Adaptive Immune Responses in the Intestinal Mucosa of Microscopic Colitis Patients
I would like to dedicate this thesis to my parents and to microscopic colitis patients
ASHOK KUMAR KUMAWAT

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Abstract


Microscopic colitis (MC) is a chronic diarrhoeal disease of unknown aetiology, comprising collagenous colitis (CC) and lymphocytic colitis (LC). The nature of the adaptive local immune responses in the mucosa of MC patients is however far from elucidated. The present study investigates phenotypic and functional characteristics of the adaptive local immune responses in the colonic mucosa of these patients.

Our immunohistochemistry and flow cytometry studies (Paper I & II) demonstrated increased frequencies of CD8+ T cells in the colonic epithelium and lamina propria of both LC and CC patients compared to controls, whereas the frequencies of CD4+ T cells were unaltered or reduced. Our flow cytometry data revealed increased local activation of both CD4+ and CD8+ T cells in the lamina propria as well as the intraepithelial compartment of CC and LC patients compared to controls, demonstrated as increased proportions of these cells expressing the active/memory marker CD45RO and the proliferation marker Ki67.

Analysis of recent thymic emigrants by measuring T cell receptor excision circle (TREC) levels in the colonic mucosa of CC and LC patients revealed reduced TRECs levels in these patients compared to controls (Paper III). These results suggests that the observed increased numbers of T cells in the mucosa of CC and LC patients is due to the expansion of local resident T cells rather than direct recruitment of recent thymic emigrants to the mucosa.

Molecular analysis of T helper (Th) cell and cytotoxic T lymphocyte (Tc) mucosal cytokines at messenger and protein levels in the colonic biopsies from CC and LC patients demonstrated a mixed Th17/Tc17 and Th1/Tc1 mucosal cytokine profile and revealed significant differences in the mucosal cytokine levels in CC and LC patients compared to controls (Paper IV).

Finally, we have set up an in vitro model to investigate how the colonic milieu affects the activation and differentiation of T lymphocytes (Paper V). Our preliminary data indicate increased production of both pro-inflammatory and anti-inflammatory cytokines by peripheral blood T cells in the presence of soluble factors from the inflamed colonic mucosa of CC patients compared to controls.

Keywords: Microscopic colitis, collagenous colitis, lymphocytic colitis, intraepithelial lymphocytes, lamina propria lymphocytes, T cell receptor excision circle, T helper cells, cytotoxic T lymphocyte and mucosal cytokines.

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Svensk sammanfattning

Mikroskopisk kolit (MC) är en kronisk diarrésjukdom med okänd etiologi, uppdelad i diagnoserna kollagen kolit (CC) och lymfocytär kolit (LC). Kunskapen om det adaptiva immunsvaret lokalt i tarmslemhinnan hos dessa patienter är fortfarande mycket begränsad. I denna avhandling har vi fenotypiskt och funktionellt karakteriserat det lokala adaptiva immunsvaret i kolonslemhinnan hos patienter med mikroskopisk kolit.

Våra immunohistokemiska och flödescytometriska studier (delarbete I & II) visade på ökade frekvenser CD8+ T-lymfocyter i epitelet och i lamina propria från kolon hos både LC- och CC-patienter jämfört med kontroller, medan frekvensen CD4+ T-lymfocyter var oförändrad eller minskad. Data från de immunohistokemiska analyserna visade signifikant ökade mängder FOXP3+ celler i både epitelet och lamina propria från CC- och LC-patienter jämfört med kontroller. Data från de flödescytometriska analyserna visade på en lokal aktivering av både CD4+ och CD8+ T-lymfocyter i såväl lamina propria som intraepithelialt i CC- och LC-patienter jämfört med kontroller, påvisat genom deras ökade uttryck av cellytemarkörerna CD45RO och Ki67, associerade med aktiverade /minnesceller respektive prolifererande celler.

Vi analyserade även mängden T-lymfocyter som nyss lämnat thymus/brässen, så kallade thymusemi granter, genom att mäta mängden ”T cell receptor excision circles (TRECs)” i kolonslemhinnan hos CC- och LC-patienter. Vi fann då minskade mängder TRECs hos dessa patienter jämfört med kontroller (delarbete III). Dessa resultat tyder på att de ökade mängder T-lymfocyter i kolonslemhinnan hos dessa patienter vi observerade i delarbete I och II beror på en lokal expansion av T-lymfocyter i tarmslemhinnan, snarare än rekrytering av thymusemigranter till slemhinnan.

För att ytterligare karakterisera immunsvaret i slemhinnan hos patienter med mikroskopisk kolit analyserade vi mängden cytokiner från T-hjälpar (Th) lymfocyter och cytotoxiska T-lymfocyter (Tc) i biopsier från kolon- slemhinnan, både på mRNA och proteinivå. Vi fann en blandad Th17/Tc17- och Th1/Tc1- cytokinprofil i slemhinnan, samt även signifikanta skillnader mellan CC-/LC-patienter och kontroller vad gäller mängden cytokiner i slemhinnan (delarbete IV). Då vi i de tidigare delarbetena visat på ökade frekvenser CD8+ T-lymfocyter, men oförändrade eller minskade frekvenser CD4+ T-lymfocyter, är det sannolikt att den ökade cytokinproduktionen kommer från CD8+ T-lymfocyter i slemhinnan, såväl som de CD4+ T-hjälpar-lymfocyterna.
För att undersöka hur den lokala miljön i tarmslemhinnan hos patienter med kollagen kolit påverkar T-lymfocyternas aktivering och differentiering, satte vi i delarbete V upp en *in vitro*-modell för att undersöka hur lösliga faktorer i tarmslemhinnan påverkar CD4⁺ T-lymfocyter från periferblod efter polyklonal aktivering. Våra preliminära resultat visar på ökad produktion av både proinflammatoriska och antiinflammatoriska cytokiner från perifera T-lymfocyter i närvaro av lösliga faktorer från den infletterade kolonslemhinnan från patienter med kollagen kolit jämfört med kontroller.
List of Publications

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals I-V:


II. **Kumawat AK**, H. Strid, K. Elgbratt, C. Tysk, J. Bohr and E. Hultgren-Hörnquist. ”Microscopic colitis patients have increased proportions of Ki67+ proliferating and CD45RO+ active/memory CD8+ and CD4+8+ mucosal Tcells”. *Journal of Crohn’s and Colitis*;doi;10.1016/j.crohns.2012.08.014

III. **Ashok Kumar Kumawat**, Kristina Elgbratt, Curt Tysk, Johan Bohr and Elisabeth Hultgren-Hörnquist. "Reduced T cell receptor excision circle (TREC) levels in the colonic mucosa of microscopic colitis patients indicate local proliferation rather than homing of peripheral lymphocytes to the inflamed mucosa" *Submitted*

IV. **Kumawat AK**, H. Strid, C. Tysk, J. Bohr, and E. Hultgren-Hörnquist. “Microscopic colitis patients demonstrate a mixed Th17/Tc17 and Th1/Tc1 mucosal cytokine profile”. *Molecular Immunology* http://dx.doi.org/10.1016/j.molimm.2013.03.007

V. **Ashok Kumar Kumawat**, Curt Tysk, Johan Bohr, Olof Hultgren and Elisabeth Hultgren-Hörnquist. “An in vitro model for analysis of the impact of the colonic milieu in collagenous colitis patients on peripheral T lymphocyte activation and differentiation”. *In Manuscript*

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LIST OF ABBREVIATIONS

6-MP – 6-mercaptopurine
APC – antigen presenting cell
AZA – azathioprine
CARD – caspase activation and recruitment domain
CC – collagenous colitis
CCR – chemokine receptor
CCL – chemokine ligand
CD – Crohn’s disease
CTL – cytotoxic T lymphocyte
CM – conditioned medium
DC – dendritic cell
DTT – dithiothreitol
DNB – denuded biopsies
DNA – deoxy-ribonucleic acid
EDTA – ethylenediaminetetraacetic acid
ECM – extracellular matrix
ECP – eosinophilic cationic protein
FAE – follicle-associated epithelium
FOXP3 – forkhead box P3
GALT – gut-associated lymphoid tissue
GATA3 – GATA binding protein
GAPDH – glyceraldehyde phosphate dehydrogenase
GUSB – glucocordinase beta
HLA – human leukocyte antigen
IBD – inflammatory bowel disease
ICOS – inducible co-stimulatory molecule
IFN – interferon
Ig – immunoglobulin
IEL – intraepithelial lymphocyte
ILF – isolated lymphoid follicle
IL – interleukin
iNOS – inducible nitric oxide synthase
LC – lymphocytic colitis
LNSCs – lymph node stromal cells
LPL – lamina propria lymphocyte
LPMCs – lamina propria mononuclear cells
MC – microscopic colitis
MCP – monocyte chemoattractant protein
MHC – major histocompatibility complex
miRNA – micro RNA
MLN – mesenteric lymph node
MMP – matrix metalloproteinase
MΦ – macrophage
NF- nuclear factor
NKT – natural killer T cell
NOD – nucleotide binding oligomerization domain
NSAID – nonsteroidal anti-inflammatory drugs
PBL – peripheral blood lymphocyte
PP – Peyer’s patch
RA – retinoic acid
RNA – ribonucleic acid
ROR – retinoic acid receptor-related orphan receptor
RSS – recombination signal sequence
RTE – recent thymic emigrant
STAT – signal transducer and activator of transcription
T-bet – T-box transcription factor
TcR – T cell receptor
TGF – transforming growth factor
Th – T helper
TIMP – tissue inhibitor metalloproteinase
TNF – tumor necrosis factor
Treg – regulatory T cell
TRECs – T cell receptor excision circles
UC – ulcerative colitis
VEGF – vascular endothelial growth factor
INTRODUCTION

Microscopic colitis (MC) is a chronic diarrhoeal disease of unknown aetiology, and is currently receiving increasing attention in the scientific community. The colonic mucosa in MC patients is macroscopically normal or almost normal and the diagnosis relies on microscopic examination of colonic mucosal biopsies. MC was previously regarded as a rare disease, but firm epidemiological studies from Europe and North America have found a dramatic increase in the incidence of MC in the population, most apparently due to an increased clinical awareness and more frequent histopathological examination of colonic biopsies from patients with chronic diarrhoea.

The aetiology of MC is believed to be multi-factorial and mostly unknown. Although the increasing but still limited pathophysiological data in microscopic colitis are insufficient to define a firm pathophysiology of MC, it is postulated that MC is caused by disturbed immune responses to luminal antigen(s) in predisposed individuals. The nature of the adaptive local immune responses in the mucosa of MC patients is however far from elucidated. The present thesis investigates phenotypical and functional characteristics of the adaptive local immune responses in the colonic mucosa of these patients.

Microscopic colitis

Microscopic colitis comprises collagenous colitis (CC) and lymphocytic colitis (LC). Collagenous colitis was first described by the Swedish pathologist C. Lindström in 1976 (1) where he described a case report of a middle-aged woman with chronic diarrhoea whose colonic biopsies showed a sub-epithelial collagen layer comparable to that observed in collagenous sprue. The term “microscopic colitis” was introduced by Read et al. in 1980 to describe patients with chronic diarrhoea who had normal colonoscopy findings but showed mucosal inflammation on microscopic examination (2). In 1989 Lazenby et al. proposed the term lymphocytic colitis, where they described chronic diarrhoea patients with a normal colonoscopy who showed increased infiltration of lymphocytes in the epithelium upon histopathological examination (3). Both CC and LC have similar clinical symptoms and share histopathological features except for epithelial collagen layer in CC. They have therefore been grouped under the common term “microscopic colitis” (4). However, it is not clear whether CC and LC are two separate disorders or different manifestations of the same disease. Transition of LC to CC or vice versa has been reported (5). Additional subtypes of MC have also been reported, which share
similar clinical features to the classical MC but differ in their histopathological appearance. These subtypes of MC include MC with giant cells, paucicellular LC, cryptal LC, incomplete MC, pseudomembranous CC and MC with granuloma infiltration, as reviewed in (6-9).

Epidemiology
Population based studies on MC have been performed in several countries but mostly in Europe and North America. Long-term epidemiological studies from Sweden and North America since the 1980s demonstrate a rising incidence in the 1980s and early 1990s followed by a stable plateau phase in some centres (6-8). In Sweden, the present annual incidence rate of CC and LC is 5-6 cases per 100,000 individuals for each disorder (6-7). The reasons for this increase in disease incidence remains unclear, but most likely it could be due to greater awareness of clinicians and pathologists when diagnosing MC. Microscopic colitis can occur at any age group but most commonly affects middle-aged or elderly individuals with a noticeable female dominance. The epidemiological studies from our group on patients from Örebro County revealed that the average age at MC diagnosis was 65 (range 53-74) years and the female:male ratio was 7:1 (6).Williams et al. have reported that patients older than 65 years were more than five fold more likely to develop MC than the younger population (8).

Clinical Features
Clinically CC and LC cannot be differentiated from each other. The main symptom of both conditions is chronic non-bloody, watery diarrhoea, and is often associated with nocturnal diarrhoea (9). Abdominal pain and weight loss is significantly common. Furthermore faecal incontinence may affect these patients, which is one of the major factors for the low quality of life of these patients (9-11). The onset of CC can be sudden in about 40% of patients (11). It has been reported that clinical symptoms in LC are milder and more likely to disappear than in CC (12). MC is often associated with other autoimmune diseases such as celiac disease, rheumatoid arthritis, thyroid disease, or diabetes mellitus (6-7, 13).

Diagnosis
Diagnosis of MC mainly relies on histopathological findings as colonoscopic examination reveals a colonic mucosa that is mostly normal or has slight edema or erythema (14). The main histological features of LC...
(Fig. 1B) are increased numbers of intraepithelial lymphocytes (IELs) (≥ 20/100 surface epithelial cells) together with surface epithelial cell damage and infiltration of lymphocytes in the lamina propria, but a normal collagen layer in contrast to CC, where an increased collagen layer of >10 µm beneath the epithelium is a characteristic feature (7, 12). In uncertain cases CD3 immunostaining is performed to assess the IEL count to confirm the diagnosis (7). The histological findings of CC are in addition to increased numbers of lymphocytes in epithelium and lamina propria, presented with a deposition of a ≥10 µm thick subepithelial collagen layer (Fig. 1C). In uncertain cases tenascin immunostaining is used to measure collagen band thickness (15).

The thickness of the collagen layer varies in the colon and is found to be most prominent in the ascending and transverse colon whereas it may be absent in the sigmoid colon or rectum (16). Therefore rectal biopsies are not sufficient for the diagnosis and collection of multiple biopsies from different parts of the colon is recommended (17).
Mechanism(s) of Diarrhoea

The precise mechanism for diarrhoea in MC patients is not clearly understood. However the severity of diarrhoea has been correlated with the intensity of inflammatory changes in the mucosa but not with the thickness of the sub-epithelial collagen layer (18-19). Inflammation in the lamina propria was the first histological change to appear after restoration of bowel continuity in a CC patient who had undergone a temporary loop ileostomy (20). These findings suggest that diarrhoea in MC is inflammatory in origin. In addition, impaired electrolyte absorption and increased secretion has been reported in MC patients (19, 21).

Treatment

Based on the currently available data from clinical trials for the treatment of MC, oral budesonide is the best documented treatment that markedly ameliorates the clinical symptoms and improves the patient’s quality of life (22-23). Budesonide is effective in both CC and LC patients for inducing clinical remission and as long-term maintenance therapy (24-25). However upon withholding the budesonide treatment symptom relapse occurred in 40-80 % of patients within six months (22).

Immunomodulators that decrease inflammatory responses, such as azathioprine (AZA) or 6 mercaptopurine (6-MP) or methotrexate have occasionally have been used in steroid dependent severe case of MC (26-27). Antidiarrhoeals, such as loperamide or cholestyramine, have not been formally studied in randomised controlled trials, but are generally recommended as the first step of treatment in the patient with mild symptoms. (7) Other options include aminosalicylates, bismuth subsalicylate, antibiotics, probiotics and Boswellia serrata extract but the evidence for these alternatives is limited. Two case reports have demonstrated that anti-TNF therapy may be effective in severe MC patients (28-29). The surgical treatment in MC is very rare, but for severe and therapy resistant cases ileostomy may be an ultimate option.

The Mucosal Immune System

The small and large intestine in humans have distinct function, where absorption of nutrition occurs in small intestine, whereas the large intestine plays vital role in the absorption of water and salt. Human intestinal mucosa is home to a vast number of commensal bacteria, and at times also pathogens. The intestinal immune system plays a major role by responding to harmful pathogens while tolerating dietary antigens and beneficial
bacteria. The intestinal immune system contains the largest amount of lymphocytes in the whole body. These lymphocytes are spread in the epithelium, the intraepithelial lymphocytes (IELs) and in the lamina propria (LP), and in organized lymphoid tissues such as the Peyers patches (PP) located predominantly in the small intestine and isolated lymphoid follicles (ILFs) in both the small and large intestine, the latter with similar structure and function as Peyers patches and in mesenteric lymph node (MLN) (30-32). In addition to the immune cells, LP also contains e.g. fibroblasts, mesenchymal stromal cells and mucosal nerves.

The intestinal epithelium acts as a physical barrier, with a single layer of epithelial cells folding into villi and crypts (small intestine) or crypts (large intestine) that covers a surface area of approximately 400 m². This physical barrier is selective, allowing regulated passage of fluids and antigens. The function of the physical barrier is achieved by complex interactions between different cellular components. The epithelium has structures named tight junctions between epithelial cells, which are made up of complex proteins such as claudin, occludin, ZO-1, ZO-2, ZO-3, cingulin and 7H6, regulating permeability between the cells (33). The mucus produced by goblet cells prevents the bacteria to reach the epithelial surface and the Paneth cells located at the base of the crypts secrete antimicrobial proteins (e.g. defensins) (34).

A specialized follicle-associated epithelium (FAE) containing M cells is overlying the PP and ILFs. These specialized areas support the transport of antigens into the lamina propria for antigen presentation by dendritic cells (DC) or macrophages. The antigens are processed by DCs, which then either activate naïve T and B cells within the Peyer’s patches (PP) or migrate to MLN to activate naïve T cells (Fig. 2).

Activated B cells undergo maturation and differentiate into plasma cells that produce large amounts of immunoglobulin A (IgA) that is transported to the lumen. IgA produced by plasma cells is a dimer and is linked via a joining (J) chain. During transepithelial transport of IgA, the J chain binds to the poly-Ig receptor (pIgR) expressed by the intestinal epithelial cells. This complex is then actively transported to the intestinal lumen. In the lumen the receptor is cleaved by proteolysis, releasing the secretory component still attached to the IgA molecule, which is now termed secretory IgA (sIgA) (35). In the lumen, IgA binds to microbes and toxins and neutralize them by blocking their entry into the host.

The antigen experienced T cells undergo maturation and starts to express the activation/memory marker CD45RO. Mucosal DCs induce expression of the integrin α4β7 and CCR7 on lymphocytes in MLN (a hallmark of LP migrating and -resident lymphocytes). These activated
lymphocytes leave the MLN through the efferent lymph via the thoracic duct into the bloodstream and enter the lamina propria.

Figure 2. Antigen uptake in the small intestinal mucosa.

The epithelium acts as a physical barrier to entry of luminal antigens into the lamina propria (LP), but it also has specialized cells like follicle-associated epithelium (FAE) and M cells, both with a less pronounced brush border. The antigen enters via M cells and FAE into the PP. The antigens are processed by dendritic cells (DCs) which then either activate naïve T cells in Peyer’s patches (PP) or migrate to mesenteric lymph nodes (MLN) to activate naïve T cells. Activated T cells leave the MLN through the draining lymphatics via the thoracic duct and into blood stream and finally back to lamina propria (LP).

**Mucosal T cells**

In humans, mucosal T cells reside in the gut associated lymphoid tissues (GALT), such as the MLN, PP, the LP and the epithelium. Whereas MLN and PP contain naïve T cells, most T cells in the LP and especially in the epithelium have an effector/memory phenotype.
Mucosal T cells are divided into two major groups based on TcR and co-receptor expression (36), which are sometimes referred as conventional and non-conventional T cells. Conventional T cells consist of TcRαβ+ major histocompatibility complex (MHC) class II-restricted CD4+ and MHC class I-restricted CD8αβ+ T cells. These conventional T cells have previously been primed in response to an antigen in the periphery and have migrated to the intestine, where they reside in the LP as effector-memory T cells. The non-conventional T cells are either CD8αε+ or CD8αε- and express TcRαβ or TcRγδ, or are “double negative” (DN) TcRαβ+ cells that lack expression of both CD4 and CD8αβ (32). Generally, conventional T cells dominate the LP compartment, whereas non-conventional cells are predominant in the epithelium. I

IELs that reside within the epithelium are mostly T cells and their frequency varies along the intestine. In humans, there is approximately one IEL per 5-10 enterocytes in the small intestine compared to one IEL per 40 enterocytes in the large intestine (32). The majority of IELs in human is dominated by TcRαβ+ T cells with only a minority being TcRγδ IELs. Of the TcRαβ+ T cells, the majority of them are conventional CD8αβ+ T cells with only few CD8αε+ and CD4+ T cells normally present.

The TcRγδ+ T cells in humans is believed to help in elimination of stressed epithelial cells by recognizing the stress induced polymorphic antigens MICA and MICB (37). In mice, the non-conventional TcRαβ+ DN cells and CD8αε+ cells are believed to contain self reactive T cell receptors (38), however their role in the human gut is yet to be fully elucidated.

T cells in the human LP are mainly conventional CD4+ T cells with lower frequencies of CD8αβ+ T cells. In addition, there are also invariant natural killer T (NKT) cells and mucosa associated invariant T cells that interact with the non-classical MHC molecules CD1d and MR1 respectively (39-40).

Despite the effector/memory phenotype of these frontline T cells in the mucosa, it is evident that LPLs have reduced proliferation rate in response to TcR/CD3 mediated stimulation compared to peripheral T cells (41). This hyporesponsiveness response seems to be selective for TcR/CD3 mediated signalling, as CD2 or CD28-driven activation resulted in strong proliferation (42).

**Immunophenotype of lymphocytes**

The T cell population can be grouped into specific subsets based on their phenotype that is defined by the expression of diverse cell surface receptors. In humans, T cell activation markers such as CD45RA or CD45RO define naïve and memory/effector T cells, respectively, together
with different co-stimulatory molecules such as CD28. The naïve or resting T cells can be defined as CCR7\(^+\)CD45RA\(^+\)CD27\(^+\)CD28\(^+\), whereas antigen experienced T cells are defined as CCR7\(^-\)CD45RA\(^-\)CD27\(^-\)CD28\(^-\) (43). Upon activation T cells express CD69 in the early stage whereas in the late stage they express CD45RO.

The B cells are most commonly characterized by expression of surface markers including CD19, CD20, CD27 and CD38. The naïve B cells are defined as CD19\(^+\)CD20\(^+\)CD27\(^-\)CD38\(^-\) and memory B cells are defined as CD19\(^+\)CD20\(^+\)CD27\(^-\)CD38\(^-\) whereas the plasma cells are defined as CD19\(^+\)CD20\(^-\)CD27\(^+\)CD38\(^+\)CD138\(^+\) (44-46). Generally in textbooks plasma cells are described as CD19 negative. However, many studies report that CD19 is indeed expressed on plasma cells (45, 47-48). Yet, Harada et al reported that malignant plasma cells are negative for CD19 expression.

**T cell differentiation**

T lymphocytes mostly consist of CD4\(^+\) and CD8\(^+\) T cells. The CD4\(^+\) T cells are the main conductors of immune responses through the production of specific cytokines. They carry out various immunological processes including promotion of maturation of B cells into plasma cells and activation of cytotoxic T lymphocytes (CTLs), macrophages and non-immune cells, but they also play a critical role in suppression of immune responses. The differentiation of different CD4\(^+\) T helper (Th) subsets is orchestrated by a complex network of transcription factors and specific cytokines.

T cells undergo differentiation when their TcR interacts with antigens bound to the MHC molecules expressed by DCs or other antigen presenting cells (APCs). Co-stimulatory molecules such as CD28 and the inducible co-stimulatory molecule (ICOS), amplifies TCR signalling, thereby promoting T cell proliferation and differentiation.

When activated, naïve CD4\(^+\) T cells differentiate into effector cells including the Th1, Th2 and Th17 subsets and induced regulatory T cells (iTreg) (see below) (Fig. 3). Each subset has specific roles in promoting immune responses towards particular pathogens. Th1 cells mainly produce interferon (IFN)-\(\gamma\), lymphotoxin and tumor necrosis factor (TNF) and activate macrophages and neutrophils to better combat intracellular pathogens, but they are also implicated in autoimmune inflammation. Th2 cells, producing e.g. interleukin (IL)-4, IL-5, IL-10 and IL-13 promote clearance of extracellular parasitic infections and they are also involved in allergic inflammation.
The Th17 cells produce mainly IL-17A but also IL-17F, IL-21, IL-22 and IL-26, and are abundant at the intestinal mucosa. Th17 cells protect the host against pathogenic bacteria and fungal infections, partly by maintaining the intestinal barrier function by inducing the tight junction protein claudin as well as promoting mucus production (49). The Th17 cells have however been implicated as important mediators in mucosal inflammation and other various inflammatory diseases, by their promotion of pro-inflammatory cytokine production as well as recruitment and activation of neutrophils.

The Treg cells suppress T lymphocytes, DCs and NK cell via cell-to-cell contact. In addition they secrete anti-inflammatory cytokines such as IL-10, transforming growth factor (TGF)-β and IL-35 that play important roles in suppression of immune responses (50-53).

The T-box transcription factor (T-bet) promotes development of the Th1 lineage, but also suppresses the development of other cell lineages (54). Both IFN-γ and IL-12 play a vital role in maturation of Th1 cells. Augmented levels of IL-12, secreted by APCs, ensure expansion of Th1 cells. T-bet induces IFN-γ production via the signal transducer and activator of transcription 1 (STAT1), whereas IL-12 induced STAT4 promotes IFN-γ production that further ensures expansion of Th1 cells (52).

The differentiation of Th2 cells is driven mainly by IL-4. IL-4 induces STAT6 that up regulates the expression of the main Th2 lineage regulator GATA binding protein (GATA3) (55). In addition GATA3 suppress Th1 differentiation by downregulating STAT4 (56).

The Th17 differentiation is mainly orchestrated by the transcription factor retinoic acid-related orphan receptor (ROR)C2 in humans and RORγt in mice as well as RORα and STAT3 (51, 57). The Th17 cells differentiates in response to the STAT3 activating cytokines IL-6, IL-21 and IL-23 together with IL-1β and TGF–β (58).
Figure 3. Development of T helper (Th) 1, Th2, Th17 and induced regulatory T cells (iTreg cells) from naive CD4\(^+\) T cells. Cytokines inducing the development of Th1, Th2, Th17 and iTreg cells are marked in red. The main effector cytokines of these four cell subsets are marked in blue. ICOS, inducible co-stimulatory molecule; T-bet, T-box transcription factor; GATA3, GATA binding protein; RORC2, retinoic acid receptor-related orphan receptor C2; FOXP3, forkhead box P3.

The natural Tregs are formed in the thymus as a distinct lineage, whereas induced Treg (iTreg) are induced in peripheral organs after antigen priming. The iTreg lineage is regulated by the transcription factor forkhead box P3 (FOXP3) (59). TGF–\(\beta\) is the master regulator of iTreg/Th17 lineage commitment (60). Abundant amounts of TGF–\(\beta\) induces FOXP3 specific iTreg differentiation, whereas at low concentration and in the presence of IL-6, TGF–\(\beta\) induces Th17 cell differentiation (52, 60). STAT5 induces IL-2 downstream signalling and enhances FOXP3 expression ensuring selective iTreg differentiation (52).

The Th subsets were previously believed to be terminally differentiated, but it has now become apparent that some Th subsets display plasticity between them. Whereas Th1 and Th2 have relatively stable phenotypes, iTreg and Th17 can readily switch to other Th subsets. iTreg cells can...
switch to Th17 cells under the influence of IL-6 and Th17 cells can switch into Th1 cells in the presence of IL-12 (61-62). Recently a subset of Th17 cells has been identified that co-produces IL-17 and IFN-γ possibly representing an intermediate Th17/Th1 phenotype (63). These IL-17/IFN-γ producing T cells have been implicated in the pathogenesis of inflammatory bowel disease (64).

CD8+ T cells differentiate into cytotoxic T cells (CTLs) upon activation by antigen. These CTLs play an important role in inducing FasLigand- or Granzyme mediated apoptosis in cells infected with intracellular bacteria or viruses, as well as (tumor) transformed cells. Different subsets of CD8+ CTLs (Tc) have also been described, such as IFN-γ producing Tc1 and IL-4, IL-5 and IL-10 producing Tc2. Tc1 and Tc2 cell development is driven by the transcription factors T-bet and GATA3 respectively (65-67). The Tc1 maturation is stimulated by IL-12 and IFN-γ while Tc2 maturation is driven by IL-4 (65-67). More recently IL-17 producing CD8+ T cells, so called Tc17 cells, have also been described (67-70). The Tc17 cells produce IL17, IL-21, IL-22, IFN-γ and TNF-α and express the IL-23R, the chemokine receptor CCR6 and the transcription factor RORC2 (humans) or RORγt (mice) (67-71).

Recent Thymic Emigrants and T cell Receptor Excision Circles (TRECs)

The thymus provides a specialized microenvironment for the maturation and selection of the vast majority of functional T cells. The T cell receptor gene rearrangement is a critical step in the development of mature T cells. The T cell receptors are associated with the CD3 subunits γ, δ, ε, and ζ. Generation of the T cell repertoires with diverse antigen specificities is achieved by random rearrangement of TcR gene segments termed V, D and J for variable, diversity and joining, respectively, in the thymus. TcR α and γ chains consist of V and J gene segments, whereas TcR β and δ chains consists of V, D and J gene segments (72). This process is initiated by recognition of recombination signal sequences (RSS) that flank the coding sequences on DNA and during this process the two signal ends are circularized, forming an extrachromosomal circular excision product, which are known as a T Cell Receptor Excision Circles – TRECs (72).

In the formation of TcR αβ, the β chain is rearranged first and then pairs with a pTα (also termed pre-T-α) chain. During the gene rearrangement for formation of a functional TcR α chain two TREC variants, one single joint TREC (sjTREC) and one coding joint TREC (cjTREC) are formed.
simultaneously resulting in the deletion of the TcR δ locus which is situated within the TcR α locus (73).

The TRECs are relatively stable but are not replicated during mitosis, and consequently they are diluted in each cell division (74). Therefore, TRECs levels are a direct reflection of the amount of recent thymic emigrants (RTE) in the periphery. The newly exported RTEs in the periphery contain high amounts of TRECs compared with the peripheral T cells that have undergone various rounds of cell division resulting in diluted TREC content. The cell division rate can be analyzed by evaluating the expression of proliferation marker Ki67, a nuclear antigen that is expressed in all dividing cells and absent in resting cells (75). Analysis of both T cell proliferation and TREC content in peripheral lymphocytes for quantification of T cells that have recently left thymus is a good measurement of thymic output.

TRECs measurements are used extensively to document T cell reconstitution following treatment of HIV infection and in patients who have undergone haematopoietic stem cell transplantation (75-76). Previous studies by our group have used TRECs measurements to quantify the amount of recently matured T cells in the periphery in both IBD patients and mouse models of colitis (77-78).

**Intestinal Homeostasis**

The normal appearance of the gastrointestinal mucosa is in state of “physiological inflammation” as it contains large number of leukocytes in the epithelium and subepithelial compartments (79). This is mostly due to an immune response to harmful pathogens while dietary antigens and beneficial bacteria are normally tolerated. At the cellular level the mucosa contains a network of complementary regulatory interactions between different types of immune and non-immune cells as well as the microbiota to maintain mucosal homeostasis in the gut (Fig 4).

The human gut is home to the largest collection of microbes. The distal gut is densely populated with approximately $10^{12}$ organisms per milliliter or gram of luminal contents (80). This microbial community is mainly dominated by bacteria and among the 100 known phyla, members of Firmicutes and Bacteroidetes are the most prevalent in the distal gut community (81). Therefore the synergistic relationship between the host immune system and its microbiota should be seen as a functional entity.

Pattern recognition receptors, e.g. Toll like receptors and NOD-like receptors that are expressed by epithelial cells, M cells and DCs can recognize conserved structures on microbes. There is a complex crosstalk
between the intestinal epithelial cells, the microbiota and the intestinal immune cells, where the microbiota defines the development and choices of immune cells, suggesting a coevolution of the immune system with the microbiota in vertebrates (50, 80). In addition, studies in mice have revealed that mice raised under germ free conditions have an underdeveloped intestinal immune system and that reconstitution of the gut microbial flora from conventionally raised mice restores the mucosal immune system (82). In addition, the commensal flora protects the host from pathogen colonization and also provides extra nutrients to the host by metabolizing a broader range of dietary components such as complex carbohydrates.

Intestinal stromal cells, important in tissue regeneration and wound repair can communicate with the epithelial and immune cells (83-84). A recent study reported that direct presentation of intestinal self antigens by lymph node stromal cells (LNSCs) resulted in activation and deletion of CD8+ T cells but not CD4+ T cells, suggesting a role of LNSCs in the regulation of CD8+ T cell mediated peripheral tolerance in the intestine (85). Human intestinal mucosal microvascular endothelial cells from patients with inflammatory bowel disease (IBD) were shown to have increased adhesiveness for leukocytes compared to the microvascular endothelial cells from normal mucosa (86).

It is now believed that the regulatory interactions in the gut mucosa occur in the midst of a complex mixture of proteins, known as the extracellular matrix (ECM). The major constituents of ECM include collagens, laminin, fibronectin, glycoproteins, tenascin, proteoglycans, elastin and others (79, 87). The ECM together with the mucosal soluble mediators such as cytokines and growth factors derived from mesenchymal cells, immune cells and epithelial cells regulate cell differentiation, proliferation and apoptotic processes (87). It has been suggested that the composition of ECM determines which type of cell surface receptors are expressed on leukocytes, thereby controlling the quantity of and state of activation of the leukocytes in local tissues (88). ECM components are degraded by proteolytic activity of matrix metalloproteinases (MMPs). Recent studies have implicated the involvement of human gut microbiota derived proteolytic activity with the capacity to engage in degradation of ECM components, adding a new branch to the network of complex interactions in intestinal homeostasis (89).
Figure 4. Components of Intestinal Homeostasis. Different types of immune and non-immune cells as well as the microbiota interact with each other to maintain intestinal homeostasis.
Pathogenesis of Microscopic colitis

The aetiology of MC is mostly unknown but believed to be multi-factorial. However it is postulated that MC is caused by disturbed immune responses to luminal antigen(s) in predisposed individuals.

Luminal Factors

The involvement of luminal agents in MC is best demonstrated by the fact that diversion of the faecal stream via loop ileostomy leads to a normalized clinical and histopathological response (20, 90). Upon restoration of bowel continuity, both clinical symptoms and histopathological findings of MC recurred (20, 90).

Drug consumption has been implicated in inducing MC. (7, 91). Nonsteroidal anti-inflammatory drugs (NSAIDs), aspirin, acarbose, cyclo3fort, lansoprazole, ranitidine, ticlopidine and sertraline have all been suggested to be strongly associated with microscopic colitis (91).

Involvement of microbes in MC pathogenesis has been discussed (92-94). Case reports on associations between MC and Clostridium difficile and Yersinia enterocolitica have been reported (92-93), but there are no consistent data available on specific pathogens or bacterial products in the MC pathogenesis.

Bile acid malabsorption can contribute to MC pathogenesis and aggravates clinical symptoms. It has been associated with MC in 27-44% of CC patients and in 9-60% of LC patients (95).

Mucosal Factors

Immunohistochemical data have revealed increased infiltration of CD3+ T cells in the lamina propria (LP) and the intraepithelial compartment of both CC and LC patients (96-97). Both these studies also reported that the infiltration in the epithelium is dominated by CD8+ T cells expressing the αβ T cell receptor (96-97). Furthermore, a Th1 cytokine profile has been reported, with elevated mucosal mRNA levels of TNF-α, IFN-γ and IL-15 but not IL-2 or IL-4 in the mucosa of both LC and CC patients (98). However, immunohistochemical analysis of the mucosa of LC patients showed that the majority of lamina propria CD4+ T cells expressed the Th2 transcription factor GATA-3, whereas CD8+ T cells expressed both the Th1 transcription factor, T-bet and GATA-3 at similar levels (99). In contrast, in the epithelium the majority of CD8+ IELs expressed T-bet (99). Recently it was demonstrated that CD4+ and CD8+ intestinal T cells had reduced expression of the activation marker CD69 in patients with active CC (100).
Active nuclear factor (NF) κB levels are increased in mucosal epithelial cells of CC patients (101). Significantly increased concentrations of luminal nitric oxide have been observed, with an increase in inducible nitric oxide synthase (iNOS) (98, 102). Infusion of the iNOS inhibitor N\textsuperscript{G}-monomethyl-L-arginine in the colon of CC patients decreased fluid secretion, suggesting that NO is involved in the diarrhoea in CC patients (103).

Apart from lymphocytic infiltration, increased numbers of functionally active eosinophils have been detected in the mucosa of CC patients (100), as well as augmented luminal levels of eosinophilic cationic protein (ECP) in perfusion fluids from the colon (104).

Several mechanisms have been proposed to explain the altered collagen deposition in CC patients, but no single mechanism has emerged. Increased numbers or activity of myofibroblasts could result in an increased collagen synthesis in CC (105). Reduced mRNA levels of the matrix degrading enzymes MMP1 and 13, and increased expression of tissue inhibitor of metalloproteinase (TIMP)-1, suggests impaired collagen degradation in CC (106).

In CC patients infiltrating eosinophilic granulocytes have increased mRNA expression levels of TGF-β1 and this may affect the connective tissue remodelling (107). Furthermore, enhanced luminal levels of the potent fibrosis enhancing vascular endothelial growth factor (VEGF) has been found in the colonic mucosa of CC patients (108).

**Genetics**

The role of genetic factors in MC still remains unclear. Occurrence of MC within families has been reported (109). An increased prevalence of human leukocyte antigen (HLA)-DQ2 has been reported in both CC and LC patients (110-111). In addition, an increased frequency of HLA-A1 and a decreased frequency of HLA-A3 have been found in LC but not in CC patients (112). Polymorphism in the gene encoding TNF-α has been reported in both CC and LC patients (111). In addition polymorphism in the MMP-9 gene has been associated with CC (113). In contrast to Crohn’s disease, no association with NOD2/CARD15 has been observed in CC patients (114). Koskela et al. analyzed the frequency of polymorphism in the IL-6, IL-1β, IL-1RA, IL-10 and CD14 genes in MC, and showed that the IL-6-174-GG allele was more prevalent in both CC and LC patients compared to the controls, but did not find any significant association between polymorphisms in the other cytokine genes and MC (115).
T cells and Inflammatory Bowel Disease

Dysregulated immune responses in the intestine can lead to chronic inflammatory diseases such as inflammatory bowel disease (IBD). IBD is mainly divided into two entities; Crohn’s disease (CD) and Ulcerative colitis (UC) and causes a chronic inflammation characterized by acute flares followed by remission. CD can affect any part of the gastrointestinal tract in a discontinuous manner, whereas UC is primarily affecting the colon and rectum. The hallmark of active inflammatory bowel disease is a pronounced infiltration of innate immune cells; neutrophils, macrophages, dendritic cells (DCs) and natural killer T (NKT) cells as well as T and B lymphocytes in the lamina propria (116). The disease is found globally, but a dramatically increased prevalence is found in Europe and North America.

Although major insights into the nature of IBD have been offered by human as well as animals studies, the etiology of IBD is still not clearly understood. Accumulating evidences suggests that the pathogenesis of IBD is characterized by exaggerated immune responses to intestinal microbes in a genetically susceptible host (116-117). Several studies of inflammatory bowel disease in mice and humans have demonstrated immune disturbances by subsets of CD4+ T-helper (Th) cells as part of the pathogenesis. Generally CD is affiliated with Th1 and Th17 cells, whereas UC display a mixed Th1/Th2/Th17 cytokine profile (118-119).

The IL-23 produced by antigen presenting cells stimulated with bacteria has been implicated in the pathogenesis of IBD (120-121), as it promotes the production of both Th1 and Th17 cytokines in the inflamed gut mucosa of CD patients and Th17 cytokines in UC patients (119, 121). Th17 cells and increased levels of the Th17 cytokines IL-17A, IL-17F, IL-21 and IL-22 have been detected in the inflamed mucosa of both human IBD and experimental models of colitis (49). Increasing evidence from T cell mediated colitis in mice shows augmented levels of proinflammatory cytokines and chemokines in the intestinal mucosa, including IL-1β, monocyte chemoattractant protein (MCP)-1, IL-6, IFN-γ, tumor necrosis factor (TNF)-α and IL-17A that were significantly attenuated in the absence of IL-23, as reviewed in (122). A recently identified subset of Th17 cells that coproduces the Th1 cytokine IFN-γ was prominent in the intestinal mucosa of active CD patients (64).

Increased numbers of CD4+CD25+ FOXP3+ regulatory T cells have been detected in the inflamed mucosa of both UC and CD patients compared to controls (123-124). However, increased apoptosis of both LP and peripheral blood regulatory T cells have also been observed in both UC and CD patients compared to controls (124).
AIMS
The overall aim of the present thesis was to investigate phenotypical and functional characteristics of the adaptive local immune responses in the colonic mucosa of microscopic colitis patients.

The specific aims for the papers were the following:

- To characterize mucosal lymphocytes in the colonic mucosa of microscopic colitis patients by immunohistochemical analysis. (Paper I)

- To characterize phenotypes of lamina propria and intraepithelial lymphocytes separately in microscopic colitis patients using flow cytometry. (Paper II)

- To investigate the amount of the recent thymic emigrants by measuring T cell receptor excision circle (TREC) levels in the colonic mucosa of microscopic colitis patients. (Paper III)

- To investigate the T helper (Th) cell and cytotoxic T lymphocyte (Tc) mucosal cytokine profile at messenger and protein levels in microscopic colitis patients. (Paper IV)

- To setup an in vitro model for analysis of the impact of the colonic milieu in collagenous colitis patients on peripheral T lymphocyte activation and differentiation. (Paper V)
METHODOLOGICAL CONSIDERATIONS

The following section gives a brief overview of the experimental approaches used in this thesis, as well as reasons for and comments on the choice of material and methods used. For a more detailed description of specific methods, see paper I-V.

Patients (Paper I-V)

Colon biopsy specimens were obtained from previously diagnosed CC, LC and UC patients with an ongoing clinically active disease. Biopsies from patients with diarrhoea, but with histologically normal mucosa and no earlier diagnosis of MC or IBD were also investigated. Finally, controls without diarrhoeal symptoms were recruited among patients undergoing examination for rectal bleeding or suspicion of malignancy; with a normal mucosal appearance and histology. Patients with a previous history of Crohn’s disease, clinical signs of gastrointestinal infection, ischemic colitis or neoplastic disease were excluded from the studies.

In study I previously collected, formalin fixed, paraffin-embedded colonic biopsies were obtained from patients with LC, CC and controls from the archive at the Dept of Pathology, Örebro University Hospital, Sweden.

In study II, III, IV & V the biopsy specimens from MC patients, diarrhoea patients and controls were taken from the right flexure, whereas in UC patients they were taken from macroscopically affected areas of the colon. The colonoscopies were performed at the division of Gastroenterology, Örebro University Hospital, Sweden.

In Study V blood samples were collected from healthy blood donors at Örebro University Hospital, Sweden.

All patients in these five studies had provided written informed consent. The studies were approved by the regional ethical committee of Örebro-Uppsala County, Sweden, with the ethical approval ID #2008/278; 081015.

Comments:
The composition of well-defined patient populations is important in clinical studies. The recruitment of microscopic colitis patients in these studies took place from October 2008 till January 2013, still resulting in a small cohort of patients. There are several difficulties in recruiting MC patients. The MC patients can only be diagnosed following a histopathological examination and patients with a confirmed diagnosis are not normally undergoing repeated colonoscopy. Most often the patients with a confirmed diagnosis are treated with immunomodulating agents,
e.g. budesonide which will most likely influence the immune responses, and therefore these patients are most of the times excluded.

Another important clinical issue encountered during the work with this thesis is a subgroup of MC patients with a previous diagnosis of LC or CC that at time of biopsy collection for this study showed no histological signs of inflammation despite clinical symptoms of active LC/CC. These patients were grouped separately, and were referred to as LC-histopathological remission (LC-HR) and CC-histopathological remission (CC-HR).

In the second and fifth study where the experiments had to be performed on fresh mucosal specimens the recruitment was further obstructed as a fraction of the new patients, recruited based on symptoms of clinically active MC but not previously diagnosed with MC, did not fulfil histopathological criteria for MC. They were therefore grouped separately as diarrhoea patients.

We chose ulcerative colitis patients as a positive control for colonic inflammation, as immunopathological data on UC is well documented. The UC patients included in this study were recruited from those referred to the outpatient Gastroenterology clinic, Örebro University Hospital, Sweden.

**Immunohistochemistry (IHC, Paper I)**

To characterize the inflammatory cells, we investigated expression of different proteins on colonic mucosal lymphocytes by immunohistochemical staining. Four µm thick sections of formalin-fixed, paraffin-embedded tissues from proximal colon were mounted and deparaffinized according to standard laboratory procedures. The stainings were performed in a routine laboratory (Department of Pathology, Örebro University Hospital) using an automated immunostaining instrument. The images were captured using a microscope with a x20 objective lens, Leica DMRXA 2 equipped with a digital camera; Leica DFC 330 FX and analysed by the macro program Leica QUIPS (Leica Microsystems, Wetzlar, Germany). This program interfaces with the Leica Qwin software; and a sequence of instructions for repetitive image analyses was set up.

**Comments:**

Immunohistochemistry is widely used to determine protein expression and to localize it within a tissue or cell. One of the major advantages with IHC is that it can be performed on preserved tissues. We had access to the archive at the Dept. of Pathology, Örebro University Hospital, and therefore we chose this technique to characterize the inflammatory cells. Traditionally, observer-dependent, semi-quantitative methods such as point counting are used for immunohistochemical evaluation. This method is time consuming and it is hard to define borders between different areas, as
there are more chances of overlap between different areas and requires highly trained individuals.

We chose the computerized image analysis that enables you to obtain results in shorter period of time compared to point counting analysis, as the computer calculates the positively stained areas based on predefined limits or thresholds. It also gives the possibility to divide the colonic mucosa in separate areas of interest without any overlap between them. In addition the images can be saved and reanalyzed by a second evaluator within the same fields of vision. However, this approach still requires manual definition of thresholds for colour intensity to set background areas and stained lymphocyte areas. It also requires manual definition of different areas of subsections of colonic mucosa for the computer to calculate positively stained areas as well as exclusion of falsely marked areas.

**Isolation of intraepithelial lymphocytes and lamina propria lymphocytes (Papers II and V)**

Fresh mucosal biopsies were treated with ethylenediaminetetraacetic acid (EDTA) to break down the epithelial layer and thus release the intraepithelial lymphocytes (IELs). The denuded biopsies were further digested with collagenase to break down peptide bonds in collagen in the lamina propria to release lamina propria lymphocytes (LPLs), with the addition of DNAse to degrade DNA, as DNA released from dead cells into the medium can cause cells to clump together resulting in clogging of released lymphocytes.

The colonic biopsies were thoroughly washed with PBS. IELs were isolated by incubation in EDTA-containing medium with constant stirring, 4 times 15 min, with collection of released cells from the first three incubations. The cells released during the fourth incubation were discarded to minimize contamination by LPLs. Isolated cells were passed twice through a 100 µm and once through a 30 µm pore size nylon mesh strainer in order to reduce mucus remnants and cell debris. They were kept on ice while the denuded biopsies were further digested with complete collagenase type VIII and DNAse I type IV for 1-1.5 hrs. Isolated LPLs were passed through strainers as described above for IELs, and both LPLs and IELs were washed twice, resuspended in PBS and kept on ice until further analysis.

**Comments:**

In general, the cell yield was lower for IELs compared to LPLs, probably at least partly due to mucus remnants in the cell suspension. Mucus remnants
in the LPL and IEL cell suspensions also likely reduced surface staining, as mucus might block the binding sites on the surface of cells for antibodies. Several strategies were employed to reduce mucus and increase cell yield as well as to obtain less viscous cell suspensions; (1) Dithiothreitol (DTT) and different types of collagenase enzymes were used to degrade mucus. However DTT and less pure collagenase markedly reduced cell surface staining. DTT and less pure collagenase containing protease contaminants might disrupt the cell surface binding sites for antibodies. Thus, DTT was excluded and more pure collagenase containing less protease contaminants was used during the IEL and LPL isolations. (2) Different cell strainers were found to reduce the mucus levels in cell suspension, so they were used during both the IEL and LPL isolations. The calcium chelating agent EDTA was used to detach the epithelial cells from the biopsies, but it also disrupts mucus. However, EDTA concentrations exceeding 1 mM inhibited the enzymatic activity of the collagenase during the LPL isolation and we were therefore restricted to use EDTA during the IEL isolation.

**Flow Cytometric Analysis (Paper II)**

The flow cytometry technique can determine the different characteristics of cells within a mixed population by quantifying cell size, granularity or irregularity, using fluorochrome labelled antibodies bound to the surface of or within the cells. Thus we choose this technique to determine different surface and intracellular markers and to characterize the immunophenotype of IELs and LPLs separately.

Cells were incubated together with different fluorochrome labelled monoclonal antibodies that binds to the protein of interest. Subsequently the cells were passed through a laser that excite the fluorochrome and emits fluorescence of specific wavelengths, which gives information on the expression of particular proteins on the surface or within each cell. We have performed 4-colour staining to identify different markers. Isolated LPLs and IELs were incubated with an anti-Fc-receptor reagent to block unspecific binding, followed by incubation with the monoclonal antibodies or their respective isotype control antibodies. The isotype-matched controls were used to estimate non-specific staining, and the unstained cells were used to predict the gating of cells negative for the staining.

Stained cells were acquired on a Coulter Epics Altra™ flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed using Kaluza software (Beckman Coulter).

**Comments:**

Exclusion of DTT and use of more pure collagenase resulted in a normal staining, that is, staining intensities comparable to peripheral blood
lymphocytes. As mentioned above, different cell strainers were found to reduce the mucus levels in the cell suspension, resulting in a much cleaner cell suspension where antibodies could bind to cell surface markers much more efficiently.

Due to technical difficulties some samples were excluded: During IELs isolation and surface staining, some stained samples had to be excluded from the analysis, due to < 1000 events for a particular marker, or samples with mucus remnants that clogged the flow cytometer during acquisition. To prevent clogging in the flow cytometer the machine was cleaned after acquisition of every two stained samples. This was due to the impure mucosal cell suspension, which is not the case while acquiring more pure cell suspensions such as from a cell line or peripheral blood lymphocytes.

Flow cytometric analysis gives information about the size and granularity of the cells, which are defined by forward scatter (FS) and side scatter (SS) respectively. During analysis the particular cell population is gated in FS and SS and analyzed subsequently for expression of other markers. Unlike peripheral blood lymphocytes, the mucosal cell suspension contains a mixture of several cell populations including T- and B lymphocytes but also plasma cells, NKT cells, epithelial cells and other innate immune cells as well as cell debris that makes it difficult to locate a specific lymphocyte population directly by FS and SS.

We excluded cellular debris based on FS and SS characteristics and the lymphocyte gates were set by “back-gating” on CD3+ lymphocytes in forward and side scatter. Analysis was performed on gated lymphocytes. With the exception of the B lymphocytes and plasma cells, all lymphocyte subpopulation characteristics were expressed as percentage of a gated, cell surface marker defined population.

**Real-time PCR (Papers III & IV)**

Real-time reverse transcription PCR analysis quantifies the amount of RNA being transcribed from the genome for protein production. Due to its sensitivity to degradation, it is convenient to reverse transcribe RNA into the complementary DNA (cDNA) which is a more stable form. Subsequently cDNA is amplified by a PCR reaction to measure the transcript levels of the genes.

In paper III, the level of T cell receptor excision circles (TRECs) was analyzed in the colonic mucosa of CC and LC patients as a measurement of recent thymic emigrants (RTE). Purified genomic DNA from colonic biopsies was analyzed for TREC content by real-time PCR. As analysis of TREC content is based on detection of genomic DNA that is expressed in all cells, and the analysis was performed on total DNA from biopsies,
rather than from purified T lymphocytes, we had to use a marker specific for T cells to relate the TREC amount. We therefore analyzed expression of the CD3 gamma gene (i.e. CD3 gamma mRNA) to estimate the total amount of T cells in the colonic biopsies, and this was used as a reference for TREC analysis. The results were presented as relative quantification of each sample as we were interested to compare TREC content in colonic tissues from MC patients and controls. Real-Time PCR was performed using the thermal cycler TaqMan 7900 Fast Real-time PCR System (Applied Biosystems) with 7900 Fast Sequence Detection and Relative Quantification software packages.

In paper IV, real-time reverse transcription PCR was used to investigate the T helper (Th) cell and cytotoxic T lymphocyte (Tc) mucosal cytokine profiles at the transcript level. The mRNA expression of the following cytokines and transcription factors was determined: IL-1β, IL-4, IL-5, IL-6, IL-10, IL-12p35, IL-17A, IL-21, IL-22, IL-23A, IFN-γ, TNF-α, T-bet, RORC2, GAPDH and GUSB.

Real-Time PCR was performed using the thermal cycler TaqMan 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with 7500 Fast Sequence Detection and Relative Quantification software packages.

**Luminex (Papers IV & V)**

A common method to analyze specific protein levels is the enzyme-linked immunosorbant assay (ELISA), where 50-100 µL of sample is required for analysis of each protein. Each mucosal biopsy homogenate yielded approximately 200-250 µL of supernatant and due to the limited amount of colonic tissues from MC patients we chose to use Luminex analysis, which only requires 50 µL of sample for analysis of several proteins.

Luminex is a fluorescent-bead based method that allows the detection of several analytes within a single sample at the same time. Color-coded polystyrene or magnetic beads are used and each bead is precoated with an antibody towards the specific protein that is analyzed in a sample. The sample is passed through two lasers in a Luminex analyzer, where one laser identifies each bead and the second laser identifies any of the reporter dyes captured during assay.

In paper IV, to complement the transcription level data on T helper (Th) cell and cytotoxic T lymphocyte (Tc) mucosal cytokine profiles, the mucosal tissue protein levels of IL-1β, IL-5, IL-6, IL-10, IL-12p70, IL-17A, IL-21, IL-23, IFN-γ and TNF-α were analysed using the xMAP technology developed by Luminex® (Austin, TX, USA).
In paper V, we were interested to investigate the possible effects of soluble factors derived from the intestinal mucosa on T cell differentiation. The cytokines related to different T cell subsets (e.g. Th1, Th2 and Th17) were analyzed in supernatants of CD4+ PBL cultures, incubated with soluble factors from colonic mucosa of MC and controls. The cytokines analyzed were IL-1β, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF-α.

All protein concentrations were determined using the Milliplex® Map Kit according to the manufacturer’s instructions (Millipore, MA, USA). Samples were read and analysed using a Luminex® 200™ with xPONENT software.

Comments:
During our optimization of the method we found that the beads were not detected while reading the samples in the Luminex analyser. We found that this was due to the viscosity of the supernatant from mucosal tissues, as centrifugation of the tissue homogenates at 10,000 rpm for 5 minutes as well as a two fold dilution of sample supernatants with assay buffer during incubation with beads resulted in appropriate bead count and analyte detection.

Compared to 1 hr incubation at room temperature, overnight incubation of the samples with beads at 4°C resulted in increased assay sensitivity for analytes and an appropriate bead count.

Although the Luminex analysis detects several analytes at the same time, there is limitation to the method as the combinatorial possibilities of different cytokines within a certain panel is restricted due to the cross reactivity of antibody with other cytokines as well as overlap in wavelengths in the different fluorescences of the different beads. Because of this we were not able to mix the desired cytokines in a single panel.

According to the manufacturers, 100 different analytes can be analyzed simultaneously by Luminex technology. However, we found difficulties to get an appropriate bead count in multiplex bioassays analysing for more than 10 analytes. The insufficient identification of beads by the Luminex analyzer could be due to the complexity of multiplexing of large number of cytokines.

Preparation of conditioned medium from the colonic mucosa
(Paper V)
In this study we were interested to investigate how the soluble factors derived from the local colonic milieu from mucosa of CC patients and controls affects peripheral T cells. Twelve fresh colonic biopsies were treated with EDTA to remove the epithelial layer and IELs. Six of these
denuded biopsies (DNB) were kept in serum free culture medium (RPMI-1640, streptomycin, gentamycin, penicillin and HEPES) on ice until further use, and the remaining six denuded biopsies were further digested with collagenase and DNAse to extract lamina propria mononuclear cells (LPMCs). Denuded biopsies and LPMCs were cultured in culture medium overnight at 37°C, 5% CO₂, to generate conditioned medium (CM). The CM was tested for endotoxin and total protein levels, and stored at -80°C until further use.

Comments:
We were interested to investigate soluble factors released by extracellular matrix (ECM) from the colonic mucosa of CC patients and controls. However we were unable to obtain cell free ECM from the colonic biopsies and we therefore chose to investigate the soluble factors released by two different preparations of the mucosa, the denuded biopsies and the LPMC fraction. Due to the fact that only two CC patients could be recruited within the time frame for this study, and thereby enabling generation of only a fairly low volume of conditioned medium, we were unable to analyze the conditioned medium separately from each individual. Therefore the conditioned medium from the DNB and LPMC fractions from CC patients and non-inflamed controls, respectively, were pooled. We tested different concentrations of total protein amounts in CM from DNB as well as LPMC from CC patients and controls to analyze the correlation between the different amounts of total proteins in CM and T cell responses.

**T cell Proliferation and Cytokine Release Assay (Paper V)**
A central point to this study was to recapitulate the *in vivo* situation as closely as possible. To determine the influence of the local intestinal milieu on newly recruited peripheral T cells into the colonic mucosa, we cultured normal CD4⁺ peripheral blood lymphocytes (PBLs) with conditioned medium derived from culture of denuded colonic biopsies and lamina propria mononuclear cells.

In order to determine the influence of soluble factors from the colonic mucosa, CD4⁺ PBLs were isolated by the Human CD4⁺ T cell Enrichment Cocktail kit (STEMCELL Technologies, Grenoble, France). Subsequently, CD4⁺ PBLs were stimulated with α-CD3/α-CD28 antibodies and were incubated in a culture medium (RPMI-1640, HEPES, L-Glutamine, AB Serum, streptomycin, gentamycin and penicillin) in either the absence or presence of CM derived from DNB and LPMC fractions from colonic mucosa of MC patients and non-inflamed controls. The cells were cultured for 3 days at 37°C, 5% CO₂, whereafter supernatants were harvested and
stored at -80°C until determination of cytokine content by Luminex. Similarly, T cell proliferation was measured after 3 days using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA).

Comments:
According to the manufacturer, the Human CD4+ T cell Enrichment Cocktail kit can be used for any volume of whole blood for CD4+ PBL isolation. However we found that although the yield of CD4+ PBLs for small volumes of whole blood (up to 4 mL) was according to the manufacturer, larger volumes of whole blood (10-15 mL) did not fulfill this pledge. In future experiments, we plan to use a buffy coat; a leukocyte-enriched fraction of whole blood. This will convert the larger whole blood volume into a smaller volume of buffy coat that can be used for CD4+ PBLs enrichment. The approach for CD4+ PBLs enrichment from buffy coat will still be the same as the one used for whole blood.

Statistical analysis
In general all data were presented as median values, as they were not normally distributed. In paper I and II, data were also expressed as median and interquartile range (IQR). The Mann-Whitney two-tailed non-parametrical test was used for analysis of statistical significances between two groups (Paper I-IV). The Kruskal-Wallis one-way ANOVA test was used to calculate the presence/absence of statistically significant differences between more than two groups (Paper I). Statistical outliers were defined by the Grubbs test (Paper III). The correlation between two variables was calculated with the Spearman correlation analysis: semi-automated image analysis and point counting (Paper I), different phenotype of lymphocytes and age (Paper II), and degree of diarrhoea and cytokine mRNA expression (Paper IV). Values of p≤0.05 were regarded as statistically significant.
RESULTS AND DISCUSSION

This section provides a brief overview of the main findings in this thesis (Fig. 5). As three of the papers are accepted but not yet published, and two are not yet accepted for publication, the results are not shown in detail, but rather generally discussed. Detailed descriptions of the results are presented in the respective papers, and are referred to by their figures within the manuscripts.

Figure 5. Schematic overview of the results in this thesis. The respective papers are indicated with roman numerals. Results from immunohistochemistry and flow cytometry analysis to characterize phenotype of mucosal lymphocytes are presented in teal coloured text (Paper I and II). Analysis of T cell receptor excision circle (TREC) levels in the CD3+ T cell compartment in the colonic mucosa is presented with blue text (Paper III). Results from analysis of Th1/Th2/Th17 and Tc1/Tc2/Tc17 associated mucosal cytokine profile are shown in black text (Paper IV). Findings of the impact of the mucosal microenvironment on differentiation of peripheral CD4+ T cells are shown in red text (Paper V). All the data presented here is in comparison to non-inflamed controls. Increase; Decrease; = no change.
Phenotypic characterization of lymphocytes in the colonic mucosa of collagenous colitis and lymphocytic colitis patients (Paper I & Paper II)

In paper one; we characterized lymphocytes in the mucosa of CC and LC patients using immunohistochemistry. There was a significant increase in the amount of CD8+ T cells in the epithelium in both LC and CC patients compared to controls, with the most pronounced rise seen in LC patients (Paper I Fig. 3), whereas the amount of CD4+ T cells were markedly reduced in the lamina propria (LP) of both LC and CC patients compared to controls (Paper I Fig. 4).

The expression of the active/memory marker CD45RO and the transcription factor Foxp3 that is involved in regulatory T cells (Treg) differentiation, was more abundant in both the epithelium and LP in both patient groups compared to controls (Paper I, Table III). Likewise, abundant amounts of the CD30 marker, expressed on activated but not resting lymphocytes, were observed in the LP of both patient groups compared to controls (Paper I, Table III). Expression of the proliferation marker Ki67 was significantly increased, but in contrast to CD45RO, this was only seen in the epithelium of both CC and LC patients compared to controls.

In this study we only performed single antibody staining and therefore we cannot say whether the increase in expression of Ki67 and CD45RO is solely due to an increase in activated T cells or by other cells such as monocytes, macrophages and granulocytes.

We therefore performed a detailed phenotypic characterization of freshly isolated lamina propria and intraepithelial lymphocytes from colonic biopsies from CC and LC patients compared to controls, using four-colour flow cytometry, in Paper II. This allows determination of the expression of particular markers on the surface or within each specific cell type. As immunopathological data on UC is well documented, we used colonic biopsies from UC patients as positive controls in this study.

The intraepithelial CD8+ T cells were markedly increased in LC patients compared to controls and UC patients (Paper II, Fig. 1A). An increase was also noted in CC and CC-HR patients (see Materials and Methods for definition of the latter patient group) compared to controls, albeit not statistically significant. The CD8+ population was also significantly augmented in LC-HR patients compared to controls (Paper II, Fig. 1A). We next analyzed the expression of the CD45RO marker within the CD8+ T cells. The proportion of CD45RO+CD8+ T cells was not significantly increased in any of the groups of MC patients compared to controls (Paper II).
II, Fig. 1B), but a trend towards increased proportions were noted in CC patients (Paper II, Fig. 1B). The proportion of Ki67+ cells within the CD8+ T cell population was significantly increased in CC patients compared to controls (Paper II, Fig. 1D).

The proportions of CD4+ IELs in MC patients were not significantly changed from controls (Paper II Fig. 2A), whereas UC patients demonstrated a significantly increased proportion compared to controls as well as to LC and CC patients.

In contrast to IELs, we did not find any significant differences in the CD8+ lamina propria T cells between the different colitis groups and controls (Paper II Fig. 4A). However, the proportions of CD45RO-CD8+ LP T cells were significantly increased in CC patients compared to controls and UC patients (Paper II Fig. 4B). CC patients also had markedly increased proportions of Ki67+CD8+ LP T cells compared to controls. There was a trend towards increased proportions of Ki67+CD8+ T cells in LC, LC-HR and UC patients, but it did not reach statistical significance (Paper II Fig. 4D).

A trend towards reduced proportions of CD4+ T cells in lamina propria were observed in CC, CC-HR and LC patients compared to controls (Paper II Fig. 5A). However the proportions of CD45RO-CD4+ LP T cells were markedly increased in CC patients compared to controls (Paper II Fig. 5B). The proportions of Ki67+CD4+ LP T cells were significantly increased in CC, LC and LC-HR patients, but this proportion was even higher in UC patients compared to controls (Paper II Fig. 5B).

Furthermore, we observed normalized proportions of CD45RO+ and Ki67+ expression within CD4+ and CD8+ IEL and LPL populations in CC patients in histological remission compared to CC patients, but not in the LC-HR patients compared to LC patients.

**Discussion:**
Generally both UC and CD are believed to be driven by aberrant CD4+ IEL and LPL responses (116, 120). In accordance, we also noted heavy infiltration of CD4+ IELs in UC patients but not in MC patients. Instead, the MC patients are presented with heavy infiltration of CD8+ IELs. Our findings of increased proportions of CD8+ IELs in CC and especially LC patients compared to controls in paper I and paper II are in agreement with previous immunohistochemical studies (96-97, 125). However, immunohistochemical studies by Mosnier et al and Armes et al state that CD4+ T cells were more numerous than CD8+ T cells in the lamina propria of MC patients; although none of these two studies present these data in their results (96, 125). In contrast we found reduced or unaltered amounts...
of CD4+ T cells in the epithelium and lamina propria compartment of MC patients compared to controls in our immunohistochemistry and flow cytometry analysis. Our data on CD4+ T cells also differ from the findings in CC patients by flow cytometric analysis by Wagner et al., but they did not analyze IELs and LPLs separately (100). The reduced proportions CD4+ IELs in LC and CD4+ LPLs in both CC and LC patients suggest that these cells might undergo apoptosis. However, in CC patients this can also partly be explained by the space occupied by the thick collagen layer. Along the same thoughts, the increased numbers of locally expanded CD8+ T cells in MC patients could be a result of decreased apoptosis of these cells. Whether there is an aberrant apoptosis of mucosal T cells in MC patients needs to be investigated, and such information is relevant to explain the disturbed T cell responses in mucosa of MC patients.

Furthermore we found increased proportions of CD8+ IELs and LPLs expressing CD45RO and the proliferation marker Ki67 in both CC and LC patients compared to controls, which was not the case in UC patients. MC patients had increased proportions of Ki67+ CD4+ IELs and CD4+ LPLs compared to controls, but theses populations were much larger in UC patients. Collectively these data suggests that UC and MC are driven by different T cell subsets.

Generally the proportion of active/memory T cells gradually increases with age. As the MC patients are mostly of old age, we were concerned that the observed increased proportions of CD45RO+ and Ki67+ T cells was due to older age in the MC patient groups compared to the controls. Our correlation analysis between CD45RO- and Ki67- T cell frequencies and age in the control group, consisting of individuals with a wider age span, demonstrated a significant correlation between higher proportions of CD45RO- within the CD4+ T cell population and increasing age (Paper II, Fig. 2C, 5C), but importantly, this was not found in the CD8-CD45RO- or CD8- Ki67- T cells nor in the CD4+Ki67- cells (Paper II, Fig. 1C, 4C), suggesting that the changes observed in the latter are indeed due to the disease itself. These findings collectively suggest that the immunopathological responses in MC are driven by CD8+ T cells. It has been demonstrated that diversion of the faecal stream via a loop ileostomy leads to a normalized clinical and histopathological response in CC patients, suggesting that luminal agent might be involved in MC pathogenesis (20, 90). Increased amounts of antibodies against the Yersinia bacterium have been detected in CC patients compared to controls (92). In addition, increased uptake and altered mucosal reactivity to non-pathogenic bacteria in CC patients compared to controls (94), suggest the involvement of microbes in MC pathology.
The cytotoxic activity of CD8⁺ T cells directed against epithelial cells containing endogenous bacteria or luminal agents may promote proinflammatory responses leading to initiation and/or exacerbation of inflammatory conditions in the intestine, as seen in MC. It is possible that these luminal agents are taken up by intestinal epithelial cells and thereby these exogenous antigens would be presented by MHC class II molecules and recognized by CD4⁺ rather than CD8⁺ T cells. The effector CD4⁺ T cells could then target these antigens as well as activate the CD8⁺ T cells through their cytokine production.

The regulatory T cells (Tregs) are key suppressors of the active immune responses. Increased frequencies of CD4⁺CD25⁺Foxp3⁺ Tregs are found in the inflamed mucosa of UC patients and their numbers increases with the disease activity in UC patients (123-124, 126). In accordance to these findings, our immunohistochemistry data (paper I) as well as others show significantly increased amounts of Foxp3⁺ cells in both the epithelium and lamina propria compartment of CC and LC patients compared to controls (127). However there are no data showing whether the increase in Foxp3 expression is from CD4⁺ or CD8⁺ T cells in MC patients and warrants further studies. Recently Veltkamp et al. reported on a significant increase in apoptosis rate of CD4⁺Foxp3⁺ Tregs in MC patients but this was even greater in both UC and CD patients compared to non-inflamed controls, resulting in reduced numbers of local Tregs (124). This suggests that despite the increased frequency of Tregs in the mucosa of IBD patients, they are relatively ineffective in suppressing the inflammation because of their much higher rate of apoptosis.

In addition to CC and LC patients, we have identified and phenotypically characterized two previously not described groups of LC and CC patients with active clinical disease, but in histopathological remission. We found clearly reduced proportions of both proliferating and activated/memory CD4⁺ and CD8⁺ IELs and LPLs in CC patients in histopathological remission compared to CC patients with histopathological changes, indicating that CC-HR patients display signs of normalized proportions of mucosal T cells. Thereby other factors seem to be involved in triggering the clinical symptoms of active CC in patients in histopathological remission. Interestingly, no significant differences was noted in the proportions of CD45RO⁺ or Ki67⁺ cells between LC-HR and LC patients, suggesting that in contrast to CC-HR, mucosal T cells in LC-HR were as active as in LC patients.
Analysis of T cell receptor excision circle (TREC) levels in the CD3+ T cell compartment in the colonic mucosa of collagenous colitis and lymphocytic colitis patients (Paper III)

After having demonstrated the heavy infiltration of CD8+ IELs in the mucosa of CC and especially in LC patients by immunohistochemistry and flow cytometry, we were interested to investigate whether the increased numbers of CD8+ IELs is due to a greater influx of T cells or a pronounced expansion of local resident T cells in the mucosa of CC and LC patients. Such information is a first step toward understanding whether the activating antigen(s) reside in the mucosa, or is rather transported via e.g. dendritic cells to the draining lymph nodes to activate naïve T cells there, thereby adding information on the nature of the chronic colonic inflammation in the mucosa of MC patients.

In this study we investigated recent thymic emigrants by measuring the TREC content in the colonic biopsies relative to the amount of the CD3+ T cells, in CC and LC patients compared to controls, using real-time PCR analysis.

We noted reduced median TREC levels in CC and LC as well as in LC-HR patients compared to controls (Paper III Fig. 1). However, the changes did not reach statistical significance. There were no differences in the median TREC levels in either CC-HR or LC-HR patients compared to CC and LC patients with histopathologically active disease.

Discussion:

Increased infiltration of T cells in the intestinal mucosa is evident in both MC and UC patients compared to non-inflamed controls. Our previous study measuring recent thymic emigrants by TREC analysis demonstrated enhanced TREC levels in the mucosal lymphocytes from UC patients compared to controls, suggesting that recent thymic emigrants are recruited to the inflamed mucosa of UC patients (78). Increased TREC levels in colonic biopsies from UC patients in paper III is in accordance with our previous findings. In contrast to UC patients, we found reduced level of TRECs in the colonic mucosa of MC patients. These reduced TREC levels and our previous observations in paper II on elevated proportions of both CD8+ and CD4+ LPLs/IELs expressing the Ki67+ phenotype, identifying proliferating cells, in both CC and LC patients strongly suggest that MC patients have local expansion of resident T cells rather than migration of peripheral lymphocytes to the inflamed mucosa. Furthermore, increased proportions of CD8+CD45RO+ IELs and LPLs in paper II and the previously shown involvement of luminal agents and bacteria in MC
pathology (90, 92, 94), suggests that constant exposure of local mucosal or luminal agents to the activated T cells could lead to proinflammatory responses resulting in epithelial damage and chronic inflammation in the intestinal mucosa, as observed in MC.

These results indicate major differences in pathogenesis between UC/CD patients and MC patients, where the latter would likely not benefit from treatment with antibodies blocking homing to the intestinal mucosa, where e.g. Natalizumab have shown good responses in CD patients (128). This also support our theory that it is indeed a local mucosal antigen triggering the T cell activation in MC pathology, possibly by one or several microbiota-derived antigens, or by drugs that may aggravate MC in some patients (91).

**T helper (Th) 1/Th17 and cytotoxic T lymphocyte (Tc) Tc1/Tc17 associated cytokine profile at messenger and protein levels in the colonic mucosa of collagenous colitis and lymphocytic colitis patients (Paper IV)**

Based on our flow cytometric data that revealed increased local T cell responses in the lamina propria and intraepithelial compartment of CC and LC patients, demonstrated as elevated expression of CD45RO and Ki67, we continued to determine the functional profile of Th and Tc in the colonic mucosa of CC and LC patients. We investigated the expression of different Th1/Th2/Th17 and Tc1/Tc2/Tc17 cell associated cytokines and transcription factors at both the messenger and protein levels in the MC patients compared to controls, with UC patients serving as positive controls, using real-time reverse transcription PCR and Luminex® analysis of multiple cytokines in the same colonic biopsies.

We found markedly up regulated mucosal mRNA levels of the Th1/Tc1 associated cytokine IFN-γ in CC, LC as well as UC patients compared to controls (Paper IV Fig. 1). Likewise, the IL-12/IL-35 subunit p35 was also significantly up regulated in CC and LC patients, although at lower magnitudes, but was not significantly altered in UC patients compared to controls (Paper IV Fig. 1). Interestingly transcript levels of both of these cytokine were higher in MC patients than in UC patients. In accordance with the Th1/Tc1 cytokine profile in CC and LC patients, the mRNA levels of T-bet, transcription factor for Th1/Tc1 maturation was also significantly upregulated in CC and to lesser extent in LC patients (Paper IV Fig. 1). However, no significant differences were noted in IFN-γ and IL-12p70 protein levels in patient groups and controls (Paper IV Fig. 2).
The proinflammatory cytokines IL-6 and IL-1β are involved in differentiation of human Th17/Tc17 cells. mRNA levels for IL-6 was significantly up regulated in CC, LC and LC-HR patients but not as much as in UC patients compared to controls (Paper IV Fig. 3). Increased levels of IL-1β transcript were noted in CC patients but less than in UC patients. In contrast, no differences were found in LC patients compared to controls (Paper IV Fig. 3). IL-23 transcript levels were also up regulated in CC and LC patients compared to controls, although at lower magnitude (Paper IV Fig. 3). The transcript levels of one of the principle cytokine produced by Th17 and Tc17 cells IL-17A, was markedly up regulated in both CC and LC patients compared to controls, but less than in UC patients (Paper IV Fig. 3). Furthermore, mucosal mRNA levels of the Th17/Tc17 associated cytokines IL-21 and IL-22 were also significantly up regulated in both CC and LC patients compared to controls, albeit less than in UC patients (Paper IV Fig. 3). In contrast, the Th17 cell attracting chemokine CCL20 was not significantly up regulated in CC and LC patients, but in UC patients compared to controls. In support of the mRNA data, significantly enhanced IL-6 protein levels were also observed in CC patients and a trend towards increased levels was noted in UC but not LC patients compared to controls (Paper IV Fig. 4). Similarly, significantly increased amounts of IL-21 protein was observed in both CC and LC patients as well as a trend towards increased levels in UC patients compared to controls (Paper IV Fig. 4).

Although the transcript levels of the pro-inflammatory cytokine TNF-α was not significantly upregulated in any of the colitis groups compared to controls, TNF-α protein expression was significantly increased in CC and UC patients an a trend towards increased levels was noted in LC patients compared to controls (Paper IV Fig. 5B).

In contrast, neither mRNA nor protein levels of the Th2/Tc2 associated cytokines IL-4, IL-5 nor IL-10 were significantly altered in any of the colitis groups compared to the controls.

Furthermore, we investigated the possible correlation between mucosal mRNA expression of cytokines and clinical activity of the disease in MC patients. We found significant positive correlations between increased frequencies of bowel movements and enhanced transcript levels of IFN-γ, IL-21 and IL-22 in MC patients (Paper IV Fig. 7).

We also noted that LC-HR but especially CC-HR patients had normalized levels of all cytokines investigated compared to LC and CC patients respectively.
Discussion:
Our mucosal mRNA analysis shows that the transcript levels of the Th1 and Tc1 associated cytokines IFN-γ and IL-12 were higher in the colonic mucosa of MC patients than in UC patients. This is likely due to the increased number of CD8+ IELs, as well as the increased proportion of CD8+ IELs and LPLs expressing the active/memory marker CD45RO and the proliferation marker Ki67 in the mucosa of both CC and LC patients, as the frequencies of CD4+ T cells were unaltered or reduced in MC patients. On the other hand, increased mucosal mRNA levels of the Th17/Tc17 associated cytokines IL-17A, IL-21 and IL-22 were more profound in UC patients than in MC patients. It is likely that Th17 cells secrete large amounts of IL-17A, IL-21 and IL-22 as seen in UC patients and there could be low number of Th17 cells in MC as CD4+ T cells are reduced or unchanged in these patients. However, these cytokine may also be derived from Tc17 cells, as increased proportions of activated CD8+ T cells are present in MC patients. So far there are no comparative studies for secretion of cytokine levels between Th17 cells and Tc17 cells. In addition, apart from Th17/Tc17 cells there are various other immune cellular sources for secretion of these cytokines, such as NKT cells, monocytes and macrophages (49).

The markedly up regulated IFN-γ mRNA levels as well as increased protein levels of TNF-α in MC patients are in accordance with the study by Tagkalidis et al. (98). Furthermore our study confirms the correlation between transcription levels of mucosal IFN-γ and the degree of diarrhoea in MC patients previously described by Tagkalidis et. al. (98). IFN-γ activates macrophages to release proinflammatory cytokines like TNF-α, IL-1β and IL-6, which in turn sustain and increase local inflammatory responses (129). IFN-γ also plays a vital role in lymphocyte infiltration in the gut, as it regulates the production of IEL-attracting chemokines like CXCL10 (IP-10) and CXCL9 (MIG) by intestinal epithelial cells (130). Significantly increased amounts of CXCL10 (IP-10) and CXCL9 (MIG) have been reported in the epithelium and lamina propria of LC patients (130). It has been shown that both these chemokines chemoattracts activated T lymphocytes (131-132). The common receptor for CXCL10 (IP-10) and CXCL9 (MIG), CXCR3, is highly expressed on IELs and activated T cells (130, 133), suggesting that IFN-γ indirectly regulates the lymphocyte infiltration into the gut. TNF-α has been shown to cause an increase in intestinal epithelial permeability in Crohn’s disease (134).

mRNA and protein levels of IL-21, being enhanced in both CC and LC patients, are secreted by Th17 and Tc17 cells. IL-21 has pleiotropic effects on immune cells; for example it enhances IFN-γ production by mucosal T
cells and NK cells (68, 135) and promotes the lytic activity of CD8+ CTLs (135). We found a positive correlation mucosal mRNA level of IL-21 and IL-22 and the number of bowel movements per day in MC patients. In addition to Th17 cells, IL-22 is also expressed by Th1 lymphocytes, CD8+ Tc17 cells and NK cells as well as CD11b+ DCs and innate lymphoid cells (49, 70, 136). IL-22 induces the production of IL-8 and TNF-α by epithelial cells and colonic myofibroblasts, and increases the epithelial barrier function by activation of proliferative and/or anti-apoptotic programs (136). Increased activity of colonic myofibroblasts by IL-22 might result in excess production and deposition of collagen in the basement membrane. Whether IL-22 is involved in repairing epithelial cell damage in CC and LC or in collagen layer deposition in CC remains unclear, but increased numbers or activity of myofibroblasts has been reported in the mucosa of CC patients (105), indicating that IL-22 might promote inflammation rather than protection in the colonic mucosa of CC patients. Our data on markedly up regulated transcript levels of the chemokine CCL20, which is induced by Th17 cytokines and attracts CCR6+ Th17 cells, in UC patients but not in MC patients, suggest that MC patients have local expansion of resident T cells rather than influx of Th17 cells in the gut mucosa. This is also supported by our findings in the studies on phenotypic characterization of the mucosal T cells (Papers I and II) as well as the study on TREC levels (Paper III). Data on whether CCL20 also attracts Tc17 cells are unknown, but they do express both CCL20 and the CCL20 receptor CCR6, indicating that they can respond to CCL20 (69, 71).

In line with our flow cytometry data on normalized proportions of activated mucosal T cells in CC-HR patients but not in LC-HR patients compared to histopathologically active CC and LC patients respectively, we found that LC-HR but especially CC-HR patients had normalized levels of all cytokines investigated. Apparently other factors have to contribute to the clinical symptoms in MC patients in histological remission: One possibility is bile acid malabsorption, as it has been shown to be associated with MC in 27-44% of CC patients and in 9-60% of LC patients (6) and aggravates clinical symptoms (95).

In this study the cytokine levels were measured in colonic biopsies rather than on isolated lymphocytes. Therefore it is difficult to draw any firm conclusions on whether the cytokines analyzed stems mainly from T helper cells or CTLs. Cytokine analysis on sorted CD4+ and CD8+ IELs and LPLs from colonic mucosa of MC patients would provide relevant information on whether the immune responses are mainly derived from CD8+ T cells. Furthermore, elucidating the cytotoxic activity of CD8+ T cells in the
The role of soluble factors from the colonic mucosa of collagenous patients in the regulation of effector T cells (Paper V)

We next analyzed the impact of the colonic milieu in collagenous colitis patients on peripheral T lymphocyte activation and differentiation. We developed a novel system that mimics the in vivo exposure of newly recruited peripheral blood T cells to the soluble factors derived from the colonic milieu of normal individuals compared to CC patients with an inflamed mucosa. Anti-CD3/anti-CD28 stimulated normal CD4+ peripheral blood T cells were incubated in the presence or absence of conditioned medium (CM) derived from the culture of denuded colonic biopsies (DNB) or isolated colonic lamina propria mononuclear cells (LPMCs). T cell proliferation as well as secretion of effector T cell cytokines was analysed.

We found reduced proliferation of peripheral CD4+ T cells exposed to CM from the colonic mucosa of both non-inflamed controls and CC patients. This inhibition in proliferation was however less pronounced with DNB-CM derived from CC patients compared to non-inflamed control mucosa (Paper V Fig. 1). In contrast, LPMC-CM derived from CC patients was more effective in inhibiting proliferation compared to LPMC-CM derived from non-inflamed controls (Paper V Fig. 2).

We next analyzed effector T cell cytokine production by α-CD3/α-CD28 stimulated CD4+ peripheral T cells in the presence of soluble factors derived from the colonic mucosa. We found increased production of the pro-inflammatory cytokines IL-17A, IFN-γ, TNF-α and IL-6 as well as the anti-inflammatory cytokines IL-4 and IL-10 by peripheral T cells in the presence of CM generated from the culture of both DNB and LPMC fractions from the colonic mucosa of CC patients compared to DNB-CM and LPMC-CM from non-inflamed controls (Paper V Fig. 3, Fig.4).

Discussion:

Generally normal human intestinal LP T cells show reduced proliferation upon TcR/CD3 stimulation compared to peripheral T cells, whereas CD2 or CD28 stimulation results in strong proliferation (42, 137). However LP T cells from patients with UC and CD showed enhanced proliferation compared with controls upon stimulation with CD3 in the presence of IL-2 (138).
This behaviour of mucosal T cells is thought to be influenced by the mucosal microenvironment (137). Our data on reduced inhibition of CD4+ peripheral T cell proliferation by soluble factors derived from denuded biopsies from the inflamed colonic mucosa of CC patients compared to controls is in accordance with the findings by Huff et al. on stroma conditioned medium from inflamed Crohn’s disease mucosa (139). Similarly, our findings on enhanced production of IFN-γ and IL-17A by CD4+ peripheral T cell in the presence of soluble factors derived from denuded biopsies and LPMC fractions from inflamed CC mucosa compared to controls are in line with the observations in that study (139). Interestingly, we also observed increased production of anti-inflammatory cytokines IL-4 and IL-10 by CD4+ peripheral T cells in the presence of both CMs derived from DNB and LPMCs fractions, from inflamed CC mucosa compared to controls. No data on the secretion of these cytokines are available in the Huff study. The increased production of IL-4 and IL-10 can be interpreted as that the local colonic milieu in CC patients makes an attempt to ameliorate the inflammatory response. IL-10 inhibits T cell proliferation as well as cytokine production (140). The recorded reduced proliferation of peripheral T cells could be due to enhanced production of IL-10 by peripheral T cells in the presence of CC-LPMC-CM but not CC-DNB-CM, suggesting that the composition in the two types of CM differ. Additional factors apart from IL-10 are also likely involved in the immunoregulatory effects of colonic CM on T cell proliferation and differentiation. As we found enhanced levels of IFN-γ, IL-17A and TNF-α, this suggests that in addition to IL-10 and other immunoregulatory molecules, there are also immune-stimulating molecules that drive the synthesis of these pro-inflammatory cytokines.

Altogether these data suggests that the local mucosal microenvironment is impaired in its’ ability to regulate the adaptive immune responses in MC patients, similarly to what has previously been demonstrated in IBD patients (139). Nevertheless, in the light of our previous studies identifying the CD8+ T cells as a more likely pathogenic T cells subset in MC, studies on the effect of soluble factors from inflamed mucosa of MC patients on CD8+ T cell activation and differentiation will provide a further understanding on whether they are the main player in driving the immunopathology in MC or not.

In the present study more patients as well as additional healthy blood donors needs to be investigated to draw any firm conclusions on the impact of the mucosal milieu on the differentiation of the peripheral CD4+ and CD8+ T cells, as alluded to above. Our in vitro model can be useful for analysis of the immunopathogenetic mechanisms behind the findings of
worsened disease in patients being treated with certain groups of drugs such as NSAIDS and proton pump inhibitors. In addition, the effect(s) of existing drugs on T cell differentiation in the intestinal mucosa can be investigated, thereby aiding in the decision on optimal doses required for suppression of T cell inflammatory responses in MC patients.

**Limitations in the studies performed in this thesis.**

The major limitation to the studies performed in this thesis is the small cohorts of MC patients. The various reasons in difficulties to recruit these patients are discussed in the materials and methods section. In addition, most of the studies were restricted due to the small amount of tissues available as colonic biopsies were used to perform experiments. Unlike IBD, surgery is very rare in MC patients, and therefore there is no resection material available for experimental purposes.

Conclusions on CD8⁺ T cell mediated pathology in MC are mainly based on the descriptive studies. However, the descriptive mapping of immune responses were (and are still) incomplete, but necessary to understand the pathophysiology in MC, Therefore we felt that this type of information is a very important step to unravel the underlying pathogenic mechanisms, as well as for planning future more mechanistic studies in MC.
GENERAL DISCUSSION

Is MC and IBD a similar disease?

Inflammatory bowel disease and microscopic colitis are both chronic inflammatory conditions of the intestine. MC is considered a mild form of colitis as the intestinal inflammation in MC is more subtle compared to ulcerative colitis and Crohn’s disease. Clinically MC is characterized by chronic non-bloody, watery diarrhoea, whereas UC is characterized by bloody diarrhoea. Despite the fact that MC has a subtler inflammation, it severely reduces the quality of life of these patients as they often have fatigue, nausea, weight loss, abdominal pain and urgency as well as nocturnal diarrhoea (11).

One important difference is that most of the cases in MC are treatable (albeit probably not curable) and hardly ever lead to life-threatening complications as are seen in UC and CD. The age at onset of IBD is relatively low, with the peak incidence at 20-30 years of age, with an almost identical disease distribution in males and females (141). In contrast, MC most commonly affects middle aged or elderly individuals with a noticeable female dominance, the peak incidence being around 60-65 year of age (6). One could think that the female dominance for this age group in MC is possibly due to the hormonal changes that occur after menopause in females. Interestingly it has been reported that the main incidence peak for CC and LC in females is 60-69 years, whereas in males the incidence peak is around 70-79 years of age. There is also a second lower incidence peak for CC in females at 80-89 years (142). To get further insight into the MC pathophysiology, it might be relevant to understand the nature of the physiological changes in the body taking place at these ages.

What are the factors involved in MC pathology?

The pathogenesis of IBD is thought to be due to exaggerated immune responses to intestinal microbes in a genetically susceptible host (116, 120). However due to the so far restricted pathophysiological data in MC, there are no firm suggested mechanism(s) on MC pathology. It has been postulated that MC is caused by disturbed immune responses to various luminal antigen(s), such as drugs, gluten or infectious agents, in predisposed individuals (6, 13). Generally, people of older age tend to consume medications for other complications, and their adverse effects might lead to diarrhoea. Nonsteroidal anti-inflammatory drugs (NSAIDs), proton pump inhibitors, aspirin and selective serotonin reuptake inhibitors have all been shown to be associated with microscopic colitis (91).
Hepatobiliary complications such as primary sclerosing cholangitis (PSC) have been associated with IBD (143). The hepatobiliary complications in MC need to be investigated. However bile acid malabsorption has been associated with a fraction of CC and LC patients (6), and shown to aggravate clinical symptoms in MC patients (95).

Are there similar immunopathological responses in MC and Celiac disease?

MC is often associated with celiac disease. It has been reported that 33% of celiac disease patients have histological changes in the colonic mucosa that are found in microscopic colitis, reviewed in (13). Celiac disease is a T cell mediated immune response against dietary gluten and causes inflammation in the small intestine whereas MC affects the large intestine. Celiac disease patients have heavy infiltration of CD8+ IELs that destroy the epithelium. In celiac disease, CD8+ IELs destroy the stressed intestinal epithelial cells expressing the MHC class I polypeptide related molecules MICA and MICB (144). Furthermore stressed intestinal enterocytes produce large amounts of IL-15, which up regulates NKG2D expression on CD8+ IELs. This enables cytotoxic killing of epithelial cells expressing the NKG2D ligand (144). Our findings in this thesis suggest that in contrast to IBD but similarly to celiac disease, immunopathological responses in MC are mainly derived from CD8+ T cells. In MC, CD8+ IELs may carry out cytotoxic activity as in celiac disease, but in contrast to celiac disease, most likely they target stressed epithelial cells or epithelial cells containing endogenous bacteria rather than dietary antigens, as the uptake of the later occurs in the small intestine.

Are local resident T cells culprits in MC immunopathology?

Although we did not find increased numbers of CD4+ T cells, they had a phenotype typical of activated lymphocytes, i.e. increased proportions of CD45RO+ and Ki67+ CD4+ IELs and LPLs were found in MC patients, as well as increased production of Th1/Th17 associated cytokines in the mucosa of these patients. In addition, our TREC data support the notion that mucosal T cells in MC is locally expanded, rather than a result of enhanced migration of peripheral lymphocytes to the inflamed mucosa. Constant exposure of the activated T cells to luminal agents such as infectious agents, drugs or dietary antigen such as gluten likely leads to proinflammatory responses resulting in epithelial damage and chronic inflammation in the intestinal mucosa, as observed in MC. However, as alluded to above, the antigens in MC are less likely to be derived from dietary antigens, as the inflammation is limited to the colon. The previously observed epithelial damage that leads to increased permeability...

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in MC (7) could be due to cytotoxic activity of CD8+ T cells or the cytokines secreted by both CD8+ and CD4+ T cells.

**Does context matter for vitamin A to regulate immune responses?**

Recently vitamin A and its derivative retinoic acid (RA) and its role in the immune system have been of interest to several researchers, as RA seems to be a key inducer of gut homing receptors (145). In addition, accumulating evidence show that retinoic acid increases the differentiation of naïve T cells into inducible FOXP3+ regulatory T cells (Treg) and inhibits their differentiation into pro-inflammatory Th17 cells (146-147). Interestingly, in contrast to this it was recently shown that RA together with IL-15 suppress Foxp3+ Treg induction and promotes differentiation of T helper cells to Th1 and Th17 cells as well as induces IL-17 and IFN-γ production (148). Future studies elucidating the role of IL-15 and vitamin A in MC are relevant to understand the basic immunopathophysiology of this disease.

**Does the mucosal microenvironment affect the T cell responses?**

Our data on increased production of both pro- and anti-inflammatory cytokines by peripheral CD4+ T cells in the presence of soluble factors derived from the inflamed mucosa of CC patients compared to controls suggest that the local colonic milieu in CC patients is in fact attempting to counteract the inflammatory response. The identity of these regulatory soluble factors from the colonic mucosa is mostly unknown, and it will be very valuable to determine the protein profile by proteomics to reveal any immunoregulatory molecules in MC that keeps the inflammation at bay in contrast to IBD patients. One obvious immunoregulatory cytokine whose abundance and activity in the colonic mucosa of MC patients should be investigated is TGF-β. Apart from proteins, the recently appreciated post transcriptional regulators in the form of non-coding microRNAs (miRNAs) has been implicated as important regulators of inflammatory responses (149), and their role in MC needs to be investigated.

**Is histological remission sufficient to get rid of the clinical symptoms in MC?**

In addition to CC and LC patients, in this thesis work we have identified previously groups of LC and CC patients with active clinical disease, but in histopathological remission that were previously not described. Our flow cytometry data and mucosal cytokine expression data show that LC-HR but especially CC-HR patients display normalized proportions of activated mucosal T cells and mucosal cytokine expression compared to LC and CC...
patients with histopathological changes. Apparently there are other factors contributing to the clinical symptoms in these patients. As discussed above, bile acid malabsorption could be one of them and therefore bile absorption would be interesting to investigate in these patient subgroups. Further studies are clearly required to investigate the mechanisms behind the clinical symptoms in MC patients with histological remission.

Is CC and LC two separate entities or different manifestation of same disease?

As CC and LC have many clinical similarities and share histopathological features, except for the subepithelial collagen layer found in CC, it has been discussed whether LC and CC are two separate but related disorders, or the same disease seen in different stages of development. Conversion of LC to CC or the opposite has been reported, but only infrequently (5). Our flow cytometry based phenotypic characterization of the mucosal lymphocytes support the notion that CC and LC are two separate entities. It has been reported that CC patients present with more aggressive clinical symptoms compared to LC patients and this was also reflected in our flow cytometry data, as CC patients had higher proportion of CD8+ IELs and LPLs expressing CD45RO and Ki67 compared to LC patients. However we did not find any significant differences in the expression of mucosal cytokines in CC and LC.

Why is the inflammation in MC subtler than in IBD?

It has been proposed that in IBD the immunopathological response are mainly orchestrated by CD4+ T cells. In contrast, our findings in this thesis support that in MC the immunopathological responses are mainly mediated by CD8+ T cells. A recently identified subset of Th17 cells that coproduces the Th1 cytokine IFN-γ have been implicated in the immunopathogenesis of IBD (64). The IL-17 cytokine activates epithelial cells, macrophages and fibroblasts to release proinflammatory chemokines and cytokines that leads to recruitment and accumulation of mainly neutrophilic granulocytes but also lymphocytes and DCs and perpetuates the inflammation in IBD (51). It is possible that CD8+ T cells do not produce the large amount of cytokines associated with Th17 and Th1 subsets. As it is believed that in IBD, aberrant immune responses are directed to intestinal microbiota, innate immune cells such as macrophages and neutrophils may initiate the immune responses against bacteria. Both macrophage and neutrophils have been implicated in tissue damage. In the majority of MC cases there is limited infiltration of neutrophils (150). In contrast, a common feature of UC is infiltration of neutrophils in the
epithelium accompanied by cryptitis, crypt abscesses leading to permanent distortion of crypts (120). This might be one explanation to why the inflammation in MC is subtler than UC.
FUTURE PERSPECTIVES

The descriptive mapping of immune responses was missing in the MC and the work in this thesis has demonstrated aberrant adaptive immune responses in the colonic mucosa of MC patients.

Our data on disturbed CD4⁺ and CD8⁺ T cell numbers indicates that this could be due to an aberrant apoptosis of mucosal T cells in MC patients, and thus this warrants investigation.

We have demonstrated increased expression of Foxp3⁺ regulatory T cells in the mucosa of MC patients, but we do not know whether the Foxp3 expression stems from CD4⁺ or CD8⁺ T cells and this warrants further studies. During our flow cytometry analysis, we were restricted in making choices for immunostaining due to the limited amounts of fresh lymphocytes, as they are isolated from colonic biopsies. Future studies analyzing both the frequency and efficiency of CD4⁺ and CD8⁺ Tregs, as well as the regulatability of the mucosal effector T cells, as performed previously in our group (151)

In addition, perforin and granzyme expression within the CD8⁺ IELs and CD8⁺ LPLs, would be highly interesting to define the Treg profile as well the pathogenic profile of CD8⁺ T cells in MC.

Our data on the Th17/Tc17 and Th1/Tc1 cell mucosal cytokine profiles in colonic biopsies needs to be validated on sorted CD4⁺ and CD8⁺ IELs and LPLs to draw a firm conclusion whether the immunopathological responses are mainly derived by T helper subsets or CTL subsets.

In the light of these studies, identifying the CD8⁺ T cells as a more likely pathogenic T cell subset in MC, studies on the effect of soluble factors from the inflamed mucosa of MC patients on peripheral CD8⁺ T cell activation and differentiation will provide a further understanding on whether they are the main players in driving the immunopathology in MC or not.

In addition, the changes in the inflammation in the colonic mucosa over time has not been elucidated in the MC and would be highly interesting to define whether the patients with longer disease duration have different types of immune responses compared to newly diagnosed MC patients.
Our *in vitro* model can be useful for analysis of the immunopathogenetic mechanisms behind the findings of worsened disease in patients being treated with certain groups of drugs such as NSAIDS and proton pump inhibitors. In addition, this model can be utilized to analyze the efficacy of existing drugs on an individual basis, enabling tailor made therapy of microscopic colitis.

I believe that my thesis work have added some important pieces of information to understand the nature of immunopathological responses in microscopic colitis and hope that the above future studies will add a valuable information in MC pathophysiology.
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