Mucosal Dendritic Cells in Inflammatory Bowel Disease

by

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To those who have played a role in my personal growth
Abstract

Crohn’s disease, a chronic inflammation of the bowel, is a multi-factorial condition where uncontrolled immune responses to luminal bacteria occur in genetically predisposed individuals. The first observable clinical signs are small ulcers that form at a specialised form of epithelium, follicle-associated epithelium (FAE). The FAE covers immune inductive sites, Peyer’s patches, which function primarily as sensory areas that sample the external gut environment. Dendritic cells are one of the key cells that are involved in sensing luminal contents and orchestrating the gut immune system.

The main aim of this thesis was to determine whether the barrier of the FAE is breached in Crohn’s disease and if dysfunctional immune regulators, namely dendritic cells, play a role in initiating and/or maintaining the chronic intestinal inflammation.

Using biopsies and surgical specimens, we were able to show that in Crohn’s disease, there was an increased transmucosal transport of Escherichia coli compared to specimens from ulcerative colitis and non-inflammatory bowel disease (IBD) controls. Dendritic cells internalised a higher percentage of bacteria that had translocated across the FAE in the Crohn’s samples. Furthermore, significantly higher concentrations of TNF-α was released upon bacterial stimulation by tissues from patients with Crohn’s disease than in controls.

We went on to characterise the dendritic cells present in the Peyer’s patches of patients with Crohn’s disease. We found an accumulation of both immature and mature dendritic cells beneath the FAE, in the sub-epithelial dome (SED). Normally, mature dendritic cells migrate towards T cell-rich areas. However, we observed mature dendritic cells accumulating in the SED because they lacked the CCR7 migratory receptor. Furthermore, they were more prone to take-up bacteria, and produced TNF-α.

To study the function of mucosal dendritic cells, we performed isolation experiments and mixed lymphocyte reactions. Dendritic cells from both the ileum and blood of patients with active Crohn’s had reduced capacity for inducing T cell proliferation than non-IBD controls. Blood dendritic cells of patients in remission had normalised function that was similar to dendritic cells from healthy controls.

The SAMP1/YitFc mice, considered an appropriate murine model for Crohn’s disease, had an inherent permeability defect that increased with the chronicity of intestinal inflammation. However unlike in human Crohn’s disease, dendritic cells did not seem to play a role in murine ileitis.

This thesis highlights the accumulation of the actively surveying dendritic cells that are prone to bacterial internalisation, and points to their possible different functional roles in active versus in-active disease; thereby confirming dendritic cells as one of the key components in the pathogenesis of Crohn’s disease.

Keywords: Blood, Crohn’s disease, dendritic cells, E.coli, FACS, follicle-associated, epithelium, human, ileum, mixed lymphocyte reaction, permeability, Peyer’s patches, SAMP1/YitFc, Ussing chambers, villus epithelium
Abbreviations

$^{51}$Cr-EDTA  $^{51}$Chromium- ethylenediaminetetraacetic acid
CD  Crohn’s disease
DC  dendritic cells
DC-SIGN  DC-specific ICAM-3 grabbing nonintergrin
E.coli  Escherichia coli
FACS  fluorescence-activated cell sorter
FAE  follicle-associated epithelium
GFP  green fluorescent protein
GM-CSF  granulocyte macrophage-colony stimulating factor
HRP  horseradish peroxidase
IBD  inflammatory bowel disease
Ig  immunoglobulin
IL  interleukin
Isc  short circuit current
LPMC  lamina propria mononuclear cells
M cell  membranous or microfold cell
MLR  mixed lymphocyte reaction
PBMC  peripheral blood mononuclear cells
PD  transepithelial potential difference
PMN  polymorphonuclear neutrophils
SED  sub-epithelial dome
slg  secretary Ig
TER  transepithelial electrical resistance
TLR  Toll-like receptor
TNF  tumour necrosis factor
UC  ulcerative colitis
VE  villus epithelium

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List of Papers

This thesis is based on the following papers which are referred to by their roman numerals.

I. Increased uptake of non-athogenic *E.coli* via the follicle-associated epithelium in longstanding ileal Crohn’s disease.


II. CD83⁺CCR7⁺ Dendritic Cells Accumulate in the Subepithelial Dome and Internalize Translocated *Escherichia coli* HB101 in the Peyer’s Patches of Ileal Crohn’s Disease.


III. T-cell expansion capacity of Dendritic cells isolated from patients with Crohn’s disease.

Sa’ad Y Salim, Karlhans Fru Che, Marie Larsson, and Johan D Söderholm. In manuscript 2009.

IV. Barrier defect in the follicle-associated epithelium of SAMP1/YitFc mice demonstrates vulnerability to adherent-invasive *Escherichia coli* LF82.

Sa’ad Y Salim, Pär Myrelid, Arlette Darfeuille-Michaud, Theresa T Pizzaro and Johan D Söderholm. In manuscript 2009.
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‘All right,’ said the Cat; and this time it vanished quite slowly, beginning with the end of the tail, and ending with the grin, which remained some time after the rest of it had gone.

‘Alice’s Adventures in Wonderland’ – 1865
I. Introduction

Inflammatory bowel diseases (IBD) result from destructive inflammations of the intestinal tract. Crohn’s disease and ulcerative colitis are two of the major types of IBD. Reports on inflammation in the bowel initially started to appear as isolated cases in Great Britain and northern Europe during the 19th and early 20th centuries. Since then, cases in IBD have steadily increased, numerically and geographically, and today it is recognised worldwide as a leading cause of chronic intestinal inflammation.

A. Crohn’s Disease

1. History

“(T)he ulcerated cecum contracted and invaginated into the ileum” – was, in 1612, one of the first histological observations of Crohn’s disease. G.F. Hildenus made these descriptions in an autopsy of a boy who had died after persistent “abdominal pain” and diarrhoea. Similar observations describing ulcerations and perforations, perianal, rectovaginal and rectovisical fistulas were recorded throughout the 16th – 19th centuries. In 1913, T. Kennedy Dalziel coined the phrase

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**Box 1 | Burrill B. Crohn (1884 – 1983)**

Burrill B. Crohn was born in New York City on June 13, 1884. By 1907, he received his M.D. degree from The College of Physicians and Surgeons at Columbia University’s Medical School. He served as an intern at the Mount Sinai Hospital in New York City. He continued to work there as a gastroenterologist at the outpatient GI clinic. It was here that Gordon Oppenheimer collected 12 patients, and Leon Ginzburg associated with A.A. Berg (who had performed surgery on all the patients) and Burrill B. Crohn collaborated on the landmark article that initiated the IBD era. Ironically, it was Berg that encouraged the collaboration but opted out his name from the alphabetically arranged authorship. Had he accepted it, today we would be writing about Berg’s disease.
“chronic interstitial ileitis” in a published case-report of a patient who had abdominal pain and diarrhoea that progressed to intestinal obstruction and death \textsuperscript{15,16}. Though this was one of the earlier scientific records, it was not until 1932 when Crohn, Ginzburg and Oppenheimer published in JAMA a “new entity” where abdominal pain, emaciation, diarrhoea and fever were symptoms of “terminal ileitis” \textsuperscript{17}. Debate over the occurrence of histological features in other locations of the intestine lead Crohn to change the name of this new entity to regional ileitis \textsuperscript{18}. Today, Crohn’s disease is described as a chronic inflammatory condition that can affect the entire digestive system, from the mouth to the anus, and affects all layers of the intestinal wall \textsuperscript{19}.

2. Epidemiology and Disease Manifestations

Epidemiological studies on IBD did not really begin until the 1950s \textsuperscript{2}. Crohn’s disease was found to be a Western world disease with highest incidences occurring in individuals from northern European origins, North America and in the United Kingdom. Crohn’s disease affected males and females at nearly equal rate; was more common amongst urban than rural dwellers; and the onset of the disease occurred at young age with the peak incidence occurring between 15-30 years \textsuperscript{12}. In Sweden, recent epidemiological studies are lacking though in 1991 the incidence was 6.1 per 100,000 inhabitants while the prevalence was 146 per 100,000 individuals \textsuperscript{20}. A more recent study that examined cases in the Stockholm County found an increase of 0.2% in the incidence of Crohn’s disease from the previous decade \textsuperscript{21}. The prevalence as of 1st of January 2002 was 213 per 100,000 inhabitants.

Though it is difficult to diagnose Crohn’s disease without radiological and histological assessments, the indicating signs of disease manifestations are abdominal pain, diarrhoea, bleeding, fever, abnormal weight loss and vomiting \textsuperscript{22}. In addition, some extra-intestinal manifestations can affect the joints, skin, eyes and the perianal region.

Histological assessments reveal Crohn’s disease to be a transmural inflammation that is segmental and discontinuous. Majority of the cases affect the ileo-cecal region \textsuperscript{23}. Microscopic observations include granulomas, lymphocyte infiltration, discontinuous distribution pattern, fibrosing of all layers of the wall, focal lymphoid hyperplasia and seldom crypt abscesses \textsuperscript{24}. 
3. Treatments

To date, there is no cure for Crohn’s disease. Crohn et al. initial recommendation was resection of the diseased segments. However, this led to short-bowel syndrome. Today, the first line of treatment consists of drug therapy that is aimed at combating symptoms of acute attack and the maintenance of remission. These include immunomodulators such as corticosteroids and mesalazine or 5-aminosalicylic acid for acute attack and mesalazine for maintenance of remission. Usually operative interventions are performed on severe cases and to avoid life-threatening situations such as obstructions and fistulae.

New medications such as infliximab (Remicade), are biological agents that are potent and can induce remission in patients with disease refractory to immunomodulators. Infliximab is a chimeric monoclonal antibody that targets tumour necrosis factor- alpha (TNF-α). Other experimental treatments include interleukin (IL)-10 and anti-IL-12 antibody therapy and use of probiotics.

4. Aetiology

![Anatomical Distribution of Crohn’s Disease](image)

**Figure 1. Anatomical Distribution of Crohn’s Disease.**
The figure illustrates the percent frequency of Crohn’s disease developing throughout the gastrointestinal tract.
The exact causal agents of the pathogenesis of Crohn’s disease are not yet known. Though evidences have suggested genetic, environmental and immunological factors all contribute to the pathogenesis $^{10,19,32}$. The earliest signs of inflammation occur at the distal ileum. Endoscopic evaluations showed the first observable signs were aphthoid lesions $^{23,33}$. These lesions were commonly found to occur in the clusters of lymphoid follicles known as the Peyer’s patches $^{23,34}$. Indeed, magnifying endoscopy and scanning electron microscopy revealed the aphthoid lesions were preceded by ultra-structural erosions of the specialised epithelium covering the Peyer’s patches, the so called follicle-associated epithelium (FAE) $^{34,35}$. Perhaps it is not a coincidence that most of cases in Crohn’s disease occur at a site where the lymphoid aggregates are more common $^{23,36,37}$. This suggests that disruption of Peyer’s patches homeostasis leads to the initiation of ileal inflammation. This is in line with the central dogma where dysregulated immune response, triggered by environmental factors in a genetically susceptible host, predisposes individuals to Crohn’s disease $^{10,19,32}$.

i. Genetic Distribution

As technology and our knowledge have increased in genome-wide association studies, so has our insight into the implicating roles of genes in the immunopathogenesis of Crohn’s disease. With genome-wide association studies, Crohn’s disease has been shown to have a higher concordance in monozygotic twins than in dizygotic twins $^{38,39}$. Along with epidemiological studies, it has been found that there is a 75-80% of concordance among individuals in families with another member affected by the disease $^{40,41}$. To date, 12 genetic loci have been associated with Crohn’s disease, and in general, it can be divided into genes that contribute to innate immune response and those that are involved with adaptive immune response $^{41}$. An example of alteration in genes in the innate immune system is the NOD2/CARD15 mutations $^{42}$. Gene polymorphisms were found in NOD (nucleotide oligomerisation domain), one of the first susceptibility gene that was found in 30% of patients with Crohn’s disease $^{43,44}$. The gene is located on chromosome 16 with three common mutations: Arg702Trp, Gly908Arg and Leu1007frame-shift, all of which are located within or near the leucine-rich repeat region of the NOD2 protein $^{45,46}$. NOD2 functions as an intracellular pattern-recognition receptor for muramyl dipeptide (MDP), a component present on both Gram-positive and Gram-negative bacteria $^{47,48}$. The precise mechanistic relationship between NOD2 and Crohn’s disease remains controversial $^{41}$. The loss-of-function hypothesis states that there is
reduced ability of the innate immune system to clear bacteria due to hyporesponsiveness of cells to bacterial stimuli \(^49^{,50}\). For example, dendritic cells derived from monocytes of Crohn’s patients with frame-shift mutation were shown to have decreased production of cytokines IL-8, TNF-\(\alpha\) and IL-12, after bacterial stimuli, even though they were phenotypically activated \(^51\). The general theme here is that alterations in intracellular processing of bacteria results in a compensatory increase in the activity of T helper (h) 1 cells, which is observed in the lamina propria of patients with Crohn’s disease.

Polymorphism in the gene encoding the IL-23 receptor (R) is the strongest association for altered adaptive immune response in Crohn’s disease \(^4\). IL-23R is composed of the IL-23R and the IL-12R\(\beta\)1 subunits (which is also part of the IL-12R), and is expressed by natural killer (NK) cells, NKT cells, CD4\(^+\) and CD8\(^+\) T cells. Mouse models have demonstrated that blockade of IL-23 resulted in increased susceptibility to enteric infections; while knockout experiments have shown the mice develop intestinal inflammation \(^52\). With this, future clinical studies are on the way to address whether antibodies against IL-23 and/or IL-23R prove to be a safe and effect treatment for Crohn’s. The hypothesis here is that IL-23R signalling pathway contributes to the pro-inflammatory state and so blocking its function could provide a novel therapeutic strategy \(^53\).

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**Box 2| Hygiene Hypothesis**

*Crohn’s disease has been associated as a Western-world disease, while its incidences are lower in developing countries. The hygiene hypothesis stipulates that lack of early exposure to certain antigens makes one susceptible to Crohn’s disease. In the West, children that were raised in a more sanitary environment were more likely to develop the disease. In support of this hypothesis, a study in Manitoba, Canada, found patients with Crohn’s disease were less likely to have lived on a farm, less likely to have drunk unpasteurized milk, be a first-generation Canadian and to have been exposed to cats. Conversely, another Canadian study in Montreal found Crohn’s disease patients were more likely to have had pets, had physician-diagnosed infections early in life, and less likely to have access to a personal towel. Furthermore, a higher ratio of household members to rooms available in the house lowered the risk for Crohn’s disease. These conflicting results highlight the complexity of the influence of environmental factors.* \(^34\)
**ii. Environmental Factors**

Several environmental factors have been proposed to be important in the pathogenesis of Crohn’s disease. Smoking has been established as a risk factor in Crohn’s disease \(^{54}\). Some studies have shown smoking to have a two-fold increase in disease risk, and it can result in an earlier recurrence of Crohn’s following surgery \(^{55,56}\). However, a few studies have also been published that show smoking having no negative effects on Crohn’s \(^{57,58}\). Diet has been suggested to influence the progression of Crohn’s disease. Increased intake of fast foods, refined sugars and fats, and the reduction of fresh fruits intake have been quoted as being important risk factors \(^{3,59}\). More empirically, it has been shown that elemental diet can be used for inducing remission as well as nutritional benefit, as absorption is usually compromised during inflammation \(^{60,61}\). Furthermore, the import of Western-style and foods has been anecdotally linked to the increase of incidences in Asia.

Other environmental factors that have been attributed to affect Crohn’s disease are psychological stress, early life influences and infection \(^{62}\). It has been suggested that individuals susceptible to stress are unduly prone to chronic inflammation due to the effect of stress on mucosal barrier \(^{63,64}\). Linked to stress, early life trauma or late exposure to environmental infections could result in an incomplete and delayed immune response that favours continued disease at a later stage in life (see Box 2| *Hygiene Hypothesis*).

Though the influences of environmental factors on Crohn’s disease are poorly understood, it is clear that there is a link to the world-wide increase in incidences. Furthermore, it is widely accepted that genetically susceptible individuals are readily influenced by factors, such as smoking, diet, stress and geo-social status \(^{3}\).

**iii. Immunological Impact**

Under physiologically normal conditions, the mucosal immune system is able to mount a response against pathogenic agents while simultaneously maintaining tolerance towards food antigens or resident bacterial flora. In Crohn’s disease, this mucosal homeostasis is disrupted resulting in a dysregulated mucosal immune response towards bacterial flora of the gut \(^{64-67}\). Generally, Crohn’s disease has been perceived as a prototypical Th 1 condition with an increase in interleukin (IL)-12, interferon (IFN)-\(\gamma\) and tumour necrosis factor (TNF)-\(\alpha\) cytokines (the classical Th1 mediating cytokines). This has been supported by the recent application of anti-TNF and ant-IL-12 therapies to treat Crohn’s disease \(^{27,53}\).
One hypothesis for the underlying dysregulation of the mucosal immune mechanism in Crohn’s disease stems from a defective mucosal effector T cells that overreact to resident microflora $^{65,67,68}$. Alternatively, it has been suggested that defective mucosal T regulatory (reg) cells that under-reacts to the resident microflora could be the contributing factor $^{69-71}$. Experiments conducted by Duchman and colleagues support this hypothesis where cells derived from inflamed intestinal IBD tissues had robust stimulation after being treated with sonicated autologous or heterologous gut microflora; whereas cells from normal individuals responded only to sonicated heterologus microflora $^{72}$. This suggests that in IBD patients, there is a loss of tolerance to one’s own gut microflora $^{72,73}$.

Several recent publications have pointed to the pivotal role of Th17 in chronic intestinal inflammation $^{52,74,75}$. T cell producing IL-17 was involved in tissue damage in many diverse pathologic conditions via the IL-17/IL-23 pathway. In the IL-10$^{-/-}$ knockout mice model, it was shown that IL-23 and not IL-12 was essential in the development of intestinal inflammation $^{75}$. IL-23 is a heterodimeric cytokine that shares a p40 subunit with IL-12, and is coupled to p19 instead of p35 which is coupled to IL-12 $^{76}$. A beneficial effect of anti-p40 antibodies in Crohn’s disease has been shown, which exerted its effect through the inhibition of IL-23 $^{75}$. Furthermore, a recent genome-wide association study reported a high association between Crohn’s disease and a gene encoding for the receptor for IL-23 $^{77,78}$.

In summary these three factors, genetic predisposition, environmental factors and immunological impact, interact together to result a complex multifactorial, heterogeneous disease whose pathogenesis has not been fully elucidated.

**Box 3 | Specific Infectious Agent in Crohn’s Disease**

In trying to determine the environmental factor behind Crohn’s disease, the search for the bacterial or viral agent has often resulted in conflicting and inconclusive results. Mycobacterium paratuberculosis has been suggested to be involved in the aetiology of Crohn’s disease. However subsequent studies measuring DNA sequences and serum antibodies against the bacteria, and controlled trials have failed to show a putative role for it in Crohn’s disease. Several other microorganisms, such as Listeria monocytogenes, Yersinia enterocolitica, Saccharomyces cerevisiae, and Chlamydia trachomatis have been proposed but none have conclusively shown to have potential aetologic role. More recently, adherent invasive Escherichia coli LF82 was isolated from damaged ileal mucosa of a patient with Crohn’s disease. Experimental studies have shown its ability to adhere and invade epithelial cells, induce and aggravate an inflammatory response in mouse colitis, and were found in 22% of patients studied with Crohn’s disease $^{5,7,79}$. 
B. Background on Intestinal Immunology
1. Intestinal Architecture

The small intestine of an adult human is approximately 5-7 meters long, and it can be divided into the duodenum, jejunum, and ileum. Though the primary function of the intestinal tract is digestion of food, it is also the largest immune organ in the body.

The surface area of the intestine is about 300-400 m², which is the size of a tennis court. The intestinal mucosa is composed of the epithelial lining, the lamina propria and the muscularis mucosa. The epithelium is comprised of a single-cell layer that digests and absorbs nutrients, while separating the external environment from the internal body proper; thus playing a prominent role in preventing noxious substances and agents from gaining access to the internal environment. The epithelium is continuous via tight junctional connections. Enterocytes that form the epithelial layer have micro-villi or brush-border composed of glycoproteins that contain digestive enzymes and are also able to retain mucus secretions.

Beneath the epithelial layer lies the lamina propria which consists of loose connective tissue. Here, several cell types can be found, such as lymphocytes, plasma cells, eosinophils, macrophages and nerves, blood vessels and smooth muscles, which border the submucosa. Meanwhile, the muscularis propria comprises of longitudinal and smooth muscles whose primary function is the peristaltic moment of digested material along the digestive tract. Finally, there is the

![Figure 3: Composition of a Lymphoid Follicle.](image)

Aggregates of lymphoid follicles form the Peyer’s patches. The follicle-associated epithelium (FAE) covers the sub-epithelial dome (SED) which contains the immune cells dendritic cells (DC) and B cells that sample contents transported by M cells. Image adopted from Neutra et al. [81].
serosa which is made up of loose connective tissues with fat and elastic tissue that prevents frictional damage on the intestine tract from rubbing against other tissues or organs.

Concentrated at the distal ileum are specialised inductive sites known as the Peyer’s patches \(^\text{82}\). It was first described by J.C Peyer over 300 years ago as “folliculi lymphatici aggregate” \(^\text{83}\). Peyer’s patches develop well before birth and are most abundant, more than 200 patches, during adolescence \(^\text{82}\). The numbers dramatically regress with age to around 50 at the age of 90 years old \(^\text{84}\). Peyer’s patches can be found throughout the gastrointestinal tract but in the distal ileum, where they are most abundant, they can be as large as 30-50cm\(^2\) \(^\text{82}\).

Peyer’s patches consists of aggregated lymphoid follicles that have specialised epithelia known as the follicle-associated epithelium (FAE). The FAE, like the regular villus epithelium (VE), is mostly comprised of enterocytes, but they differ from ones found in the VE \(^\text{81}\). FAE enterocytes have lower amounts of membrane-associated hydrolases, which are involved in digestion, and have different glycosylation patterns, making this region susceptible to microbial binding and internalisation \(^\text{85}\). This susceptibility is further facilitated by the decreased number of Paneth cells found in the FAE crypts.

In contrast to the VE, the FAE contains specialised enterocytes known as membranous or microfold (M) cells whose primary function is to sample and transport luminal antigens to its underlying pocket \(^\text{86}\). Unlike regular enterocytes, M cells have ruffles or microfolds, and reduced expression of digestive enzymes, such as sucrose, isomaltase and alkaline phosphatase \(^\text{81}\). M cells’ basolateral membrane forms deep invaginated pocket that contains T cells, B cells or dendritic cells. This ensures sampled luminal antigens are readily available for antigen presentation and immune induction. Though there has been several attempts to identify histological markers on human M cells, to date there is no acceptable marker hence making its identification difficult \(^\text{83,87}\).

Beneath the FAE is the sub-epithelial dome (SED). Here many of the lymphoid cells specialised in immune induction are present, such as T and B cells, and phagocytic dendritic cells, macrophages and monocytes (see Figure 3) \(^\text{79,81}\). At the centre of the follicles lies the B cell germinal centre that is surrounded by the marginal zone constituting of proliferating B cells expressing IgM and IgG. Between the follicles is a region known as the interfollicular region where it is mainly comprised of T cells and dendritic cells. Here is also where dendritic cells program the T lymphocytes.
2. Intestinal Barrier Function

The first line of defence in the intestinal immunology is the epithelial layer. This single layer is comprised of enterocytes interspersed with Goblet cells, Paneth cells, enterochromaffin cells, M cells and intraepithelial lymphocytes (IELs) \(^{79}\). The epithelial barrier is maintained by the physical defence mechanism associated with the mucosal surface (via secretion of mucus and cryptidins) and the junctional complexes that link adjacent epithelial cells. Goblet cells maintain steric defence by secreting mucus, a gel-like substance that can trap pathogens and antigens. Paneth cells, generally found at the base of the crypt, secrete a number defensin peptides (cryptidins) that have antimicrobial activity. Meanwhile, the tight junctions are highly regulated filaments that allow for the passage of ions and water, but exclude, under physiological conditions, macromolecules that are larger than 500 Daltons \(^{88}\). Finally, in the lamina propria there are immune cells, such as plasma cells that produce secretory immunoglobulin (sIg) A and dendritic cells that sample luminal contents, that actively protect via the mucosal immune response mechanism \(^{83}\).

Tight junctions, also known as zonula occludens, form part of the physical barrier of the epithelia by forming a continuous cell-cell contact \(^{89}\). They are located at the apical area and as well as serving as a gate function, they maintain polarity of lipids and receptors. The tightness of the gate is affected by the number of strands composition of claudins comprising the tight junction and intracellular second messengers (cAMP, Ca\(^{2+}\), PKC, PLC, and calmodulin) \(^{90, 91}\). There is a size and charge selectivity where small positively charged molecules and ions pass more readily. Structural and functional changes of tight junctions can be found in Crohn’s disease patients. Furthermore, inflammatory cytokines, such as IFN-γ, TNF-α and IL-4, and several pathogens, are known to disrupt the tight junctions and hence compromising the intestinal barrier \(^{89}\).

3. Intestinal Immunology

The gut mucosal immune system, being the largest immune organ in the body, contains more than \(10^{12}\) lymphocytes and produces more sIgA than any other site \(^{92}\). The gut-associated lymphoid tissues (GALT) are comprised of organised lymphoid follicles, known as Peyer’s patches, mesenteric lymph nodes, and a large number of lymphoid cells scattered throughout the lamina propria and within the epithelium (such as the IELs). The GALT not only serves as the first line of immune defence against external luminal antigens, but it also capable of modulating the epithelial function \(^{91}\).
The intestine, in addition to maintaining a physical barrier, actively induces immune surveillance, especially at the lymphoid follicles. M cells, with their high endocytic activity, sample particles and bacteria and transfer them to underlying dendritic cells and/or lymphocytes. A small number of bacteria in the dendritic cells can survive and induce T-cell-independent IgA responses, which are part of the regulation of the endogenous bacteria. Dendritic cells are also capable of directly sampling luminal bacteria by extending their dendrites between epithelial cells via the tight junctions. As there are ~500 different commensal bacterial species in the gut lumen, it is thus imperative for the GALT to distinguish between pathogens and normal flora. Under physiological conditions, dendritic cells with their luminal load, travel to immunocompetant sites and induce T regulatory responses. These T cells produce the anti-inflammatory cytokine, IL-10 and/or transforming growth factor (TGF)-β. Neutralising any of these two cytokines abolishes the suppressive effect of the GALT, as it is seen in IL-10 deficient mice that develop enterocolitis. Pathogens are further distinguished from commensals by pathogen-associated molecular patterns that are recognised by pathogen recognition receptors found on antigen presenting cells, such as epithelial cells, dendritic cells and macrophages.

C. Background on Murine Models of Crohn’s Disease

Unlike patient-orientated experiments, animal models provide us several advantages in facilitating our understanding of the pathogenic mechanisms underlying Crohn’s disease. With animal models, we are able to study specific pathophysiological events that occur even prior to disease onset. Genetic and immunologic manipulations allows for the identification of specific pathways, while contribution of bacteria to disease pathogenesis can be used in these mice models. Another advantage to the use of murine models is the possibility to rapidly test novel treatments in an in vivo system.

1. Genetic Manipulation

Advancements in transgenic and knockout methodologies have greatly expanded our knowledge and application in animal models of IBD. With genetic models, investigators have been able to identify the role of key immune-related molecules in the pathogenesis of chronic intestinal inflammation. Examples of knockout models of intestinal inflammation include interleukin (IL)-10<sup>−/−</sup>, IL-2<sup>−/−</sup>, T cell receptor
(TCR)α/β−/− and tumour necrosis factor (TNF)ΔARE 102. Most genetic models develop colitis but the TNFΔARE is a model of ileitis 103. This model supports the key role of TNF in the pathogenesis of Crohn’s disease where patients respond to anti-TNF antibody treatment. The heterozygote TNFΔARE+/− mutant mice develop ileal inflammation in the distal ileum and occasionally at the proximal colon by eight weeks. The TNFΔARE mice bare a 69 base-pair deletion at the 3′-AU-rich region of the TNF gene. The mucosal abnormalities include villus hyperplasia, thickening of intestinal wall due to infiltration of acute and chronic inflammatory cells such as mononuclear leukocytes, plasma cells, and neutrophils. Though this model provides the unique opportunity to study the precise mechanisms underlying TNF-induced ileitis, it is fairly limited in trying to understand causative factors of Crohn’s disease. Furthermore, no mutation in the 3′-AU-rich region of the TNF gene has been identified in patients with Crohn’s disease 102.

Ironically, the advantage of using genetic models of intestinal inflammation – identify the role of key immune-related molecules – is precisely its limitations. These models give insights only into single causative factors and not the complexity in investing the underlying causes of Crohn’s disease.

2. Chemical Stimulation

Intestinal inflammation in animal models can also be induced by administering exogenous chemical agents. These include trinitrobenzene sulfonic acid (TNBS), dextran sodium sulphate (DSS), and oxazolone. The advantages in the use of chemically-induced intestinal inflammation is in studying the biochemical pathways of inflammation, as provided in the TNBS model, and in studying the events in the breakdown of immunological tolerance, as seen in the DSS model. In the DSS model, epithelial disruption leads to luminal bacterial translocation and subsequent infiltration of neutrophils and other acute immune cells 102. However, inflammation can occur even in the absence of lymphocytes, hence not representing the chronic phase of inflammation. Chemically-induced models of intestinal inflammation recapitulate the events that lead to acute mucosal inflammation due to mucosal injury, but they do not follow the clinical course of human IBD. Furthermore, epithelial disruptions primarily lead to colitis hence not being ideal models to study ileal chronic inflammation.
3. Immunological Manipulation

Immunological models of intestinal inflammation involve the transfer of T cells or bone marrow precursors into immunodeficient recipient mice. Two classical examples of immunological manipulations are the CD45RB$^{\text{high}}$ and bone marrow chimera transfer models. In the CD45RB$^{\text{high}}$ T cell transfer model, recipient mice develop intestinal lesions with presence of polarised helper T cell type 1 (Th1) $^{104}$. This could be prevented with treatment of anti-interferon (IFN)-γ or TNF-α monoclonal antibodies. Interestingly, transfer of CD45RB$^{\text{low}}$ CD4$^+$ T cells along with CD45RB$^{\text{high}}$ cells prevented the development of disease in the immunodeficient mice $^{102}$. This indicates that in normal healthy animals, intestinal inflammation is prevented by distinct T regulatory cells. The advantage of the immunologically manipulated models has been the elucidation of the role of pathogenic and regulatory T cells in mucosal immunity. They provided strong evidence of the role polarised Th1 plays in Crohn’s disease. However, the prime limitation of these models is the use of immune deficient mice thus inducing abnormalities in investigating the innate factors causing Crohn’s disease.

4. SAMP1/YitFc Mice: Spontaneous Ileitis

Spontaneous models of intestinal inflammation are attractive in studying human diseases cause of the occurrence of disease without any apparent exogenous manipulations. Two models of spontaneous inflammation are the C3H/HeJ Bir and SAMP1/YitFc murine models. In the C3H/HeJ Bir mice, inflammation develops in young mice in the right ascending colon. However, the colitis generally resolves with age and without recurrence. In the SAMP1/YitFc mice, spontaneous inflammation develops in mice at a young age and continues to chronic stage in older mice, with the formation of anal fistulas. In fact, inflammation in the SAMP1/YitFc mice occurs at the distal ileum, which is the hallmark location for Crohn’s disease.

SAMP1/YitFc model was generated by >20 generations of brother-sister mating of senescence-accelerated mouse line at the University of Virginia. Along with developing spontaneous ileitis at the distal ileum, as earlier as 10 weeks old, histology feature of the SAMP1/YitFc also closely resembles terminal ileal human Crohn’s disease. They demonstrate segmental inflammation, transmural disease presentation along with the presence of mucosal and submucosal granulomas. The SAMP1/YitFc mice also demonstrate the novel characteristics of active and chronic inflammatory elements that respond to administrations of corticosteroids or
antibodies against TNF $^{108}$. Furthermore, experiments have illustrated the development of ileitis to be a bacteria-related $^{109}$, Th1-mediated phenomenon followed by the up-regulation of Th2 cytokines in the chronic stages $^{110}$. The SAMP1/YitFc murine model supports the hypothesis that Crohn’s disease is caused by dysregulated immune response to luminal antigen in genetically susceptible hosts.

D. Background on Dendritic Cells

Dendritic cells (DCs) were first identified in 1868 in the skin as Langerhans cells by a medical student named Paul Langerhans. The Langerhans cells were approximately 3% of the total epidermal cells, interspaced throughout the epidermis, and formed a constituent part of the skin immune system $^{111,112}$. It was not until 1973 when Ralph M. Steinman and Zanvil A. Cohn at Rockefeller University, observed a novel cell type in peripheral lymphoid organs of mice $^{113}$. They were coined as ‘dendritic’ cells because they were able to form “pseudopods of varying length, width, form, and number, resulting in a variety of cell shapes”. Since then, significant advances in the understanding of dendritic cell biology and functional implications in the pathology of many immune-related disorders such as allergies, autoimmunity, infectious diseases, inflammatory disorders, transplants and cancer, have been described $^{114,115}$.

DCs are professional antigen-presenting cells. They are continuously produced from haematopoietic stem cells within the bone marrow $^{116}$. There are variations in ontogeny and function of DCs found in humans and mice $^{117}$. Key differences relevant to this thesis will be discussed below. In humans, there are two distinct types of DCs: myeloid and plasmacytoid DCs $^{118}$. Myeloid DCs (MDCs) include epidermal Langerhans cells, dermal and interstitial DCs and can be found in circulating blood or in peripheral tissues mainly as immature DCs $^{119,120}$. Plasmacytoid DCs (PDCs) are primarily distinguished by their capacity to release type I interferons after viral challenge $^{121,122}$.

Generally in the gut, DCs serve as sentinels in the periphery, surveying the intestinal lumen, and sensing for tissue necrosis and local inflammation $^{123,124}$. Signals obtained after encountering pathogens induces DCs to undergo a maturation process while migrating through the afferent lymphatics into the T cell areas of the draining lymph nodes $^{125}$. Here, the mature DCs present the processed antigens to T cells via both the classical (MHC class I and class II) and non-classical (CD1 family) antigen-presenting molecules. This results in T cell proliferation and differentiation into helper and/or effector cells with unique function and cytokine profiles $^{117,126,127}$.
DCs also have the capacity to activate B cells, NK cells and NK T cells. Non-activated immature DCs with self-antigens, for example from apoptotic cells, present to T cells in the absence of appropriate costimulatory molecules, leads to the induction of tolerance \(^98;128;129\). Meanwhile, mature antigen-loaded DCs can launch antigen-specific immunity \(^130\).

1. Physiological Role of Dendritic Cells in Intestinal Immuno-Homeostasis

i. Studies in Mice

Initially, distinct DC subsets were evident in mice due to the relative accessibility of different murine lymphoid tissues. Generally all murine DCs express CD11c (the integrin-\(\alpha_x\) chain) \(^117;118\). DCs found in the intestinal lamina propria were CD11b\(^+\) CD8\(^+\), CD11b\(^-\) CD8\(^+\), CD11b\(^-\) CD8\(^-\) DCs, as well as plasmacytoid DCs \(^124\). In the distal ileum, DCs expressed the receptor CX3CR1 which was essential for dendrites formation into the intestinal lumen \(^98;131;132\). Mice deficient for CX3CR1 failed to extend DC dendrites into the lumen and sample *Salmonella* \(^133\). Following oral gavage with *Salmonella*, the FAE in the Peyer’s patches express higher levels of CCL20 chemokine which recruits CCR6\(^+\) DCs to the epithelial monolayer \(^134;135\). Indeed, CCR6-deficient mice had reduced numbers of DCs in the SED \(^136\) and were unable to respond to bacterial invasion of the Peyer’s patches and also failed to initiate T cell activation \(^136\). Furthermore, it was found that in the CCR6-deficient mice, there was loss of regulatory CD4\(^+\) CD45RB\(^-\) T cells. During the maturation process, DCs express IL-12, which stimulates CD4\(^+\) T cells to produce Th1 type cytokines \(^125\), and CCR7, which is required for DC migration towards T cell regions \(^137\) – as CCR7 knockout mice had impaired DC migration from the intestine to the draining mesenteric lymph nodes \(^138\). Under physiologically normal conditions, however, freshly isolated Peyer’s patches CD11c\(^+\) DCs produced high levels of IL-10 that promoted the differentiation of IL-10 and IL-4 producing CD4\(^+\) T cells \(^99\). This indicates that Peyer’s patches DCs inherently generate non-inflammatory and tolerogenic conditions since neutralising antibody against IL-10 resulted in significantly enhanced IFN-\(\gamma\) secretion from CD4\(^+\) T cells.
Intestinal DCs have been found to harbour live commensal bacteria \cite{139}. This results in the induction of protective immunity via secretory IgA, which limit bacteria penetrating the mucosal barrier. Furthermore, DCs with live commensal bacteria are found restricted to mucosal sites and draining lymph nodes thus preventing the unnecessary systemic immunity to the normal gut flora \cite{140}. DCs with pathological bacteria can be found in the spleen which could result in a systemic immune activation.

**ii. Human Dendritic Cell Studies**

In humans, myeloid DCs located mainly in the intestine can be recognised by their expression of DC-SIGN/CD209 \cite{141,142}, and CD83 \cite{143} while plasmacytoid DCs express CD123 \cite{117}. There are very few studies looking at human ileal DCs. In situ studies of Peyer’s patches have illustrated DC-SIGN$^+$ DCs to be located in the SED and interfollicular regions \cite{142}. Meanwhile colonic DCs were found to express CD11c and CD1a \cite{143,144}. Recently, Verstege \textit{et al.} \cite{145} conducted immunohistochemical staining of the human colon and found that all the different DC markers used gave variable staining and that there were no CD1a positive cells.

Blood is the more readily available source of human DCs and they have been found to express CD4, CD11b and CD11c \cite{116,117,119,146}. Several studies have generated DCs from monocytes by culturing blood isolated monocytes with IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) \cite{147-149}. These studies revealed that immature DCs expressing CCR6, migrated towards the CCL20/MIP-3\(\alpha\) chemokine. Maturation by stimulating DCs with LPS, TNF-$\alpha$ or CD40L resulted in the decreased expression of CCR6 mRNA and a sharp upregulation of CCR7, which in turn initiated DC migration towards CCL19/MIP-3\(\beta\) \cite{126,150,151}.

During the maturation process, DCs undergo cytoskeletal reorganisation; they have reduced phagocytic uptake capacity; acquire cellular motility and migrate towards lymphoid tissues; and have enhanced T cell activation potential. Mature DCs, in addition to expressing the lymph migratory receptor CCR7, begin to express a number of markers that distinguish them from their immature counterparts, such as CD80, CD86 and CD83 \cite{114,124,126}. CD83 is a cell surface molecule that is involved in cell-cell interactions and CD4$^+$ T cell development \cite{152}. The maturation process induces DCs to release different cytokines thus determining the type of ensuing immune response \cite{153}. Congruently, the type of stimuli (viral versus bacteria), the subtype of DCs triggered (mDC versus pDC), and the origin of the DC (interstitial versus blood) determines the immune response.
The type of cytokines released by activated DCs also determines the direction of T cell polarisation \(^{126}\). For example, low or absence of IL-12, presence of IL-4 and IL-10 skew T cells towards a Th2 profile. Meanwhile IL-12 released by DCs results in the Th1 type immune response and the generation of cytotoxic T lymphocyte (CTL). DCs also convert vitamin A into retinoic acid, which it uses to differentiate suppressor T cells that block inflammatory conditions \(^{115}\). DCs ability to govern the type of immune response initiated depends partly on its detection of microbe-associated molecular patterns (MAMPs). MAMPs are conserved molecular motifs found in commensals or pathogenic bacteria. DCs utilise their pattern-recognition receptors (PRRs) to sense the bacteria and mount appropriate immune responses \(^{154}\). Toll-like receptors (TLRs) are one of the most essential PRRs used by DCs in detecting extracellular bacteria. Myeloid DCs have been found to express TLRs 1 through 5, while plasmacytoid DCs express TLRs 1, 7 and 9 \(^{155}\). Triggering TLRs leads to the activation of nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways, and to the eventual expression of pro-inflammatory genes \(^{156}\). TLR-4, TLR-5 and TLR-9 are known to be activated by lipopolysacchide (LPS), flagellin (the subunit of flagellum) and bacterial DNA, respectively \(^{157}\). Coupled to its PRRs, DCs are the key cells that can mediate innate immune regulation as well as initiate adaptive immunity through T and B cell activation \(^{155,158}\).

2. Dendritic Cells in Intestinal Pathology

The role of DCs in human IBD has not been fully elucidated. Studies have found DCs to be located at sites of inflammation which has been in agreement with animal studies \(^{114}\). DCs isolated from colonic mucosa were found to express TLR-2 and TLR-4 along with the activation marker CD40 \(^{159}\). Furthermore, a subset of DCs tagged MDC8\(^+\) was found to release significant levels of TNF-α, and treatment with anti-TNF-α antibodies resulted in reduced DC activation \(^{160}\). We have also shown ileal DCs expressing TLR-4 and producing TNF-α (see Paper II). TNF-α has been demonstrated to increase the expression of CCL20 in colonic explants cultures form normal patients \(^{161}\). This suggests that activated DCs in mucosal inflammation recruit more CCR6\(^+\) DCs hence exacerbating local inflammation. In Crohn’s disease tissues, increased numbers and mature DCs have been reported in colonic lamina propria \(^{162,163}\). In addition, DC-SIGN\(^+\) DCs produced IL-12 and IL-18, which can promote Th1 development \(^{163}\). DCs generated from the peripheral blood monocytes of IBD patients had increased abilities to stimulate immune responses \(^{164,165}\). Taken together, this suggests the presence of activated DCs in tissues and blood of patients with IBD.
Intestinal mucosal DCs have unique features that allow them to tightly regulate immune function that mediate tolerance while simultaneously protecting against pathogenic organisms. Under inflammatory conditions, it is possible that DCs maturing within the lamina propria may be activating local T cells. This is supported by observations showing mucosal DCs producing inflammatory cytokine. However, intestinal human DCs have proven to be difficult to study due to the relative difficulty in obtaining tissue samples and in isolating DCs. In this thesis, we have tried to overcome this obstacle by using surgical tissues and blood samples from patients and healthy controls and performed immunofluorescence staining, mucosal isolation of DCs, examining immune activation through mixed-lymphocyte reactions, and performed animal studies on SAMP1/YitFc mice.

**Box 4** How do DCs maintain or distinguish from being tolerogenic to becoming immunogenic?

The intestinal tract is a major induction and effector site of the immune system. The presence of a vast number of both harmless and harmful microorganisms begs the question as to how the immune system distinguishes ‘friend’ from ‘foe’. Part of the answer lies in the expression of pathogen-associated molecular patterns (PAMPs). A vast majority of the PAMPs expressed by harmful microorganisms are utilised by the bugs to bind and to invade epithelial tissues. As such, DCs sensing PAMPs initiate an inflammatory response. Conversely, commensal bacteria produce non-inflammatory molecular patterns in addition to colonising luminal niches that keep them away from innate defences. However, dyregulated tolerogenic responses can lead to exaggerated immune responses against commensals, thus leading to destructive inflammation. How and why this occurs remains poorly understood.
II. Aims and Hypothesis of the Thesis

The main aim of this thesis has been determining whether the barrier function of FAE in Crohn’s disease and if dysfunctional immune regulators, namely dendritic cells, played a role in initiating and/or maintaining the chronic intestinal inflammation.

As mentioned earlier, past evidences have shown the development of aphtoid lesions at the FAE, while dendritic cells have the capacity to directly sample luminal antigens. Taken together, we hypothesized that dysfunctional dendritic cells in the SED further contribute to a defective FAE barrier, activate and maintain intestinal inflammation.

Specific aims of each of the four separate studies:

I. To investigate the FAE barrier and role of DCs in the uptake of non-pathogenic bacteria in patients with CD.

II. To characterise the DCs present in the Peyer’s patches of patients with CD.

III. To isolate DCs from blood and ileum of patients with CD and elucidate functional capabilities in mixed-lymphocyte reactions.

IV. To elucidate the permeability defects and the role of DCs in an ileitis model of SAMP1/YitFc mice.
II. Aims and Hypothesis of the Thesis
III. Materials and Methods

In this section, the pros and cons of the chosen methodology and reason of choice where applicable will be discussed. For details on the subjects and methods, refer to the individual articles.

A: Patients

In the first paper, 23 patients with Crohn’s disease (CD) were included in the study. Samples were obtained from the terminal or neo-terminal ileum from 17 patients that underwent surgery while biopsies were obtained from 6 patients who were having routine follow-up colonoscopy. This study also included 6 patients with ulcerative colitis that served as inflammatory controls, and 21 patients with non-inflammatory intestinal conditions that served as non-IBD controls. Meanwhile paper II included 6 patients from the first paper and 23 additional patients. Six non-IBD controls patients from the paper I and 12 additional patients were included in paper II, plus 15 additional patients with ulcerative colitis (UC).

In paper III, distal ileal tissue samples and blood were obtained from 5 patients with CD that underwent surgery due to obstruction or fistula. Distal ileal tissue and blood was also obtained from 4 non-IBD patients that underwent right-sided hemicolecctionomy. In addition, blood was collected from 5 patients with CD that came to the clinic for routine colonoscopy examination. Blood was also collected from 5 age-matched healthy volunteers.

Samples were collected at the University Hospital of Linköping and Vrinnevi Hospital in Norrköping. All the studies were approved by the local committee for Human Ethics in Linköping, Sweden, and all subjects gave their informed consent.

The advantage to using patient samples is the relevance of material to disease. With the use of CD specimens, we directly observe the mechanisms involved during disease progression and the sites and mode of dysfunction. We are able to dissect each layer, for example by studying the barrier function to bacteria and assessing the role of immune cells. With the human samples, we are also able to measure the cytokine production after certain stimuli and compare it with control samples.

However, the disadvantage to using surgical tissue samples is that specimens are obtained when the disease has progressed to a later stage. Patients that undergo surgery usually have mild to high intestinal inflammation, though this is usually
offset by the colonoscopic samples with little to no inflammatory indicators. Another limitation to using patient samples is that we cannot obtain specimens at an early stage of the disease therefore it is difficult to elucidate the early events of Crohn’s disease. Variability amongst patients usually results in spread in results, unlike genetically reared mice or cultured transformed cell-lines. Due to obvious ethical reasons, we are unable to perform in vivo studies in humans, and this is where disease-relevant murine model has the advantage.

B. SAMP1/YitFc Mice

The SAMP1/YitFc mice were bred and provided by Dr. Pizarro, University of Virginia Health System, Charlottesville, VA, and were shipped to the University Hospital of Linköping in 2 batches. The first batch had 10 males that were between 22–25 weeks old, while the second batch of shipment included 10 males aged 9–10 weeks. The mice were acclimatised for one week while being maintained in specific-pathogen free environment with 12 : 12 hour light : dark cycle and fed ad libitum.

SAMP1/YitFc mice were chosen for our experiments because they closely resemble Crohn’s ileitis. Our research focus has been on ileal Peyer’s patches of Crohn’s disease and the SAMP1/YitFc mice spontaneously develop ileitis without any chemical, immunological or genetic (such as gene-knockout) manipulations. Another advantage to using this murine model of ileitis is the possibilities of performing in vivo ileal loops studies that examines the translocation of bacteria and DC trafficking within the host. In addition, we gain the advantage of determining the initial events of ileitis by studying mucosal barrier and immunological markers prior to disease development.

Initially the SAMP mice were developed from brother-sister mating of AKR/J mice. The original intent was to develop a senescence-accelerated mouse (SAM) line to study senescence or aging. Sub-strains of these mice were shown to develop enteric inflammation. AKR mice were used as controls as they were the background strains on which the SAMP mice were originated.

The obstacle of shipping 2–3 week pups hampered parts of our aim. Past study has shown epithelial barrier defect in 3 week old mice even prior to the onset of ileal inflammation. Our aim was to determine whether the permeability defects originated in the FAE. The initial observations of recurrent CD are the development of aphthoid ulcers at the FAE. This would elucidate whether barrier defect and the subsequent triggering of underlying immune cells are the initial key events of ileitis.
C. Permeability Studies

To study the passage routes and mechanisms involved during barrier defects in intestinal inflammation, we used Ussing chambers. The technique was first developed by a pair of Danish physiologists, Ussing and Zerahm
\footnote{167}. It was subsequently modified and simplified by Grass \textit{et al.}\footnote{168} and since then it has been primarily used to study ion transport, drug and protein absorption, bacterial internalisation, and immunological response to stimuli\footnote{169}.

The mucosa is mounted between two half-chambers filled with physiological buffer and continuously oxygenated. The gas port provided buffer circulation, mixing of reagents and reduces the thickness of the unstirred water. A pair of Ag / AgCl electrodes measured electrophysiological parameters. These parameter measurements depend mainly on the Na$^+$ / K$^+$ - ATPase found on the epithelial membrane and the barrier function of the junctional complexes. The electrogenic ion pumps create a potential difference (PD) across the epithelia. A current is passed through the Ag / AgCl electrodes to nullify the PD which is represented as the short circuit current (Isc). The Isc is a function of the ion pump activity. The barrier function of the junctional complexes represents the electrical resistance or the passive ion transport between epithelial cells. This is known as the transepithelial electrical resistance (TER).

By passing a current (I) through the epithelia, one can determine the change in PD. Through the use of Ohm’s law, PD = I x TER, one obtains the resistance of the epithelia. In order for this calculation to work, one relies on viewing the epithelium as a parallel circuit that consists of paracellular and transcellular routes. These measurements also determine the viability of the tissue as energy is required for active ion transport and maintaining of a PD across the epithelium.

The over simplification of the calculations is a limitation of the model. The thickness of the tissue could affect the TER measurements. Biopsies, compared to FAE surgical specimens, have a lower TER. This could be contributed by the resistance generated over the connective tissues. Newer techniques have been developed that can separate electrophysiological measurements of the epithelia from subepithelia\footnote{170}. 
D. Immunological Markers for Dendritic Cells

To date, there is no single accepted universal marker for human mucosal DC. A recent publication by Verstege et al.\textsuperscript{145} used several different markers on colonic biopsies and found that all the markers gave variable staining.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-SIGN</td>
<td>44-kDa type II lectin membrane protein involved in antigen recognition, uptake and presentation</td>
<td>141, 171</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Heterodimeric transmembrane protein compose of $\alpha$ and $\beta$ subunit involved in antigen presentation</td>
<td>172, 173</td>
</tr>
<tr>
<td>CD123</td>
<td>Also known as IL-3 receptor alpha (low affinity)</td>
<td>174, 175</td>
</tr>
<tr>
<td>CD11c</td>
<td>Type 1 transmembrane glycoprotein involved in cell adhesion</td>
<td>176, 177</td>
</tr>
<tr>
<td>CD83</td>
<td>45-kDa glycoprotein and member of Ig superfamilly involved with antigen presentation and T cell development</td>
<td>152</td>
</tr>
<tr>
<td>CD1c</td>
<td>Part of the CD1 transmembrane glycoproteins that are structurally related to MHC involved in the presentation of primary lipid and glycolipid of self and microbial antigens to T cells identical to neuropilin-1, neuronal receptor; also co-receptor for vascular endothelial growth factor A no known function on DCs but useful for purification</td>
<td>178, 179</td>
</tr>
<tr>
<td>CD304</td>
<td>CC chemokine receptor, 6 transmembrane protein cells expressing it migrate towards its ligand, CCL20</td>
<td>180</td>
</tr>
<tr>
<td>CCR6</td>
<td>CC chemokine receptor, 7 transmembrane protein cells expressing it migrate towards the ligand CCL19</td>
<td>137</td>
</tr>
</tbody>
</table>

### Table 2 | Selected Murine Dendritic Cell Markers

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c</td>
<td>Integrin alpha X chain protein involved in adherence and stimulation of endothelial cells</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>Integrin alpha E, part of adhesion molecules containing β7 subunit involved in cell adhesion</td>
<td>181;182</td>
</tr>
<tr>
<td>CD103</td>
<td>Type 1 membrane protein; member of Ig superfamily involved with co-stimulatory signalling of T cells</td>
<td>183</td>
</tr>
<tr>
<td>CCR7</td>
<td>G protein coupled receptor family CC chemokine receptor migrates cells towards CCL19 ligand</td>
<td>184;185</td>
</tr>
</tbody>
</table>

### E. Microscopy

In our studies, we used both light and confocal microscopy. For light microscopy, we stained in Paper I, naïve tissue using standard Giemsa staining. In Paper II, we used immunohistochemistry to visualise TLR-4 expression and TNF-α production. Giemsa staining provided us the opportunity to visualise commensal bacteria found bound to or associated with the FAE epithelia of the human specimens. Immunohistochemistry for TLR-4 and TNF-α was performed because the techniques were established and to avoid non-specific staining. The remainder of the studies involved the use of confocal microscope.

Difficulties that can be faced during the microscopy studies are the non-specific stainings of antigens. This was avoided by using appropriate blocking reagents and by performing antibody serial dilution to determine the optimal concentrations for staining. Negative and isotype-matched control stainings were also performed to determine the specificity of the secondary antibodies. During image acquisition from the confocal microscope, individual fluorochrome was acquired in order to avoid fluorescence spilling over to another gate. This ensured that each fluorochrome was excited and detected individually.

The advantage to using confocal microscope is the possibility of illuminating and detecting a thin section of the tissue. This avoids out-of-focus images and we obtain information from thin section of the specimens. With the confocal microscope we
are also able to combine several sections and render it into a 3-deminsional image, as in Paper I figure 2A and B.

**F. Fluorescence Activated Cell Sorter (FACS)**

FACS operates by analysing individual cells in a liquid stream. Cells pass through a laser beam and deflect it 2 ways, as forward scatter (FSC) and side scatter (SSC) \(^{168}\). FSC depends on the size of the cell while SSC depends on the particular elements or granules within the cells. This can be used to distinguish different cell types within a sample. FACS also uses lasers to excite antibodies bound to cells while detectors register the signals generated. This technique permits one to identify different antigen markers found on cells in a sample. In Paper II, we stained isolated lamina propria mononuclear cells with HLA-DR, Lineage 1 antibody cocktail, CD11c and CD83 antibodies. While in Paper III, anti-CD123, CCR7 and TLR-4 antibodies were used in addition to the ones in Paper II. The data were presented in Dot plots which permit one to visualise individual sub-populations within a sample.

Using FACS provides the advantage to analyse individual cells based on size, structure and antigens expressed by cells. With FACS, one is able to quantify sub-populations and the intensity of antigen expression. However, the limitation of FACS is that the sample must contain cells in suspension. This means that tissues must be digested. Furthermore with FACS, one looses information as to which compartment the isolated cells were located. Therefore, the optimal approach would be performing FACS studies in conjunction with microscopic studies, thus allowing one to quantify the type of sub-populations within tissues and the compartmental locations of these cells, respectively.

**G. Dendritic Cell Isolation**

We used two different techniques to isolate ileal mucosal DCs; magnetic cell sorting via AutoMACS and using FACS Aria. Past study has shown that colonic DCs are about 0.6% of the cell population \(^{143}\). As such, the isolation protocol needed to be efficient. The initial steps involved the digestion of the ileal tissue with collagenase D. It was imperative that digestion of connective tissue did not result in the digestion of surface antigens on DCs, and subsequent activation. Previous publications performed tissue-digested DC isolation using collagenase D \(^{143,159}\). In a collaborative comparison with Marie Larsson’s group at Medical Virology, we
found that tissue digestion using collagenase D or collagenase IV resulted in similar number of lamina propria mononuclear cells (LPMCs; unpublished observations).

The digestion enzyme choice, temperature, and duration of digestion are essential in rapid release of cells from tissues. Poor enzyme choice could result in the digestion of surface antigens and possibly the subsequent activation of DCs and the inefficient release of DCs from the tissue. Regulating the temperature ensures effective enzyme activity as low or too high temperatures results in ineffective tissue digestion. The optimal digestion activity for collagenase D was 37°C, as per manufacturer’s instructions. We optimized the duration of digestion and found that increasing digestion for more than 2 hours did not result in a significant gain of cells. Conversely, there were almost twice as many cells in 2 hours of digestion than 1 hour. We also learned that cutting the tissues into small pieces gave us the higher numbers of LPMCs.

Upon obtaining LPMCs, the cells were incubated with the appropriate antibodies for characterisation in Paper II or for each isolation protocol in Paper III. Selecting antibodies meant not only obtaining pure DC population, but to also ensure the DCs were not prematurely activated and that there were antigens available for subsequent characterisation stainings.

1. *via* AutoMACS

We chose CD1c for myeloid DCs and CD304 for plasmacytoid DCs selection and isolation. These choices were restricted because of the lack of antibodies available. The initial step of the separation involved the removal of CD19+ B cells and pre-enriching DCs. The separation involved the positive selection of DCs (see Paper III for details).

The principle behind autoMACS is that the cells of interest are tagged with antibodies conjugated to magnetic beads. During the automated separation, magnets align the column and labelled cells remain within the column while the unlabelled cells elute through. Subsequently, the magnets position away from the column and the labelled cells were collected. With this protocol, we were able to isolate 6,000-25,000 cells from ileal tissues. The variability in cell numbers depended on the size, weight, location and the level of inflammation of the tissues. The larger the surgical specimen and the closer to the ileo-cecal valve, the greater the number of cells obtained during isolation. However, chronically inflamed tissues with fibrosis usually resulted in lower DC isolation numbers. More DCs were isolated from
Crohn’s tissues than non-IBD ilea, even when normalised for tissue weight (unpublished observations).

Limited antibody choices were one of the limitations of this technique. Low numbers of purified DCs was another. The repeated number of wash cycles involved in the protocol may have contributed to the loss of cells. Furthermore, we observed via trypan-blue staining that 30-40% of the cells purified via AutoMACS were dead cells. However, these cells were not included in the cell counts. Another possible limitation with this technique is that labelling CD1c may have contributed to the low MLR activity of DCs compared to cells purified by FACS Aria (see Paper III).

2. *via FACS Aria*

In Paper III, isolated lamina propria mononuclear cells were stained with anti-HLA-DR, Lineage 1 cocktail (CD3, CD14, CD16, CD19, CD34, CD56) antibodies and cells within the gate HLA-DR\(^+\) Lineage 1\(^-\) were selected as DCs, as was previously published by Hart *et al.*\(^{159}\). The FACS Aria sorting and purification is based on charged water droplets that are deflected into different wells depending on the selection criteria. Initially, the cell found in a droplet is identified via the antibodies bound onto it, followed by the application of a charge to that droplet. If the droplet contains the cell of interest, it is deflected into the collection tube; otherwise it is regarded as waste. Using this technique, we were able to attain \(\geq99\%\) purity in the HLA-DR\(^+\) Lineage 1\(^-\) DCs.

The FACS Aria provided flexibility in selection criteria to purify cells. Cells can be purified based on morphology and on antibodies bound to surface antigens. This technique can also sort cells to achieve a high purity. However, with purity there was loss of desired cells due to abortion of droplet. This occurs when an unwanted cell is found within the same droplet as the cell of interest, or within another droplet that is too close to the desired droplet. Therefore to avoid contamination, the system aborts the droplet. Another limitation to this technique is that unlike the AutoMACS, cells in the FACS Aria are exposed to higher pressures as they travel through narrower tubes to achieve single cell passage. This pressure stress may affect the function of some cells. Conversely, the FACS Aria provides the advantage to ‘sort-out’ dead cells, unlike the Auto-MACS. Dead cells have different morphological characterisation and are hence excluded from the sort and purification.
IV. Results

Detailed descriptions of the results obtained are given in their respective papers. This section will only highlight the significant findings.

A. Paper I

As the first observable signs of recurrent CD are minute ulcerations of the FAE, our aim in this paper was to characterise FAE in diseased state. We used samples from patients with CD while specimens from non-inflammatory bowel disorders and ulcerative colitis served as controls and inflammatory controls, respectively. With the bacterial passage studies, we found CD tissues had increased transepithelial passage of both dead and live *E.coli* compared to controls and UC (CD = 4.9 [4.2–5.3]; controls = 2.8 [2.4–3.4] and UC = 2.7 [2.1–3.8]; *P* = 0.002). Bacterial passage was confirmed with immunofluorescence staining that found greater number of bacteria within the SED of CD than controls. In fact, 45 [20–67] % of *E.coli* found in the SED of CD tissues were found to co-localise with DC-SIGN⁺ DCs versus 14 [0–33] % in controls. In addition, bacterial exposure increased TNF-α secretion in CD and not controls.

In conclusion, we found greater transepithelial barrier defect to non-pathogenic *E.coli* in the FAE of patients with CD, and not UC, when compared to controls. Almost half the numbers of bacteria taken-up within the SED of patients with CD were found inside DCs which further resulted in immune activation, via TNF-α release.

B. Paper II

Finding the involvement of DCs in *E.coli* uptake led us to characterise its presence in the SED of patients with CD. In ileal FAE specimens, immunofluorescence staining illustrated the presence of a greater number of both DC-SIGN⁺ and CD83⁺ DCs in CD than both inflammatory and non-IBD controls. The DCs present in the SED of Crohn’s tissues had greater propensity to take-up *E.coli* HB101 than the non-IBD controls, confirming our previous observations.

Further characterisation of the SED DCs illustrated 71 [66–92] % of the mature CD83⁺ DCs in CD were CCR6⁺ versus 28 [27–52] % that were CCR7⁺.
IV. Results

Immunohistochemistry illustrated DCs present in the SED of patients with CD expressed TLR-4 and released TNF-α.

In conclusion, DCs accumulated in the SED of patients with CD. Less than a third of these DCs expressed the migratory receptor, CCR7. The accumulated DCs expressed TLR-4 and had greater propensity to take-up bacteria.

C. Paper III

Upon characterising the DCs present in the SED, our next aim was to study the functional properties of mucosal DCs by isolating and running a mixed lymphocyte reaction (MLR). Initially we digested the ileal tissue with collagenase D, and DCs from the LPMC and blood were isolated using AutoMACS magnetic beads separation. The purified DCs were incubated with lymphocytes at a 1:30, DC: T cells ratio for 5 days. Tissue and blood DCs from non-IBD controls had numerically higher T cell proliferation capacity (tissue DCs: 0.19±0.11; blood DCs: 0.25±0.17) than DCs isolated from Crohn’s disease samples (tissue DCs: 0.05±0.02; blood DCs: 0.07±0.04). MLR reactions done on DCs isolated from blood of healthy controls and CD samples from ‘healthier’ patients via FACS Aria had equal T cell stimulatory capacity. However, isolated DCs that were pre-incubated with E.coli LF82 had reduced stimulation of T cell proliferation (see Figure 3 in Paper III).

These results highlight 2 factors, firstly, the isolation technique has an effect on the DC function and secondly, E.coli LF82 decreases DC functionality. DC isolation using FACS Aria seems to preserve functionality compared to DCs isolated using AutoMACS. Pre-incubation with E.coli LF82 could be inducing DC cell death that may be causing the reduced T cell stimulation capacity.

In conclusion, DCs from patients with active CD have lower T cell stimulatory capacity than non-IBD controls, while this activity is restored to normal levels in ‘healthier’ CD patients that are in long-term remission.

D. Paper IV

Our next approach to understanding the role of mucosal DCs in IBD was to determine whether results obtained using human specimens could be reproduced in the murine model of Crohn’s ileitis, SAMP1/YitFc mice. The SAMP1/YitFc mice closely resemble CD and if our results could be confirmed, then this would open
new possibilities in not only studying DCs, but in determining *in vivo* mechanisms and ultimately the development of novel therapeutic strategies that directly target the mucosal immune system.

We found higher mucosal permeability in SAMP1/YitFc mice than AKR controls. There was greater transmucosal permeability to *E. coli* LF82 in both the FAE and VE in the SAMP1/YitFc mice aged 27 weeks (0.205±0.109 and 0.108±0.092 apparent bacterial passage (cm/min), respectively) than in the FAE and VE of AKR controls (0.0005±0.0003 and 0.0004±0.0003 apparent bacterial passage (cm/min), respectively). However, we did not find any difference in the number nor in the type of DCs found in the SED of SAMP1/YitFc and AKR control mice. This indicates that unlike in CD, DCs do not play a major role in intestinal inflammation in the SAMP1/YitFc mice.

In conclusion, SAMP1/YitFc mice have inherent ileal permeability defect that is exacerbated by *E. coli*. DCs might not be playing a key role in inflammation.
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V. General Discussion

Having a chronic disorder such as Crohn’s disease (CD), can be felt as a burden to the patient, with an inability to lead a fully normal life. Ever since the start of the IBD era in 1932, no cure for CD has been discovered yet. This has been hampered partly by the complexity of the disease (being multi-factorial) and the difficulty in understanding key initiating events in the pathogenesis of the disease.

As past evidences have pointed to the Peyer’s patches being the initial site for recurrent disease pathogenesis, we have focused our research in this area. It is not surprising that the distal ileum, where Peyer’s patches are most abundant and where there is an increase of bacterial colonisation, is also the site which is most affected by CD. The main question we posed was if there was a barrier compromisation effect at the FAE and/or if dysfunctional immune regulators, namely dendritic cells, played a key role in initiating chronic intestinal inflammation.

Our initial focus was on FAE barrier. In Paper I, we highlighted an increase in transmucosal permeability in ileal FAE of patients with CD to non-pathogenic E.coli, than in ulcerative colitis and controls. Furthermore in naïve surgical tissues (not exposed to experimental bacteria), we found higher frequency of bacteria adherent to the FAE surface in CD than in controls. This indicates that there is a defective FAE barrier in CD. CD ileal sensitivity to bacteria was also demonstrated by the increase of paracellular permeability via conductance and $^{51}$Cr-EDTA passage (see Paper I Figure 4). This defect and sensitivity of the FAE ultimately exposes the underlying immune system to a barrage of luminal antigens thus not only triggering immunity, but also maintaining it.

To determine whether intestinal barrier defect preceded an inflammatory response, we utilised the SAMP1/YitFc mice model of ileitis. Past clinical and epidemiological studies have shown that first degree relatives of patients with CD have increased intestinal permeability in the absence of clinical symptoms. Our aim with the murine model was to determine whether this permeability defect initiated at the FAE. In Paper IV we used two different age groups of SAMP1/YitFc mice; 11 weeks old which represented acute phase inflammation and 27 weeks old representing chronic phase. Unlike our data obtained using human specimens, we did not find permeability differences between FAE and VE within the groups. Though we did find greater passage of the respective paracellular and transcellular markers in both FAE and VE in the chronically inflamed SAMP1/YitFc mice compared to the AKR controls (see Paper IV Figure 2). We also found higher transmucosal passage of pathogenic E.coli LF82 across FAE and VE of the 27 weeks old SAMP1/YitFc mice. These results suggest that in this model of ileitis,
there was no difference in permeability between the two different ileal segments; FAE versus VE. However, \textit{in vitro} permeability increased as the chronicity of the ileitis progressed.

Though SAMP1/YitFc mice posed as a promising model to study CD it, however, did not corroborate our past observations using human specimens, especially in regards to the FAE. Yes, there was an inherent permeability defect in the SAMP1/YitFc mice, as illustrated by our electrophysiological measurements (see Paper IV Table 2) and TER measurements by Olson \textit{et al.}\textsuperscript{166}, but it was not limited to the FAE. Furthermore, Olson \textit{et al.} were able to show that epithelial defect did indeed precede ileal inflammation.

Under non-inflammatory conditions, our group previously illustrated human FAE, compared to VE, has higher transmucosal permeability\textsuperscript{189}. This difference in permeability between FAE and VE was also not evident in the AKR control mice. It is clear that in the murine model permeability precedes inflammation but the FAE does not play a significant role. However in humans, clear evidence of the FAE contributing to the initiation of intestinal inflammation remains elusive.

One of the novel findings in Paper I was the internalisation by DC, of \textit{E.coli} that had translocated across the FAE. This was corroborated by findings in Paper II. Furthermore, in Paper II we found that mucosal DCs were expressing TLR-4, as previously demonstrated\textsuperscript{159}. TLR-4, being one of the most studied Toll-like receptors, recognises LPS, and its stimulation results in a potent activation and maturation of DCs. Furthermore, previous studies have illustrated peritoneal macrophages expressing TLR-4 had greater phagocytic activity of \textit{E.coli} than mutants\textsuperscript{190}, while \textit{in vitro} M cell model showed TLR-4 and not TLR-2 to be essential in Gram-negative bacteria uptake\textsuperscript{191}. Likewise, the mucosal DCs identified in our studies, present beneath the FAE, maybe utilising TLR-4 to take up \textit{E.coli}. The result is DC immune activation, via TNF-\(\alpha\) production. This could be one of the key initial events that lead to an intestinal inflammation.

DCs accumulating within the SED may be contributing to the high levels of TNF-\(\alpha\). In Paper II (which can also be seen in Paper I Figure 5E), we found an accumulation of DCs within the SED. This is abnormal since DCs, upon activation, mature and migrate away from mucosal sites and towards T cell-rich areas. This is accomplished by the down-regulation of CCR6 and up-regulation of CCR7 expression\textsuperscript{126}. However in the SED of patients with CD, we found significant infiltrate of CD83\textsuperscript{+} DCs and that more than 70\% of them were still expressing CCR6. Reasons as to why the mature DCs do not express the emigrating receptor
remains to be elucidated. Other studies have also shown DCs retention within the intestine \(^{159,162,163}\), though expression of migratory receptors was not studied.

A possible explanation for finding accumulated CD83\(^+\) CCR6\(^+\) DCs is the presence of the CCL20 (MIP-3\(\alpha\)) chemokine in the SED. It is known that the FAE, and in particular in IBD tissues, express CCL20 and recruit DCs to the mucosal compartments \(^{161,192}\). Furthermore, stimulation with TNF-\(\alpha\) exacerbates CCL20 expression. In Paper II, we have shown the release of CCL20 by the FAE and that there was a higher concentration of TNF-\(\alpha\) release in CD tissues, partly being contributed by DCs. As such, a vicious cycle is created where recruited DCs that are activated, release TNF-\(\alpha\), thus causing the release of more CCL20 and further recruitment of DCs into the SED. However, the question that remains unanswered is why the recruited DCs do not subsequently begin to express the emigrating receptor, CCR7.

In addition to the immuno-histochemistry and -fluorescence staining of ileal mucosal tissues, we performed FACS analysis of isolated mucosal DCs. Both methods confirmed high numbers of myeloid DCs present in the distal ileum of patients with CD. The significance of this finding relates to the barrier properties of the diseased tissue. In CD, there is a flawed barrier function and as such, there is an abundance of translocated luminal contents into the host. A major role of the mucosal immune system is deciphering innocuous substances/organisms from harmful pathogens. Perhaps it would have been inevitable that in CD, the constant exposure to luminal antigens would eventually result in the activation of an immune response to a non-pathogen; such as commensal bacteria. Furthermore, it has been shown that under metabolic stresses, the intestine becomes vulnerable and even a non-pathogenic \textit{E. coli} HB101 can become invasive \(^{193,194}\). Therefore, it is not surprising that the host’s immune system recruits DCs, one of the primary cells that orchestrate mucosal immunology, to this site.

To study the functional role of the recruited mucosal DCs, we performed isolation studies of ileal DCs and compared it to blood DCs. The mixed lymphocyte reactions (MLR) performed in Paper IV revealed an unexpected event. Initially we observed, via microscopy, that the non-emigrating matured (CD83\(^+\) CCR7\(^-\)) DCs recruited to the SED, sampled bacteria and produced TNF-\(\alpha\). We expected that these DCs in a MLR reaction would stimulate a dramatic T cell proliferation. However, the contrary was true. DCs from both the ileum and blood of patients with CD had reduced T cell proliferative capabilities than non-IBD controls. One possible explanation could be that DCs from patients with active CD are in a semi-mature state.
Past study have shown DCs having the capacity to become partly activated by not expressing all the co-stimulatory markers\textsuperscript{195}. These DCs usually have regulatory activity via inducing tolerogenicity and CD4\textsuperscript{+} T cells producing anti-inflammatory cytokine, IL-10. Therefore, one could speculate that DCs from inflamed tissues may be attempting to dampen an immune response rather than exacerbating it. It would be an interesting continuation of Paper IV to characterise the type of T cells generated and cytokines produced in the MLR. It is possible that in chronically inflamed CD, the key drivers of intestinal inflammation are activated CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells while DCs are attempting to create a non-inflammatory environment. This has been observed in murine studies where isolated DCs exposed to commensal bacteria normally conferred an anti-inflammatory environment, via IL-10 production, unlike DCs exposed to pathogens\textsuperscript{99}.

In Paper IV, one of the objectives was to determine whether there was a difference between DCs found in the periphery (in the distal ileum) versus DC circulating in the blood. We did not find any functional difference between ileal and blood DCs within the patient groups. This means that DCs found in the ileum had similar capacity to induce T cell proliferation as circulating DCs. However, FACS analysis illustrated a greater percentage of myeloid DCs found in both the ileum (see Paper II Figure 1) and blood (see Paper IV Figure 4A) of patients with CD compared to controls. In addition, there was a difference in the surface antigen expression between ileal and blood DCs isolated from patients with CD. In Paper I, we showed that a significant proportion of the ileal DCs were CD11c\textsuperscript{+} CD83\textsuperscript{+} while in Paper IV, we found a significant percentage of CD11c\textsuperscript{+} CD83\textsuperscript{+} DCs. In addition, greater proportion of CD83\textsuperscript{+} CCR7\textsuperscript{+} DCs was found in the blood as opposed to the CD83\textsuperscript{+} CCR7\textsuperscript{−} DCs observed in the ileum. This indicates that mature blood DCs are set to migrate towards T cell-rich areas whereas their ileal counterparts remain within the mucosal compartment. Furthermore, this also indicates that examining surface expression alone does not provide insight into the functional capabilities of DCs.

The question that still remains is: what is the significance of the accumulated mucosal DCs in CD?

To begin to understand this, one must look at chronic inflammation as a separate entity to acute inflammation. The process can be looked at as an analogy of a series of beads bound to a string. The string represents key stimulators or factors that lead to a cluster of events that is exemplified by the beads. At first, 3 string events typified by environmental factors (bacteria and/or stress) and immunological dysfunction, come together along with a genetically susceptible host. This precipitates a cluster of events that is immune triggering and priming of T cells by
DCs. Exactly how this occurs in CD – what environmental events or factors; which genes (note that no major genetic associations, so far, have been linked with the rise in IBD seen in Asia); and which immune cells contribute to the development of intestinal inflammation – is still not fully clear. What we do know is that activation and priming of T cells leads to the development of effector and memory cells that, along with the constant antigen exposure, maintains the inflammation (the connecting string between acute and chronic inflammation). During chronic inflammation, patients that are in long-term remission have ‘normal’ functioning DCs (as shown by the restoration of DC activity in the MLR reaction in Paper IV Figure 3A). However, in the cluster of events that occurs during an inflammatory flare-up, the DCs might be attempting to counter-balance the inflammation.

Therefore, it is tempting to speculate that DCs might be playing different roles, depending on the stage of inflammation. And as such, one must be mindful of not only the cluster of events surrounding the cells, but also the preceding string of events and the eventual outcomes.
VI. Conclusions

- There is an increased transmucosal transport of *E. coli* through the FAE of patients with Crohn’s disease compared to patients with ulcerative colitis and non-IBD controls

- A higher percentage of the *E. coli* that had translocated into the SED were found to be internalised by dendritic cells in Crohn’s disease than in non-IBD controls

- An accumulated number of both immature and mature dendritic cells were found within the SED of surgical specimens from Crohn’s disease versus specimens from ulcerative colitis and non-IBD controls

- The accumulated mature CD83⁺ dendritic cells found in Crohn’s were lacking the migrating CCR7 receptor expression

- The CD83⁺ CCR7⁺ dendritic cells were more prone to *E. coli* uptake and expressed TLR-4 and produced TNF-α

- Functional dendritic cells isolated from both the ileum and blood of patients with Crohn’s disease showed reduced capacity for activating T cell proliferation

- Patients under long-term remission had normalised dendritic cell function as activation of T cell proliferation was similar to dendritic cells isolated from healthy controls

- The SAMP1/YitFc murine model of Crohn’s disease has an inherent permeability defect that increases with chronicity of inflammation

- Unlike in Crohn’s disease, dendritic cells did not seem to play a key role in the SAMP1/YitFc murine model of ileitis
VII. Summary

This section describes the series of events that we have observed to occur within the sub-epithelial dome (SED) of patients with Crohn’s disease (CD), as depicted by Figure 4. Initially, bacteria found within the intestinal lumen can stimulate the production and release of CCL20 by the follicle-associated epithelium (FAE). The CCL20 chemokine recruits immature DC-SIGN$^+$ CCR6$^+$ dendritic cells (DCs) to the SED. In the SED, DCs take up translocated bacteria. The result is the maturation of DCs via the expression of CD83. However, the mature DCs do not express the CCR7 receptor which aids in its migration towards T cell-rich areas (for example towards the lymph nodes). The result is the accumulation of mature, TNF-α producing DCs within the SED. Depending on the inflammatory status, these DCs may be triggering T cell expansion or tolerance.
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IX. Svensk Sammanfattning

Crohns sjukdom är en kronisk inflammatorisk tarmsjukdom där individer drabbas av ett okontrollerat immunsvår mot bakterier i tarmen. De tidigast observerbara sjuksomstecknen är små sår i en specialiserad del av tarmepiteliet som kallas follikelassocierat epitel (FAE). FAE täcker ansamlingar av immunceller i tarmen som kallas Peyerska plack. I dessa områden sker en ständig interaktion mellan bakterierna i tarmen, epitelet och immuncellerna. De s.k. dendritiska cellerna har här en viktig funktion som sensorer av tarminnehållet och styr därigenom tarmens immunsystem.

Huvudsytet med detta avhandlingsarbete var att studera hur det lokala immunsystemet i Peyerska placken och särskilt dendritiska celler interagerar med FAE, och vilken roll detta samspelet har vid initiering och bibehållande av tarminflammationen vid Crohns sjukdom.

Inledningsvis kunde vi genom studier av tunntarmsslemhinnan (ileum) visa att det vid Crohns sjukdom fanns en ökad bakterietransport över tarmen jämfört med kontroller. Dessutom togs en större andel av bakterierna som passerade över FAE upp av de dendritiska cellerna och tarm från Crohns sjukdom frisatte högre halter av TNF-α efter bakteriell exponering.

Vidare identifierades en anhopning av både omogna och mogna dendritiska celler i vävnaden nedanför FAE, den s.k. subepiteliala domen (SED). Normalt ska mogna dendritiska celler efter exponering för främmande ämnen migrera till lymphkörtlarna. Vi observerade dock att mogna dendritiska celler ansamlades i SED vid Crohns sjukdom. Detta kunde förklaras av att de såkna migrationsreceptorn CCR7. Dessutom var dessa celler mer benägna att ta upp bakterier och producera TNF-α.


Avslutningsvis fann vi att möss som spontant utvecklar en tarminflammation som liknar Crohns sjukdom upptäckte en defekt tarmbarriär som ökade vid kronisk inflammation. Till skillnad från inflammationen vid Crohns tycks de dendritiska cellerna inte spela någon stor roll i denna musmodell.

Denna avhandling belyser betydelsen av aktiva dendritiska celler som ansamlas nedanför FAE och tar upp bakterier, och pekar på skillnad i funktionalitet vid inaktiv kontra aktiv sjukdom. Dessa fynd bekräftar att dendritiska celler är viktiga komponenter i sjukdomsutvecklingen vid Crohns sjukdom.
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