die op dit moment deel uit maken van de behandeling van UC en CD, zijn onder andere 5-ASA, steroiden, methotrexaat, ciclosporine, azathioprine en anti-tumor-necrosis-factor-alpha (anti-TNFα) waaronder infliximab en adalimumab. Laatstgenoemden zijn antilichamen gericht tegen TNFα, een pro-inflammatoir stofje (cytokine) dat in grote mate aanwezig is in de darm van IBD patienten. Een anti-TNFα bindt TNFα en kan op deze manier TNFα neutraliseren.

Idealiter is een behandeling 100% effectief en zijn er geen bijwerkingen. Dit is zelden het geval. Tevens zou het in de ideale situatie mogelijk zijn om patiënten op basis van hun ziektekarakteristieken en/of genetisch profiel een behandeling-op-maat aan te bieden. Op de wijze zou een hogere effectiviteit bereikt kunnen worden en nadelige bijeffecten geminimaliseerd. Om dit in de toekomst te bewerkstelligen, is het belangrijk dat er meer bekend is over hoe CD en UC precies ontstaan, zodat gezocht kan worden naar nieuwe aangrijpingsmechanismen. Daarnaast is het van belang dat er meer inzicht wordt verkregen in het werkingsmechanisme van bestaande therapieën, opdat deze verder geoptimaliseerd kunnen worden. Verder is het noodzakelijk dat nadelige bijeffecten goed in kaart worden gebracht en dat mogelijke risicogroepen geïdentificeerd kunnen worden. Tot slot moet gezocht blijven worden naar nieuwe behandelingsstrategieën met mogelijk minder bijwerkingen die een uitkomst kunnen bieden voor patiënten die niet of slecht reageren op de huidige therapieën.

In dit proefschrift worden verschillende aspecten van huidige en toekomstige therapie bij IBD belicht.
In **hoofdstuk 2** worden verschillende behandelstrategieën besproken. Hierbij wordt onderscheid gemaakt tussen de step-up en top-down benadering. Bij een step-up behandeling wordt de behandeling op geleide van klachten en symptomen aangepast; indien de ziekte verslechterd wordt er een "sterker" medicijn aan de behandeling toegevoegd. Recente onderzoeken tonen echter aan dat in een bepaalde groep CD patienten het de voorkeur geniet om top-down te behandelen. Dit houdt in dat in een vroeg stadium van de ziekte wordt behandeld (early intervention) met medicijnen die anders pas later in de behandeling geintroduceerd zouden worden, dit betreft met name behandeling met infliximab en azathioprine. Vooral patienten die een verhoogd risico hebben op een gecompliceerd ziektebeloop zouden van de early intervention benadering kunnen profiteren. Een overzicht van de verschillende aspecten van deze behandelingsstrategieen alsmede mogelijke factoren die patienten met verhoogde risico op een gecompliceerd ziektebeloop kunnen identificeren worden in dit hoofdstuk besproken.
In **hoofdstuk 3** wordt een nieuwe rol voor autofagie in het ontstaan van de ziekte van Crohn beschreven. Autofagie ("autophagos" – zelf-eten) is van oorsprong een proces dat een belangrijke rol speelt bij de overleving van een cel. Uit verschillende GWAS bleek dat een bepaalde mutatie in het gen *ATG16L1* geassocieerd is met het ontstaan van CD. Het is bekend dat *ATG16L1* belangrijk is voor het proces van autofagie, maar nog onvoldoende hoe dit mogelijk gerelateerd is aan CD. In dit hoofdstuk onderzoeken wij hoe een defect in dit gen kan leiden tot een overmatige afweerreactie. Verlaagde levels van ATG16L1 in dendritische cellen (DCs, cellen die belangrijk zijn in de herkenning van een pathogeen en voor de aansturing van het adaptieve afweersysteem) bleek te resulteren in een hyperactieve DCs. Hierdoor werden T cellen extra geactiveerd hetgeen kan resulteren in een afweerreactie. Dit mechanisme zou in de toekomst meer inzicht kunnen verschaffen in nieuwe aangrijpingspunten bij patienten die een mutatie in dit gen hebben, bijvoorbeeld met medicijnen die het autofagieproces stimuleren of die de DC – T cel interactie verkorten.
In **hoofdstuk 4** wordt een nieuw werkingsmechanisme van infliximab beschreven. Het belangrijkste werkingsmechanisme van infliximab lijkt niet te berusten op de neutralisatie van TNFα, aangezien andere medicijnen die TNFα kunnen neutraliseren niet effectief

zijn gebleken in de behandeling van IBD. In dit hoofdstuk beschrijven we de inductie van macrophagen met wondgenezende eigenschappen die een rol kunnen spelen bij het genezen van ulcers in de darm. Deze inductie bleek afhankelijk te zijn van het Fc gedeelte van infliximab. Het Fc gedeelte is de "achterkant" van het antilichaam (dus niet de kant waar TNFα wordt gebonden en geneutraliseerd), en kan een interactie aangaan met Fc receptoren op onder andere monocyten. Deze interactie bleek cruciaal te zijn in de inductie van regulatoire macrophagen. Tevens kan dit model verklaren waarom sommige anti-TNFs wel effectief zijn in IBD en anderen niet. Deze observatie verschaft meer inzicht in de factoren die belangrijk zijn voor de effectiviteit van anti-TNF en kan mogelijk in de toekomst leiden tot een nieuwe generatie anti-TNF met grotere effectiviteit.
In **hoofdstuk 5** wordt verder gekeken naar de rol van infliximab-geinduceerde regulatoire macrophagen in het proces van wond genezing. We laten zien dat patiënten die een goede respons hebben op infliximab, grotere hoeveelheden regulatoire macrophagen in hun darm hadden na infliximab therapie dan patiënten die niet reageerden op infliximab. Tevens zijn infliximab-geinduceerde regulatoire macrophagen in staat om wondgenezing te bevorderen in een *in vitro*-model. Deze data suggereren dat regulatoire macrophagen geïnduceerd door infliximab inderdaad een rol spelen bij de wond genezing. Tevens is het bekend dat een hogere effectiviteit bereikt wordt indien infliximab in combinatie met azathioprine wordt gebruikt. In dit hoofdstuk laten wij zien dat er grotere hoeveelheden regulatoire macrophagen ontstaan indien azathioprine wordt toegevoegd aan infliximab. Daarnaast hebben de macrophagen die geïnduceerd zijn door de combinatietherapie sterkere immuunsuppressieve eigenschappen.
In **hoofdstuk 6** wordt een Fase I studie beschreven waarin 9 CD patiënten behandeld werden met mesenchymale stamcellen (MSC). Het doel van deze studie was om te onderzoeken of het haalbaar en veilig was om patiënten met MSCs te behandelen. MSCs zijn relatief gemakkelijk te isoleren en op te kweken, en hebben, net als regulatoire macrophagen, immuunsuppressieve eigenschappen. Door deze kenmerken zijn MSCs een potentiële kandidaat voor een nieuwe behandeling van CD. De behandeling met MSCs bleek haalbaar en veilig te zijn. Omdat deze studie patiënten includeerde die niet meer reageerden op de gangbare therapieën, is het moeilijk om te speculeren over effectiviteit; meer inzicht in de effectiviteit zal verkregen moeten worden uit toekomstige studies.
In **hoofdstuk 7** wordt onderzocht of patiënten met IBD een vergrote kans hebben op het ontstaan van kwaadaardige (maligne) lymfomen. In deze studie die ongeveer 18000 IBD patiënten includeert, werd geen algeheel verhoogd risico op lymfoom in IBD patiënten gevonden. Wel werd een verhoogd risico waargenomen in de leeftijdscategorieën 35 – 39 en 45 – 49. Daarnaast bleek er een sterke associatie te zijn tussen het ontstaan van EBV+ (Epstein-Barr positieve) lymfomen en het gebruik van azathioprine.
Samengevat, verschaffen de resultaten beschreven in dit proefschrift nieuwe inzichten in verschillende aspecten van de behandeling van IBD, welke besproken worden in **hoofdstuk 8**. De beschreven rol van autofagie in het ontstaan van CD en de inductie van regulatoire macrophagen door infliximab en infliximab/azathioprine combinatie therapie, kunnen leiden tot nieuwe aangrijpingspunten in de behandeling. Tevens is het risico op maligne lymfoom bij de IBD-patienten in kaart gebracht; welke in het algemeen niet verhoogd is maar in bepaalde leeftijdsgroepen toch moet leiden tot awareness. Tot slot is de experimentele behandeling met MSCs haalbaar en veilig gebleken, hetgeen aanleiding geeft tot verder onderzoek naar de effectiviteit van MSCs in de behandeling van CD.

List of publications

Anne Christine W. Vos, Manon E. Wildenberg, Marjolijn Duijvestein, Auke P. Verhaar, Gijs R. van den Brink and Daniel W. Hommes. Anti-TNF antibodies induce regulatory macrophages in an Fc region dependent manner. ***Gastroenterology***. 2011 Jan;140(1):221-230.e3

Anne Christine W. Vos, Noor Bakkal, Robert C. Minnee, Mariel K. Casparie, Dirk.J. de Jong, Gerard Dijkstra, Pieter Stokkers, Ad van Bodegraven, Marieke Pierik, C.Janneke van der Woude, Bas Oldenburg, Daniel W. Hommes for the 'Initiative on Crohn's and Colitis (ICC). Risk of Malignant Lymphoma in Patients with Inflammatory Bowel Diseases, a Dutch Nationwide Study. ***Inflammatory Bowel Diseases***, *in press*

Anne Christine W. Vos, Manon E. Wildenberg, Ingrid Arijs, Marjolijn Duijvestein, Auke P. Verhaar, Gert de Hertogh, Severine Vermeire, Paul Rutgeerts, Gijs R. van den Brink and Daniel W. Hommes. Regulatory macrophages induced by infliximab are involved in healing *in vivo* and *in vitro*. ***Inflammatory Bowel Diseases,*** *accepted for publication*

Anne Christine W. Vos, Herma H. Fidder, Gijs R. van den Brink and Daniel W. Hommes. Management of lack and loss of response to anti-tumor necrosis factor therapy. ***Inflamm Bowel Dis Monit 2009***; 10(2);43-52.

Anne Christine W. Vos and Daniel W. Hommes. Early intervention therapy in Crohn's disease. ***Springer IBD text book***, *under review*

Marjolijn Duijvestein, Anne Christine W. Vos, Helene Roelofs, Manon E. Wildenberg, Barbara B. Wendrich, Henricus W. Verspaget, Engelina M.C. Kooy-Winkelaar, Frits Koning, Jaap Jan Zwaginga, Herma H. Fidder, Auke P. Verhaar, Willem E. Fibbe, Gijs R. van den Brink, Daniel W. Hommes. Autologous bone marrow derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. ***Gut***. 2010;59(12):1662-9.

Manon E. Wildenberg, Anne Christine W. Vos, Simone C.S. Wolfkamp, Marjolijn Duijvestein, Auke P. Verhaar, Anje A. te Velde, Gijs R. van den Brink and Daniel W. Hommes. Dendritic cell autophagy attenuates the adaptive immune response by destabilizing the immunological synapse. *Manuscript in preparation*

Anne Christine W. Vos and Marjolijn Duijvestein. Report from the United European Gastroenterology Week/World Congress of Gastroenterology (Gastro 2009). ***Inflamm Bowel Dis Monit*** 2010;11(3);106-109

Anne Christine W. Vos and Marjolijn Duijvestein. Report from the United European Gastroenterology Week Barcelona 2010. ***Inflamm Bowel Dis Monit***. 2011;10(3);134-137

Marjolijn Duijvestein, Manon E. Wildenberg, Mick M. Welling, Anne Christine W. Vos, Simone Hennink, Tjalling Bosse, Eveline S.M. de Jonge-Muller, Helene Roelofs, Hein W. Verspaget, Willem E. Fibbe, Anje A. te Velde, Gijs R. van den Brink, Daniel W. Hommes. Pretreatment with interferon-gamma enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. ***Stem Cells***, *accepted for publication*

Marjolijn Duijvestein, Ilse Molendijk, Helene Roelofs, Anne Christine W. Vos, Auke P. Verhaar, Marlies E.J. Reinders, Willem E. Fibbe, Hein W. Verspaget, Gijs R. van den Brink, Manon E. Wildenberg, Daniel W. Hommes. Mesenchymal Stromal Cell Function is Not Affected by Current IBD Drugs. ***Cytotherapy***, *accepted for publication*

Curriculum Vitae

Christine Vos werd geboren op 18 juli 1985 te Utrecht. In 2003 behaalde zij haar diploma aan het Christelijk Gymnasium Utrecht. In hetzelfde jaar startte zij met de studie Geneeskunde aan de Universiteit Leiden. In het vierde jaar van haar studie deed zij een wetenschappelijk stage op de afdeling Maag-Darm-Leverziekten van het Leids Universitair Medisch Centrum onder supervisie van Prof. dr. D.W. Hommes naar de incidentie van maligne lymfomen bij IBD patiënten in Nederland, welke later ook onderdeel werd van haar proefschrift. In augustus 2007 begon zij onder supervisie van Prof. dr. D.W. Hommes met haar promotieonderzoek naar therapeutische aspecten van inflammatoire darmziekten in het laboratorium van de afdeling Maag-Darm-Leverziekten van het Leids Universitair Medisch Centrum, wat geresulteerd heeft in verschillende publicaties en de totstandkoming van dit proefschrift. Op dit moment is Christine bezig met co-schappen, en zij hoopt in het voorjaar van 2013 het arts-examen te behalen.

Christine Vos was born in Utrecht on the 18th of July 1985. In 2003 she finished her secondary education at the Christelijk Gymnasium Utrecht. She started Medical School at Leiden University in September 2003. During her fourth year, she did a research-internship at the department of Gastroenterology and Hepatology at the Leiden University Medical Center under the supervision of Prof. Dr. D.W. Hommes, investigating the incidence of malignant lymphoma in inflammatory bowel disease patients in the Netherlands, which became part of her thesis later on. In August 2007, she started her PhD at the research laboratory of the Department of Gastroenterology and Hepatology of the Leiden University Medical Center, investigating therapeutic aspects of inflammatory bowel diseases, which resulted in several publications and this thesis. At this moment, she is doing her clinical internships, and she hopes to graduate in Spring 2013.

Nawoord

Dit proefschrift is tot stand gekomen met hulp van velen. Door de aanwezigheid van zoveel leuke en talentvolle mensen om me heen is het gelukt om dit proefschrift succesvol af te ronden, en bleek promoveren behalve uitdagend vooral heel erg leuk te zijn! Om die reden wil ik graag een aantal mensen bedanken.
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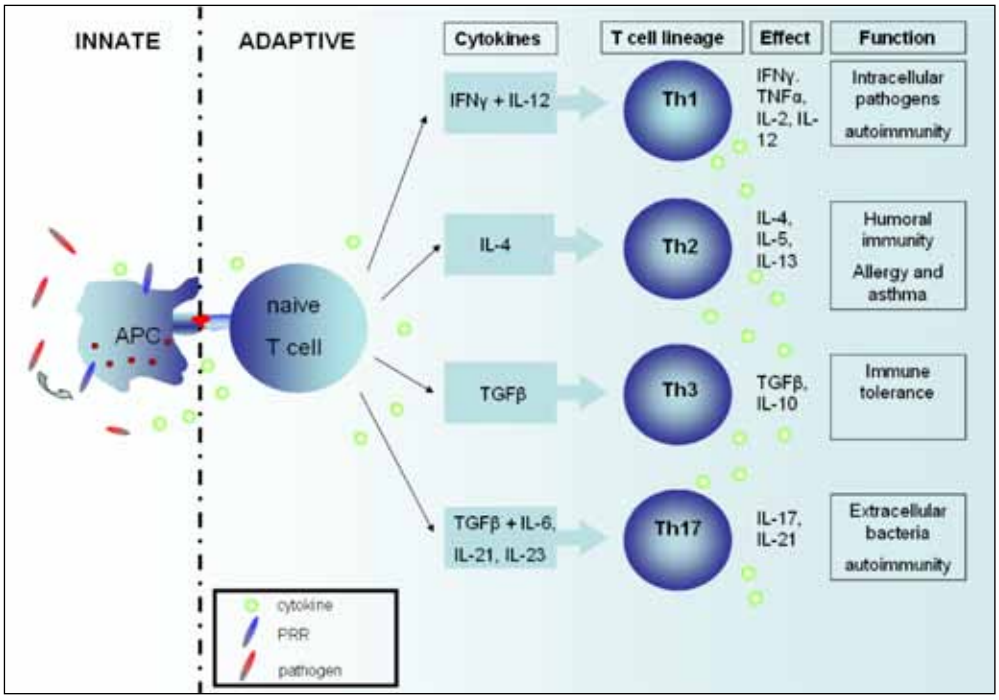
Tot slot wil ik graag mijn vrienden en familie bedanken. Mijn favoriete slogan “gezelligheid kent geen tijd” veranderde het laatste jaar soms in “tijd kent geen gezelligheid” en ik heb sommigen een beetje verwaarloosd. Dank voor jullie steun, vriendschap, gezelligheid en vertrouwen, hetgeen geen vanzelfsprekendheid is. Ik prijs me enorm gelukkig met zoveel lieve mensen om me heen! Dear Einhorn family: thanks for giving me a second home in the US! An, Atie en Rik: jullie support was en is van onschatbare waarde. Lieve Alexander, pap en mam, dank voor alles en nog heel veel meer. Het boekje is af, tijd voor een feestje!

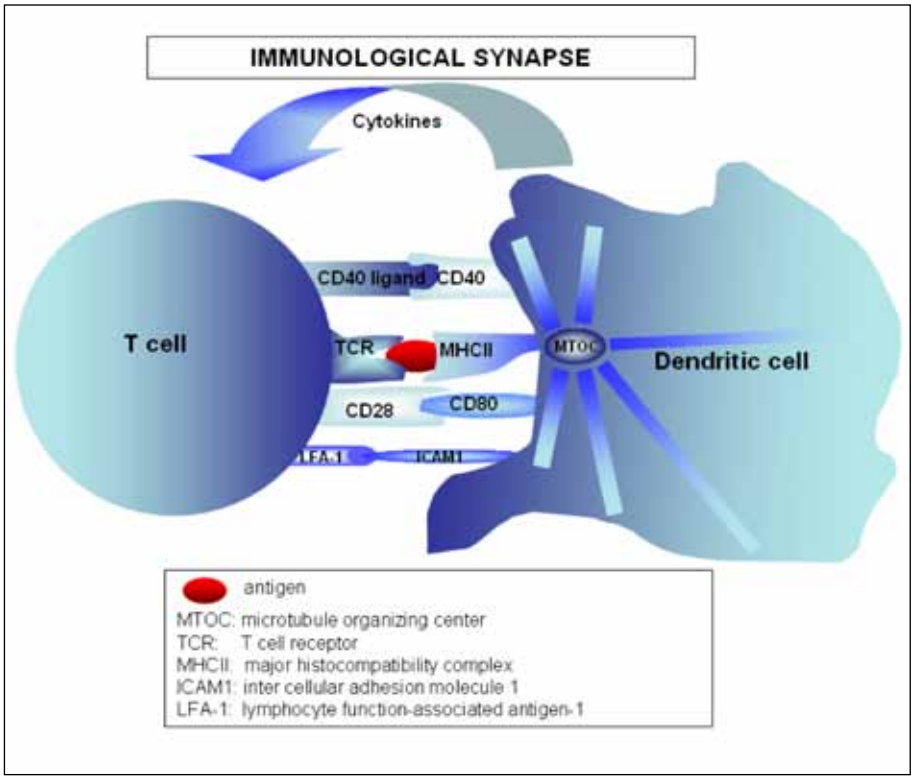
Anne Christine Wilhelmina Vos
Leiden, 8 september 2011

Full colour illustrations

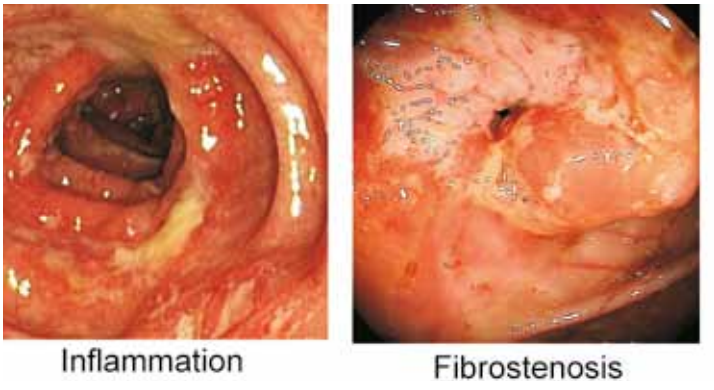
Chapter 1: Figure 1 Innate and adaptive immunity.



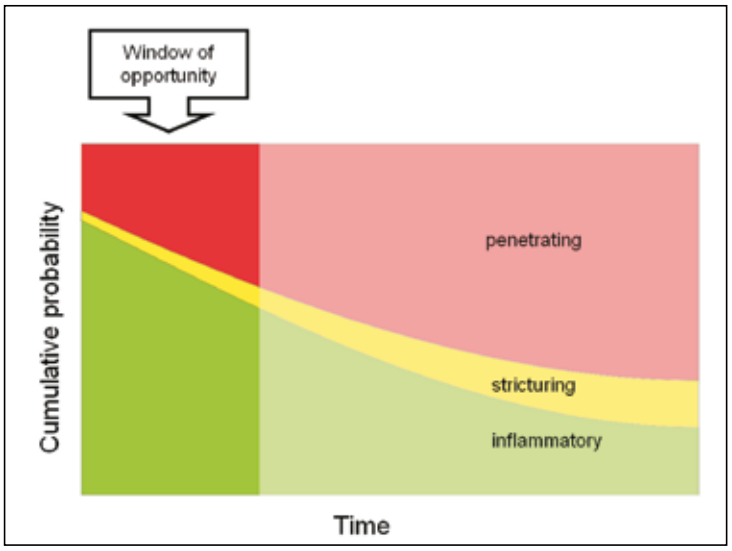
Chapter 1: figure 2 The immunological synapse.



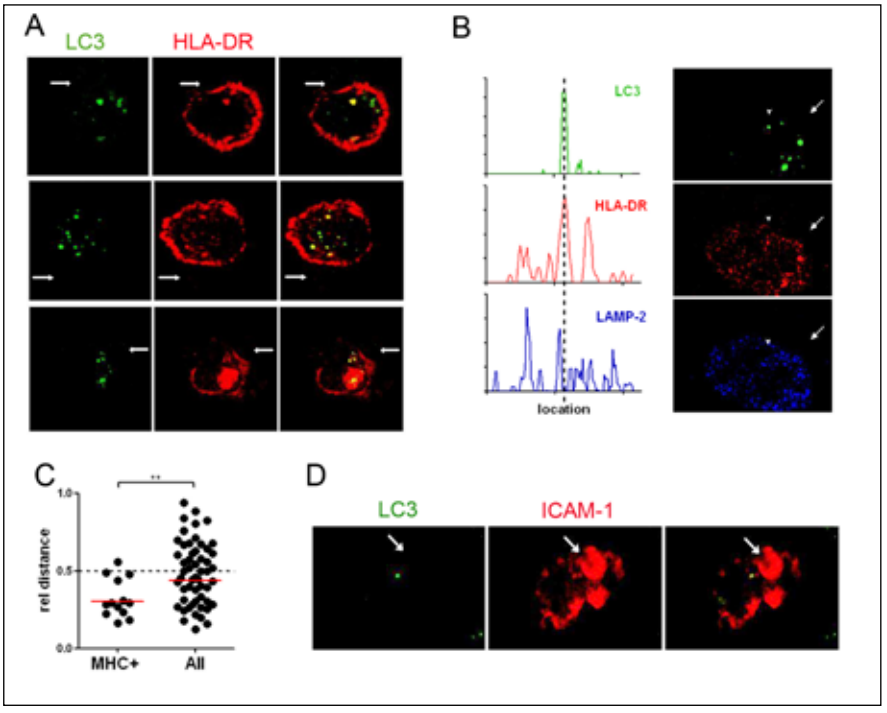
Chapter 2: figure 1 Inflammation (left panel) and fibrostenosis (right panel).



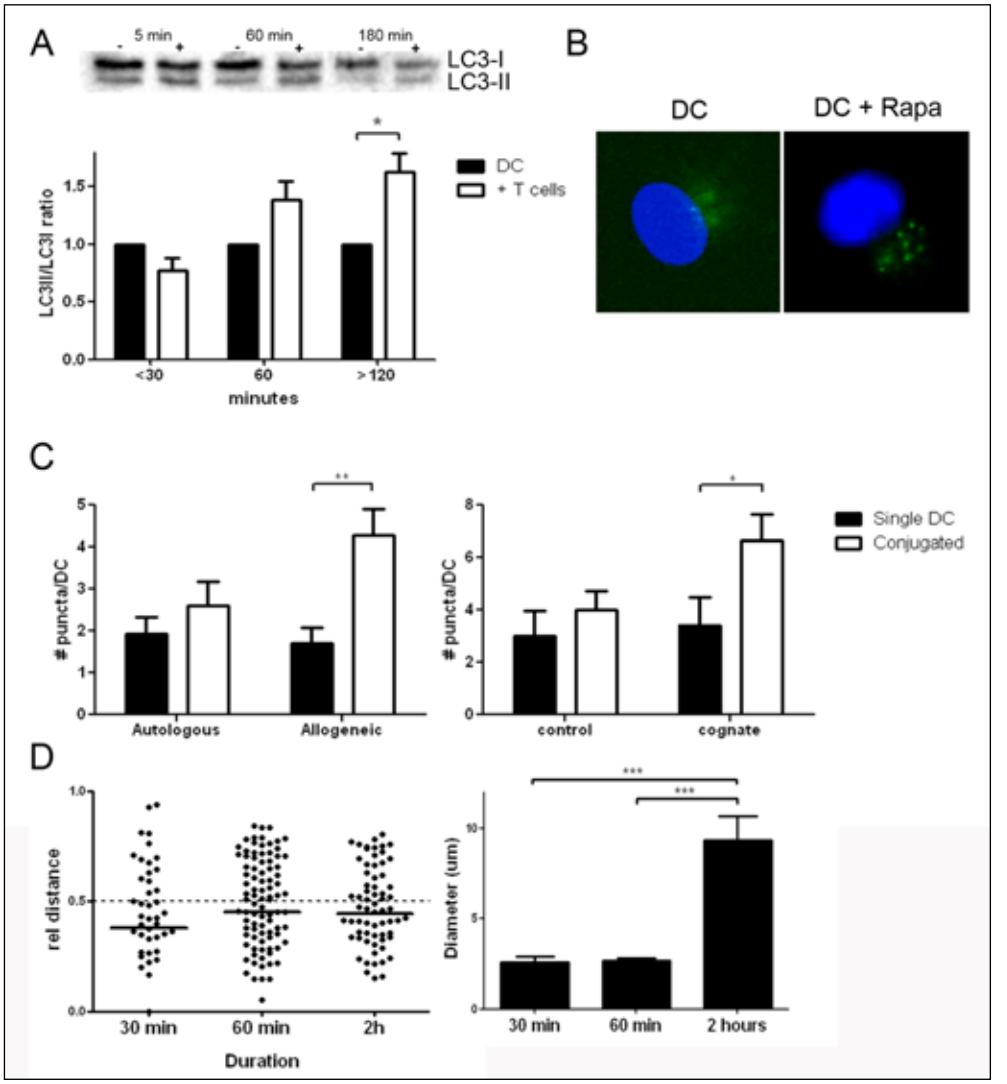
Chapter 2: figure 2 Window of opportunity.



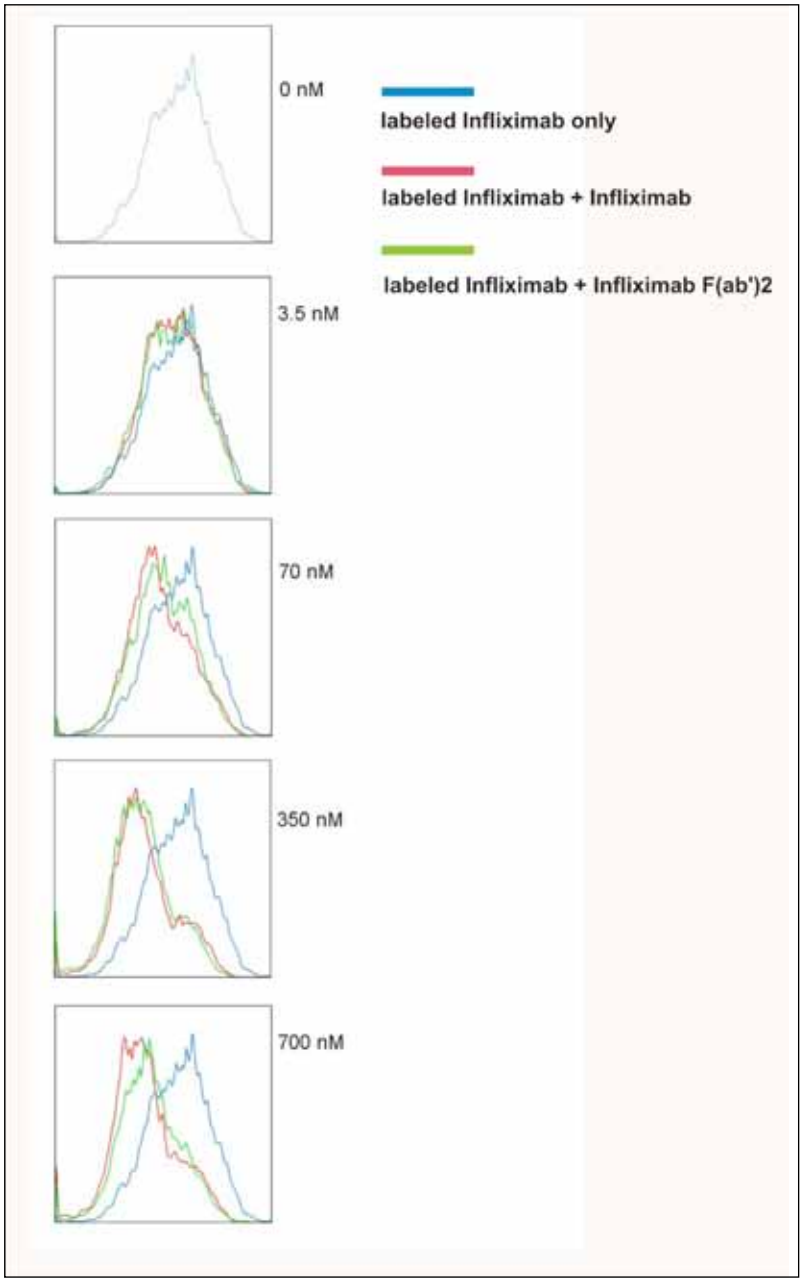
Chapter 3: figure 6 Autophagosomes are involved in degradation of synaptic components.



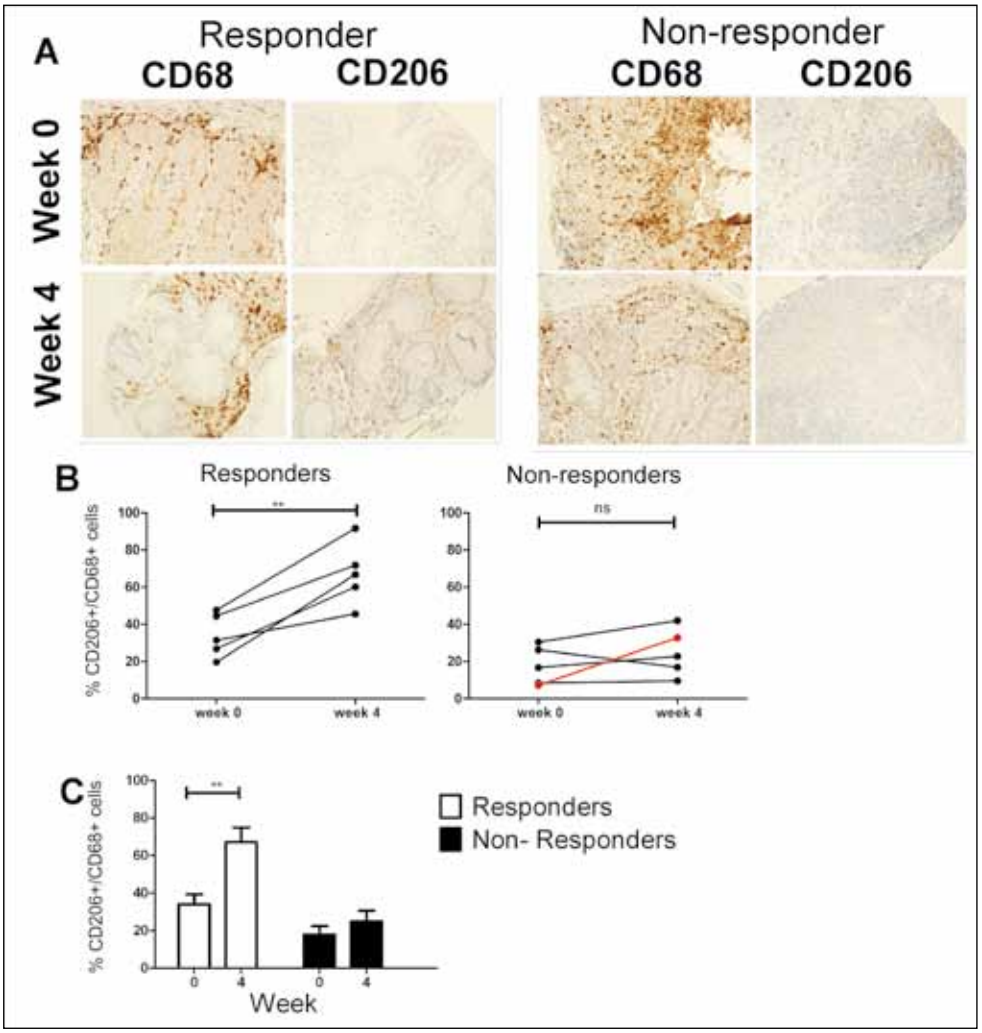
Chapter 3: figure 5 Immunological synapse formation induces autophagy in DC.



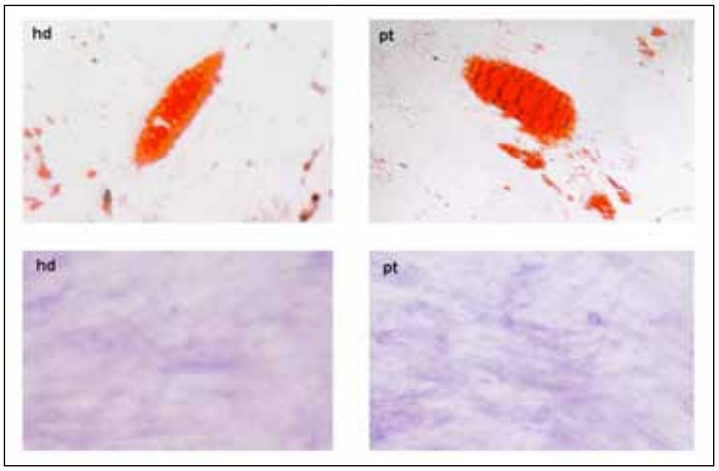
Chapter 4: supplementary figure 3.



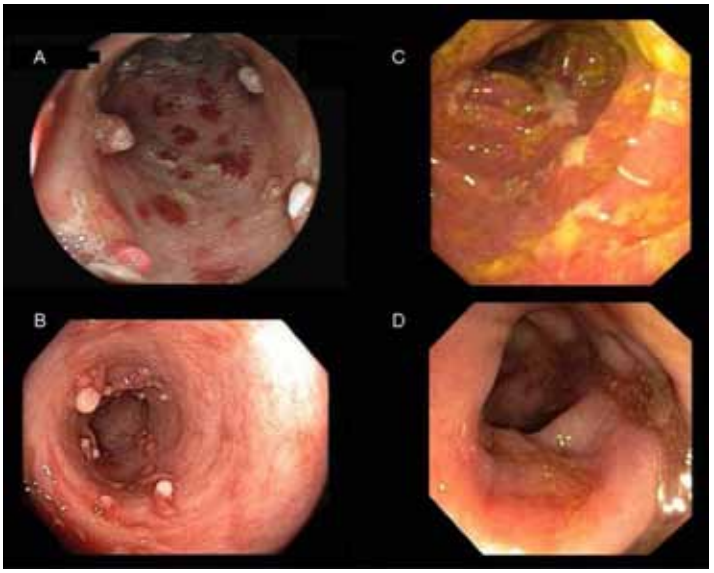
Chapter 5: figure 1 Infliximab induces regulatory macrophages in responders but not in non-responders.



Chapter 6: figure 2.



Chapter 6: figure 5 Endoscopy 0 (upper panels) and at 6 weeks (lower panels) after mesenchymal stromal cell (MSC) treatment (two administrations of 2x10∧6 autologous bmMSCs) shows clear mucosal healing. Pictures A and B are from patient 2, pictures C and D from patient 3.



Chapter 6: supplementary figure 1.