A thesis presented for the degree of Doctorate of Medicine (Research)

Aberrant expression of homing markers on dendritic cells drives inflammation in Crohn’s disease

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The candidate confirms that all work presented in this thesis is the result of his own investigations except where reference has been made to the work of others
“Success is the ability to go from one failure to another with no loss of enthusiasm”

Sir Winston Churchill
1874 - 1965
Abstract

CD is a chronic transmural inflammatory disease of the gut. The aetiology of CD is unknown, but is likely to result from dysregulated innate immune responses to gut microbiota leading to over activation of the acquired immune system in a genetically susceptible host. Inflammation occurs anywhere from mouth to anus, in addition to extra-intestinal sites. This compartmentalisation of the inflammatory process is linked to tissue-specific innate immune mechanisms and immune cell homing. DCs play a key role in discriminating between gut commensal microbiota and harmful pathogens, directing T-cell polarisation and directing immune cells to specific anatomical locations by expressing and imprinting homing markers.

Improved understanding of the immunopathogenic basis of inflammation in CD has led to the development of more efficacious medical therapy, in particular, targeting the pro-inflammatory cytokine TNFα. The precise mechanism of action of TNFα blockade in CD remains unclear.

We found homing marker expression on circulating DCs in patients with CD closely correlates with anatomical phenotype of inflammation. When immune cells are cultured with IFX we found homing marker expression on monocytes and T-cells changed from a gut-homing to skin-homing phenotype, perhaps explaining the phenomenon of ‘paradoxical inflammation’ seen in some patients treated with anti-TNFα therapy.

Blood-enriched DCs isolated from patients with active CD had higher levels of pro-inflammatory cytokines in culture medium. IFX culture resulted in reduced concentrations of pro-inflammatory cytokines and decreased gut-homing marker expression.
Finally, we examined the functional effects of IFX-pre-treated-LDCs on T-cells. There was a dose-dependent reduction in LDC stimulatory capacity with increasing concentrations of IFX, but no changes in T-cell phenotype.

The effect of IFX on the immune system goes beyond TNFα blockade alone. This work has identified several mechanisms by which IFX interacts with immune cells \textit{in vitro}, which may result in changes to CD inflammatory process \textit{in vivo}.

\textbf{Abbreviations:}

- CD \hspace{1em} Crohn's disease
- DCs \hspace{1em} Dendritic cells
- TNFα \hspace{1em} Tumour necrosis factor alpha
- IFX \hspace{1em} Infliximab
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Chapter 1

Introduction
1.1 Crohn’s disease

Crohn’s disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract characterised by a relapsing-remitting course. The earliest description of chronic transmural inflammatory bowel disease was provided by Dalizel in 1913, but the disease was first recognised as a pathological and clinical entity in 1932 by Burril Crohn and colleagues, who described a regional ileitis (Crohn, Ginzburg et al. 1984). The inflammatory process in CD can involve any part of the digestive tract from the mouth to the anus, but predominantly affects the distal ileum and colon (figure 1). It is unclear why different patients develop Crohn’s disease in different anatomical locations.

![Anatomical distribution of Crohn's disease in the human gastrointestinal tract. The ileocaecal region is the most commonly affected location in the adult Crohn's disease population. Isolated colonic disease is the most common phenotype in paediatric populations (approximately 80% of patients under the age of 16 years).](image)

Figure 1. Anatomical distribution of Crohn’s disease in the human gastrointestinal tract. The ileocaecal region is the most commonly affected location in the adult Crohn’s disease population. Isolated colonic disease is the most common phenotype in paediatric populations (approximately 80% of patients under the age of 16 years).
1.1.1 Epidemiology

The incidence of CD varies depending on the region studied. The highest incidence is seen in the United Kingdom, North America and northern parts of Europe. Studies have consistently identified a North-South gradient in the incidence of CD within Europe, with higher disease incidence in Northern countries, and furthermore the same incidence trends are seen within individual countries, including the UK (Economou, Zambeli et al. 2009). More recently, the ECCO-EpiCom inception cohort has shown the existence of an East-West gradient in the incidence of CD, with higher incidence rates in West Europe compared to East Europe (Burisch, Pedersen et al. 2014).

The incidence of CD has been increasing over time in most countries, although it now seems to have reached a plateau in the North America (Loftus, Loftus et al. 2007), Sweden (Lapidus 2006) and in the UK (Gunesh, Thomas et al. 2008).

CD can occur at any age, although there are two peaks of incidence. The first peak is seen in teens and early twenties with a second peak in the fifties to seventies (Baumgart and Sandborn 2007). There is a slight female predominance in CD, which has been shown by several studies (Moum, Vatn et al. 1996; Vind, Riis et al. 2006; Ramadas, Gunesh et al. 2010).

The prevalence of CD in North America is estimated as ranging from 26 to 198 cases per 100,000 persons, equating to 400,000 to 600,000 patients with CD (Loftus, Schoenfeld et al. 2002). Prevalence estimates from Northern Europe are lower, ranging from 27 to 48 persons per 100,000 (Bernstein, Wajda et al. 2006). A study carried out in the UK, using data obtained from primary care, reported the prevalence of CD to be 145 cases per 100,000 population (Rubin, Hungin et al. 2000).
1.1.2 Aetiology

The exact aetiology of CD remains unclear. It is likely that the disease is caused by a complex interplay of genetic, microbiological, immunological and environmental factors.

Genetic factors
There is now ample evidence that CD is, in part, the result of a genetic predisposition. Familial aggregation of IBD was first reported in the 1930s (Russell and Satsangi 2004). A positive family history is still the largest independent risk factor for the disease. The estimated lifetime risk of developing IBD for a first-degree relative of a CD patient is 4.8-5.2% (Russell and Satsangi 2004). This figure rises to 7.8% in the Jewish population, which has the highest prevalence of CD of any ethnic group and displays the phenomenon of genetic anticipation. Identical twin studies have shown a concordance rate of 50-60% (Orholm, Binder et al. 2000; Halfvarson, Jess et al. 2006).

CD is a polygenic disease and there are now many genes that have been identified as being linked to the condition. Of major interest is the CARD15/NOD2 gene, located on chromosome 16, first described by Economou et al. in 2004 (Economou, Trikalinos et al. 2004). Mutations in this gene (including amino acid substitutions or a frame shift resulting in insertion of a cysteine residue) account for 10-15% of patients with CD (Hugot, Chamaillard et al. 2001), and homozygous expression of variant NOD2 confers a 20 fold increased risk of developing CD (Cuthbert, Fisher et al. 2002). CARD15/NOD2 mutations interfere with the ability of NOD2 to recognise ligand and a reduced capacity to activate nuclear factor kappaB (NF-κB) in response to stimulation with muramyl dipeptide (Inohara, Ogura et al. 2003). It is not clear exactly how these mutations give rise to developing CD, although they are thought to result in a disturbance in the equilibrium maintained between the mucosal immune system and commensal intestinal microbiota. Furthermore, NF-κB mediates the
transcription of numerous inflammatory cytokines, including tumour necrosis factor alpha (TNFα), interleukin-1 (IL-1), IL-6 and IL-12.

To date, 163 loci have been identified as being associated with the development of IBD, mainly involved with mucosal homeostasis and innate immune responses (Jostins, Ripke et al. 2012). A meta-analysis of all genome-wide association studies in CD has identified 71 regions of susceptibility to CD (Franke, McGovern et al. 2010), including IL-23R (dysregulated adaptive immunity), ATG16L1 and IRGM (defects in autophagy) and XBP1 and ORMDL3 (endoplasmic reticulum stress).

**Microbiological factors**

Microbial agents appear to be intimately involved in the pathogenesis of CD. Several microbes have previously been implicated, including *Mycobacterium paratuberculosis*, the measles virus and *Listeria monocytogenes*. However, despite extensive investigations over the past four decades, no single, specific pathogen has been shown to be critical in the pathogenesis of CD.

The intestine harbours the largest and most diverse microbiota in the human body, consisting of more than 500 species of bacteria. Sequencing of 16S RNA has shown that patients with CD have an overall reduction in the diversity of gut microbiota with increased abundance of Fusobacteriaceae, Veillonellaceae and Enterobacteriaceae Pasteurellaceae and reduced levels of Clostridiales, Bacteroidales and Erysipelotrichales (Gevers, Kugathasan et al. 2014). Interestingly, this dysbiosis was only seen in mucosal biopsies and not in stool samples, indicating that CD pathogenesis involves mucosa-associated microbiota more than luminal bacteria. Commensal enteric bacteria provide constant antigenic stimulation, thereby continuously activating pathogenic T-cells to cause chronic intestinal injury in CD (Eckburg and Relman 2007).
Immunological factors
The gut houses a large part of the mucosa-associated lymphoid tissue in the human body. The mucosal surfaces are the physical interface between the immune system and the high antigenic load within the gut lumen. Under normal conditions, the intestinal mucosa is in a state of ‘controlled inflammation’, being regulated by a delicate balance maintained by the innate and acquired immune systems.

In CD, this well-controlled balance of the intestinal immune system is disturbed at all levels. Luminal antigens gain access to the underlying mucosal tissue via a ‘leaky’ luminal barrier. There are deficiencies in the innate immune system and an exaggerated T-cell driven adaptive immune response. In active CD, effector T-cells (Th1 and Th17) predominate over T-cells with a regulatory phenotype (Treg). Activated effector T-cells produce pro-inflammatory cytokines and influence macrophages to do the same. Tissue damage results from the release of numerous noxious mediators.

Other environmental factors
Cigarette smoking is associated with an increased risk of developing CD (Lindberg, Tysk et al. 1988). Smoking also negatively influences the clinical course of CD and is associated with clinical recurrence of CD after surgical resection in CD patients (Kane, Flicker et al. 2005).

Vitamin D is increasingly being recognised as important in the pathogenesis of CD. A large prospective study of 72,719 women enrolled in the Nurses’ Health Study in the USA examined vitamin D status and the risk of developing IBD. The authors showed that higher predicted levels of serum vitamin D were associated with a reduced risk of developing CD, but not UC (Ananthakrishnan, Khalili et al. 2012). Another study has shown that lower plasma vitamin D levels in patients with CD is associated with increased risk of hospitalisation and surgery (Ananthakrishnan, Cagan et al. 2013).
The relationship between appendicectomy and the risk of developing CD has also been investigated. A systemic review of the literature in 2008 found the relative risk (RR) of having CD diagnosed following an appendicectomy was significantly elevated (Kaplan, Jackson et al. 2008). Within the first year after surgery, the RR was 6.69 (95% CI: 5.42-8.25). An increase risk in developing CD was also seen 1-4 years after appendicectomy. However, data from 4 years post-appendicectomy and onwards shows no increase in the risk of developing CD.

A recent population-based case-control study in Asia-Pacific identified several environmental factors that confer protection against developing CD (Ng, Tang et al. 2015). These included being breast fed (aOR 0.10; 95% CI 0.04 to 0.30), antibiotic use (aOR 0.19; 0.07 to 0.52), having dogs (aOR 0.54; 0.35 to 0.83), daily tea consumption (aOR 0.62; 0.43 to 0.91) and daily physical activity (aOR 0.58; 0.35 to 0.96).

Ultimately, it is likely that CD is the result of continuous microbial antigenic stimulation of pathogenic immune responses as a consequence of host genetic defects in mucosal barrier function, innate bacterial killing and immunoregulation.
1.1.3 Clinical Features

CD is a disease with a broad spectrum of clinical manifestations, and the initial presentation is seldom a good predictor of the clinical course (Katz 1994). It is clinically useful to classify CD according to the site, extent and pattern of disease, since this influences medical management. This has been formalised by the Montreal Classification (table 1).

The site of the disease influences presentation; however symptoms of abdominal pain, diarrhoea and weight loss are ubiquitous among all phenotypes of CD. When patients are experiencing active disease, patients may also experience symptoms of fever, malaise and anorexia. The frequency of anatomical locations of inflammation in CD is shown in figure 1 (section 1.1), with the terminal ileum being the most commonly affected region.

Small bowel (SB) CD is often associated with colicky abdominal pain, which may also suggest a local stenotic lesion. Extensive ileal disease (>100cm), is associated with more profound weight loss and poor nutrition. Specific vitamin deficiencies, such as vitamin B12 and folic acid, may also be seen.

Colonic CD is associated with diarrhoea more commonly than SB CD. Perianal Crohn's disease is common, occurring in approximately 50% of patients (Cosnes 2008). Perianal disease is characterised by recurrent abscesses, fistulae and fleshy skin tags, with or without ulceration. This occurs more commonly in patients with ileocolonic disease and less so in isolated SB disease. Anal and rectal stenosis are complications of perianal CD and may cause symptoms of constipation and spurious diarrhoea.

The pattern of CD also influences patient presentation. Fibrostenotic disease pattern is commonly associated with disease in the ileocaecal region or SB. This can lead to localised strictures and obstructive symptoms. Inflammatory disease pattern is more commonly colonic, causing profuse diarrhoea, pronounced
weight loss and marked elevation in serum inflammatory markers. CD is a transmural inflammatory disease and therefore has the capability to penetrate the full thickness of the gut wall, leading to severe clinical consequences.

**Table 1. The Montreal Classification for Crohn’s disease.** This classification system takes into account the patient’s age at diagnosis, site of disease and behaviour (pattern) of disease. It is useful in the clinical setting as these factors influence the medical management, likelihood of surgery and patient’s prognosis. L4 is a modifier and can be added to L1-3 if the patient has concomitant upper GI CD. ‘P’ is also a modifier and can be added to B1-3, if concurrent perianal disease is present.

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>Site of disease</th>
<th>Disease behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1: &lt;16yrs</td>
<td>L1: Ileal</td>
<td>B1: Non-stricturing, non-penetrating</td>
</tr>
<tr>
<td>A2: 17-40yrs</td>
<td>L2: Colonic</td>
<td>B2: Stricturing</td>
</tr>
<tr>
<td>A3: &gt;40yrs</td>
<td>L3: Ileocolonic</td>
<td>B3: Penetrating</td>
</tr>
<tr>
<td></td>
<td>L4: Upper GI tract</td>
<td>P: Perianal disease</td>
</tr>
</tbody>
</table>

1.1.4 **Extra-intestinal manifestations**

Extra-intestinal manifestations (EIMs) occur in about 30% of individuals with IBD (Veloso 2011). They are slightly more common in CD compared to UC, and patients with CD affecting the colon have a higher incidence of EIMs. The clinical spectrum of EIMs varies from mild transitory to very severe lesions, the latter of which can be more incapacitating than the intestinal disease itself.

Immune-mediated EIMs can occur in the joints (peripheral and axial arthritis), skin (erythema nodosum and pyoderma gangrenosum), mouth (apthous stomatitis), eyes (episcleritis and uveitis) or liver (primary sclerosing cholangitis, PSC). Often, immune-mediated EIMs are related to disease activity and therefore will respond to treating the underlying disease process. However, some patients
experience EIMs, which are unrelated to disease activity and run their own course. For example, PSC, a chronic inflammatory liver disease characterised by progressive bile duct destruction, is a known EIM of IBD. In PSC, liver and bowel inflammatory are rarely concomitant, to the extent that PSC may develop in IBD patients whose diseased colons have been removed many years previously. A summary of EIMs associated with CD is shown in table 2.

<table>
<thead>
<tr>
<th>Common (5-20%)</th>
<th>Unusual (&lt;5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Relate to disease activity</strong></td>
<td><strong>Unrelated to disease activity</strong></td>
</tr>
<tr>
<td>Erythema nodosum</td>
<td>Gallstones</td>
</tr>
<tr>
<td>Finger clubbing</td>
<td>Sacroilitis</td>
</tr>
<tr>
<td>Ocular involvement:</td>
<td>Arthralgia (small joints)</td>
</tr>
<tr>
<td>- conjunctivitis</td>
<td>Nutritional deficiency</td>
</tr>
<tr>
<td>- episcleritis</td>
<td></td>
</tr>
<tr>
<td>- iritis</td>
<td></td>
</tr>
<tr>
<td>Arthritis (large joints)</td>
<td></td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>Liver disease (fatty liver, PSC)</td>
</tr>
<tr>
<td></td>
<td>Ankylosing spondylitis</td>
</tr>
<tr>
<td></td>
<td>Renal stones (oxalate)</td>
</tr>
<tr>
<td></td>
<td>Osteomalacia</td>
</tr>
<tr>
<td></td>
<td>Sweet’s syndrome</td>
</tr>
<tr>
<td></td>
<td>Systemic amyloidosis</td>
</tr>
</tbody>
</table>

*Table 2. Extra-intestinal manifestations associated with Crohn’s disease.*
1.1.5 Diagnosis

There is no single test for CD, and a positive diagnosis requires several different modalities including clinical assessment and endoscopic, radiological and histological investigation. Internationally accepted criteria for the diagnosis of CD were defined by Lennard-Jones in 1989 (Lennard-Jones 1989). The criteria can be applied after excluding infection, ischaemia, irradiation and malignancy as causes for intestinal inflammation and comprises a variety of clinical examination, radiological, endoscopic and histological findings. The presence of 3 or more of these features is diagnostic of CD.

Initial assessment of CD needs to establish the site, extent and pattern of inflammation, as well as the presence of disease complications, as these factors directly influence management and prognosis.

1.1.6 Medical therapy

The medical treatment of patients with CD is difficult. The heterogeneity of patients, including the disease location and pattern, presence of EIMs and response to therapy – promotes challenges when recommending optimal treatment. The principles of management are a consistent approach, attention to nutrition, medical treatment of active disease and surgical management of complications.

Dietary therapy is an established treatment option for CD. The use of elemental or polymeric diets has been shown to be effective in reducing gut inflammation in CD. Unfortunately, this treatment modality is frequently unpalatable and is restrictive to the patient, and therefore its use is limited. Dietary therapy is more widely used in paediatric populations where adherence is better and it offers a good alternative to the use of corticosteroids.
Historically, the medical treatment of CD has been with non-specific immunosuppression, including corticosteroids and immunomodulator therapies (such as thiopurines and methotrexate). These medications are used to modify the pattern of disease, but expose patients to systemic side effects of immunosuppression.

The discovery of immunological mechanisms and pathways that underlie inflammation in CD, has led to the development of therapies that target specific molecules and pathways in the inflammatory cascade, thereby maximising benefit and minimising harm to patients (Melmed and Targan 2010). This form of therapy is currently available in the form of the anti tumour necrosis factor alpha (anti TNFα) drugs. This class of drug has revolutionised the medical management of CD thanks to their ability to induce and maintain remission, heal mucosa, reduce hospitalisations and surgical operations and restore quality of life (Di Sabatino, Liberato et al. 2011). These therapies, their mechanisms of action and therapeutic targets are discussed further in section 1.3 of this introduction.

1.1.7 Complications

In addition to EIMs, CD can give rise to a variety of complications within the gut, some of which can be potentially life-threatening. Small intestinal obstruction can result from active disease and also by a food bolus becoming impacted on a fibrostenotic stricture. Toxic dilatation (toxic megacolon) can occur, although this is much less common than in UC. Bowel perforation rarely presents acutely because an abscess cavity is likely to form and usually requires surgical intervention.

Massive rectal bleeding is rare (1% of patients with colonic disease and very rare in SB CD). Colonic carcinoma occurs in <5% of patients with colonic disease and
is related to the extent and duration (>10 years) of Crohn’s colitis. The risk of small intestinal carcinoma is increased, but remains very rare.

Abdominal, pelvic or ischiorectal abscesses can occur and are usually treated with radiological or surgical drainage and antibiotics. Fistulae can occur at any point along the GI tract, including from gut to gut, skin, bladder, vagina and perianal fistulae. The management of fistulae involves ensuring local sepsis has been excluded by imaging and/or surgical examination under anaesthetic and then biological drug therapy with or without immunomodulator therapy should be considered along with surgical options.
1.2 Dendritic cells

1.2.1 Overview of the innate and adaptive immune systems

The key to a healthy immune system is the interaction between the innate and adaptive immune response to foreign and self-antigens. The initial encounter with an antigen occurs within the innate immune system. Innate immunity is a primitive system which produces a rapid, but non-specific response. Cellular mediators include macrophages, dendritic cells (DCs), granulocytes and natural killer (NK) cells. Macrophages and DCs produce high levels of cytokines when foreign antigens are encountered, resulting in increased vascularity, permeability and elevated expression of adhesion markers and leukocyte recruitment. The function of the innate response is to limit the early replication and spread of infectious agents.

The adaptive immune system responds to signals from the innate response and is antigen-specific. After a lag period of several days, there is a clonal expansion and differentiation of naïve lymphocytes into effector T-cells and antibody-secreting B-cells, usually eliminating the pathogen. On repeated exposure to a particular antigen, the response adapts and becomes more rapid and specific – a phenomenon known as ‘immunological memory’. The nature of the adaptive immune response is dependent on the quality and size of the innate response.

DCs are professional antigen presenting cells and therefore play a pivotal role in the immune system by linking the innate and adaptive immune responses. Antigen presentation by DCs can occur in one of two ways. Endogenous antigens (including viral antigens), processed by the endoplasmic reticulum, are presented by Major Histocompatibility Complex (MHC) class I-bearing cells. These MHC/antigen complexes are recognized exclusively by CD8+ (cytotoxic) T-cells. Once activated, CD8+ T-cells release the cytotoxins perforin, granzymes and granulysin, which enter the cytoplasm of the target cell and trigger the caspase cascade, ultimately resulting in apoptosis (programmed cell death).
CD8+ T-cells can also induce apoptosis via cell surface interactions with the infected target cell.

Exogenous antigens are processed by the lysosomal (endosomal) route and are presented by MHC class II-bearing cells to CD4+ (helper) T-cells. Following activation, CD4+ T-cells differentiate into several distinct subgroups depending on the cytokine profile (Th1, Th2, Th17, regulatory T-cells). The characteristics and functions of these subgroups is discussed later in the introduction (section 1.2.7).

By bridging the innate and adaptive immune responses, DCs act as immunological guardians of the immune system. Although monocytes, macrophages and B-cells are also able to present antigens to stimulate T-cells, it is the ability of DCs to stimulate naïve T-cells that makes them unique (Banchereau, Briere et al. 2000).

1.2.2 Definition and origin of dendritic cells

In 1868, the German anatomist Paul Langerhans identified Langerhan’s cells in the skin, which he mistook for nerve endings. These are now known to be epithelial DCs, crucial in the initiation of immune responses to epidermal pathogens. Later, in 1973, Steinman and colleagues found cells in the mouse spleen that were potent stimulators of the immune system (Steinman and Cohn 2007). These were called DCs because of their appearance of spikey arms or dendrites. This morphology is ideally suited to facilitate interaction with T-cells.

DCs originate from haematopoietic stem cells in the bone marrow, and are widely present throughout all tissues in the human body with the exception of the cornea and testes (Steinman 1991). Despite this, they constitute only a fraction of circulating leucocytes in human blood with values in the literature
varying from 0.1% - 2% (Van Voorhis, Hair et al. 1982; Knight, Farrant et al. 1986; Hart 1997).

Progenitors of DCs in bone marrow migrate via the blood stream to peripheral tissues where they encounter several essential growth factors such as GM-CSF, IL-4, IL-15, TNFα, TGFβ, and IL-3 secreted by various cell types (including endothelial cells, mast cells, keratinocytes and fibroblasts). Such growth factors determine the fate of the progenitor and what type of DC it will become. The capacity to react to innate immune factors places DCs in a pivotal position at the interface between the innate and adaptive immune systems allows DCs to shape the ultimate immune response to a vast range of antigenic stimuli.

1.2.3 Dendritic cell subsets

Human blood contains two major DC subsets, identified by the presence or absence of the adhesion molecule, CD11c.

Plasmacytoid dendritic cells
The CD11c- DC subset are called plasmacytoid DC (pDC). In addition to the absence of CD11c, they are phenotypically distinguished from CD11c+ DCs by expression of the IL-3 receptor (CD123), CD45RA, CD62L, BDCA2, BDCA4 and by lack of expression of CD33 and CD16 (Dzionek, Fuchs et al. 2000). pDCs also exclusively express toll-like receptors (TLRs) 7 and 9 (Ng, Kamm et al. 2010).

Unlike their CD11c+ counterparts, pDCs develop fully within the bone marrow and then travel to the thymus and secondary lymphoid organs via the blood in the healthy state (Colonna, Trinchieri et al. 2004). Prior to antigenic stimulation, pDCs are often referred to as ‘pre-pDCs’ (Shortman and Naik 2007) and migrate to specific tissues (such as LNs or gut) under the control of chemokine receptors such as CCR7 and CCR9 respectively.
Myeloid dendritic cells

The CD11c+ DC subset follows a myeloid differentiation pathway and are therefore termed myeloid DC (mDCs). In addition to CD11c, mDCs express CD13, CD33, CD11b and a high level of HLA DR. They also express TLR1 to 6 and TLR8 (Ng, Kamm et al. 2010). Monocytes are thought to serve as a circulating reservoir of mDC precursors.

Recently, mDCs have been further subdivided into two groups. Type 1 mDCs are identified by BDCA1+ (also known as CD1c+) and represent the vast majority of mDC in the human body (>90%). These DCs are involved in presenting MHC class II antigens, leading to activation of CD4+ T-cell responses. Type 2 mDCs (<10% total mDC) are BDCA3+ (also known as CD141+) and carry out MHC class I antigen presentation to CD8+ T-cells with antigen-cross presenting capabilities (Collin, Bigley et al. 2011).

![Figure 2. Human dendritic cell subsets and their distribution in blood, epithelial tissues and lymph nodes (Collin, McGovern et al. 2013).](image-url)
1.2.4 Antigen detection by intestinal dendritic cells

A single layer of epithelial cells separates the intestinal lamina propria from the gut lumen. On the luminal surface of the epithelial layer is a thick layer of mucus containing bactericidal defensins, neutrophils and large amounts of antigen specific IgA (Sansonetti 2004). There are several mechanisms by which intestinal DCs can sample the antigenic material within the intestinal lumen. Microfold (M) cells transport luminal antigens to the sub-epithelial dome of Peyer’s patches for DCs to sample. Lamina propria DCs can directly sample luminal antigenic material by extending dendrites through the epithelial layer under the control of CX3CR1 expression. DCs can internalise apoptotic epithelial cells and thereby acquiring antigens contained within the apoptosed cell. In addition, breakdown of the epithelial barrier may allow DCs direct access to the luminal antigenic load (Stagg, Hart et al. 2003).

DCs are able to sense microbes by a series of surface receptors (pattern recognition receptors, PRRs), which recognise microbial molecular patterns known as pathogen-associated molecular patterns (PAMPs). Examples of PRR families on DCs include TLRs, cell surface c-type lectin receptors and intracytoplasmic NOD-like receptors. Microbes can directly activate DCs through PRRs, or indirectly, through the internalisation of apoptotic or necrotic cells dying in response to microbial stimuli.

To date, at least ten TLRs have been identified in humans. These are largely characterised by their cellular location (surface or intracellular), differential expression by DC subsets and the microbial product they detect (Akira 2001). Of particular importance are TLR2 and TLR4. TLR2 recognises peptidoglycan lipoteichoic acid from gram-positive bacteria and lipoproteins from both gram-positive and gram-negative organisms and stimulates DCs to secrete IL-10, leading to a Th2 response. TLR4 is required for recognition of lipopolysaccharide (LPS) from Escherichia Coli and stimulates IL-12 production from DCs, giving rise to a Th1 response.
A study by Hart et al. has shown that DCs isolated from human lamina propria express lower TLR2 and TLR4 compared with blood from the same healthy individuals. This may serve to limit recognition of commensal flora in healthy gut and prevent aberrant immune responses. Expression of both TLR2 and TLR4 was raised in inflamed IBD gut tissue, likely leading to increased recognition of bacterial products and an enhanced response to them (Hart, Al-Hassi et al. 2005).

1.2.5 Dendritic cell maturation and migration

In the steady state, DCs are immunologically immature and therefore do not have the capacity to prime naïve T-cells (Banchereau, Briere et al. 2000). Immature DCs have a high endocytic potential with MHC class II molecule accumulation in the endosomal compartments and a low T-cell activation potential with low surface expression of co-stimulatory markers (CD80 and CD86) (Villadangos and Schnorrer 2007).

DC activation and subsequent maturation occurs via the PRRs, cytokine and chemokine receptors that recognise potential stimulatory molecules. Factors that can stimulate DCs include:

- Host-derived molecules such as CD40 ligand, TNFα, IL-1, IL-6 and IFNα.
- Microbial products stimulating TLRs expressed on the surface of DCs.
- Signals released from distressed or dying cells.

The result of DC maturation is down regulation of materials involved in antigen acquisition and upregulation of MHC Class II, MHC Class I and co-stimulatory molecules (CD40, CD80 and CD86). The exact nature of the maturation process and the cytokines secreted vary depending of the type of PRR encountered and are specific to each pathogen (Guermonprez, Valladeau et al. 2002). Phenotypic properties of immature and mature DCs are shown in table 3.
Simultaneously, the maturing DCs up-regulate the chemokine receptor CCR7 and therefore acquire responsiveness to the CCL19 and CCL21 ligands. This process promotes the migration of lamina propria DCs to mesenteric lymph nodes (MLN) via the afferent lymph system (Dieu, Vanbervliet et al. 1998). The lymph nodes are rich in memory and naïve T-cells. Indeed, both memory and naïve T-cells also express CCR7, encouraging their own migration to the MLN.

The chemokine receptor, CCR5, controls the DC’s ability to migrate to areas of inflammation. Immature DCs are CCR5\textsuperscript{high}CCR7\textsuperscript{low} and are therefore likely to migrate to sites of inflammation where they are required to sample antigenic material. Following antigen acquisition, the maturation process reverses the ratio of expression of these two chemokine receptors (becoming CCR5\textsuperscript{low}CCR7\textsuperscript{high}), thereby encouraging migration to draining lymph nodes to trigger the adaptive immune response (Sallusto, Schaerli et al. 1998).

<table>
<thead>
<tr>
<th>DC characteristic</th>
<th>Immature</th>
<th>Mature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Function</td>
<td>Antigen capture</td>
<td>Antigen presentation</td>
</tr>
<tr>
<td>Morphology</td>
<td>Stellate</td>
<td>Multiple veil-like projections</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>Low CD54</td>
<td>High CD54</td>
</tr>
<tr>
<td>Co-stimulatory molecules</td>
<td>Low CD40, CD80, CD86</td>
<td>High CD40, CD80, CD86</td>
</tr>
<tr>
<td>Maturation marker</td>
<td>Low CD83</td>
<td>High CD83</td>
</tr>
<tr>
<td>Chemokine receptors</td>
<td>Low CCR7, High CCR5</td>
<td>High CCR7, Low CCR5</td>
</tr>
<tr>
<td>Macropinocytosis</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Stimulatory capacity</td>
<td>Low</td>
<td>High</td>
</tr>
</tbody>
</table>

Table 3. Immature and mature dendritic cell phenotype. Immature DCs capture antigens in the peripheral tissue. As they migrate to lymphoid organs, they mature and prepare for antigen presentation to T-cells.
1.2.6 Antigen presentation

Naïve T-cells require two signals from DCs to become fully activated. The antigen (bound to MHC-II molecule) provides the first signal and interacts with the T-cell receptor (TCR). The second signal is antigen non-specific and is provided by co-stimulatory molecules on the surface of the DC binding with their corresponding receptors on the naïve T-cell. Examples of such co-stimulatory molecules are CD40, CD80 and CD86. This double signal is necessary for T-cell proliferation, differentiation, survival and cytokine secretion. Activation of T-cells without co-stimulation results in suppression of the immune response, and, in some cases, induces antigen-specific tolerance (Lenschow, Walunas et al. 1996).

Matured or activated DCs also provide a third signal to the T-cells via secretion of cytokines. The cytokines produced by DCs determine the type of effector T-cell response that occurs. This signal is the most important factor inducing the effector functions in T-cells (de Jong, Smits et al. 2005).

Figure 3. Antigen presentation by dendritic cells. Signal 1 is provided by the antigen itself, bound to MHC-II molecule. Signal 2 represents the co-stimulatory molecules and signal 3 is cytokine production. The quality and quantity of these 3 signals regulates the subsequent T-cell response.
1.2.7 T-lymphocyte differentiation and effector function

DCs can contribute to the expansion and differentiation of most classes of lymphocytes. In addition to T-cells, they also play a role in the differentiation of B-cells and NK cells (section 1.2.8). The type of DC and its maturation status influences the subsequent T-cell response and therefore the effect on adaptive immunity. DCs drive both T-cell proliferation and the functional differentiation of T-cells. The type of T-cell differentiation depends on multiple factors, such as the antigen dose, types of cytokines secreted by the DC and co-stimulatory molecule expression.

Figure 4. Proposed mechanisms of control of T-cell responses by intestinal dendritic cells. Once antigen acquisition by the DC has occurred, the DC begins to mature and migrates to the MLN to interact with the T-cell population. Cytokines produced by the activated DC play a role in polarising the T-cell response resulting in distinct effector CD4+ T-helper cell subsets.
Production of IL-12 by DCs promotes a Th1 response (Macatonia, Hosken et al. 1995). Two further members of the IL-12 family have been described; IL-23 which induces proliferation of memory T-cells (Oppmann, Lesley et al. 2000) and IL-27 which induces proliferation of naïve T-cells (Pflanz, Timans et al. 2002). Both IL-23 and IL-27 are produced by DCs and polarise T-cells to a Th1 response (Smits, van Beelen et al. 2004).

Prior to the characterisation of Th17 lymphocytes, Th1 T-cells were thought of as the main mediators of the pathogenesis of CD. Evidence for this includes the observation that DCs and macrophages in CD produce large amounts of IL-12 (Monteleone, Biancone et al. 1997). In addition, there is enhanced production of IL-18 (a cytokine that perpetuates Th1 cell responses) in the intestinal mucosa of patients with active CD (Pizarro, Michie et al. 1999), and T-cells isolated from areas of active CD have been shown to express increased amounts of IFNγ and decreased amounts of IL-4 (a Th2 cytokine) compared with healthy control subjects (Fuss, Neurath et al. 1996). Further evidence for the importance of Th1 cells in the pathogenesis of CD comes from a study of fontolizumab (an anti-IFNγ-antibody) in patients with CD, where the study drug was associated with a clinical response in CD patients (Hommes, Mikhailova et al. 2006).

Effector Th1 cells produce high levels of IFNγ and TNFβ cytokines, which are required for cell-mediated immunity against intracellular pathogens through macrophage activation and the promotion of cytotoxic activity in NK and CD8+ T-cells. While mucosal T-cells mount a strong Th1 response in the early stages of CD with high IFNγ production and IL-12Rβ2 chain expression, in the latter stages of CD, this appears to be lost (Kugathasan, Saubermann et al. 2007). This suggests that mucosal T-cell immunoregulation varies with the course of IBD and may have implications on the therapeutic efficacy of drug treatment in different stages of CD.

The production of IL-4 and IL-10 by DCs, polarise a Th2 cell response. Th2 cells drive humoral immunity directed towards clearing extracellular pathogens. They
stimulate B-cells to produce IgE, enhance the maturation of eosinophils and promote degranulation of mast cells and basophils. Th2 cells produce high levels of IL-4, IL-5 and IL-13 cytokines.

More recently, a new subset of IL-17-producing Th17 cells with different effector functions from Th1 and Th2 cells have been identified (Bettelli, Korn et al. 2008). In contrast to Th1 and Th2 cell differentiation, which depend on effector cytokines for differentiation (IFNγ and IL-4 respectively), Th17 cells do not require IL-17 for their differentiation (Brand 2009). Murine Th17 cells originate from naïve CD4+ T-cells in the presence of IL-6 and TGFβ and their ongoing development is supported by IL-21 and IL-23 (Aggarwal, Ghilardi et al. 2003; Bettelli, Carrier et al. 2006). Wilson et al. demonstrated that Th17 cells are characterised by an increased expression of IL-17a, IL-17F, IL-22, IL-26, IFNγ and CCL20 (Wilson, Boniface et al. 2007). These cytokines and chemokines are highly expressed in active CD, therefore supporting their role in CD pathogenesis.

Regulatory T-cells (Tregs) express the phenotype CD4+CD25high and the intracellular forkhead box transcription factor p3 (FoxP3). Tregs play a crucial role in the maintenance of self-tolerance and prevention of autoimmune disease. Treg differentiation occurs via a mechanism dependent on retinoic acid and TGFβ (Coombes, Siddiqui et al. 2007). Cytokines that induce differentiation of Th1, Th2 and Th17 cells antagonise Treg cell differentiation (Zhou, Bailey-Bucktrout et al. 2009). The production of immunosuppressive cytokines TGFβ and IL-10 have been implicated in the effector function of Tregs, with immature DCs secreting IL-10 on contact with Tregs which in turn induces the Tregs to suppress immune activity by secreting IL-10 (Jonuleit, Schmitt et al. 2000). However, some recent evidence suggests that Tregs phenotype, or at least the expression of their regulatory-associated FoxP3 transcription factor, may be more dynamic than was initially thought (Bernardo, Al-Hassi et al. 2012).
1.2.8 Effect of dendritic cells on other lymphocytes

DCs also have the capacity to stimulate other lymphocytes, including NK T-cells, by secretion of IL-12p70 upon microbial contact (Munz, Steinman et al. 2005) and naïve B-cells, by producing pro-inflammatory cytokines such as IL-12, thereby stimulating B-cell antibody production.

1.2.9 Completing the dendritic cell lifecycle

Following migration to LN, DCs have a life expectancy of up to 72 hours. The lifecycle of DCs ends by a process of cellular apoptosis, mediated by T-lymphocytes. The process of DC apoptosis triggers signaling pathways that lead to the next wave of DCs migrating from local tissues to the lymphoid tissue (Parajuli, Nishioka et al. 1999). The lifecycle of DCs is shown in figure 5. A small percentage of DCs leave the secondary lymphoid organs via efferent lymphatics and accumulate in the thoracic duct. From here they are seeded back into the systemic circulation. These DCs have an antigen load and therefore spread the antigenic stimulus to the thymus, spleen and the bone marrow. This results in adequate adaptive immunity with the ability to mount a future effective defence as well as contribute to long term immunological memory.
Figure 5. The lifecycle of the dendritic cell. Immature DCs in the circulation enter the tissues and encounter pathogens directly or indirectly to induce cytokine secretion. DC maturation and migration occurs resulting in mature DCs arriving at the lymphoid organs displaying peptide-MHC complexes and recruiting antigen-specific T lymphocytes (Ueno, Schmitt et al. 2010).

1.2.10 Principles of T-lymphocyte migration

Every day, nearly a trillion lymphocytes leave the circulation, enter the tissues and then re-enter the blood. By continually migrating around the body, lymphocytes optimise the chances of encountering an antigen. Cell migration on this massive scale must be tightly controlled. Lymphocytes express homing molecules which recognise specific ligands on the vascular endothelium, thereby enabling lymphocytes to migrate to their target tissue.

Naïve T-cells tend to migrate to lymphoid organs and are generally excluded from peripheral tissues due to high levels of CCR7 expression. Once activated by antigen, naïve T-cells alter their homing marker expression, thereby enabling migration to peripheral tissues.
Recruitment of immune cells to sites of inflammation is a complex process mediated by adhesion molecules, chemokines and receptors. This interaction can be divided into several steps, including: rolling and tethering on the vascular endothelium; activation and adhesion to the endothelium; locomotion from the site of adhesion to the endothelial cell junction; and transmigration across the endothelial barrier into the tissue compartment (figure 6). This series of steps happen in sequence and not in parallel and occur rapidly, with a leukocyte stuck to the endothelium only taking a few minutes to reach the subendothelial basement membrane (Muller 2003).

The recruitment of leukocytes to a specific site of inflammation is dependent upon localised endothelial cell activation, a major part of which is an increase in the expression of adhesion molecules (Carlos and Harlan 1994). Examples of these adhesion molecules are the endothelial selectins (E- and P-selectin) and endothelial immunoglobulin superfamily proteins (ICAM-1, VCAM-1, PECAM-1 and MadCAM). The expression of adhesion molecules is rapidly increased in response to inflammatory stimuli. Chemokines also play an important role in the conversion of rolling behaviour to firm integrin-dependent cell arrest (Campbell, Hedrick et al. 1998). This change in behaviour occurs in seconds following chemokine stimulation of leukocytes (Alon and Feigelson 2002).
Leukocyte recruitment to sites of inflammation. Leukocyte recruitment from blood to sites of inflammation occurs in a stepwise process: Rolling and tethering on the vascular endothelium, mediated by selectins and their respective ligands; Activation and adhesion to the endothelium, mediated by the interaction of leukocyte integrins with ICAMs and VCAM-1; Locomotion from the site of adhesion to the endothelial cell junction; Diapedesis across the endothelial cell and basement membrane into the tissue (Garton, Gough et al. 2006).

1.2.11 Dendritic cells influence T-lymphocyte homing

An efficient system would ensure that responder cells return to the site where the antigen was originally acquired in order to carry out their response. In addition to activating naïve T-cells, DCs also direct T-cells to the site where the antigen was encountered. This is done at the time of antigen-acquisition by way of a fourth signal; a ‘homing’ signal.

Studies have shown that murine DCs obtained from mesenteric LN, but not from peripheral LN, induced the gut-homing molecule α4β7 on T-cells following stimulation, therefore targeting those T-cells back to the gut tissue (Stagg, Kamm et al. 2002). Other experiments have shown a similar picture when using murine intestinal DCs obtained from Peyer’s patches and gut lamina propria, resulting in expression of gut-homing α4β7 and CCR9 on activated T-cells (Mora, Bono et al. 2003; Johansson-Lindbom, Svensson et al. 2005). Conversely, DCs obtained from skin-draining LN specifically induce expression of skin-homing markers on
activated T-cells (Campbell and Butcher 2002). These studies have shown that DCs are essential for imprinting lymphocytes with tissue-specific properties. A variety of homing molecules on immune cells have been identified:

**Gut-homing molecules**

**Beta7 (β7) integrin**

Effector T-cells homing to intestinal sites express high levels of the gut-homing molecule α4β7 integrin (Lefrancois, Parker et al. 1999). The ligand for this integrin is mucosal vascular addressin cell-adhesion molecule 1 (MAdCAM-1) and is expressed by post-capillary endothelial cells in the small intestinal lamina propria (Berlin, Berg et al. 1993) and the colonic lamina propria (Berg, McEvoy et al. 1993). Studies performed with β7-deficient mice or utilising antibodies to α4β7 or MAdCAM-1 have shown β7 integrin expression is mandatory for lymphocyte recruitment to intestinal tissues (Wagner, Lohler et al. 1996).

In addition to mediating lymphocyte homing to the gut, the presence of β7-expressing DCs in the gut is required to induce Treg and IL-10 producing T-cells, resulting in tolerogenic properties. Furthermore, lack of the α4β7 integrin in the innate immune compartment leads to a reduction in retinoic acid-producing DCs and accelerates T-cell-mediated colitis (Villablanca, De Calisto et al. 2014).

The αε integrin (also known as CD103) serves as a mucosal DC subset marker. These cells mediate essential immune activities such as antigen presentation (del Rio, Rodriguez-Barbosa et al. 2007) and Treg induction. Although only 2% of circulating lymphocytes express CD103, expression is greater than 90% on intraepithelial lymphocytes (IELs) (Cepek, Parker et al. 1993). The ligand for αεβ7 is e-cadherin, which is expressed on epithelial surfaces, including the gut, lungs and skin. CD103+ DCs are able to synthesise retinoic acid (RA), resulting in the generation of Tregs at the expense of Th17 cells (Coombes, Siddiqui et al. 2007).
Throughout this thesis, cells which have positive β7 expression have been assumed to be expressing the molecule α4β7 and therefore taken to have a gut-homing phenotype. It is accepted that a proportion of these β7 positive cells will actually be expressing αεβ7 and therefore have a different homing phenotype. However, in view of the tiny proportion of αεβ7 expression on circulating lymphocytes (2%, as described above), cells staining positive for β7 in the experimental work in this thesis are assumed to have gut-homing properties.

**CCR9 chemokine receptor**

CCR9 is a chemokine receptor, selectively expressed on T-cells homing towards intestinal sites and on the majority of T-cells in the small intestine (Zabel, Agace et al. 1999). The ligand for CCR9 is CCL25/TECK, which is expressed by epithelial cells within the small intestine (Wurbel, Philippe et al. 2000). Together, CCR9 and CCL25/TECK play a selective role in effector T-cell homing to the small intestine. This has been demonstrated by Stenstad et al., who showed the reduced ability of CCR9-deficient CD4+ murine T-cells to enter the small intestinal lamina propria compared to their wild-type counterparts (Stenstad, Ericsson et al. 2006).

Despite CCR9 being considered a small bowel homing marker, we have recently shown that it is expressed on DC from the small bowel and the colon, albeit in higher quantities in the former group (Mann, Landy et al. 2013). Furthermore, differences in CCR9 expression have also been reported on a gradient between human DC isolated from the ascending and descending colon (Dr David Bernardo, personal communication).

Studies have shown the expression of α4β7, MAdCAM-1 and CCR9 to be dysregulated in humans with IBD (Souza, Elia et al. 1999; Hart, Kamm et al. 2004). We recently demonstrated that human colonic DCs generate gut-homing T-cells when cultured together (Mann, Bernardo et al. 2012). Although murine studies have identified the default pathway for stimulated T-cells is skin-trophism, we found that in the presence of signals provided by intestinal DCs,
gut-homing properties can be acquired by T-cells. Furthermore, we have demonstrated that, in humans, blood and cutaneous DCs, but not intestinal DCs, induced expression of cutaneous lymphocyte antigen (CLA) on stimulated T-cells, a molecule involved in leucocyte homing to the skin (Mann, Bernardo et al. 2012).

**Lymph node homing molecules**

As previously described, CCR7 expression is dramatically increased on DC after stimulation and maturation (Sallusto, Scherli et al. 1998). CCR7 expression promotes homing to local draining lymph nodes by acquired responsiveness to the CCR7-ligand, macrophage inflammatory protein 3β (MIP-3β), also known as CCL19, and CCL21. These ligands attract CCR7-expressing mature DC and naïve T-cells to the lymph nodes. CCL19 expression is spread over the T-cell zone, driving maturing DC and naïve T-cells into the paracortical areas for antigen presentation and subsequent T-cell stimulation (Dieu, Vanbervliet et al. 1998).

**Skin homing molecules**

**Cutaneous lymphocyte antigen**

CLA arises from specialised glycosylation of P-selectin glycoprotein ligand-1 (CD162) (Fuhlbrigge, Kieffer et al. 1997). CLA interacts with E-selectin (endothelial receptor) which is expressed on post-capillary venules in the skin. Under physiological conditions, the majority of T-cells in the skin compartment express high levels of CLA (Clark, Chong et al. 2006). Recent work in our laboratory has shown aberrant CLA expression on γδ T-cells in patients with cutaneous manifestations of IBD (Mann, McCarthy et al. 2012).

**Skin-associated chemokine receptors**

CCR4 (ligand CCL17) is likely to confer skin-homing of immune cells under physiological conditions (Campbell, O’Connell et al. 2007). Under physiological conditions, the vast majority of T-cells in the skin compartment are CCR4+ (Clark,
Chong et al. 2006). CCR10 (ligand CCL27) also promotes skin-homing (Sigmundsdottir, Pan et al. 2007). However, some evidence shows CCR10 is implicated in homing to mucosal sites. The mucosal epithelium within the gut expresses an alternative CCR10 ligand (CCL28), which mediates CCR10-dependent recruitment of IgA secreting cells to the gut mucosa (Lazarus, Kunkel et al. 2003).

CCL17 and CCL27 have been identified in inflamed and non-inflamed skin endothelium (Campbell, Haraldsen et al. 1999), further supporting the skin-homing function of these chemokine receptors.

The role of CCR8 (ligand CCL1) remains poorly understood. It is thought that CCR8 expression on lymphocytes confers a homing preference towards human skin (although lung-homing properties also remain a possibility). CCR8-expressing, skin-derived lymphocytes promote the production of the pro-inflammatory cytokines IFN\(\gamma\), TNF\(\alpha\) and IL-12 after \textit{ex-vivo} stimulation (Schäerli, Ebert et al. 2004).
<table>
<thead>
<tr>
<th>Cell surface receptor</th>
<th>Receptor/Ligand</th>
<th>Target tissue</th>
</tr>
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<tbody>
<tr>
<td>α4β7</td>
<td>MadCAM1</td>
<td>Gut</td>
</tr>
<tr>
<td>CLA</td>
<td>E-selectin</td>
<td>Skin</td>
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<tr>
<td>CCR4</td>
<td>CCL17</td>
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<td>CCR7</td>
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<td>CCR8</td>
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<td>Skin</td>
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<td>CCR9</td>
<td>CCL25</td>
<td>Small bowel</td>
</tr>
<tr>
<td>CCR10</td>
<td>CCL27/28</td>
<td>Skin / mucosal surfaces</td>
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</tbody>
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*Figure 7. Induction of T-lymphocyte homing by dendritic cells. DCs deliver 4<sup>th</sup> homing signal to T-cells at the time of antigen presentation in the MLN. This signal is a reflection of where the DC has originated from and directs T-cell migration back to that site to enact their response (Courtesy of Dr Elizabeth Mann, APRG, Imperial College London). The incorporated table shows the molecules and receptors involved in lymphocyte homing.*
Other factors promoting immune cell homing

The vitamin A metabolite, retinoic acid (RA), plays an important role in gut-homing. This was demonstrated by Iwata and colleagues, who identified that mucosal DCs, but not spleen or skin DCs, express relatively high levels of retinal dehydrogenase (RALDH), an enzyme required for the production of RA. They went on to demonstrate that, while vitamin A deficiency in mice results in decreased expression of α4β7 integrin on effector and memory T-cells and reduced lamina propria lymphocyte numbers, it has no effect on E- or P-selectin ligand expression on skin-homing effector cells. The authors also showed that pharmacological inhibition of RALDH inhibits DCs originating in GALT from imprinting gut trophism (Iwata, Hirakiyama et al. 2004).

The presence of RA has a similar effect on B-cell homing. Mora et al. studied this by stimulating B-cells with and without RA and then re-stimulated the cells under reversed imprinting conditions. B-cells that were activated with RA expressed few gut-homing receptors, but became α4β7highCCR9+ upon re-stimulation with RA. Conversely, B-cells initially exposed to RA and then re-stimulated without RA down-regulated CCR9 expression but remained α4β7high. Thus, effector B-cells adjust their homing phenotype according to changing microenvironmental conditions (Mora, Iwata et al. 2006).

Furthermore, RA in combination with TGFβ has been implicated in the generation of T-cells with a regulatory phenotype (Tregs) from naïve T-cells (Sun, Hall et al. 2007). Peripheral induction of Tregs is also accompanied by up-regulation of α4β7 and CCR9 homing markers, directing Treg migration to gut tissue (Benson, Pino-Lagos et al. 2007).

Vitamin D influences DC-mediated homing marker expression in activated human T-cells, favouring a skin-homing phenotype. Human in vitro studies have shown the vitamin D3 precursor is efficiently processed to the active molecule 1,25(OH)2D3 by DCs in the skin. This active form of vitamin D up-regulates the
expression of the skin-homing marker CCR10 on activated T-cells while down-regulating the expression of the gut-homing markers, α4β7 and CCR9 (Sigmundsdottir, Pan et al. 2007).

1.2.12 The role of dendritic cells in intestinal homeostasis

The intestine represents the largest and most complex component of the human immune system. In health, the gut mucosal immune system maintains a disease-free state in the presence of a highly antigenic environment (commensal microbiota and food antigens). However, the intestinal immune system also has the capacity to respond appropriately to pathological organisms. Any defects in this delicate balance could lead to an overly aggressive immune response to normal gut flora or food antigen, resulting in inflammation and tissue damage. At the other end of the spectrum, a lack of or absent immune response to pathological organisms in the gut lumen could lead to unimpaired infection. In order to achieve this balance between ‘tolerogenic’ and ‘immunogenic’ responses, there is a tightly regulated state of restrained immune activity termed ‘physiological inflammation’.

In view of their ability to shape an immune response on the basis of signals received from the local environment, DCs are critical in ensuring that active inflammatory processes are not triggered by harmless or commensal antigenic material within the gut lumen. This tolerogenic function is performed by migration of DCs within the LP to MLNs where they perform antigen presentation to T-cells under the influence of CCR7 expression. This migration increases in the presence of inflammatory stimuli, but has also been shown to occur in the absence of such a stimulus (Turnbull, Yrlid et al. 2005). Furthermore, impaired migration of DCs from the LP to MLN in a CCR7-/- murine model leads to defective tolerance to luminal antigens (Worbs, Bode et al. 2006). There are several mechanisms in the literature pertaining to induction of tolerance by DCs:
• The level of DC maturity at the time of antigen presentation is likely to play a role in the development of tolerance. Repetitive stimulation with allogeneic immature human DCs results in induction of IL-10-producing, non-proliferating CD4+ T-cells with regulatory properties (Jonuleit, Schmitt et al. 2000).

• Down-regulation of CD80 and CD86 (Steinman, Hawiger et al. 2003). The reduction in CD80 and CD86 molecules also causes suppression of the production of indoleamine-2,3-deoxygenase (IDO). IDO is an enzyme which functions as the rate-limiting step in the production of tryptophan (an amino acid, essential for T-cell proliferation).

• Signalling through novel receptor-ligand interactions, such as serrate 1-induced notch signalling. This may regulate the decision between immunity and tolerance (Hoyne, Dallman et al. 2000).

• Production of a ‘tolerogenic’ cytokine profile by DCs (IL-10, TGFβ or IFNα) may also play a role in polarising T-cells towards a regulatory phenotype (Levings, Sangregorio et al. 2001; Yamagiwa, Gray et al. 2001). Furthermore, production of TGFβ by epithelial cells also results in downregulation of IL-12/23 production by DCs (Ng, Kamm et al. 2010).

1.2.13 Role of DC in intestinal inflammation

Intestinal inflammation in IBD occurs when there is dysregulation of the immune system to intestinal flora. In a murine model of CD4+ T-cell-induced colitis, Krajina et al. demonstrated lamina propria DCs had an activated phenotype, with up-regulation of CD40, CD80 and CD86 and increased IL-12 expression, compared with non-colitic mice (Krajina, Leithauser et al. 2003). In this model of colitis, this suggests that DCs are driving a pro-inflammatory Th1 response.
Hart et al. demonstrated DCs from inflamed CD tissue express higher levels of CD40 than DCs from non-inflamed CD tissue or healthy tissue (Hart, Al-Hassi et al. 2005). These elevated levels of CD40 returned to normal after successful treatment with anti-TNF alpha therapy. The finding of elevated co-stimulatory molecules on DC in patients with active IBD is supported by other immunohistological studies which have shown increased numbers of DCs expressing CD40, CD86 in active IBD (Ikeda, Akbar et al. 2001; Vuckovic, Florin et al. 2001).

DCs appear in the colon early in the process of intestinal inflammation, before the development of epithelial lesions and tissue degradation (Karlis, Penttila et al. 2004). In another animal model of colitis, a 15-36 fold increase in the number of activated DC were observed in the MLN compared with normal mice or mice that had been administered regulatory T-cells to prevent the development of disease (Malmstrom, Shipton et al. 2001). All together, these observations highlight the role of intestinal DC in controlling the dysregulated immune response in CD.
Figure 8. Homeostatic and immunogenic properties of human dendritic cells. Immature DCs in the peripheral tissues develop from these DC precursors. In the steady state, they take up self-antigens, mature and follow a tolerogenic pathway of development. Upon exposure to infectious/inflammatory antigens, immature DCs mature and migrate to lymph nodes when antigen presentation leads to T-cell expansion and differentiation (Quah and O’Neill 2005).
1.3 Anti tumour necrosis factor alpha therapies for Crohn’s disease

1.3.1 Biology of the TNF alpha cytokine

TNF alpha was first described by Carswell et al. in 1975 as an endotoxin-induced glycoprotein, which caused haemorrhagic necrosis of solid tumors (Carswell, Old et al. 1975). At low concentrations, TNFα has beneficial effects in the human body, initiating defense responses to local injuries and playing a central role in host defense against infections (Chowers and Allez 2010). At high concentrations, TNFα contributes to excessive inflammation, resulting in tissue damage (Kollias 2005). In patients with CD, there are elevated concentrations of TNFα in inflamed tissue (Braegger, Nicholls et al. 1992), and hence it is considered a pro-inflammatory cytokine.

TNFα is predominantly synthesised by lymphocytes and activated macrophages (Vassalli 1992) but also by other immune cells (dendritic cells, eosinophils, neutrophils, and mast cells), non-immune cells (glial cells, astrocytes, fibroblasts, granuloma cells and keratinocytes) and many kinds of tumour cells (Bazzoni and Beutler 1996).

TNFα is generated as a 26-kDa transmembrane type II polypeptide precursor (tmTNF). The 17-kDa soluble TNFα (sTNF) is released from tmTNF by the action of TNFα converting enzyme (TACE) (Black, Rauch et al. 1997). Both sTNF and tmTNF are biologically active by binding to one of two structurally distinct receptors: TNF receptor type I (TNFR1, subsequently referred to as the p55 TNF receptor) and TNF receptor type II (TNFR2 or p75 TNF receptor) (Vandenabeele, Declercq et al. 1995). TNFR1 and TNFR2 are found on all cell types with the exception of erythrocytes. The receptors share the same extracellular TNF-binding domain structure, but induce different cytoplasmic signaling pathways (Wallach, Varfolomeev et al. 1999). sTNF binds to TNFR1 with a high affinity. TNFR1 contains a death domain which mediates the induction of apoptosis.
Historically this action was considered to be the principal mechanism through which TNFα induces its pro-inflammatory mechanism. However, the role of tmTNF and subsequent TNFR2 signaling is becoming increasingly relevant. Indeed, tmTNF induces colitis in the absence of sTNF (Corazza, Brunner et al. 2004).

In addition to its action on effector cells (‘forward signaling’), tmTNF also functions as a cellular receptor on TNFα-producing cells, resulting in ‘reverse signaling’. By this process, the membrane-integrated ligands can receive signals, acting as receptors that can transmit positive and negative feedback signals into the ligand-bearing cell. Eissner et al. demonstrated that reverse signaling through tmTNF in human monocytes and macrophages confers resistance to LPS (Eissner, Kirchner et al. 2000). Reverse signaling by tmTNF also induces E-selectin expression in activated human CD4+ T cells (Harashima, Horiuchi et al. 2001). Therefore, current evidence suggests that drugs can target tmTNF in different ways: by acting as an antagonist (blocking tmTNF interaction with TNFR1/2), or as an agonist by initiating reverse signaling, leading to apoptosis, cell activation or cytokine suppression. Reverse signaling is an emerging area of interest regarding the mechanisms of action of anti-TNFα antagonists.

Figure 9. The synthesis, biology, cellular receptors and effector functions of the TNFα cytokine.
1.3.2 Anti-TNFα therapies for CD

The era of biological therapy for IBD began in 1998 with the approval of the anti-TNFα agent, infliximab, for the treatment of luminal CD. Later, it was approved for the treatment of fistulising CD and for patients with acute severe UC. Adalimumab was subsequently approved in 2007 and certolizumab pegol in 2008 for luminal CD. To date, certolizumab pegol has not received approval for use by the European Medicines Agency and therefore it is only available for use in the United States and Switzerland.

Biological therapies have offered a powerful new weapon in the treatment of IBD thanks to their ability to induce and maintain remission, heal mucosa, reduce admissions to hospital, prevent surgical operations and restore quality of life (Di Sabatino, Liberato et al. 2011). The use of biological therapy in CD is currently reserved for patients with disease not responding to conventional therapy (corticosteroids and/or immunosuppressants).

Infliximab

Infliximab (IFX) is a mouse chimeric monoclonal antibody that targets the human TNFα cytokine. It is composed of a human constant region IgG1k light chain that accounts for approximately 75% of the antibody, and a mouse variable region (25%). It is administered intravenously (usually 5mg/kg body weight) typically at 6-8 week intervals (following an accelerated induction course).

IFX has a rapid onset of action, and disease response can be evaluated after only 3 doses. Week 4 response rates after a single dose of IFX was 66% and remission rate was 33%, both being superior to placebo (Targan, Hanauer et al. 1997).

This early response rate to IFX was further demonstrated in the large ACCENT I trial (A Crohn’s disease Clinical study Evaluating infliximab in a New long term Treatment regimen), which demonstrated that patients who responded to a single dose of IFX were more likely to stay in remission up to week 54 if they
continued with maintenance IFX treatment compared to placebo (Hanauer, Feagan et al. 2002).

A Cochrane Database System Review in 2008 examined three randomized control trials examining maintenance of remission of CD with IFX treatment (Behm and Bickston 2008). IFX was better than placebo for maintenance of remission (relative risk, RR, = 2.50), clinical response (RR = 2.19), steroid-sparing effects (RR = 3.13) and complete fistula healing (RR = 1.87) to week 54.

**Adalimumab**

Adalimumab (ADA) is a fully human IgG1 monoclonal antibody to TNFα. It is administered subcutaneously, usually every 2 weeks at a dose of 40mg (following an induction course).

The CHARM trial (Crohn’s Trial of the Fully Human Antibody Adalimumab for Remission Maintenance) (Colombel, Sandborn et al. 2007) showed ADA to be superior to placebo in maintenance of remission of moderate to severe CD at week 26 (40% versus 17% respectively) and week 56 (36% versus 12% respectively). There was no significant difference in clinical efficacy when comparing ADA 40mg every other week to 40mg weekly dosing.

There are no clinical trials directly comparing efficacy of IFX to ADA, but clinical remission rates appear comparable. Remission rates with ADA were 40% at week 26 in the CHARM trial, compared to IFX which achieved 41.8% remission at week 30 in the ACCENT I trial. The two therapies also have similar rates of complete fistula closure; 36% for IFX (Sands, Anderson et al. 2004) and 33% for ADA in subgroup analysis of the CHARM trial at week 54, although there is no trial involving ADA looking at fistula closure as the primary outcome.
Certolizumab

Certolizumab (CZP) is a humanised TNFα Fab’ monoclonal antibody fragment linked to polyethylene glycol. The PEGylation increases its plasma half-life and reduces the requirement for frequent dosing, possibly reducing immunogenicity as well. CZP is administered subcutaneously, usually every 4 weeks at a dose of 400mg (following an induction course).

The Pegylated Antibody Fragment Evaluation in Crohn’s Disease: Safety and Efficacy 1 (PRECISE I) study (Sandborn, Feagan et al. 2007) examined the efficacy of CZP in 662 patients with moderate to severely active CD, and concluded that induction and maintenance therapy with CZP was associated with a modest improvement in response rates compared with placebo, but with no significant improvement in remission rates.

The PRECISE 2 study was designed differently by giving all patients an induction course of CZP and those with a defined clinical response at week 6 were randomly assigned to receive either CZP maintenance therapy or placebo and followed up to week 26 (Schreiber, Khaliq-Kareemi et al. 2007). The study showed that patients with moderate to severe Crohn's disease who had a response to CZP induction therapy were more likely to have a maintained response and be in remission at 26 weeks with maintenance CZP treatment than with a switch to placebo.

Etanercept

Etanercept is a genetically engineered fusion protein consisting of two recombinant human TNF p75 receptors linked to an Fc portion of human IgG1 fragment. It is administered subcutaneously at a dose of 25mg twice weekly.

Etanercept is effective in the treatment of rheumatoid arthritis, but is ineffective in the treatment of CD (Sandborn, Hanauer et al. 2001), although this may be the result of insufficient dosing of etanercept in the study as opposed to fundamental differences in the mechanism of action of the drug.
1.3.3 Mechanisms of action of anti-TNFα therapies in Crohn’s disease

Studies in CD have shown an increased number of lamina propria sTNF-producing cells and high concentrations of sTNF can be detected in the stool of patients with active inflammatory responses (Braegger, Nicholls et al. 1992; Breese and MacDonald 1995). All the available anti TNF antibodies effectively neutralise the bioactivity of sTNF (ten Hove, van Montfrans et al. 2002). However, the apparent failure of etanercept in phase II clinical trials in the treatment of CD means that neutralisation of sTNF in isolation is unlikely to represent the only mechanism of action of anti-TNFα antibodies, with the proviso that inadequate dosing of etanercept was not responsible for the lack in clinical benefit observed.

Binding of anti-TNFα antibodies to tmTNF also occurs and suppresses pro-inflammatory cytokines, cell activation and upregulation of adhesion molecules in different tmTNF transfected cell lines (Tracey, Klareskog et al. 2008), resulting in a down-regulation of inflammation. IFX, ADA and CZP are comparable with regards their tmTNF-antagonist activity when compared directly in vitro (Gramlick, Fossati et al. 2006).

Although all anti-TNFα antibodies bind to tmTNF, there is evidence for differential induction of cytokine suppression through reverse signaling (Scallon, Cai et al. 2002; Mitoma, Horiuchi et al. 2004; Mitoma, Horiuchi et al. 2005). This may be, in part, the result the phenomenon of ‘crosslinking’. IFX and ADA are bivalent antibodies that recognize each monomeric component of trimeric TNF (Scallon, Cai et al. 2002). Therefore, a single sTNF or tmTNF is capable of being bound by three different molecules of IFX or ADA. As each IFX or ADA molecule has two antigen-binding sites, a single antibody might bind different soluble and/or transmembrane TNF molecules (crosslinking). These qualities enable IFX and ADA to bind to tmTNF at much higher levels than etanercept (Scallon, Cai et al. 2002) or CZP.
Neutralisation of TNFα is clearly important in the clinical effect of anti-TNFα therapies in the treatment of CD. However, the different degrees of efficacy of different anti-TNFα drugs mean that this is not the only mechanism of action of anti-TNFα agents. Other biological effects on barrier function, lymphocyte apoptosis, mucosal angiogenesis and immunological mechanisms have also been demonstrated, as outlined below.

1.3.3.1 Barrier function

The gastrointestinal epithelial lining consists of a monolayer of columnar cells held together by circumferential intercellular junctions. It forms a selectively permeable barrier between the luminal contents and the body, preventing potentially harmful macromolecules and microorganisms exiting the intestinal lumen. The structural and functional properties of the epithelium regulate the quantity of antigenic material that reaches the epithelial surface (mucus secretion) and also control the antigenic exposure to M-cells and DC that sample antigenic material and provide signals to the mucosal immune system, thereby guaranteeing permanent immunosurveillence.

Tight junctions (TJs) are located at the apical end of the intercellular space and play a vital role in regulating paracellular movement of fluid and solutes (Turner, Rill et al. 1997). TJs are made up of many different proteins, including ZO-1, occludins and the claudins. Increased permeability of TJs is associated with the pathogenesis of CD (Gassler, Rohr et al. 2001). Patients with CD have increased intestinal permeability and reduced amounts of occludin in the diseased intestine (Poritz, Lynch et al. 1997). Therefore, in active disease, there is increased exposure of the mucosal innate and acquired immune system to pro-inflammatory molecules, and is a major factor in the development of mucosal inflammation.

TNFα and the intestinal epithelial barrier function are intimately related. In vitro models have demonstrated that TNFα influences barrier function by inducing
disassembly of TJs in epithelial cells (Bruewer, Luegering et al. 2003; Wang, Graham et al. 2005). TNFα may also act to increase barrier permeability by induction of epithelial apoptosis (Zeissig, Bojarski et al. 2004).

More recent work in murine models of colitis found that TNFα is involved in early TJ rearrangement and its effects are mediated through TNFR1. The study investigated the effects of both IFX and etanercept in this model and found them to be equally effective despite their clinical differences in efficacy in CD (Fries, Muja et al. 2008).

A study by Suenaert et al. examined intestinal permeability (by measurement of urinary 51Cr-EDTA excretion after oral intake) in refractory CD before and 4 weeks after a single infusion of IFX (5mg/kg). The authors found that following IFX infusion, there was a significant reduction in gut inflammation and a largely restored gut barrier (Suenaert, Bulteel et al. 2002). It remains unclear whether this observation simply reflects healing of the injured mucosa independently of the mechanism by which it was achieved.

Furthermore, epithelial apoptosis was up regulated in the colon of patients with active CD and was restored to normal in 10 of 11 patients, 2 weeks following IFX treatment (5mg/kg). Interestingly, the expression of TJs (occludin, claudin 1 and claudin 4) did not alter following IFX therapy and therefore did not contribute to this therapeutic effect (Zeissig, Bojarski et al. 2004).

Restoration of epithelial barrier integrity in CD by anti-TNFα drug treatment is likely to play a major role in the long-term anti-inflammatory therapy effect, and indeed such 'barrier healing' may represent the first step towards 'mucosal healing' that is seen in some patients receiving anti-TNFα therapy.
1.3.3.2 Apoptosis

Apoptosis is a distinct mode of cell death and represents a crucial regulatory mechanism to remove redundant cells in many physiological events. CD is characterised by mucosal T-cell proliferation that exceeds T-cell apoptosis (Ina, Itoh et al. 1999), resulting in an accumulation of activated lamina propria T lymphocytes.

Apoptosis is regulated by trimerisation of specific death receptors, including TNF-R1, Fas/APO-1, TRAIL-R1 and TRAIL-RI (Arnold, Brenner et al. 2006). This leads to the formation of a death-inducing signaling complex and proteolytic cleavage of procaspases, ultimately activating caspase-3, a key effector of apoptosis. An alternative pathway of apoptosis involves molecules released from the mitochondria. Two such proteins are Bax (pro-apoptotic) (Brady, Gil-Gomez et al. 1996) and Bcl-2 (anti-apoptotic) (Kroemer 1997). The intracellular ratio of Bax:Bcl-2 determines whether the cell will respond to apoptotic signals (Adams and Cory 1998). A reduced intracellular ratio of Bax:Bcl-2 correlates with resistance of lamina propria lymphocytes to Fas mediated apoptosis in CD in several studies (Boirivant, Marini et al. 1999; Ina, Itoh et al. 1999).

Lugering et al. used Annexin V staining by flow cytometry to demonstrate increased apoptosis of peripheral blood monocytes in patients with chronic active CD, 4 hours after being administered IFX (5mg/kg). IFX-induced apoptosis in monocytes was dependent on proteolytic activation of different members of the caspase family, such as the initiator caspase-8 and caspase-9, as well as the effector caspase-3 (Lugering, Schmidt et al. 2001). T-lymphocyte apoptosis results from a caspase-dependent phenomenon following IFX treatment (Di Sabatino, Ciccocioppo et al. 2004).

Ten Hove et al. demonstrated that IFX restores the inappropriate T-cell accumulation present in CD by induction of T-cell apoptosis in vivo (ten Hove, van Montfrans et al. 2002). Ten patients with steroid-refractory Crohn’s disease
were treated with IFX (5mg/kg) and a clinical response was achieved in 9/10 patients. The expression of activation markers, homing receptors, memory cells, Fas expression or Bax:Bcl-2 expression on peripheral blood T-lymphocytes did not change. However, there was a significant increase in CD3 and terminal dUTB nick end labeling (TUNEL) positive cells in colonic biopsies 24 hours following IFX administration. IFX-induced apoptosis, and an increase in the Bax:Bcl-2 ratio, of CD3/CD28 stimulated Jurkat cells, but not in unstimulated Jurkat cells. This suggests that IFX stimulates a rapid and specific increase in apoptosis of T lymphocytes in the gut mucosa.

A recent study isolated CD14+ macrophages and CD4+ T-cells from peripheral blood and lamina propria mononuclear cells in patients with IBD and healthy control subjects (Atreya, Zimmer et al. 2011). Immunohistochemistry and fluorescence-activated cell sorting techniques were used to assess cell surface markers and apoptosis. All the clinically effective anti-TNFα antibodies studied (IFX, ADA and CZP) resulted in a significant induction of T-cell apoptosis in IBD when lamina propria CD4+ T-cells expressing TNFR2 were cultured with mTNF+ CD14+ intestinal macrophages. In contrast, the anti-TNFα antibodies did not induce significant apoptosis in mono-cultured or co-cultured CD14+ and CD4+ cells from healthy control subjects. T-cell apoptosis was critically dependent on TNFR2 signaling and could be prevented by IL-6 signal transduction. The findings highlight the important role of the mTNF/TNFR2 pathway in anti-TNFα-induced apoptosis.

The clinical relevance of anti-TNFα-induced apoptosis of activated lamina propria T-lymphocytes in patients with active CD has been shown in a study by Van den Brande et al (Van den Brande, Koehler et al. 2007). This study used 99mTechnetium-annexin V single photon emission computer tomography (SPECT) to study the effect of anti-TNFα treatment on apoptosis in the intestine in patients receiving IFX therapy. They found that colonic uptake of 99mTc-annexin V correlated with the clinical benefit of anti-TNFα therapy in vivo, and therefore may be predictive of therapeutic success.
While these studies support the ‘apoptosis theory’ as a mechanism of action of anti-TNFα antibodies, there is conflicting data. Induction of apoptosis in monocytes and/or T-lymphocytes by IFX has been demonstrated in multiple in vitro and in vivo studies. ADA is also effective in inducing apoptosis of human monocytes (Shen, Assche et al. 2005), and both of these anti-TNFα therapies are effective in CD. However CZP, despite its clinical efficacy in CD, does not induce apoptosis (Nesbitt, Fossati et al. 2007). Etanercept does not induce apoptosis and is not effective in the treatment of CD (Van den Brande, Koehler et al. 2007); however Onercept (a soluble p55 TNF receptor) does induce apoptosis but, again, is not effective in the treatment of CD (Lugering, Lebiedz et al. 2006). It is therefore likely that apoptosis, although an important mechanism of action of anti-TNFα antibodies, is not the only mechanism by which these treatments exert their effect in CD.

1.3.3.3 Mucosal angiogenesis

Angiogenesis is the formation of new capillaries from pre-existing blood vessels, and is a fundamental component of complex biological processes such as chronic inflammation (Szekanecz, Besenyei et al. 2010), including contributing to the pathogenesis of IBD (Danese, Sans et al. 2006). Vascular endothelial growth factor-A (VEGF-A) is the predominant cytokine regulating angiogenesis in IBD (and other chronic inflammatory disorders). Overexpression of VEGF-A occurs in patients with IBD, and neutralisation of VEGF-A correlates with the inhibition of angiogenesis (Scaldaferrari, Vetrano et al. 2009).

A recent study by Rutella et al. examined mucosal angiogenesis by staining endoscopic biopsies with CD31 and Ki-67 in CD patients prior to IFX treatment and again, following 54 weeks of IFX therapy (Rutella, Fiorino et al. 2011). VEGF-A release was also studied by enzyme-linked immunosorbant assay (ELISA). In vitro studies were performed in cultures of human intestinal fibroblasts stimulated with TNFα in the presence or absence of IFX. The authors found that the administration of IFX down regulates mucosal angiogenesis in patients with
CD and restrains the production of VEGF-A by mucosal fibroblasts. These phenomena did not occur in patients who showed no clinical response to IFX treatment. The results of this study indicate that IFX ameliorates inflammation-driven angiogenesis in the gut mucosa in patients with CD.

1.3.3.4 Cytokine production

Cells of the innate immune system, such as macrophages, monocytes and DC, secrete several pro-inflammatory cytokines in response to a danger signal, for example an infectious agent. The resulting cytokine milieu directs the development of adaptive immunity mediated by T- and B- lymphocytes (Papadakis and Targan 2000). As previously discussed, the immune response in CD is a Th1/Th17 response, with the production of the stimulatory cytokines IFN\(\gamma\), IL-2, IL-12, IL-17 and TNF\(\alpha\).

IFX acts on multiple constituents of the cytokine network in order to exert its action. To examine the effect of IFX on cytokine production, Ringheanu et al. isolated monocytes from healthy individuals and pre-treated the cells \textit{in vitro} with IFX before stimulation with LPS or staphylococcal enterotoxin A (SEA) to assess gene expression for pro-inflammatory cytokines (TNF-alpha, IL-1\(\beta\), IL-6, and IL-8) by reverse transcription polymerase chain reaction (Ringheanu, Daum et al. 2004). The effect of \textit{in vivo} IFX treatment of monocytes was similarly determined by comparing the responses of monocytes from CD patients before and immediately after IFX infusion. The authors found that, following pre-treatment with IFX \textit{in vitro}, monocytes produced significantly less TNF\(\alpha\) after stimulation with the bacterial products LPS and SEA. Peripheral blood mononuclear cells isolated from patients 1 hour following IFX infusion had fewer copies of IL-1\(\beta\), IL-6, IL-8 and TNF\(\alpha\) following stimulation with LPS and SEA than prior to the IFX infusion.
Other *in vitro* studies have supported these findings by showing complete suppression of TNFα and IL-1β production by monocytes by IFX, ADA and CZP, but only partial suppression with etanercept (Nesbitt, Fossati et al. 2006).

Agnholt et al. cultured *in vitro* colonic T-cells from 25 patients with Crohn's disease and 10 healthy controls, using medium supplemented with IL-2 and IL-4 (Agnholt and Kaltoft 2001). T-cells from CD patients produced significantly higher levels of IFNγ and TNFα than T-cells from healthy controls. When cultured in the presence of IFX, colonic T-cells from CD patients decrease their expression of IFNγ, demonstrating the effect of IFX on the secretion of pro-inflammatory cytokines by intestinal T-cells.

Mizutani et al. have further demonstrated the effect of IFX on cytokine levels in patients with CD *in vivo*. In this study, 37 patients with active CD were enrolled and administered IFX 5mg/kg intravenously. A Bio-Plex suspension array system was used before treatment and 4 and 8 weeks after IFX treatment to quantify serum levels of 17 cytokines. They found serum levels of IL-6, IL-7, IL-8 and MIP-1β were statistically significantly decreased in CD patients following IFX treatment compared to before treatment (Mizutani, Akasaka et al. 2011).

The evidence above suggests that therapeutic blockade of TNFα may lead to an interruption in the cascade of inflammatory cytokines and ultimately reduce the inflammatory response.

**1.3.3.5 CD40/CD40L**

Naïve T-cells require contact with activated dendritic cells in order to be primed and cause an inflammatory response. Intestinal DCs act as professional antigen presenting cells (APCs) by sampling antigenic material within the gut lumen, DCs sense microbes by a series of surface receptors (including TLRs), which recognise structural elements displayed on the surface of microbes (Kadowaki, Ho et al. 2001). After interaction with the antigenic material (or other
maturation stimuli such as cytokines), immature DCs migrate to the draining lymphoid tissue under the influence of alterations in surface expression of chemokine receptors (Banchereau and Steinman 1998). During this process, DCs down-regulate their antigen-acquisition machinery and up-regulate cell surface expression of molecules required for the activation of naïve T-cells. At the time of antigen presentation, DCs deliver 3 signals to the T-cell. The first signal is the antigen itself, expressed on the surface of the DC by MHC class II molecules (such as HLA DR). The second signal is provided by surface expression of co-stimulatory molecules, such as CD40, CD80 and CD86, which are recognised by CD40L and CD28 respectively on T-cells. The third signal refers to the cytokine milieu (produced by DCs) in which antigen presentation takes place. The quantity and quality of the 3 signals determines the ultimate T-cell response in terms of immune response or immune tolerance.

The CD40-CD40L interaction is one of the most effective APC-activating signals. In patients with active CD, CD40 is up regulated in several cell types including microvascular endothelial cells resulting in vascular inflammation. In the same patients, CD40L expression is up regulated by lamina propria T-cells and platelets, with the soluble form (sCD40L) being increased in the circulation of IBD patients (Tilg, Moschen et al. 2007). These observations indicate that the CD40/CD40L pathway plays a key pathogenic role in intestinal inflammation (Danese, Sans et al. 2004).

Danese et al. evaluated 18 patients with CD and found that IFX treatment decreases serum levels of sCD40L and CD40L expression on peripheral T-cells and platelets (Danese, Sans et al. 2006). They also demonstrated that following IFX treatment, there was reduced colonic tissue expression of CD40 and vascular cell adhesion molecule-1 (VCAM-1). In vitro IFX prevented TNFα-induced CD40 and VCAM-1 expression by human intestinal microvascular endothelial cells (HIMEC) and reduced T-cell surface CD40L expression and sCD40L release. In addition, IFX decreased T-cell-induced VCAM-1 expression in HIMEC by down-regulating CD40L in T-cells and promoting T-cell apoptosis.
These findings have been mirrored when examining CD40 expression on DCs in CD. Hart et al. demonstrated significantly higher levels of CD40 expression on intestinal DCs taken from inflamed mucosa in CD patients when compared to non-inflamed CD tissue or healthy controls. In 7 patients with CD receiving treatment with IFX, levels of CD40 expression on colonic DCs was evaluated before and after infusion. There was a significant reduction in CD40 expression on DCs after treatment with IFX, which was irrespective of resolution of inflammation (Hart, Al-Hassi et al. 2005).

1.3.3.6 Cell-mediated and complement-dependent cytotoxicity

TNFα antagonists also bind to tmTNF-bearing cells and therefore have the potential to induce cell cytotoxicity by Fc-dependent mechanisms including; i) antibody-dependent cell-mediated cytotoxicity (ADCC) and/or ii) complement-dependent cytotoxicity (CDC).

ADCC is a mechanism of cell-mediated immune defense whereby an effector cell actively lyses a target cell that has been bound by specific antibodies. In CDC, the pathway is initiated by binding of C1q to the C_{II2} domain of the Fc region of cell-bound antibodies or Fc-fusion proteins. Cross-linking of these molecules can result in activation of the complement cascade and the formation of the membrane attack complex, pore formation and cell lysis.

Only a few in vitro studies exist to show the effects of anti-TNFα agents on CDC and ADCC potential. The Fc regions of IFX, ADA and Etanercept are all of the IgG1 isotype and therefore capable of mediating CDC and ADCC. Although IFX, ADA, CZP and Etanercept all bound to a tmTNF transfected cell line, IFX and ADA were more active than Etanercept in mediating ADCC and CDC. CZP was inactive, as would be expected in a molecule that lacks an Fc region (Scallon, Cai et al. 2002; Gramlick, Fossati et al. 2006).
However, there are no in vivo studies to show the effect of these agents on ADCC and CDC, and therefore whether anti-TNFα therapies mediate these effects in patients is unknown.

1.3.3.7 Regulatory T-cells

Several studies have demonstrated data on the interaction of IFX and putative Tregs in rheumatoid arthritis. TNFα inhibits the suppressor function of Treg cells through the TNFR2 receptor. Treatment with IFX restored the capacity of Tregs to inhibit cytokine production and to convey a suppressive phenotype to ‘conventional’ T-cells. Furthermore, IFX treatment led to a significant rise in the number of T-cells with a regulatory phenotype in patients with rheumatoid arthritis responding to treatment, correlating with a fall in C-reactive protein ( Ehrenstein, Evans et al. 2004).

Nadkarni et al. demonstrated that IFX therapy leads to the generation of a specific population of regulatory T cells (Nadkarni, Mauri et al. 2007). This population of T-cell exerts its suppressor function through the anti-inflammatory cytokines TGFβ and IL-10. These cells lack expression of CD62L, thereby distinguishing them from natural Treg cells present in healthy individuals and patients with active rheumatoid arthritis.

A recent study examined the immunosuppressive effects of different anti-TNFα therapies on activated T-cells in vitro (Vos, Wildenberg et al. 2012). The authors found that for an anti-TNFα therapy to inhibit T-cell proliferation, the compound needed to bind to tmTNF on activated T-cells. They demonstrated that IFX and ADA bind efficiently to tmTNF, with intermediate binding of CZP and low binding of etanercept and IgG control. Furthermore, the compound must possess an Fc region to interact with the Fc receptor on APC. The result of this binding induces formation of a distinct macrophage subset with an immunosuppressive phenotype. These macrophages inhibit the proliferation of activated T-cells,
produce anti-inflammatory cytokines and express the regulatory macrophage marker CD206.

Figure 10. The mechanisms of action of anti-TNFα therapies in Crohn’s disease.

A) General mechanisms of action of anti-TNFα therapy, including; 1 neutralisation of soluble and transmembrane TNFα; 2 restoration of epithelial tight junction integrity; 3 inhibition of mucosal angiogenesis in gut mucosa; 4 induction of T-cell apoptosis.

B) Immunologically-mediated mechanisms of action of anti-TNFα therapy, including; 5 apoptosis of tmTNF-expressing cells (cell and/or complement-mediated); 6 down regulation of co-stimulatory molecules on DCs (CD40); 7 reduced production of pro-inflammatory cytokines; 8 induction of Treg population.
1.3.4 **Anti-TNFα therapy-induced paradoxical inflammation**

Although proven to be clinically efficacious in the treatment of IBD, anti-TNFα agents are associated with rare side effects, including opportunistic infections (Toruner, Loftus et al. 2008) and lymphoproliferative diseases (Siegel, Marden et al. 2009). The phenomenon of paradoxical inflammation (PI) has recently been described in patients with various chronic inflammatory diseases, following treatment with an anti-TNFα agent. It is defined as the development of inflammatory lesions in patients with immune mediated inflammatory disease after initiation of treatment with anti-TNFα agents, normally used to treat them (Chowers, Sturm et al. 2010). For example, a patient with CD can paradoxically experience the onset of another immune mediated inflammatory process (eg psoriasis), suggesting the ability of anti-TNFα agents to promote inflammation at remote sites in some patients.

The development of inflammatory skin disorders following anti-TNFα therapy for IBD is an emerging problem. A recent prospective study was performed from January 2006 to January 2010 of all patients treated with an anti-TNFα therapy and presenting with eczematiform or psoriasiform lesions to a centre in Lille, France (Rahier, Buche et al. 2010). Over the study period, 562 patients were initiated on anti-TNFα treatment (341 IFX, 221 ADA) and 28 patients (5%) developed an inflammatory skin lesion (3% eczematiform and 2% psoriasiform). Other studies have found the incidence rate of paradoxical skin lesions to be higher; Fidder et al. 9% of 743 patients with IBD treated with IFX (Fidder, Schnitzler et al. 2009); Esmailzadeh et al. 16% patients receiving IFX for various inflammatory disorders including 59 patients with IBD (Esmailzadeh, Yousefi et al. 2009).

There is also evidence of new onset IBD in patients with other immune mediated inflammatory diseases receiving anti-TNFα therapy. Toussirot et al. published a French nationwide series in patients with inflammatory rheumatic disease who have developed IBD following anti-TNFα therapy (Toussirot, Houvenagel et al.)
During the two-year study period, 16 rheumatology patients developed IBD with a mean onset of 29.3 ± 20.1 months. Fourteen of these patients had received etanercept and 2 had received IFX. According to endoscopic and histological findings, the IBD was classified as CD in 8 cases, Crohn’s-like disease in 6, indeterminate in one case and definite ulcerative colitis in one case. In all cases, the anti-TNFα agent was discontinued and replaced by another monoclonal anti-TNFα agent without any evidence of recurrent IBD.

The mechanism(s) responsible for PI in patients treated with anti-TNFα therapy are unknown. Evidence suggests that all the anti-TNFα agents have the potential to induce PI, thereby indicating that a class effect may be responsible.

One hypothesis is that some patients have a genetic predisposition to developing PI (such as NOD2/CARD15 gene variants), and the introduction of the anti-TNFα agent may modify the cytokine balance leading to circumstances where PI can develop (Toussirot, Houvenagel et al. 2011).

Another possibility is that some inflammatory pathways in CD may be TNFα-independent, and subsequently are up regulated during TNFα blockade. For example, IFNα production by pDCs is down regulated by the presence of TNFα, and vice versa (Palucka, Blanck et al. 2005). pDCs infiltrate the skin of psoriatic patients and become activated to produce IFNα early in the disease process (Nestle, Conrad et al. 2005). Hence, the administration of anti-TNFα agents has the effect of increasing the production of IFNα (Schmitt, Zhang et al. 2008). This has been shown by De Gannes et al. who found increased levels of IFNα in the dermal vasculature and perivascular lymphocytic infiltrate of lesional skin of patients with TNFα antagonist therapy (de Gannes, Ghoreishi et al. 2007).

Murine models of joint disease have shown TNFα blockade to increase the populations of Th1 and Th17 cells in the periphery, while inhibiting their accumulation in the joints (Notley, Inglis et al. 2008). This finding of increased Th1 and Th17 cells in organs other than those that are inflamed further supports
the suggestion that TNFα blockade is up-regulating other pro-inflammatory cytokines, and this may be partly responsible for the phenomenon of PI.

Altered lymphocyte homing profiles may also play a role in PI, with some evidence suggesting this may occur through the expression of chemokine receptor CXCR3 ligands (Seneschal, Milpied et al. 2009). Other homing markers on DCs and/or T-cells may also be implicated, as indicated by work described in this thesis.

PI remains a relatively rare phenomenon but is now a recognised adverse event of anti-TNFα therapy, and it appears that the broad impact on the immune system of anti-TNFα agents exceeds the blockade of TNFα itself (Chowers, Sturm et al. 2010). The mechanism of PI is not fully understood, but it seems likely that a number of TNFα-independent inflammatory pathways may be responsible.
1.4 Hypothesis and Aims

Hypothesis
We hypothesise that aberrant expression of homing markers on DCs drives inflammation in CD, and that these homing markers give rise to different clinical phenotypes of CD and are altered by the presence of a clinically efficacious anti-TNFα therapy in vitro.

Specific aims

Chapter 3
To characterise the phenotype of circulating DCs in patients with CD in whom inflammation is confined to specific anatomical locations. Study groups include:

- Active small bowel CD
- Active colonic CD
- Extra-intestinal manifestations of CD, ie skin manifestations
- Inactive ileocolonic CD
- Healthy controls

Chapter 4
To examine the in vitro effects of anti-TNFα therapy on homing marker expression of peripheral blood mononuclear cell (PBMCs).

Chapter 5
To examine the in vitro effects of anti-TNFα therapy on blood-enriched DCs or low density cells (LDCs). The following parameters will be investigated:

- LDC homing marker expression
- LDC activation and maturation marker expression
- Ongoing LDC intracellular cytokine production
- Cell-free supernatant cytokine profile
Chapter 6
To examine the effects of anti-TNFα-treated LDCs on subsequent T-cell responses in both patients with active CD and healthy controls, characterising the phenotype of stimulated T-cells through:

- T-cell homing marker expression
- T-cell primed cytokine profiles
Chapter 2

Materials and Methods
2.1 Ethical Approval

Ethical approval has been obtained under the title ‘Prospective study of immunological and microbiological factors in inflammatory bowel disease’ by Brent Research and Ethics Committee, for the use of human blood.

REC reference: 08/H0717/24
Protocol number: cro1043.

All patients and healthy volunteers recruited for research were given information sheets and provided written consent for samples to be taken.

Local NHS Research and Development approval and sponsorship to conduct the study was secured from Imperial College London and the North West London Hospitals NHS Trust.

2.2 Materials

2.2.1 Human blood samples

Venesection was performed on patients and healthy volunteers according to the study protocol. Volumes of blood taken ranged between 20ml and 50ml per venesection, depending on the experiment design. Blood samples were collected in 10ml lithium heparin vacutainer tubes (BD Biosciences) and processed within 30 minutes of venesection in the Antigen Presentation Research Group (APRG) laboratory at Imperial College London.

The diagnosis of patients with CD was made using clinical parameters, radiological, endoscopic and histological criteria. Demographical information was obtained and full medication histories recorded. Disease activity was assessed using the Harvey Bradshaw Index for CD (see section 3.3.1).
2.2.2 Patient inclusion and exclusion criteria

Strict inclusion and exclusion criteria were used during all patient recruitment throughout the research project, to ensure the accuracy of data obtained. The following criteria were applied to patient recruitment:

Inclusion criteria:

- Age ≥ 18 years
- Able to provide informed, written consent
- Histology compatible with a diagnosis of CD
- Endoscopic and radiological study within 6 months of recruitment
- No medication or dose change within last 3 months

Exclusion criteria:

- Local or systemic sepsis
- Co-existing inflammatory condition (eg tuberculosis, sarcoid, diverticulitis)
- Perianal CD
- Previous intestinal resection surgery
- Polyposis syndrome
- Current or any past history of cancer
- Biological, corticosteroid or methotrexate therapy within 3 months

2.2.3 Culture medium

Dutch modification RPMI-1640 medium (Sigma Aldrich, UK) was used for cell dilution and suspension during whole blood processing. Dutch modified RPMI 1640 medium incorporates both HEPES buffer for partial pH control and bicarbonate for gas exchange.
2.2.4 Complete medium

For washing and culture of both peripheral blood mononuclear cells (PBMCs) and DC-enriched low density cells (LDCs), cells were suspended in RPMI 1640 Dutch modification, supplemented with 2mM L-glutamine, 100μg/ml streptomycin, 100units/ml penicillin, 50μg/ml gentamicin and 10% foetal calf serum (FCS, Harlan Laboratories).

2.2.5 Buffers

2.2.5.1 FACS Buffer

Cells were washed and re-suspended in FACS buffer in preparation for antibody labeling and analysis by flow cytometry. FACS buffer was prepared from phosphate buffered saline (PBS), supplemented with FCS (2%), sodium azide (0.02%, Sigma, UK) and EDTA (1mM, Sigma, UK).

2.2.5.2 MiniMACs buffer

Sterile PBS supplemented with 0.5% bovine serum albumin (BSA) and 2mM EDTA was used for T-cell separation using negative magnetic cell separation columns (see section 2.3.4).

2.2.6 Reagents

2.2.6.1 5-carboxyfluorescein-diacetate-succinimidyl ester (CFSE)

CFSE (Invitrogen Ltd, UK) is a fluorescent cell staining dye and was used to fluorescently label human T-cells prior to co-culture with allogeneic DC. CFSE is incorporated into cells and is used to identify cell proliferation assays due to
progressive halving of CFSE fluorescence within daughter cells following cell division.

2.2.6.2 Ficoll-Plaque

Ficoll-Plaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) is a polysaccharide dissolved in an aqueous solution and creates a density gradient at centrifugation. This gradient was used to isolate PBMCs from fresh venous blood samples.

2.2.6.3 Flow-count™ fluorospheres

Flow-count™ fluorospheres (Beckman Coulter, Buckinghamshire, UK) with a known density (depending on the batch) were used as a reference during flow cytometry analysis of human cells for calculation of absolute cell numbers.

2.2.6.4 Foetal calf serum (FCS)

FCS (TCS Cellworks, Buckingham, UK) was added to cell culture media to provide positive growth factors for cells. FCS also was used during monoclonal antibody labeling to block non-specific binding.

2.2.6.5 Leucoperm A

Leucoperm A (Serotec, UK) was used as a fixative reagent and is used for intracellular cytokine staining of LDCs. It contains paraformaldehyde.

2.2.6.6 Leucoperm B

Leucoperm B (Serotec, UK) is a cell permeabilisation reagent used to assess the intracellular cytokine staining of LDCs by flow cytometry.
2.2.6.7 Monensin

Monensin (Sigma, UK) was prepared in ethanol at a stock concentration of 3mM and stored at -70°C. Monensin was further diluted and added to cell cultures to give a final concentration of 3μM. Monensin is an ionophore originating from *Streptomyces cinnamomensis* that disrupts protein transportation from the Golgi apparatus leading to the trapping of newly synthesised protein within the cell cytoplasm (Mollenhauer, Morre et al. 1990).

2.2.6.8 Nycoprep

Nycoprep (Axis-Shield, Oslo, Norway) was used to create a density gradient to separate LDCs from the non-adherent fraction of PBMCs.

2.2.6.9 Paraformaldehyde (PFA)

Paraformaldehyde (BDH chemicals, UK) was dissolved in saline (0.85%) through slow heating at 60°C at pH 7.0-7.4 to make a 1% stock solution which was added directly to antibody-labelled cells as a fixative reagent.

2.2.6.10 Trypan blue

Trypan blue (Sigma, Poole, UK) is a diazo dye used to selectively colour dead cells and tissue, visible by optic microscopy. Live cells with intact membranes are not coloured by the dye and therefore this allowed counting of live cells. Trypan blue was used at a working concentration of 0.4%.
2.2.7 **Infliximab**

Infliximab (MSD Pharmaceuticals, Hertfordshire, UK) was used in all relevant experiments as the study anti-TNFα drug. The drug was stored in powder form at 4°C. When required, it was re-constituted from powder with sterile water to a concentration of 10mg/ml (10ml total volume) as per manufacturer’s instructions.

Stock was stored at 4°C and used within eight hours of reconstitution. The stock was further diluted with complete medium to create the study concentrations (1μg/ml, 10μg/ml and 100μg/ml).

2.2.8 **Antibodies**

Antibodies with specificities and fluorochrome-conjugated labels were used. Matched isotype-controls were derived from the same manufacturer. Table 4 lists the antibodies and isotype-controls used.
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<td>IL-15</td>
<td>151303</td>
<td>PE</td>
<td>Mse IgG2b</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
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<td>35409</td>
<td>PE</td>
<td>Mse IgG1</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
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<td>JES3-19F1</td>
<td>APC</td>
<td>Rat IgG1</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>IL-12</td>
<td>C11.5</td>
<td>APC</td>
<td>Mse IgG1</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>

*Table 4. Fluorochrome-conjugated antibodies including isotype, clone, conjugated fluorochrome and manufacturer*
2.2.9 Immunomagnetic beads and cell separation columns

Anti-CD14, anti-CD19 and anti-HLA DR immunomagnetic beads (Miltenyi Biotech, Bisley, UK) were used in conjunction with LD magnetic columns and MidiMACs apparatus (Miltenyi Biotech, Bisley, UK) for T-cell separation prior to mixed leukocyte reaction, as described in section 2.3.4.
2.3 Methods

2.3.1 Isolation of PBMCs

PBMCs were obtained by centrifugation of human peripheral venous blood over Ficoll Plaque at 650g for 30min at room temperature and harvesting the buffy coat layer from the interface. As previously described, Ficoll Plaque separates whole blood into a top layer of plasma, followed by a layer of PBMCs and a bottom fraction of polymorphonuclear cells (eg neutrophils and eosinophils) and erythrocytes. PBMCs were washed twice in culture medium by centrifugation at 650g for 5min.

PBMCs were used for direct phenotypic analysis, culture in the presence of IFX, dendritic cell enrichment or for T-cell enrichment.

2.3.2 Enrichment of dendritic cells

To obtain blood-enriched DCs or low density cells (LDCs), PBMCs were suspended in complete medium and incubated for 24 hours at 37°C, 5% CO2 and high humidity in T75 tissue culture flasks (35-50 million PBMCs/flask). Non-adherent cells from overnight cultures were centrifuged at 600g for 15min over Nycoprep to obtain a density gradient. LDCs were recovered from the interface and washed twice in complete medium.

2.3.3 Viable cell counts

Viable cell counts were performed by adding known volumes of cells, medium and trypan blue dye to a mixing well at the ratio 1:3:1 (cells:medium:dye). Viable cells exclude the blue dye from their cytoplasm and are therefore easily recognisable at light microscopy. The solution was pipetted onto a counting slide
and cell count was performed under a light microscope. The numbers of cells/ml solution was calculated (number of viable cells) counted x $10^4$ (number of cells/ml) x 5 (corrects dilution of mixing cell) x total volume of cell suspension (ml).

2.3.4 **Mixed leucocyte reaction**

The stimulatory capacity of blood LDCs was assessed in an allogeneic mixed leukocyte reaction (MLR). The following steps were performed to carry out an MLR:

**Step 1. T-cell enrichment**

PBMCs from healthy volunteers were washed twice in miniMACs buffer before being incubated with anti-CD14, anti-HLA DR and anti-CD19 immunomagnetic beads (30μl each) on ice for 20 minutes following manufacturer's instructions. Cells were then washed twice with miniMACs buffer. During this time, the magnetic cell sorting system (VarioMACSTM Separation System, Miltenyi Biotech, Bisley, UK) was setup and the columns washed through twice with MiniMACS buffer. Cells were kept at 4°C throughout the entire T-cell enrichment process by use of ice and pre-cooled centrifuges.

Cells that had been incubated with the immunomagnetic beads were added directly into the column, followed by 4ml of miniMACS buffer at 4°C. The resultant solution is depleted of CD19+, CD14+ and HLA DR+ cells. In order to increase T-cell purity, a new depletion column was setup and the T-cell solution was run through the column again.

The final solution has a CD3+ T-cell purity of >98% (Bernardo, Al-Hassi et al. 2012). The cells were washed twice in cold PBS and re-suspended in 3ml PBS.
**Step 2. T-cell CFSE labeling**

CFSE (5μL) was suspended in 10ml PBS (tube CFSE1). 3ml of solution from CFSE1 was then diluted in a further 3ml PBS (tube CFSE2). This was then added to the enriched T-cell suspension and gently mixed. The solution was then left for exactly 3 minutes, with one further gentle mix performed at 1½ mins. The reaction was quenched by addition of 6ml of sterile FCS. The CFSE-labeled T-cells were then washed twice by adding complete medium and centrifuging (600g, 5min). The supernatant was discarded and the pellet re-suspended in 1ml of complete medium. A trypan blue cell count was then performed to determine the T-cell concentration. Once the cell count was known, the solution was further diluted with complete medium, to give a final concentration of 4 million T-cells/ml.

**Step 3. MLR and proliferation assays**

LDCs obtained from the blood were cultured with various concentrations of IFX in 5ml polystyrene round-bottom (FACS) tubes (BD Falcon) in 1ml complete medium at a concentration of 0.5 million cells/ml. Three different IFX concentrations were used (as previously described) and LDCs in basal conditions served as an internal control. Cells were washed twice in complete medium (600g, 5 mins) to ensure any remnants of the active drug had been removed prior to addition of T-cells.

CFSE-labelled T-cell solution (100μl, 4x10⁵ cells) were placed in separate chambers in a 96-well round bottom plate. The pre-treated LDCs were then added to each T-cell aliquot at known concentrations (0% or no LDCs, 1% or 4000 LDCs, 2% or 8000 LDCs and 3% or 12,000 LDCs). These test conditions were performed for each concentration of IFX being studied.

Cells were then incubated for 5 days at 37°C, 5% CO₂ and high humidity. Cells were recovered at the end of this time and T-cell proliferation was quantified by way of CFSE dilution. Homing marker expression and intracellular cytokine profile was measured by antibody staining and flow cytometry.
2.3.5 Antibody labeling

For phenotypic analysis, cells were washed twice in FACS buffer and divided into 100µL aliquots in FACS tubes (minimum of 5x10^4 cells per FACS tube). 15µL of FCS was added to each tube to reduce non-specific antibody binding. Cells were labeled with antibodies (5 or 10µL of antibody as per manufacturer instructions) on ice and left in the dark for 20min (a method pre-determined in the laboratory to optimise cell antibody labeling).

After 20 minutes, cells were washed twice in FACS buffer to remove excess fluorochrome. Flow count fluorospheres (20µL) were added to appropriate tubes. Finally, cells were fixed in 1% PFA, and stored at 4°c prior to acquisition on the flow cytometer. Acquisition was performed within 48 hours of cell fixation.

2.3.6 Conjugated antibody labeling

In the absence of a directly conjugated antibody, secondary labeling was performed. To study the expression of the skin-homing marker, CLA, on T-cells, the cells were incubated with a monoclonal antibody, anti-CLA, conjugated with Biotin (CLA-biotin). Cells were then washed and re-incubated with the APC fluorochrome conjugated to Strepavidin (Strepavidin-APC). Finally, cells were washed twice in FACS buffer and fixed in 1% PFA as described in the previous section prior to acquisition.

2.3.7 Intracellular cytokine staining

LDCs (0.5x10^6 cells/ml) were cultured in complete medium with 7.5µL of monensin or an additional 7.5µL of complete medium (controls) for 4 hours at 37°C, 5% CO₂, high humidity. Cells were then washed in FACS buffer and the cell
pellet re-suspended in 100μL Leucoperm A fixative reagent and incubated at room temperature for 30min. The fixed cells were then washed in FACS buffer and re-suspended in 100μL Leucoperm B reagent which permeabilises the cells. The cell suspension was then incubated with 5μL anti-cytokine antibody for 30 minutes at room temperature in the dark. The labeled cells were then washed twice in FACS buffer and fixed in 300μL 1% PFA prior to acquisition.

2.3.8 Multiplex

BD Cytometric Bead Array (CBA). Human Th1/Th2/Th17 Cytokine Kit. Catalogue no 560484
Includes: IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ, IL-17A

The BD CBA Human Th1/Th2/Th17 Cytokine Kit uses bead array technology to simultaneously detect multiple cytokine proteins in research samples. Seven bead populations with distinct fluorescence intensities had been coated with capture antibodies specific for their specific cytokine (IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ and IL-17a). The seven bead populations are then mixed together to form a bead array, which is analysed in the APC channel of the flow cytometer.

During the assay procedure, the cytokine capture beads were mixed with the recombinant standards and research samples and incubated with PE-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex reveals the concentration of that cytokine (figure 11).

Detection limits were calculated by taking the mean + 2 standard deviations of the blank (control) and plotting this onto the standard curve for each cytokine. Results falling below this value were given the detection limit as their result.
Figure 11. CBA Human Th1/Th2/Th17 Cytokine Kit. Graph showing the different cytokine peaks taken directly from the flow cytometer. The Y axis identifies the different cytokines and the X axis quantifies their relative concentration.

2.3.9 Flow cytometry

Flow cytometry allows simultaneous multiparametric analysis of the physical characteristics of cells, as they pass through one or more focused light and laser beams in suspension. As the cells pass through the light and laser beams, they disrupt and scatter light in two different planes. This degree of scatter correlates with the cell’s morphology with forward scatter (FSC) giving a representation of the cell’s size, and side scatter (SSC) correlating with granularity. This allows cell populations to be identified based on their physical characteristics (figure 12).
Monoclonal antibodies conjugated to fluorescent dyes, which are conjugated to cells, can be detected by colour-specific detectors (channels) of the flow cytometer. Each fluorochrome emits light at a different wavelength when excited by the laser beam (table 5). The machine registers the fluorescence generated as cells pass through it, enabling analysis of the phenotypic properties of cells. This method of analysis is widely used for:

- Analysing expression of cell surface and intracellular molecules
- Defining different cell types in heterogenous cell populations
- Assessing purity of isolated subpopulations
- Analysing cell volume, size and granularity

Figure 12. Identification of cell populations by flow cytometry. Histogram to identify cell populations by way of physical characteristics of cell granularity (SSC) and cell size (FSC).
<table>
<thead>
<tr>
<th>Channel</th>
<th>Name</th>
<th>Excitation laser line (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Colour</th>
</tr>
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<tbody>
<tr>
<td>FL1</td>
<td>FITC</td>
<td>488</td>
<td>519</td>
<td>Green</td>
</tr>
<tr>
<td>FL2</td>
<td>PE</td>
<td>488</td>
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<td>Yellow</td>
</tr>
<tr>
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<td>PE-Cy5</td>
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<td>667</td>
<td>Red</td>
</tr>
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<td>FL4</td>
<td>APC</td>
<td>635</td>
<td>660</td>
<td>Red</td>
</tr>
</tbody>
</table>

*Table 5. Fluorochromes used in flow cytometry in this study. Monoclonal antibodies are conjugated to fluorochromes to allow for detection at flow cytometry. When the cytometer laser is shone at the fluorochrome, a specific wavelength of light is emitted which is recorded by the machine.*

Two flow cytometry machines were used during this project. Data contained in results chapters 3 and 4 of this thesis were analysed using a 4-colour FACSCalibur flow cytometer (BD Biosciences). The cytometer is capable of detecting the fluorochrome dyes Fluorescein (FITC), Phycoerythrin (PE), PE-Cy5 and Allophycocyanin (APC), corresponding to wavelengths FL1, FL2, FL3 and FL4 respectively (table 5).

Data contained in results chapters 5 and 6 of this thesis were analysed using a 6-colour FACSCantoII flow cytometer (BDBiosciences), which has two further channels (PE-Cy7 and APC-Cy7) in addition to the four channels above. These additional two channels were not used to obtain data during this project.

**2.3.9.1 Compensation**

DCs only constitute a small proportion of total leucocytes from peripheral blood and cells from different samples emit different levels of innate/background autofluorescence. Therefore, compensation was carried out separately of each individual experiment.
CellQuest software (BD Biosciences) was used for partial online compensation prior to cell acquisition, and generation of listmode data files. Compensation was completed and listmode data files analysed offline using WinList™ software (Verity Software House, Maine, US).

In order to perform partial online compensation, 5 compensation tubes are made, each containing PBMCs in FACS buffer suspension. One tube is left unlabelled and the remaining 4 are labelled with a fluorochrome conjugated antibody to either CD3+ or CD8+ (one tube for each fluorochrome; FITC, PE, PECy5 and APC). The unlabelled PBMC compensation tube was initially used to set the amperes and voltages on the machine, based on the cell’s background or autofluorescence. The FSC:SSC plot was adjusted using the FSC gain and range and the SSC voltage to place the cell population of interest in the centre of the FSC:SSC plot and therefore eliminate most of the fine subcellular debris. The cell suspensions, labelled with each fluorochrome were then analysed and compensation adjusted accordingly. Samples were then acquired and saved as listmode data files.

Compensation was completed offline using the compensation toolbox on the WinList™ software program by identifying lymphocyte populations labelled with antibodies specific for T-cells. Each individual experiment has its own unique compensation settings, saved as compensation files.

2.3.9.2 Identification of cell populations

PBMCs are defined as any blood cell containing a round nucleus and includes several cell types, including lymphocytes, monocytes, DC, macrophages etc.

Cell populations were identified by expression of their specific antibody within a gated leukocyte population (T-cells CD3+ve; B-cells CD19+ve and monocytes CD14+ve). The example below demonstrates this method to identify T-cells (figure 13).
Figure 13. Identification of T-cell population in PBMCs. A) Light scatter plot showing region R1 which contains all lymphocytes. B) One-parameter histogram identifying cells in region R1 with positive staining for CD3, gated in region R2.

Unlike most other cell types, there is not a single marker for DCs that easily allows for their identification. Our laboratory identifies DCs by a double gating strategy. Initially a light scatter plot is performed of all PBMCs and a gate is drawn around all living cells, thereby excluding erythrocytes, platelets and cell debris (figure 14A). A second two-way histogram is then created, including events from within the viable cell gate, to identify cells positive for HLA DR expression, but negative for lineage cocktail (a cocktail of other non-DC cell lineage markers, including CD3, CD14, CD16, CD19 and CD34), as shown in figure 14B.

Figure 14. Identification of DC population from peripheral blood by flow cytometry. A) Light scatter plot showing all viable cells within the gated region. B) Two parameter histogram of HLA DR (x axis) and Lineage cocktail (y axis) gated on all viable cells. Region 2 (R2) contains the DC population to be studied.
2.3.9.3 Region gating

One method for determining the surface marker expression on cells is to calculate the proportion of antibody stained cells falling beyond the distribution of staining from an isotype matched control antibody. For example, a region is drawn that excludes irrelevant, isotype matched control antibodies or non-specific staining. All events that fall within this region exceed isotype control staining intensity and are therefore regarded as positively labelled cells (figure 15). The proportion of positive cells amongst the total cell population can then be calculated by WinList software.

Figure 15. Cell surface labelling by region gating. A) A region (R3) is drawn on the isotype-matched control monoclonal antibody staining that excludes non-specific staining. B) The same region is then applied to the phenotype staining histogram (R4). The result gives the total percentage positive cells for that marker (71.4% of cells expressing CCR8 in the example above).

2.3.9.4 Enhanced normalised subtraction method

Region gating, described above, is the widely accepted method for identifying cell types. However, it can be inaccurate when assessing quantitative markers due to overlapping between marker expression and the isotype. In this situation, different operators performing analysis may obtain different results, depending on where the region is placed. An alternative strategy, which avoids this potential issue, is the use of super-enhanced $D_{max}$ (SED) normalised subtraction.
In this method, single parameter histograms are created for both the antibody-specific staining and the negative (isotype) control staining. The isotype control is then subtracted from the antibody staining using the super-enhanced $D_{\text{max}}$ (SED) normalised subtraction facility in WinList, as shown in figure 16.

This method provides values for total percentage expression of the antibody and the intensity ratio (IR) of staining (ie the amount of marker that is being expressed on positive cells), irrespective of the operator. The $D_{\text{max}}$ is given as a percentage following subtraction of the test and control histograms. If the two histograms are identical and overlap perfectly, a $D_{\text{max}}$ of 0% is the result. If the test histogram has a higher fluorescent intensity than the control and there is no overlap, then the $D_{\text{max}}$ is 100%. An example of such subtraction is shown in figure 16.

![Figure 16. Enhanced normalised subtraction method. A) FACS histograms demonstrating marker expression ($\beta7$ in this example) and matched isotype control (Rat IgG 2a). B) Subtraction histogram, values indicating total percentage $\beta7$ expression on cells (88.1% in this example) and intensity ratio (IR) of $\beta7$ expression on positive cells (22.4 in this example).](image)

In order to calculate the proportion of ongoing cytokine production in cells, the staining of cells cultured in the absence of monensin was subtracted from staining of cells cultured in the presence of monensin, again using SED normalised subtraction. The result is representative ongoing cytokine production in the absence of exogenous stimulation over the 4-hour assay period. The same antibody is used to label cells from both monensin-treated and untreated cultures, therefore giving the technique a high degree of sensitivity for
identifying small changes in antibody binding (Hart, Al-Hassi et al. 2005). This process determines natural or spontaneous cytokine production, within a 4 hour time period, in the absence of external stimulatory factors. An example of cytokine labelling and subtraction is shown in figure 17.

**Figure 17. Intracellular cytokine staining by subtraction.** A) Staining of intracellular TNFα with (red) and without (blue) monensin. B) Subtraction histogram created by subtracted total staining of TNFα without monensin from the staining of TNFα with monensin. Result shown as percentage of cells producing TNFα and intensity ratio.

### 2.3.9.5 WinList statistics

In order to determine if two histograms in a pair were significantly different, Kolmogorov-Smirnov (K-S) statistics were used to calculate the critical D value ($D_{crit}$), using the following equation:

$$D_{crit} = \frac{D_{max}}{\left\{ \frac{(n_1 + n_2)}{(n_1 \times n_2)} \right\}}$$

- $D_{max}$: The value calculated by WinList when subtracting the control histogram from the test histogram
- $n_1$: The number of events in the region on which the test histogram is gated
- $n_2$: The number of events in the region on which the control histogram is gated

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**2.3.9.6 Absolute cell counts**

Absolute cell counts were obtained by simultaneous acquisition of Flow-Count fluorospheres. Fluorescent counting beads were identified and gated on a FL1:SSC histogram plot. The absolute cell count is calculated by taking the number of events in the gated region of interest, the number of events in the bead gate and a known concentration of beads used at a 1:5 ratio during cell acquisition. The figures are entered into the equation below and the final cell count is expressed as number of cells per µL of blood:

\[
\text{Number of cells} (\mu L) = \frac{\text{Number of cells in gated region}}{\text{Number of beads in gated region} \times 5} \times \text{Bead concentration} (\mu L)
\]

**2.3.10 Statistical analysis**

Statistical analysis was carried out using Prism software (Graphpad Software Inc, San Diego, USA).

Statistical analysis was performed with either paired t-test, unpaired t-test, one-way and two-way repeat measures analysis of variance (ANOVA). In the case of multiple comparisons, subsequent ad-hoc Dunnett’s multiple comparison test was applied. Studies correlating patient demographical data with laboratory data were performed with Pearson’s rank correlation coefficient test.

P values of <0.05 were considered significant in all cases.
Chapter 3

Circulating dendritic cell phenotype in patients with Crohn’s disease
3.1 Abstract

**Background**

CD is a chronic transmural inflammatory disease of the gut, which can occur anywhere from mouth to anus. Although the precise aetiology of CD is unknown, a dysregulated immune response to gut microbiota and aberrant immune cell trafficking play a central role in the disease pathogenesis.

DCs play a key role in discriminating between commensal microorganisms and potentially harmful pathogens, thereby creating a balance between immune tolerance and active immunity. DCs also direct T-cell differentiation and imprint specific homing properties on stimulated T-cells, sending them to the effector site (eg, gut, skin). Despite this, information about homing marker expression on human DCs is scarce. We set out to investigate homing molecule expression on blood DCs in different phenotypes of active CD.

**Methods**

Blood from patients with active isolated small bowel (SB) CD (Harvey Bradshaw Index, HBI ≥ 7), isolated colonic (LB) CD (HBI ≥ 7) and active extra-intestinal manifestations of CD was obtained. Control groups included patients with inactive (IA) ileocolonic CD (HBI ≤ 3) and healthy subjects (HC).

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood following Ficoll gradient separation. DCs were identified as HLA DR +ve, Lineage (CD3, CD14, CD16, CD19, CD34) -ve. Expression of gut (β7, CCR9), skin (CLA, CCR4, CCR10) and lymph node (CCR7) markers on DC were quantified. Unpaired t-test was applied.

**Results**

CCR9 (small bowel homing marker) was up regulated on DC from patients with SB CD compared with HC (p=0.03), IA CD (p=0.04) and LB CD (p=0.013), while CLA expression was down regulated compared with expression in HC (p=0.002),
IA (p=0.03) and LB (p=0.021). CCR9 expression was also down regulated on DC isolated from patients with active colonic CD compared to HC (p=0.016).

Beta7, a gut-homing integrin, was up regulated on DC from LB CD compared with IA (p<0.0001) and HC (p<0.05).

CCR10, a skin-homing marker, was up regulated on DC from patients with EIMs compared to HC (p=0.01) and IA (p=0.03). CCR4 (another skin-homing marker) was down regulated on patients with EIMs compared to IA CD (p=0.0002) and patients with isolated active SB CD (p=0.0005).

**Conclusion**

We demonstrate dysregulation of human DC phenotype in CD. Furthermore, DC obtained from patients with CD isolated to particular anatomical locations express specific homing marker phenotypes, which are likely to correlate with infiltrates of effector cells at those sites.

*This abstract was presented at BSG conference 2012 and subsequently published in Gut journal with the following reference. Permission to reprint in Appendix, section 10.2.*

Altered homing molecule expression on blood dendritic cells in different phenotypes of Crohn’s disease

**Peake ST**, Bernardo D, Mann ER, Al-Hassi HO, Landy J, Knight SC, Hart AL

*Gut* 2012; 61(S3): A390
3.2 Introduction

3.2.1 Anatomical location of inflammation in Crohn’s disease

Crohn’s disease represents a heterogenous entity. Wide variations are seen between disease patient demographics, disease behaviour and anatomical location of inflammatory lesions. This diversity of disease phenotype contributes towards the difficulty in developing efficacious and safe therapies.

Classification systems have been developed to incorporate the phenotypical characteristics of Crohn’s disease, and therefore allow for the identification of patient subgroups and guide therapeutic interventions. Such classification systems include the Montreal classification system (see table 1, section 1.1.3) and the Vienna classification, proposed at the 1998 world gastroenterology meeting in Vienna (Gasche, Scholmerich et al. 2000). As well as helping to guide treatment, these standardised classification systems aid the ability to correlate putative aetiological factors (genetic and environmental) with particular disease phenotypic characteristics (Lichtenstein, Hanauer et al. 2004).

Disease location is a major determinant of symptoms, clinical course and prognosis of disease. Studies have shown that most patients with CD will develop complications of stricturing or penetrating disease and that the initial location of disease will determine the time and type of these complications (Cosnes, Cattan et al. 2002). In a historic study of 615 CD patients, Farmer et al. found that complications such as perianal fistulas, internal fistulas and intestinal obstruction were associated with ileocolonic disease. Patients with colonic disease had complications such as rectal bleeding, perianal fistulas, toxic megacolon and arthritis. In addition, 73% of patients with ileocolonic CD required surgery, compared with 51% of patients with isolated colonic disease. Patients who had disease confined to the small bowel only had the best prognosis with lower rates of surgery (Farmer, Hawk et al. 1975).
The anatomical location of CD remains broadly stable over time (Peyrin-Biroulet, Loftus et al. 2010). One study found that over a 10 year period, 85% of patients did not show any change in the anatomical location of CD (Louis, Collard et al. 2001). During the course of CD, extension or regression within the same region may occur, but only rarely does the disease change in classification, as defined by Montreal or Vienna classification. When it does occur, the main change appears to be from ileal (L1) or colonic (L2) to ileocolonic (L3) disease. The incidence of CD affecting the upper gastrointestinal tract (L4) at diagnosis is rare and very few patients develop this over the course of their disease (Louis, Collard et al. 2001).

**Genetics**

The factors determining disease location in individual patients with CD remains unclear, however there is likely to a significant genetic component. A study by Bayless et al. examined the records of 554 consecutive patients with CD, 95 of which had a family history of CD (17%). Sixty families were then analysed for concordance for site and type of CD. The authors found a high rate of concordance of 2 family members of 85% and 90% for site and type respectively (Bayless, Tokayer et al. 1996).

Multiple genetic mutations have been identified that are associated with clinical disease locations. Mutations in the NOD2/CARD15 (IBD 1) gene on chromosome 16 are associated with ileal disease and their frequency in colonic CD is no greater than unaffected individuals (Ahmad, Armuzzi et al. 2002). Localisation of CD to the colon has been associated with a low human beta-defensin 2 (HBD-2) gene copy number (Fellermann, Stange et al. 2006), in contrast to ileal disease, which is associated with reduced alpha-defensin expression (Wehkamp, Harder et al. 2004).

Other genotypes have been associated with ileal CD, such as ATG16L1T300A GG genotype (Prescott, Fisher et al. 2007) and other ATG16L1 risk alleles (Van Limbergen, Russell et al. 2008). Similarly, the carriage of the HLA-DRB1*0103
allele is strongly associated with isolated colonic CD (Beckly, Hancock et al. 2008).

**Ethnic background and smoking**

It is well documented that anatomical location of CD is linked to a patient’s ethnic background. One study investigated differences in CD phenotype between patients originating from South Asia and those with a Northern European background, but now all residing in Northwest London. The authors found a higher rate of isolated colonic disease in the South Asian patient cohort compared with the Northern European group (46.8% versus 34.8%, p=0.048) in whom ileocolonic disease was the most frequently affected location (40.5%) (Walker, Williams et al. 2011).

Differences in CD phenotype between ethnic groups does not necessarily imply a genetic cause but may reflect variations in environmental factors related to lifestyle in different groups. One environmental factor that may be of importance in determining CD phenotype is smoking. The study above reported a significantly lower number of (self-reported) smokers in the South Asian cohort of patients compared to the Northern European cohort (10.6% versus 31.6%, p<0.0001).

Smoking has been reported to be closely associated with disease location with a higher prevalence of ileal disease and a lower prevalence of colonic involvement in smokers (Lindberg, Jarnerot et al. 1992). Smoking is also associated with increased risk of complications, such as strictures or fistulae, and an increased risk or requiring surgery.

Having identified that smoking leads to increased prevalence of ileal CD, and that smoking is more common in North European than South Asian communities, it seems that differences in the anatomical location of CD may be the result of genetic or environmental factors.
**Age at diagnosis**

A study from 1996 found that a younger age of diagnosis (less than 20 years of age) correlated significantly with an increased frequency of ileal disease compared with patients diagnosed at an older age in whom colonic disease was more common (Polito, Childs et al. 1996). Despite this, more recent studies agree that isolated colonic CD is more common in paediatric patients with 76.5% of children < 5 years of age having Crohn's disease limited to the colon (Paul, Birnbaum et al. 2006), dropping to 63% of children < 8 years of age (Heyman, Kirschner et al. 2005).

**Serological markers**

Specific disease locations have also been linked to serological markers such as serum antineutrophil cytoplasmic antibodies (ANCA). In UC, expression of pANCA occurs in 50-80% of patients and is associated with treatment-resistant left sided colitis and patients with aggressive disease.

Serum ANCA has also been detected in patients with CD, albeit with a lower prevalence (between 10 and 30% in most studies). Vasiliauskas et al. investigated whether pANCA expression in patients with CD correlates with an identifiable clinical subgroup. The authors found that expression of pANCA is associated with left sided colonic CD and symptoms thereof (rectal bleeding and mucus discharge), while having an absence of small bowel inflammation. This ‘UC-like’ CD phenotype correlated with pANCA expression in all cases (Vasiliauskas, Plevy et al. 1996).
3.2.2 Hypothesis and aims

We hypothesised that alterations in the phenotype of circulating peripheral blood DC may contribute to the dysregulated immune response that underlies CD. In particular, an abnormal pattern of homing marker expression on circulating DC may correspond to the clinical phenotype (ie disease location) of the patient.

Aims

- To identify patients with active CD limited to specific anatomical compartments within the gastrointestinal tract. In addition, patients with cutaneous EIM of CD were identified, along with those with inactive CD.

- To analyse homing marker expression of peripheral circulating blood DC on these groups of patients and relate the findings to their clinical disease phenotype.

- To identify DC sub-populations (myeloid and plasmacytoid DC) and quantify relative expression of homing markers on DC sub-populations.
3.3 Methods

Patients were recruited from IBD outpatient clinics and the endoscopy unit at St Mark’s Hospital between November 2010 and December 2011. All patients were recruited directly by the candidate to ensure standardisation of patient selection. A Microsoft Excel database was created containing phenotypical data for each patient recruited, including the following parameters:

- Age
- Gender
- Number of years since diagnosis with CD
- Montreal classification of CD
- Disease activity (measured using Harvey Bradshaw Index, HBI)
- Current medications
- Date of most recent endoscopic examination
- Date of most recent radiological examination

Inclusion and exclusion criteria (outlined in methods, section 2.2.2) were applied to all patient recruitment.

3.3.1 Crohn’s disease activity

The Crohn’s Disease Clinical Activity Index (CDAI) was developed by Best and colleagues at the Midwest Regional Health Center, Illinois in 1976 (Best, Becktel et al. 1976). It is essentially a research tool used to quantify symptoms being experienced by a patient. The CDAI is considered the ‘gold standard’ for assessing disease activity in CD, although validation of the index has been varied. It is difficult to use in daily practice, for example in a busy outpatient clinic, as it requires complex calculations, biochemical and physical measurements (height/weight). In addition, patients need to record all the parameters in a diary for 7 days prior to assessment. A further criticism is that it does not incorporate
a subjective assessment of quality of life, endoscopic features of inflammation or systemic manifestations (such as fatigue).

The Harvey Bradshaw Index (HBI) was devised in 1980 as a simpler and more user-friendly version of the CDAI (Harvey and Bradshaw 1980). It contains five simple clinical parameters as shown in table 6.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of liquid stools per day</td>
<td>Score 1 per liquid stool</td>
</tr>
<tr>
<td>General well-being</td>
<td>0 = very well, 1 = slightly below par, 2 = poor, 3 = very poor, 4 = terrible</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>0 = none, 1 = mild, 2 = moderate, 3 = severe</td>
</tr>
<tr>
<td>Abdominal mass</td>
<td>0 = none, 1 = dubious, 2 = definite, 3 = definite and tender</td>
</tr>
<tr>
<td>Complications</td>
<td>Arthralgia, uveitis, erythema nodosum, pyoderma gangenosum, oral apthous ulceration, anal fissure, new fistula, abscess (score 1 per item)</td>
</tr>
</tbody>
</table>

*Table 6. The Harvey Bradshaw Index of disease activity in Crohn’s disease. The first three parameters are scored over the 24-hour period prior to assessment.*

The HBI has been shown to correlate well with the CDAI (Best 2006). For these reasons, the HBI was chosen as the disease activity index throughout this research project. Patients scoring \( \leq 3 \) were classified as ‘in remission’ and those scoring \( \geq 7 \) were classified clinically as having active inflammation.
3.3.2  Crohn’s disease phenotype

Crohn’s disease phenotype was recorded according to the Montreal Classification of CD (table 1, section 1.1.3). All types of disease behaviour were included (inflammatory, stricturing and penetrating), as long as there were no complications relating to these (ie localised perforation or abscess formation in the case of penetrating CD).

Disease location (the parameter being investigated) had been confirmed by endoscopic and radiological studies within 6 months of patient recruitment to the study. Patients with skin-related EIMs (erythema nodosum or pyoderma gangrenosum) were assessed by 2 separate consultant gastroenterologists, who agreed on the diagnosis, prior to patient recruitment. These patients did not have active luminal symptoms of CD.

The following groups were recruited (individual demographic and clinical patient details provided later):

- Active isolated small bowel (SB) CD, HBI ≥7
- Active isolated colonic CD, HBI ≥7
- Patients with active skin EIMs of CD
- Patients with inactive ileocolonic CD, HBI ≤3
- Healthy control subjects
3.3.3 Sample collection and processing

Following venesection from the patient, samples were taken to the laboratory and processed within 30 minutes. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood and labeled with fluorochrome conjugated antibodies, as described in sections 2.3.1 and 2.3.5 respectively. Four colour flow cytometry was used to identify peripheral blood DC populations and gating techniques (described in section 2.3.9) were used to quantify expression of homing molecules on total DC, mDC and pDC. Data was expressed as percentage homing molecule expression on DC (or DC subset) for each homing molecule studied.

Homing molecules examined were β7 integrin, CLA, CCR4, CCR7, CCR9 and CCR10.

3.3.4 Statistical analysis

Statistical analysis was carried out using unpaired t-test. Correlation studies were performed using Pearson’s rank correlation coefficient test.

P values of <0.05 were considered statistically significant.
3.4 Results

3.4.1 Patient characteristics

PBMCs were obtained from 33 subjects (9 with isolated SB CD, 5 with isolated colonic CD, 3 with skin EIMs, 7 with inactive ileocolonic CD and 9 healthy control subjects). Table 7 on the following page outlines the clinical and demographical patient information.
<table>
<thead>
<tr>
<th>Case number</th>
<th>Gender</th>
<th>Age</th>
<th>Years since diagnosis</th>
<th>CD location</th>
<th>HBI</th>
<th>Current medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>56</td>
<td>4</td>
<td>Small bowel</td>
<td>10</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>46</td>
<td>15</td>
<td>Small bowel</td>
<td>7</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>53</td>
<td>36</td>
<td>Small bowel</td>
<td>7</td>
<td>6-MP, ENT</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>43</td>
<td>8</td>
<td>Small bowel</td>
<td>9</td>
<td>6-MP</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>19</td>
<td>9</td>
<td>Small bowel</td>
<td>8</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>34</td>
<td>11</td>
<td>Small bowel</td>
<td>12</td>
<td>5-ASA, 6-MP</td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>27</td>
<td>2</td>
<td>Small bowel</td>
<td>10</td>
<td>AZA</td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>32</td>
<td>2</td>
<td>Small bowel</td>
<td>9</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>29</td>
<td>5</td>
<td>Small bowel</td>
<td>11</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>31</td>
<td>24</td>
<td>Colon</td>
<td>8</td>
<td>AZA</td>
</tr>
<tr>
<td>11</td>
<td>Male</td>
<td>55</td>
<td>12</td>
<td>Colon</td>
<td>14</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>12</td>
<td>Female</td>
<td>26</td>
<td>6</td>
<td>Colon</td>
<td>14</td>
<td>5-ASA</td>
</tr>
<tr>
<td>13</td>
<td>Male</td>
<td>22</td>
<td>2</td>
<td>Colon</td>
<td>12</td>
<td>6-MP, PROB</td>
</tr>
<tr>
<td>14</td>
<td>Male</td>
<td>41</td>
<td>9</td>
<td>Colon</td>
<td>10</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>15</td>
<td>Male</td>
<td>75</td>
<td>41</td>
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<td>0</td>
<td>NIL</td>
</tr>
<tr>
<td>16</td>
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<td>31</td>
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<td>6-MP</td>
</tr>
<tr>
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<td>52</td>
<td>26</td>
<td>Ileocolonic</td>
<td>2</td>
<td>AZA</td>
</tr>
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<td>5-ASA</td>
</tr>
<tr>
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<td>Ileocolonic</td>
<td>0</td>
<td>NIL</td>
</tr>
<tr>
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<td>48</td>
<td>17</td>
<td>Ileocolonic</td>
<td>2</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>22</td>
<td>Female</td>
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<td>AZA</td>
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<td>41</td>
<td>12</td>
<td>EN (legs)</td>
<td>4</td>
<td>5-ASA</td>
</tr>
<tr>
<td>24</td>
<td>Male</td>
<td>62</td>
<td>26</td>
<td>PG (legs)</td>
<td>4</td>
<td>5-ASA, AZA</td>
</tr>
<tr>
<td>25</td>
<td>Female</td>
<td>35</td>
<td>-</td>
<td>HC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>Female</td>
<td>28</td>
<td>-</td>
<td>HC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>Female</td>
<td>53</td>
<td>-</td>
<td>HC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>28</td>
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<td>-</td>
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</tr>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
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<td>-</td>
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<tr>
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<td>33</td>
<td>-</td>
<td>HC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>33</td>
<td>Male</td>
<td>34</td>
<td>-</td>
<td>HC</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 7. Clinical and demographical information of patients and healthy controls. 

EN = erythema nodosum, PG = pyoderma gangrenosum, HC = healthy control, 5-ASA = 5-aminosalicylic acid, AZA = azathioprine, 6-MP = 6-mercaptopurine, ENT = polymeric diet, PROB = probiotic therapy,
3.4.2 Identification of dendritic cell sub-populations

DCs were identified in the PBMC pool as previously described in section 2.3.9.2 (HLA DR+, lineage cocktail-). In order to identify DC subpopulations (mDC and pDC), a one parameter histogram was created identifying positive and negative expression of CD11c within the previously gated DC population (figure 18). Cells expressing CD11c are myeloid DC and those not expressing CD11c are the putative plasmacytoid population.

Figure 18. Identification of mDC and pDC within total dendritic cell population. A) A single parameter histogram is created, identifying CD11c expression by DCs. B) Plasmacytoid DCs are negative for CD11c and are gated within region 4 (R4). C) Region 5 (R5) contains DCs expressing CD11c and therefore are taken as the mDCs.

The WinList software programme allows 'backgating' of cell populations onto other histogram plots. Therefore it is possible to demonstrate the location of mDC and putative pDC within the total DC population (figure 19). The backgating plot shows the two cell subpopulations to be quite distinct when looking at HLA DR expression, with mDC expressing higher levels of HLA DR compared to pDC.
Figure 19. Distribution of mDC and pDC within the total dendritic cell population.  
A) CD11c- DCs are gated in region R4 (red) and CD11c+ DCs are gated in region R5 (blue).  
B) Backgating of DC subpopulations onto HLA DR vs lineage cocktail plot.  
Myeloid DCs are seen to express higher levels of HLA DR compared to pDCs.
3.4.3 Expression of homing molecules on circulating dendritic cells

3.4.3.1 β7 expression

Expression of β7 surface integrin on circulating DC was high (80.6 ± 9.9%). DCs isolated from patients with active colonic CD had up-regulated β7 expression compared to DCs from HC or patients with inactive ileocolonic CD (figure 20). In view of its gut-homing significance, we were surprised to see that there was no significant difference in β7 expression on DCs isolated from patients with active SB CD.

![Figure 20](image.png)

Figure 20. β7 expression on dendritic cells in different phenotypes of CD. A) FACS histograms demonstrating β7 expression on DCs and matched isotype control (Rat IgG 2a). B) Subtraction histogram, values indicating percentage β7 expression on all DCs and intensity ratio (IR) of expression on positive cells. C) Pooled results of β7 expression on DCs.

HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD.

Statistical analysis performed with unpaired t-test.
3.4.3.2 CLA expression

We found quite wide variation in CLA expression across the different CD phenotypes, ranging from 1% up to 85%. DCs from patients with active SB CD have down regulated CLA expression compared to HC, patients with IA CD and patients with active colonic CD (figure 21). There are 4 patients in the EIM group (compared to 3 in the other markers) because an extra case of EIM was included in which only the CLA staining was successful.

Figure 21. CLA expression on dendritic cells in different phenotypes of CD. A) FACS histograms demonstrating CLA expression on DC and matched isotype control (Rat IgM). B) Subtraction histogram, values indicating percentage CLA expression on all DCs and IR of expression on positive cells. C) Pooled results of CLA expression on DCs.

HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD. Statistical analysis performed with unpaired t-test.
3.4.3.3 β7/CLA co-expression

To further investigate gut versus skin homing phenotypes of DC in the clinical groups, we looked at co-expression of β7 and CLA homing markers on circulating DCs in each cohort of patients.

This method of analysis demonstrated that, despite there being no increased expression of β7 on DCs from patients with active SB CD as there was on DCs from patients with active colonic CD, there was a reduction in CLA expression. This reduction in CLA expression renders increased single positive β7 expression on DCs from patients with active SB CD compared to HC (figure 22).
Figure 22. β7 and CLA co-expression on DCs in different phenotypes of CD. A) Representative histogram plot demonstrating β7 and CLA on DC in a healthy control subject. B) Representative histogram demonstrating β7 and CLA expression on DCs in a patient with active SB CD. C) Pooled results of single positive β7, single positive CLA expression, double positive β7/CLA expression and double negative β7/CLA expression on DCs in different phenotypes of CD.

HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD.

Statistical analysis performed with unpaired t-test.
3.4.3.4  CCR9 expression

CCR9 expression on circulating DCs was found to be relatively low (<40% in all groups except active SB CD). There was greater expression of CCR9 on DCs isolated from patients with active SB CD compared to HC, IA CD and active colonic CD. Patients with active colonic CD had very low CCR9 expression (1% in 4 out of 5 patients), which was statistically significant when compared to the HC group.

\[ \text{HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD.} \]

\[ \text{Statistical analysis performed with unpaired t-test.} \]

**Figure 23.** CCR9 expression on dendritic cells in different phenotypes of CD. A) FACS histograms demonstrating CCR9 expression on DCs and matched isotype control (Mouse IgG2a). B) Subtraction histogram, values indicating percentage CCR9 expression on all DCs and IR of expression on positive cells. C) Pooled results of CCR9 expression on DCs.
3.4.3.5 CCR4 expression

CCR4 expression was found to be >50% in most subjects studied. Circulating DCs isolated from patients with skin-related EIMs were found to have down-regulated expression of CCR4 compared to the IA CD group and patients with active SB CD. CCR4 is a skin homing marker, and therefore this represents a surprising and paradoxical result. However, only 3 patients were studied in this group and therefore further work is needed to confirm this finding.

Figure 24. CCR4 expression on dendritic cells in different phenotypes of CD. A) FACS histograms demonstrating CCR4 expression on DCs and matched isotype control (Mouse IgG2b). B) Subtraction histogram, values indicating percentage CCR4 expression on all DCs and IR of expression on positive cells. C) Pooled results of CCR4 expression on DCs.

HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD.
Statistical analysis performed with unpaired t-test.
3.4.3.6 CCR10 expression

CCR10 expression was found to be very low on circulating DC across all the clinical groups (<16%). Despite this, CCR10 expression was found to be up-regulated on DC from patients with skin EIMs compared to HC and patients with inactive CD (figure 25), albeit patient numbers in the EIM cohort were low.

![Image of FACS histograms demonstrating CCR10 expression on DC and matched isotype control (Rat IgG2b). Due to low CCR10 expression on DCs, the region gating method was utilised (region R6) to obtain values for percentage CCR10 expression on all DCs and IR of expression on positive cells. Pooled results of CCR10 expression on DCs. HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD. Statistical analysis performed with unpaired t-test.]

Figure 25. CCR10 expression on dendritic cells in different phenotypes of CD. A) FACS histograms demonstrating CCR10 expression on DC and matched isotype control (Rat IgG2b). B) Due to low CCR10 expression on DCs, the region gating method was utilised (region R6) to obtain values for percentage CCR10 expression on all DCs and IR of expression on positive cells. C) Pooled results of CCR10 expression on DCs. HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD. Statistical analysis performed with unpaired t-test.
3.4.3.7  CCR7 expression

CCR7 expression was found to vary between 30% and 80% in all subjects. There were no statistically significant differences in CCR7 expression between the clinical study groups (figure 26).

Figure 26. CCR7 expression on dendritic cells in different phenotypes of CD. A) FACS histograms demonstrating CCR7 expression on DCs and matched isotype control (Mouse IgG2a). B) Subtraction histogram, values indicating percentage CCR7 expression on all DCs and IR of expression on positive cells. C) Pooled results of CCR7 expression on DCs.

HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD.

Statistical analysis performed with unpaired t-test.
3.4.4 Expression of homing molecules on circulating dendritic cell sub-populations

Having found altered homing marker expression on total DCs in different phenotypes of CD, we examined whether the homing markers were expressed on both subpopulations of DC. Expression of the adhesion integrin, CD11c, was used to positively identify mDCs (figure 18, section 3.4.2). Putative pDCs were taken as the CD11c- DC population. Relative expression of each homing marker was quantified for both mDCs and pDCs in the 5 clinical CD groups.

3.4.4.1 Myeloid dendritic cells

Myeloid DC expressed greater levels of Beta7, CLA and CCR9 compared to pDC. This trend occurred regardless of the presence or location of CD (figure 27).
Figure 27. Expression of A) β7, B) CLA and C) CCR9 homing markers on mDCs and putative pDCs in different clinical phenotypes of CD.

HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD.

Statistical analysis performed with unpaired t-test.
### 3.4.4.2 Plasmacytoid dendritic cells

With the exception of CCR9, chemokine receptors were expressed in greater amounts on pDCs (CCR4, CCR7 and CCR10), as shown in figure 28. Again, the differences did not alter according to the clinical phenotype of CD or healthy and diseased states. The only exception to this was CCR10 expression in the EIM group. However, as there was only one patient studied, this may represent an outlying result.

*Figure 28. Expression of A) CCR7, B) CCR4 and C) CCR10 chemokine receptors on mDCs and putative pDCs in different clinical phenotypes of CD.*

*HC = healthy control, SB = active small bowel CD, Colon = active colonic CD, EIM = extra-intestinal manifestations of CD, Inactive = inactive CD.*

*Statistical analysis performed with unpaired t-test.*
3.4.5 Correlation of homing marker expression with clinical patient data

After finding differences in the homing profile of DCs in different clinical phenotypes of CD, we studied whether such changes correlated with the demographical and/or clinical data collected on the patients. In order to study this, the expression of homing markers in all DCs, mDCs and pDCs was correlated with each clinical and demographical parameter, in each clinical phenotype of CD. Correlation studies on patients in the EIM patient group were not performed due to the low numbers in the EIM patient cohort.

Small bowel CD correlation studies

Correlation studies in patients with active SB CD indicated that there was reduced CLA expression on circulating mDCs with increasing age. Furthermore, as disease severity increased (measured by clinical indices making up the HBI), circulating mDCs had greater expression of CCR9 and pDCs had reduced expression of CCR4. Table 8 displays all correlation data in active SB CD.
<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age</th>
<th>Years since diagnosis</th>
<th>HBI</th>
<th>Gender: % expression (mean ± SD) male; female; p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homing marker ↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = -0.54 **</td>
<td>r = -0.27 **</td>
<td>r = -0.23 **</td>
<td>- 75.9±8.1; 73.4±6.5; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>r = -0.03 **</td>
<td>r = +0.46 **</td>
<td>r = -0.51 **</td>
<td>- 94.7±1.8; 93.5±1.8; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = +0.28 **</td>
<td>r = +0.42 **</td>
<td>r = -0.53 **</td>
<td>- 67.9±4.2; 56.2±13.2; ns</td>
</tr>
<tr>
<td>CLA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = +0.23 **</td>
<td>r = -0.30 **</td>
<td>r = -0.08 **</td>
<td>- 19.1±7.6; 33.7±12.8; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>r = -0.71 **</td>
<td>r = -0.29 **</td>
<td>r = +0.18 **</td>
<td>- 36.0±13.6; 38.1±13.7; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = -0.59 **</td>
<td>r = -0.35 **</td>
<td>r = +0.11 **</td>
<td>- 31.4±10.9; 30.3±14.6; ns</td>
</tr>
<tr>
<td>CCR9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = +0.64 **</td>
<td>r = +0.52 **</td>
<td>r = -0.05 **</td>
<td>- 33.4±10.0; 37.5±10.6; ns</td>
</tr>
<tr>
<td>- mDCs</td>
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<td>r = +0.73 **</td>
<td>- 39.0±7.2; 53.9±8.5; ns</td>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CCR4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = -0.49 **</td>
<td>r = +0.34 **</td>
<td>r = -0.15 **</td>
<td>- 62.1±4.4; 66.1±3.7; ns</td>
</tr>
<tr>
<td>- mDCs</td>
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<td>r = -0.03 **</td>
<td>r = -0.43 **</td>
<td>- 59.3±4.3; 59.1±5.7; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = +0.17 **</td>
<td>r = +0.65 **</td>
<td>r = -0.71 **</td>
<td>- 74.6±5.7; 63.0±3.2; ns</td>
</tr>
<tr>
<td>CCR10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = +0.13 **</td>
<td>r = -0.32 **</td>
<td>r = +0.14 **</td>
<td>- 2.0±0.5; 6.5±2.5; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>- pDCs</td>
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<td>r = -0.08 **</td>
<td>r = -0.03 **</td>
<td>- 4.7±2.1; 2.6±1.1; ns</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = +0.39 **</td>
<td>r = +0.17 **</td>
<td>r = +0.28 **</td>
<td>- 49.0±6.1; 53.9±2.3; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>r = +0.17 **</td>
<td>r = -0.40 **</td>
<td>r = +0.25 **</td>
<td>- 10.6±9.6; 40.4±19.6; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = +0.12 **</td>
<td>r = +0.24 **</td>
<td>r = -0.26 **</td>
<td>- 70.6±2.1; 63.1±13.8; ns</td>
</tr>
</tbody>
</table>

**Table 8.** Correlation studies between homing marker expression and clinical parameters in patients with active small bowel CD. Statistical analysis of age, years since diagnosis and HBI was performed using Pearson's correlation. Correlations in gender was performed using unpaired t-test.

n/a = expression of homing marker falls below the detection limit in >80% of data points.
Colonic CD correlation studies

Correlation studies in patients with active colonic CD identified a positive correlation between age and CCR4 expression on circulating mDCs (see table 9).

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age (r value p value)</th>
<th>Years since diagnosis (r value p value)</th>
<th>HBI (r value p value)</th>
<th>Gender: % expression (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homing marker</td>
<td></td>
<td></td>
<td></td>
<td>male; female; p value</td>
</tr>
<tr>
<td>Beta7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>-0.85 ns</td>
<td>-0.62 ns</td>
<td>-0.05 ns</td>
<td>86.9±3.5; 87.2±2.3; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>0.08 ns</td>
<td>+0.02 ns</td>
<td>0.18 ns</td>
<td>94.3±1.2; 94.9±0.7; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>-0.81 ns</td>
<td>-0.39 ns</td>
<td>+0.21 ns</td>
<td>71.7±3.6; 80.9±7.0; ns</td>
</tr>
<tr>
<td>CLA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>+0.80 ns</td>
<td>-0.24 ns</td>
<td>+0.35 ns</td>
<td>72.4±8.9; 40.8±4.4; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>+0.55 ns</td>
<td>-0.47 ns</td>
<td>+0.32 ns</td>
<td>62.5±18.2; 55.2±37.0; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>+0.63 ns</td>
<td>+0.38 ns</td>
<td>+0.37 ns</td>
<td>41.7±16.9; 42.6±4.6; ns</td>
</tr>
<tr>
<td>CCR9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>- mDCs</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>- pDCs</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CCR4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>+0.10 ns</td>
<td>+0.52 ns</td>
<td>+0.19 ns</td>
<td>48.0±8.1; 68.9±0.8; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>-0.89 p&lt;0.04</td>
<td>-0.59 ns</td>
<td>-0.02 ns</td>
<td>66.8±3.5; 66.9±1.6; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>-0.64 ns</td>
<td>-0.17 ns</td>
<td>-0.60 ns</td>
<td>73.9±4.6; 72.2±2.8; ns</td>
</tr>
<tr>
<td>CCR10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>- mDCs</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>- pDCs</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CCR7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>-0.24 ns</td>
<td>-0.57 ns</td>
<td>0.84 ns</td>
<td>55.7±1.7; 58.6±7.5; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>0.65 n.s.</td>
<td>+0.54 ns</td>
<td>-0.26 ns</td>
<td>39.8±9.3; 23.8±22.8; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>-0.17 ns</td>
<td>-0.40 ns</td>
<td>+0.83 ns</td>
<td>64.6±6.2; 70.4±11.1; ns</td>
</tr>
</tbody>
</table>

Table 9. Correlation studies between homing marker expression and clinical parameters in patients with active colonic CD. Statistical analysis of age, years since diagnosis and HBI was performed using Pearson’s correlation. Correlations in gender was performed using unpaired t-test.

n/a = expression of homing marker falls below the detection limit in >80% of data points.
Inactive CD correlation studies

There were no correlations seen between homing marker expression and clinical parameters in patients with inactive ileocolonic CD (see table 10).

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age</th>
<th>Years since diagnosis</th>
<th>HBI</th>
<th>Gender: % expression (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homing marker ↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>( r = -0.31 ) ns</td>
<td>( r = -0.30 ) ns</td>
<td>( r = +0.41 ) ns</td>
<td>70.5±1.1; 74.3±1.5; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>( r = -0.44 ) ns</td>
<td>( r = -0.19 ) ns</td>
<td>( r = +0.22 ) ns</td>
<td>91.8±2.9; 91.0±2.9; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>( r = -0.03 ) ns</td>
<td>( r = -0.28 ) ns</td>
<td>( r = -0.47 ) ns</td>
<td>67.2±5.9; 65.2±17.6; ns</td>
</tr>
<tr>
<td>CLA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>( r = -0.05 ) ns</td>
<td>( r = +0.26 ) ns</td>
<td>( r = +0.27 ) ns</td>
<td>40.2±6.3; 57.3±12.3; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>( r = 0.21 ) ns</td>
<td>( r = +0.36 ) ns</td>
<td>( r = +0.02 ) ns</td>
<td>50.5±23.5; 43.3±22.9; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>( r = +0.29 ) ns</td>
<td>( r = +0.63 ) ns</td>
<td>( r = +0.23 ) ns</td>
<td>21.6±11.9; 27.0±17.8; ns</td>
</tr>
<tr>
<td>CCR9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>( r = +0.44 ) ns</td>
<td>( r = +0.65 ) ns</td>
<td>( r = -0.09 ) ns</td>
<td>19.2±4.7; 13.8±8.0; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>( r = -0.02 ) ns</td>
<td>( r = -0.14 ) ns</td>
<td>( r = -0.38 ) ns</td>
<td>22.4±10.7; 17.4±11.6; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>( r = -0.15 ) ns</td>
<td>( r = -0.09 ) ns</td>
<td>( r = -0.20 ) ns</td>
<td>14.1±6.6; 11.8±10.7; ns</td>
</tr>
<tr>
<td>CCR4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>( r = +0.21 ) ns</td>
<td>( r = +0.49 ) ns</td>
<td>( r = -0.14 ) ns</td>
<td>71.7±5.0; 67.2±3.1; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>( r = +0.59 ) ns</td>
<td>( r = +0.68 ) ns</td>
<td>( r = -0.35 ) ns</td>
<td>66.3±7.0; 59.4±1.7; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>( r = +0.03 ) ns</td>
<td>( r = +0.29 ) ns</td>
<td>( r = -0.04 ) ns</td>
<td>78.2±3.7; 72.3±4.6; ns</td>
</tr>
<tr>
<td>CCR10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>( r = -0.03 ) ns</td>
<td>( r = -0.28 ) ns</td>
<td>( r = -0.47 ) ns</td>
<td>1.5±0.3; 1.9±0.6; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>- pDCs</td>
<td>( r = +0.51 ) ns</td>
<td>( r = +0.65 ) ns</td>
<td>( r = -0.02 ) ns</td>
<td>8.7±2.8; 3.2±0.9; ns</td>
</tr>
<tr>
<td>CCR7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>( r = -0.10 ) ns</td>
<td>( r = -0.03 ) ns</td>
<td>( r = -0.29 ) ns</td>
<td>56.4±3.9; 60.3±6.0; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>( r = -0.33 ) ns</td>
<td>( r = +0.08 ) ns</td>
<td>( r = -0.02 ) ns</td>
<td>41.5±7.1; 38.1±10.1; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>( r = -0.34 ) ns</td>
<td>( r = -0.47 ) ns</td>
<td>( r = -0.19 ) ns</td>
<td>65.9±9.5; 81.8±6.8; ns</td>
</tr>
</tbody>
</table>

Table 10. Correlation studies between homing marker expression and clinical parameters in patients with inactive ileocolonic CD. Statistical analysis of age, years since diagnosis and HBI was performed using Pearsons correlation. Correlations in gender was performed using unpaired t-test. n/a = expression of homing marker falls below the detection limit in >80% of data points.
Healthy control correlation studies

There were no correlations seen between homing marker expression and clinical parameters in healthy control subjects (table 11). These findings are the same as those in patients with inactive ileocolonic CD.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age</th>
<th>Gender:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homing marker ↓</td>
<td>(r value × p value)</td>
<td>% expression (mean ± SD)</td>
</tr>
<tr>
<td>Beta7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = +0.29</td>
<td>71.4±3.6; 81.7±4.2; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>r = +0.52</td>
<td>80.4±8.7; 87.1±5.4; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = +0.16</td>
<td>51.9±6.7; 63.5±2.8; ns</td>
</tr>
<tr>
<td>CLA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = -0.34</td>
<td>54.2±6.5; 60.7±8.4; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>r = +0.20</td>
<td>68.2±9.9; 71.4±4.5; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = -0.21</td>
<td>54.2±4.6; 46.0±6.3; ns</td>
</tr>
<tr>
<td>CCR9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = -0.15</td>
<td>22.2±3.7; 16.6±2.2; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>r = -0.35</td>
<td>21.1±5.1; 15.1±5.4; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CCR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = +0.17</td>
<td>42.9±14.1; 59.7±22.8; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>r = +0.02</td>
<td>57.7±2.0; 49.6±4.5; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = +0.29</td>
<td>63.4±3.5; 62.8±4.4; ns</td>
</tr>
<tr>
<td>CCR10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = -0.42</td>
<td>1.6±0.6; 1.3±0.2; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = +0.07</td>
<td>2.6±0.9; 2.1±0.5; ns</td>
</tr>
<tr>
<td>CCR7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- All DCs</td>
<td>r = +0.35</td>
<td>47.3±3.7; 46.7±4.4; ns</td>
</tr>
<tr>
<td>- mDCs</td>
<td>r = -0.19</td>
<td>34.8±8.1; 31.5±2.9; ns</td>
</tr>
<tr>
<td>- pDCs</td>
<td>r = -0.38</td>
<td>75.9±6.9; 69.2±6.6; ns</td>
</tr>
</tbody>
</table>

Table 11. Correlation studies between homing marker expression and clinical parameters in healthy control subjects. Statistical analysis of age was performed using Pearson's correlation. Correlations in gender was performed using unpaired t-test.

n/a = expression of homing marker falls below the detection limit in >80% of data points.
### 3.4.6 Summary of results

<table>
<thead>
<tr>
<th>CD phenotype</th>
<th>DC phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isolated active colonic CD</strong></td>
<td>Increased expression of β7</td>
</tr>
<tr>
<td></td>
<td>Reduced expression of CCR9</td>
</tr>
<tr>
<td><strong>Isolated active SB CD</strong></td>
<td>Increased expression of CCR9</td>
</tr>
<tr>
<td></td>
<td>Reduced expression of CLA (renders increased single positive β7 expression)</td>
</tr>
<tr>
<td><strong>EIMs</strong></td>
<td>Increased expression of CCR10</td>
</tr>
<tr>
<td></td>
<td>Reduced expression of CCR4</td>
</tr>
<tr>
<td><strong>Inactive ileocolonic CD</strong></td>
<td>DC phenotype same as in HC</td>
</tr>
</tbody>
</table>

*Table 12. Summary of DC phenotype in different clinical phenotypes of CD.*

- Myeloid DCs expressed greater levels of CLA, beta7 and CCR9 compared to plasmacytoid DC.
- Plasmacytoid DCs expressed greater levels of CCR4, CCR7 and CCR10 compared to myeloid DC.
- There was a positive correlation between CCR9 expression on mDCs and negative correlation between CCR4 expression on pDCs with HBI in patients with active SB CD.
- There was a negative correlation between CLA expression and age on mDCs in SB CD, and a positive correlation between CCR4 expression on mDCs and age in colonic CD.
3.5 Discussion

Dendritic cell phenotype in Crohn’s disease

We found distinctive phenotypic properties of circulating DCs in patients with active CD. DCs isolated from patients with active colonic CD expressed high levels of the β7 integrin. In these experiments, positive expression of β7 is taken to represent α4β7 integrin expression on cells, although this has already been identified as a limitation in the methodology (section 1.2.11). In healthy mucosa, MAd-CAM1 (the ligand for β7) is expressed on intestinal lamina propria venules, however this expression substantially increases in IBD. Furthermore, in CD, expression of MAd-CAM-1 is increased in inflamed tissue compared to non-inflamed tissue (Arihiro, Ohtani et al. 2002). Indeed, studies have shown an increased density of α4β7+ cells in inflamed colonic lamina propria of patients with CD compared to controls (Souza, Elia et al. 1999).

DCs from patients with isolated active SB CD, were found to have a different gut-homing phenotype. These cells up-regulated CCR9 and down-regulated CLA expression. The reduced expression of CLA rendered the DCs with increased single positive β7 expression.

In the small bowel, both the α4β7/MAd-CAM-1 and CCR9/CCL25/TECK pathways are important for lymphocyte migration. However, in the colon, expression of CCR9 and CCL25/TECK is limited. CCL25/TECK expression on epithelial cells in the small bowel has been shown to follow a gradient, with highest expression in the proximal small intestine and lowest expression in the ileum (Ericsson, Kotarsky et al. 2006). Studies have shown only a small subset of cells in the colon express CCR9 and there is no expression of the CCL25/TECK ligand (Papadakis, Prehn et al. 2000). This highlights an important role for CCR9 and CCL25/TECK in small bowel homing, but only a limited role in colonic homing. This was demonstrated in a study using a T-cell receptor transgenic transfer model, which showed activated CCR9+ lymphocytes selectively localised
to the small intestinal mucosa, but following *in vivo* neutralisation of CCL25, there was a marked reduction in the ability of the lymphocytes to populate the small intestinal epithelium (Svensson, Marsal et al. 2002). Another study used both de-sensitisation of CCR9 as well as anti-CCL25 antibodies to demonstrate reduced migration of lymphocytes to the small bowel, but not the colon, in both inflamed and uninflamed conditions. They also identified increased expression of CCL25 in the presence of TNFα in the lamina propria of the small intestine, further underlining the importance of the CCR9-CCL25 interaction in CD (Hosoe, Miura et al. 2004).

Overall, we have identified two DC homing markers that are associated with gut-homing; β7 and CCR9. It also seems likely that the relative expression of β7 and CCR9 on circulating DCs and MAd-CAM-1 and CCL25/TECK expression in the intestinal tissue may determine preferential homing to either large or small bowel. These markers controlling anatomical compartmentalisation of the immune response in CD are increasingly being recognised as important therapeutic targets for new therapies, in the form of Vedolizumab (anti-α4β7) and Traficet-EN (anti-CCR9) currently undergoing trials for the treatment of CD.

DCs isolated from patients with skin-related EIMs of CD had increased CCR10, reduced CCR4 and no change in CLA expression. Skin-related EIMs in CD often occur during episodes of active luminal CD, and therefore it has been suggested that primed effector immune cells are being recruited to the skin and away from the gut. CCR10 was traditionally thought of as a skin-homing marker, but more recent evidence suggests that it is a more ‘general’ mucosal homing receptor (Mora and von Andrian 2009), which may be more related to retaining T-cells in the skin after they have reached it, rather than actually guiding their migration (Mora 2008). This may be relevant in skin-related EIMs in CD, which can be refractory to treatment and chronic in their nature.

Other skin-homing markers do exist on DCs (for example CCR8), which were not studied in this section. Furthermore, it is feasible that it is not the DC receptor
that is dysregulated in EIMs, but rather the ligand for that receptor. For example, in PSC (a liver-related EIM of CD), MAd-CAM-1 and CCL25 are upregulated in the liver (Eksteen, Grant et al. 2004) and provide a mechanism by which long-lived populations of memory lymphocytes are able to migrate to and infiltrate the liver, resulting in inflammatory damage. This may also be the case in other EIMs of CD, including the skin.

Overall, it is difficult to draw any substantial conclusions from the EIM data in view of the low patient number recruited for the study. This is a reflection on the rarity of the condition and the rapid nature in which treatment is initiated in this patient group (usually with corticosteroids). Future work could increase patient recruitment in this area, as well as studying any differences between EN and PG in terms of DC phenotype and immune cell homing in other EIMs (eg eyes, joints).

**DC subsets**

When examining homing marker expression on the two DC subsets (mDCs and pDCs), we found consistent differences. mDCs expressed relatively more β7, CLA and CCR9, while pDCs expressed relatively higher levels of the remaining chemokine receptors (CCR4, CCR7 and CCR10). These changes in relative marker expression were independent of the clinical disease phenotype, or indeed in the absence of a disease state. Both mDCs and pDCs have different distributions in vivo, with mDCs being found in both tissues and LN, while pDCs being mainly resident in LN. This may explain why the cell types have different expression of homing markers.

It is also important to note that, the overall changes in homing marker expression between the different cohorts of patients and HC, were seen consistently on both mDC and pDC. For example, in the case of increased expression of CCR9 in patients with active SB CD, increased CCR9 expression was identified on both mDC and pDC groups separately.
Correlation studies

The subject of changes in the immune system with increasing age is currently the source of much interest and research. The progressive deterioration in immune function that accompanies age (immunosenescence), is the result of alteration to both innate and adaptive immunity. Murine studies have demonstrated migration of DCs to secondary lymphoid organs (an essential function for DCs to exert their effect on T-cells) is significantly reduced in ageing. This is the result of both defective CCR7-signalling by mature DCs extracted from old mice compared to young mice and reduced chemotactic efficacy of CCL21 in old mice (Grolleau-Julius, Harning et al. 2008). In addition, recent work carried out in our laboratory has identified reduced CCR7 expression on intestinal DCs with increasing age in a healthy control population [Dr David Bernardo, personal communication].

We found that expression of CLA and CCR4 on circulating mDCs is reduced in patients with active small bowel and active colonic CD respectively, with increasing age. Changes in homing marker expression on circulating DCs may represent one way in which the immune system ‘ages’ with time and further work is needed to confirm these findings.

In the active SB CD group, there were correlations between homing marker expression on DCs and disease severity (as measured by HBI). In addition to CCR9 expression being significantly increased on circulating DCs in patients with active SB CD compared to the other clinical groups, we found that CCR9 expression on circulating mDCs positively correlates with disease severity. CCR9 expression on pDCs is negligible, and this is likely to be masking a statistical correlation between CCR9 expression and disease severity in all DCs in the active SB CD group. These findings appear to underline the importance of CCR9 in playing a central role in the disease process in SB CD. Indeed, figure 23 (section 3.4.3.4) identifies 4 patients with especially high CCR9 expression (>40%) and this correlated with the 4 patients with the highest disease severity (HBI ≥10).
Furthermore, CCR4 expression on circulating pDCs has an inverse correlation with clinical disease severity, indicating a reduced skin homing potential of these cells in the presence of increased inflammation in the SB. We have previously shown that pDCs express relatively greater amounts of CCR4 compared to mDCs (figure 28, section 3.4.4.2).

Finally, there were no correlations seen in the inactive ileocolonic CD group, perhaps expected in view of low disease burden. These findings were mirrored in the HC group, again, perhaps reflecting a return to 'resting state' of DCs in CD once the disease enters clinical remission.

**Tissue specificity of circulating DCs**

One striking observation from the results in this chapter is that circulating DCs in patients with CD display tissue specificity, which generally correlates with the anatomical location of inflammation. DCs originate from haematopoietic stem cells in the bone marrow and enter the blood stream. Fresh circulating DCs express both gut (β7) and skin (CLA) homing molecules and are therefore have the potential to migrate to any tissue and become ‘tissue-specific’ once there (Mann, Bernardo et al. 2012). When they encounter an antigen at the tissue site, they then migrate to the draining LN and present the antigen to the effector cell. Only a tiny minority of DCs will then pass via efferent lymphatics back to the thoracic duct. The vast majority of DCs end their lifecycle following antigen presentation.

Despite this, our results indicate a high level of tissue specificity on circulating DCs in the disease state. In the case of gut homing, this specificity is not just to the gut, but also to different locations within the gut (ie small versus large bowel). There are two possible mechanisms by which this may occur:
It may be that there are systemic changes in circulating plasma that cause or aid the imprinting of tissue specificity on DCs in CD. The cytokine milieu in plasma is the prime example, and it is possible that alterations in cytokine levels in circulating plasma is leading to changes in DC phenotype. An example of this is the function of IL-6 in patients with ulcerative colitis. Work in our laboratory has shown that elevated levels of IL-6 in plasma leads to a skin-homing phenotype on DCs and makes them more efficient at imprinting skin-homing onto T-cells (Bernardo, Vallejo-Diez et al. 2012). In CD, the presence of leptin has been shown to increase expression of CCR7 on intestinal DCs in a dose dependent manner (Al-Hassi, Bernardo et al. 2013).

Furthermore, studies have identified other factors which are important for imprinting homing phenotype on cells. Retinoic acid (RA) can be generated by intestinal DCs by metabolising vitamin A and induces the expression of gut-homing molecules α4β7 and CCR9 on responding T-cells, as well as concomitant suppression of skin-homing molecules E- and P-selectin (Iwata, Hirakiyama et al. 2004). Recent work in our laboratory has shown that, when cultured with different doses of RA, monocyte-derived DC (MoDC) acquire expression of both β7 and CCR9 in a dose-dependent manner, and increased phagocytic capacity (an important characteristic of immature DCs to allow for antigen sampling) (Bernardo, Mann et al. 2013). Vitamin D has also been shown to promote the development of DCs that stimulate T-cells to express skin-homing markers (Sigmundsdottir, Pan et al. 2007).

A further potential explanation for tissue-specificity of circulating DCs in CD centres on the regional draining lymph nodes. In healthy conditions, DCs do not generally recirculate via efferent lymphatics, however, this may not be the case in disease states. In the presence of cancer promoters, large numbers of epidermal Langerhans' cells have been identified in efferent lymphatics, having traversed the regional lymph node (Dandie,
Watkins et al. 1994). The development of ‘leaky lymph nodes’ may also occur in other pathologies, such as CD, and lead to increased recirculation tissue-specific DCs. Further studies would be needed to confirm this.

Another question raised by these results is whether CD occurs because of dysregulated homing of DCs or do DCs have dysregulated homing profiles as a consequence of CD? The latter explanation seems the most plausible, and is supported by several observations:

- Recent work carried out in our laboratory has shown that the microenvironment of the gut (cell-free supernatant obtained following culture of colonic biopsies) can be used to condition fresh human blood DCs to acquire a gut-specific phenotype (Mann, Bernardo et al. 2012). Therefore, in the diseased state, the cell-free microenvironment of the inflamed gut will be polarising DCs towards developing a gut-homing phenotype, encouraging further recruitment of immune cells to the site of inflammation. Further studies conducted in our lab, using patients with active UC, used colonic biopsies from inflamed and non-inflamed areas of colon and cultured in vitro to obtain the cell-free supernatants. Human blood enriched DCs from HC subjects were conditioned with the cell-free supernatants. The results showed that DCs conditioned with non-inflamed culture supernatants acquired a regulatory phenotype with decreased stimulatory capacity, whereas DCs cultured with inflamed biopsy supernatants acquired a pro-inflammatory phenotype with higher levels of stimulatory capacity (Bernardo, Vallejo-Diez et al. 2012). These studies indicate that the inflammatory process in IBD has a direct effect on DC phenotype and function.

- During my work in the laboratory, one HC subject was recruited for research examining blood DC homing phenotype. The results of DC phenotyping showed high levels of CCR9 expression on circulating DCs and almost completely absent CLA expression (a significantly outlying
result). It subsequently transpired that the individual was suffering with viral gastroenteritis at the time the blood was taken, and repeat samples taken 2 weeks following the resolution of the illness showed complete normalisation of these results. These interesting but anecdotal results are shown in figure 29.

Figure 29. Dendritic cell phenotype in an individual with viral gastroenteritis. A) A typical DC phenotype for a healthy individual, showing CLA/β7 expression in a two-parameter histogram (percentage expression values shown) and one-parameter histograms showing CCR9 and CCR7 expression. B) The same DC phenotype data sets obtained from the individual with active viral gastroenteritis. C) DC phenotyping from same individual shown in B, performed 2-weeks following the resolution of viral gastroenteritis symptoms.
Finally, our results showed no differences between patients with inactive ileocolonic CD and HC, in any of the homing markers studied. It seems very likely that patients with active ileocolonic CD would have dysregulation of DC homing phenotype (likely a combination of the active SB and active colonic CD groups from the previous results). This group of patients had inactive disease (HBI≤3) for at least three months prior to recruitment, and it seems that DC phenotypic changes have resolved over that period of time, and returned to a ‘resting state’.
Chapter 4

Effect of infliximab on the phenotype of peripheral blood mononuclear cells
4.1 Abstract

Background
In health, the immune system maintains immune homeostasis in the gut. In inflammatory bowel disease, there is dysregulated immune activity resulting in mucosal inflammation. The inflammatory process may also involve other organs, such as the skin, eye, liver and joints (EIMs). The cause of EIMs is poorly understood. However compartmentalisation of inflammatory processes to specific organs may be linked to abnormalities in immune cell trafficking. Anti-tumour necrosis factor alpha (anti-TNFα) therapies are effective treatments for IBD, but their precise mechanism of action is unclear. Paradoxical skin inflammation (e.g. psoriasis, eczema) has been reported in up to 20% of patients treated with anti-TNFα therapy. Antigen presenting cells and effector cells (T-cells) are involved in tissue homing. In this study, we investigated the in-vitro effect of anti-TNFα on homing marker expression on peripheral blood mononuclear cells from healthy control subjects.

Methods
PBMCs were isolated from fresh blood from 8 healthy volunteers by performing a Ficoll gradient separation and cells were cultured in-vitro (1million PBMC/ml) with and without Infliximab (IFX, 100µg/ml) at 37.0°C for 24hours. Previous dosimetry studies had used 10ng/ml, 1µg/ml, 10µg/ml and 100µg/ml. DC (HLA DR+, CD3-, CD14-, CD16-, CD19-, CD34-), monocytes (CD14+), T-cells (CD3+) and B-cells (CD19+) were identified by flow cytometry after culture. Expression of skin (CLA, CCR4, CCR10), gut (β7, CCR9) and lymph node (CCR7) homing markers were quantified after culture. Paired t-test was applied.

Results
Monocyes cultured with IFX increased CCR7 expression (p=0.023). T-cells increased CCR4 (p=0.028) and decreased β7 (p=0.029) and CCR7 (p=0.011) expression after culture with IFX. There were no significant differences in homing marker expression on DCs or B-cell populations.
Conclusions
Up-regulation of CCR4 and reduced expression of β7 on T-cells incubated with IFX shows the acquisition of a skin-homing profile by T-cells, suggesting a possible mechanism for paradoxical skin inflammation observed in patients treated with anti-TNFα therapy. Increased expression of CCR7 on monocytes following incubation with IFX suggests an increased lymph node homing capacity. Targeting tissue-homing pathways provides a specific approach to IBD therapy.

This abstract was presented at ECCO conference 2012 and subsequently published in JCC journal with the following reference. Request for permission to reprint in Appendix, section 10.3.

Anti-TNFα induces a dysregulated tissue-homing profile on human immune cells in-vitro

Peake ST, Bernardo D, Mann E, Al-Hassi HO, Landy J, Tee CT, Knight SC, Hart AL

JCC 2012; 6(1):S15
4.2 Introduction

4.2.1 Clinical application of infliximab in Crohn’s disease

The development of monoclonal antibodies (mAb) to the TNFα cytokine over the last 15 years has revolutionised the medical treatment of immune diseases, including CD. IFX was the first mAb used for the treatment of patients with CD, approved by the US Food and Drug Administration in 1998 and subsequently introduced to the UK in 1999.

The ability of IFX to induce and maintain remission, heal mucosa, reduce hospital admissions and surgical procedures and restore quality of life in patients with CD has led to the rapid adoption of IFX into clinical practice. It is now a widely used and efficacious treatment for CD and is an important addition to the physician’s medical armory.

Subsequently, there has been great interest in the development of additional mAbs to block TNFα activity and other pro-inflammatory cytokines that play central roles in the activating and perpetuating inflammation in patients with CD. Despite this, IFX remains the drug of choice in the treatment of CD unresponsive to conventional therapies in most IBD units in the UK.

4.2.2 Pharmacokinetic properties of infliximab

IFX is administered intravenously. This enables administration of large volumes of the drug, achieves immediate central distribution (extracellular fluid) and reduces variability in drug levels between patients. The large loading doses and intravenous administration of IFX results high peak concentrations and low trough levels (Fasanmade, Adedokun et al. 2011). The exact mechanism of clearance of IFX from the system is not well understood, but is likely to be via proteolytic catabolism after receptor-mediated endocytosis in cells of the
reticuloendothelial system (Wang, Wang et al. 2008). Table 13 outlines the pharmacokinetic properties of IFX in CD.

<p>| | |</p>
<table>
<thead>
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<tr>
<td>Maximum concentration</td>
<td>118 μg/ml</td>
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<tr>
<td>Half Life</td>
<td>7.7 – 9.5 days</td>
</tr>
<tr>
<td>Time to reach maximum plasma concentration</td>
<td>&lt;1 hour</td>
</tr>
<tr>
<td>Volume of distribution</td>
<td>4.5 – 6 L</td>
</tr>
<tr>
<td>Volume of distribution in central compartment</td>
<td>52.4 ml/kg</td>
</tr>
<tr>
<td>Volume of distribution in peripheral compartment</td>
<td>19.6 ml/kg</td>
</tr>
<tr>
<td>Clearance</td>
<td>5.42 ml/kg/d (15.8ml/hr)</td>
</tr>
</tbody>
</table>

Table 13. Pharmacokinetic properties of infliximab in Crohn’s disease (Ordas, Mould et al. 2012).

4.2.3 Effects of infliximab on immune cells

Our current knowledge of the mechanisms of action of IFX in CD has been detailed previously in the introduction section of this thesis (section 1.3). This subject remains the centre of much interest and ongoing work. To date, TNFα blockade by IFX has been shown to have beneficial effects on epithelial barrier integrity, immune cell apoptosis, mucosal angiogenesis, cytokine production, co-stimulatory molecule expression and induction of cells with a regulatory phenotype.

Despite all this data, there is still much work needed to clarify the modes of action of anti-TNFα agents. One area of potential interest is the effect of these therapies on homing marker expression. To date, the effect of anti-TNFα therapies on homing marker expression on DCs in CD has not been investigated.
4.2.4 Hypothesis and Aims

Hypothesis

We hypothesised that, following culture with IFX, there would be dysregulation of homing markers on circulating PBMCs. This change in homing marker expression may represent one novel mechanism of action of IFX therapy, by directing immune cells away from the site of inflammation.

Aims

- To identify changes in homing marker expression on circulating DCs, monocytes, B-lymphocytes and T-lymphocytes following culture with IFX in vitro.

- To investigate the degree of IFX-induced immune cell cytotoxicity in vitro.
4.3 Methods

In order to investigate these aims, PBMCs were isolated from 8 healthy control subjects as previously described (section 2.3.1).

IFX was stored in manufacturer vials at 4°C and made up as per instructions immediately prior to use, to create a 10ml stock of IFX (100mg/ml). This was further diluted with complete cell medium to create aliquots of the test concentration (100μg/ml). Previous dosimetry experiments had been performed at other concentrations of IFX (10ng/ml, 1μg/ml, 10μg/ml and 100μg/ml).

PBMCs (1 million PBMC/ml) were cultured with IFX and in complete medium alone as a paired internal control for 24hours at 37°C, 5% CO₂, high humidity. Following culture, specific cell populations were identified and surface marker expression was quantified.

Statistical analysis

Statistical analysis was performed with paired t-test. P values of <0.05 were considered statistically significant.
4.4 Results

Different cell populations within the PBMC group express different homing markers. For example, our studies have shown that circulating DCs co-express β7 and CLA (Mann, Bernardo et al. 2012). However, the primary homing markers of interest in monocytes has been identified to be CCR9 and CCR7 (Bernardo, Mann et al. 2013). Therefore, based on previous experience from the laboratory, we have selected a different array of homing markers to be studied in each cell population.
4.4.1 Dendritic cells

DCs were identified by a double gating strategy as described in the methods chapter (figure 14, section 2.3.9.2). Six homing markers were examined, including gut-homing markers β7 and CCR9, skin-homing markers CCR4, CCR10 and CLA and the lymph node homing marker CCR7. All the markers studied had significant expression on DCs in basal conditions; β7 50.6±9.9%; CLA 56.1±11.3%; CCR9 67.0±4.4%; CCR4 20.8±6.3% and CCR7 49.5±9.2%. The skin-homing marker CCR10, was the only exception to this, and was expressed in very low numbers on circulating DCs (1.7±0.5%). There were no significant differences in homing marker expression on DCs following culture with IFX (figure 30).

Figure 30. Expression of homing markers on dendritic cells following 24-hour culture with IFX (100μg/ml).

Statistical analysis performed using paired t-test.
4.4.2 Monocytes

Monocytes were identified by positive staining of CD14 on all PBMCs (figure 31).

![Figure 31. Identification of monocytes in PBMCs. A) Light scatter plot showing region R1 which contains all viable cells. A second histogram is made identifying cells with positive staining for CD14, gated in region R2. B) Histogram displaying the location of CD14+ve cells within the viable cell region by backgating.](image)

Previous work in our laboratory has identified CCR9 and CCR7 as being the primary homing markers in interest when studying monocytes (Bernardo, Mann et al. 2013). However, our results show low levels of CCR9 expression on circulating monocytes (3.9±2.9%). There were no changes in CCR9 expression following IFX culture (figure 32).

There was greater expression of CCR7 on monocytes in basal conditions (13.1±3.8%). CCR7 expression was up-regulated on circulating monocytes following IFX culture (p=0.023), as shown in figure 32.
The increased expression of CCR7 on monocytes following culture with IFX indicates an increased lymph-node homing capacity. To further investigate the cell phenotype, we have examined level of HLA DR expression on CCR7+ monocytes following culture with IFX.

Our results show that, following IFX culture, CCR7+ monocytes have a high expression of HLA DR (see figure 33), and therefore have a high capacity for antigen presentation. It is likely that this population of CD14+CCR7+HLA DR^{high} cells are differentiating into DC-like cells of a myeloid lineage. Despite this, there were no significant changes in the intensity ratio of HLA DR expression on monocytes following culture with IFX.

Figure 32. Expression of CCR7 and CCR9 on monocytes following 24-hour culture with IFX (100μg/ml).

Statistical analysis performed using paired t-test.

Figure 33. HLA DR expression on CCR7+ monocytes following IFX culture. A) Two-parameter histogram showing CCR7 and HLA DR expression on monocytes in basal conditions B) The same staining histogram on monocytes following culture with IFX (100μg/ml) showing 38.3% of monocytes co-express CCR7 and the level of HLA DR expression on these cells is also high.
4.4.3 B lymphocytes

B-cells were identified by positive staining for CD19 on cells within the gated lymphocyte population (figure 34). We examined the expression of two gut-homing markers (β7 and CCR9) and two skin-homing markers (CLA and CCR4) on circulating B-cells following IFX culture.

![Figure 34. Identification of B-cell population in PBMCs. A) Light scatter plot showing region R1, which contains all lymphocytes. A one-parameter histogram is made identifying cells with positive staining for CD19, gated in region R2. B) Histogram displaying the location of CD19+ve cells within the lymphocyte region by backgating.](image-url)
We found high levels of β7 expression on circulating B-cells (81.4 ± 3.2%). However, there was low level of expression of CLA, CCR4 and CCR9 on B-cells in basal conditions with values of 4.1±3.1%, 2.4±0.6% and 2.3±0.7% respectively. There were no significant changes in homing marker expression on B-cells following culture with IFX (figure 35).

Figure 35. Expression of gut and skin homing markers on B-cells following 24-hour culture with IFX (100μg/ml).
Statistical analysis performed using paired t-test.
4.4.4 T lymphocytes

T-cells were identified by positive staining for CD3 on cells within the gated lymphocyte population (figure 36). We examined the expression of the gut-homing marker β7, three skin-homing markers (CLA, CCR4 and CCR10) and the lymph node homing marker CCR7 on circulating T-cells following IFX culture.

Figure 36. Identification of T-cell population in PBMCs. A) Light scatter plot showing region R1, which contains all lymphocytes. A one-parameter histogram is made identifying cells with positive staining for CD3, gated in region R2. B) Histogram displaying the location of CD3+ve cells within the lymphocyte region by backgating.
There was high levels of homing marker expression on circulating T-cells (β7 70.2±2.7%; CLA 12.2±4.4%; CCR4 18.7±3.6% and CCR7 87.9±2.4%). Again, CCR10 was the exception, with low levels of expression on T-cells (6.2±4.5%).

β7 and CCR7 expression were found to be down-regulated following 24-hour culture with IFX (p=0.029 and 0.011 respectively). CCR4 expression was significantly up-regulated on T-cells following IFX culture (p=0.028). These changes in the homing profile of circulating T-cells following 24hr IFX culture, appear to suggest a shift from a gut-homing profile (β7^high CCR4^low) to a skin-homing profile (β7^low CCR4^high).

![Figure 37](image_url). Expression of gut and skin homing markers on T-cells following 24-hour culture with IFX (100μg/ml).

Statistical analysis performed using paired t-test.
4.4.5 Cytotoxicity of infliximab in vitro

In order to confirm whether the changes in homing marker expression following IFX culture are a true finding, we needed to check that the IFX drug was not toxic to cells in vitro, and that cell death was not responsible for our findings by inducing selective apoptosis on different cell populations expressing different homing markers.

This was achieved by calculating and comparing absolute cell numbers in the basal and IFX conditions. Flow-count™ fluorospheres with a known density (depending on the batch) were used as a reference during flow cytometry analysis of human cells for calculation of absolute cell numbers. The fluorosphere counting beads can be identified on a light scatter plot and gating around this event allows us to calculate absolute cell numbers. Figure 38A-C demonstrates this method and also confirms that the presence of counting beads does not affect the results, as the beads are never located within the viable cell region when initial cell gating is performed.

There were no changes in cell counts in basal conditions compared to IFX culture and therefore no significant IFX-associated cell cytotoxicity was detected (see figure 38D).
Figure 38. Calculation of absolute cell numbers of cell populations within PBMC group. A) Identification of monocytes as CD14+ cells in region R2. B) Two-parameter histogram (FL1:FL2) with monocytes backgated onto plot (red) and the fluorosphere counting beads (blue) gated within region R3. C) Backgating of both the monocytes (red), which show up within the viable cell gate, R1, used for experiments, and the counting beads (blue) which are clearly seen outside the viable cell gate and therefore not interfering with results. D) Absolute cell numbers for each cell population in basal conditions and following 24-hour culture with IFX (100μg/ml).

Statistical analysis performed using paired t-test.
4.4.6 Summary of results

- No change in DC or B-cell phenotype post culture with IFX

- Increased expression of CCR7 on monocytes following IFX culture

- Reduced β7 and CCR7 expression and increased CCR4 expression on T-cells following IFX culture
4.5 Discussion

Human circulating DCs display a multi-homing β7+ and CLA+ phenotype. This is not shared by circulating monocytes which have previously been shown to express CCR9 and CCR7 as their main homing markers and virtually no β7 or CLA (Bernardo, Mann et al. 2013). These differences may account for the different tissue distributions of the cell types in vivo. In these experiments, positive expression of β7 is taken to represent α4β7 integrin expression on cells, although this has already been identified as a limitation in the methodology (section 1.2.11).

Despite this, we found low levels of CCR9 expression on the circulating monocyte population. This may reflect on the HC status of the donor subjects and individual variability. The low levels of CCR9 expression on monocytes may also be explained by the 24hr time period that the cells have spent in culture prior to staining and acquisition. Studies which use monocyte-derived DCs have shown that during the 5-day cell culture phase required to produce DCs, monocytes gradually lose homing expression (see section 5.2.1, figure 39) (Bernardo, Mann et al. 2013). Therefore, it may be that the monocytes have down regulated CCR9 expression during the period of time spent in cell culture in the absence of external stimuli.

Our results showed up regulation of CCR7 on monocytes following 24-hour culture with IFX, indicating an increased lymph node homing capacity. Retention of APCs in the LN and tissue is an essential factor to maintaining immune homeostasis. Although the homeostatic/tolerogenic status of the monocytes was not studied, it is likely that they express a ‘regulatory’ phenotype in view of the fact that they were isolated from HC subjects. If this were found to be the case, the increased capacity for monocytes to migrate to the LN following culture with IFX may represent one way in which IFX acts. These findings would need to be confirmed in a larger study, including monocyte phenotype and functional studies in both HC and active CD.
We identified a significant change in T-cell phenotype following culture with IFX for 24 hours. CCR7 expression was down regulated, and therefore T-cells have a reduced LN homing capacity following IFX culture. This may serve to direct naïve T-cells away from the LN and therefore avoid exposure to antigen presenting cells and subsequent stimulation and proliferation of T-cells.

Furthermore, T-cells down regulated their gut homing potential (reduced expression of β7) and up regulated skin-homing affinity (increased CCR4 expression) following IFX culture. This appears to represent an overall change in T-cell migration, away from the gut and towards the skin. These findings are interesting and may represent a novel mechanism by which the phenomenon of paradoxical inflammation (PI), seen in some patients with CD treated with anti-TNFα therapy, occurs.

PI is a poorly understood process, although there have been several possible mechanisms described in the literature by which it may occur, including a genetic predisposition, autoimmune factors, effect of TNFα blockade on IFNα production by pDCs in the skin and the role of other cytokines. The effect of IFX on immune cell homing has not been previously studied with respect to PI. Future larger studies would need to identify the cellular and molecular mechanisms through which IFX is having its effect on T-cells. It is unclear whether the effect of IFX on T-cells is a direct action or via a different primary responding cell. It would also be interesting to examine the effect of IFX on T-cells in the in vivo setting, using patients who develop PI while on anti-TNFα therapy. Indeed, this approach could be expanded to evaluate the evolution of homing marker expression in time in different phenotypes of CD, as well as the effects of different therapies on homing marker expression.

We did not see any changes on DC or B-cell phenotype following IFX culture. In this chapter, these cell populations were examined in the setting of PBMCs. In the next chapters, we set out to enrich DCs in fresh blood and examine the effect of IFX on the phenotype and function of low density cells.
Chapter 5

Effect of infliximab on the phenotype, intracellular cytokine production and cytokine profile of low density cells in Crohn’s disease and healthy controls.
5.1 Abstract

Introduction
DCs play a key role in discriminating between commensal microorganisms and potentially harmful pathogens. DC phenotype and cytokine production determine the type of immune response (immunity/tolerance) elicited by T-cells following antigen presentation. DCs also direct the T-cells to target tissues to perform their function via imprinting tissue-specific homing markers. In CD, dysregulation of the immune response to gut microbiota & aberrant immune cell trafficking play a central role in disease pathogenesis. IFX is a widely used and effective treatment for CD, but its mechanism of action is unclear.

In this study, we investigated the *in-vitro* effect of IFX on phenotype and ongoing cytokine production of human blood-enriched DCs from patients with active CD and HC.

Methods
Low density cells (LDCs), which are enriched for DCs, were obtained following Ficoll and Nycoprep gradient separation of blood obtained from patients with active ileocolonic CD (HBI ≥7) and HC. LDCs were cultured (0.5x10^6 cells/ml) with IFX (1μg/ml, 10μg/ml, 100μg/ml & basal) for 24 hours. Activation marker (CD40, CD80, HLA DR), TLR receptor (TLR2 and TLR4) and homing marker (CCR4, CCR5, CCR7, CCR8, CCR9, CCR10 and β7) expression was quantified by flow cytometry. Natural ongoing intracellular cytokine production (TNFα, TGFβ and IL-6, IL-10, IL-12 and IL-15) was assessed via intracellular staining and flow cytometry. Cytokine secretion was measured on cell-free culture supernatants via Multiplex.

Unpaired t-test and one-way ANOVA statistical analysis with Dunnett’s multiple comparison tests were applied.
Results
TNFα and IL-6 were increased in culture supernatants from patient with active CD (p=0.031 and 0.033 respectively) although their intracellular ongoing cytokine production was decreased (p=0.004 and 0.006 respectively). LDCs from CD had increased β7 (gut-homing integrin) expression compared to HC (p=0.032).

Following IFX culture, LDCs decreased β7 expression (p<0.05) and reduced CCR9 intensity ratio (p<0.01). There was a trend towards reduction in TLR2 and 4 expression in CD. IL-12 production by LDCs from HC was increased following IFX culture (p<0.01). There was a marked reduction in TNFα in cell supernatant following culture with IFX (p<0.01) and a reduction in IL-2 at the highest IFX concentration in HC (p<0.05).

Conclusions
Increased TNFα and IL-6 in culture supernatants from CD patients coupled with a decrease in ongoing production by DC may suggest a negative cytokine feedback system. Reduced expression of β7 and CCR9 and elevated production of IL-12 in LDCs cultured with IFX, shows reduced affinity for gut-homing and increased immunogenicity and may suggest a possible mechanism for IFX-induced paradoxical inflammation. The most dramatic effect of IFX was a reduction in TNFα in the supernatant, which is likely to represent neutralisation of the TNFα cytokine.

This abstract was presented at BSG and ECCO conferences 2013 and subsequently published in Gut and JCC journals with the following references. Permissions to reprint in Appendix, section 10.2 and 10.3 respectively.

Unravelling the mechanism of action of infliximab in Crohn's disease and healthy controls following in vitro culture with blood-enriched dendritic cells
Peake ST, Bernardo D, Mann ER, Landy J, Al-Hassi HO, Knight SC, Hart AL
Gut 2013; 62: A254
JCC 2013; 7(1): S140-141
5.2 Introduction

In the previous chapter, we have examined the effect of IFX on the phenotype of several cell populations in vitro. We found no changes in the phenotype of DCs following culture with IFX. However, that work had been carried out by identifying DCs from PBMCs as HLA DR +ve and lineage cocktail –ve cells. In order to specifically investigate the effect of IFX on DCs in vitro, we isolated and purified DCs from peripheral blood (low density cells, LDCs), and subjected this cell population to in vitro IFX culture.

5.2.1 Low density cells

Most studies to date investigating DCs have used DCs derived from either murine bone marrow or human monocyte-derived DC (MoDC). The latter are generated by in vitro culture of purified CD14+ peripheral blood monocytes for several days in the presence of GM-CSF and IL-4 at supra-physiological conditions. This drives DC differentiation, with up-regulation of HLA DR and down regulation of CD14. However, due to prolonged period of time in vitro, the cells lose homing marker expression (figure 39), likely due to the lack of ongoing exposure to tissue microenvironment required to condition DCs (Bernardo, Mann et al. 2013). In view of this, the use of MoDC to study DC homing markers and phenotype would likely yield inaccurate data.
Figure 39. Effect of differentiation of monocytes into MoDC on homing marker expression. Graphs showing the effect of prolonged culture (5 days) on MoDC homing marker expression in the absence of external stimuli (Bernardo, Mann et al. 2013).

In order to study homing marker expression, we have used LDCs as our in vitro 'cell model' for human peripheral blood DCs. Despite the fact LDCs do not provide as pure a cell population as MoDC, because they have been in culture for a much shorter period of time, they provide a more physiological model to accurately study the effects of IFX on phenotype and function of DCs.

LDCs are obtained from PBMCs, which initially undergo a period of in vitro culture, allowing immature DCs (small cells that resemble lymphoid cells) to acquire increased density buoyancy and monocyte-like morphology. This period of in vitro culture also allows for depletion of monocytes, as these cells will adhere to the culture flask. DCs from human peripheral blood do also adhere transiently, but become non-adherent within 16 hours (Van Voorhis, Hair et al. 1982), and therefore, following in vitro culture, the non-adherent cell fraction contains a greater proportion of lymphocytes and DCs.

In order to further enrich for DCs, the non-adherent cell fraction is exposed to a Nycoprep gradient. Nycoprep is a hyperosmolar solution that has useful
properties for DC enrichment. Lymphocytes are sensitive to Nycoprep and upon contact with Nycoprep, they undergo significant water loss from the cell cytosol and shrink. This has the effect of making them denser and hence they pass rapidly through the Nycoprep layer to form a pellet for discarding.

Cell analysis has shown the isolated LDC population to be highly enriched for DCs with a high surface expression of HLA DR, CD11c, CD83 and CD86 (Knight, Farrant et al. 1986). Previous work from our laboratory has also shown that the enriched DC population isolated by this method is 98-100% positive for HLA DR expression and has morphological features of DC at both optical and electron microscopy (Holden, Bedford et al. 2008). Although the final cell population is only approximately 80% purified for cells with DC phenotype and morphology, the LDC population offers a ‘physiological’ model for in vitro experimental work with blood DCs, especially when compared with MoDC.

A third method of obtaining human circulating DCs for in vitro work is by enriching circulating blood DCs via flow or magnetic sorting of the different DC populations. As well as being able to obtain ‘pure’ DCs, this method also allows the extraction of DC subpopulations, ie pDCs (CD11c-, CD123+, BDCA2+), type 1 mDCs (CD11c+, CD123-, BDCA2-, CD1c+, CD141-) and type 2 mDCs (CD11c+, CD123-, BDCA2-, CD1c-, CD141+). At the time this work was carried out, our laboratory did not have a flow sorter, and the method of magnetic sorting had not been optimised. Therefore, these approaches were not possible.

5.2.2 Activation and maturation markers on LDCs

As previously discussed, TLRs are one type of pattern recognition receptor. TLRs are proteins expressed either on the cell surface or in the intracellular compartment of cells such as DCs and macrophages. They play a vital role in recognition of specific microbial products and therefore aid antigen acquisition. We have concentrated our studies looking at two cell-surface TLRs; TLR2 (gram
positive bacteria) and TLR4 (gram negative bacteria). Work conducted in our laboratory has shown that, in CD, intestinal DCs isolated from areas of active inflammation express higher levels of TLR2 and TLR4 than uninflamed tissue (Hart, Al-Hassi et al. 2005).

At the time of antigen presentation to naïve T-cells, DCs express the antigen presenting molecule (such as the MHC class II molecule, HLA DR) in addition to co-stimulatory molecules (eg CD40, CD80, CD86). The quality and quantity of these two markers (in addition to cytokines) will determine whether the response of tolerogenic (ie tolerance to food antigens and commensal microbiota) or immunogenic. Immature DC express low levels of these co-stimulatory molecules (Banchereau, Briere et al. 2000), and up-regulation occurs after antigen acquisition in the intestine and during DC migration to the draining LN.

5.2.3 Cytokines in Crohn’s disease

Thirty years ago, two T-helper cell subsets (Th1 and Th2) were described (Mosmann, Cherwinski et al. 2005). Traditionally it was assumed that CD was mediated by Th1 cells, while UC was the result of Th2 cells. Th1 differentiation is mainly driven by IL-12 and IFNγ and Th1 cells produce IFNγ, with their primary role being protection against intracellular microbes (Brand 2009). At the onset of CD, mucosal T-cells appear to mount a typical Th1 response that resembles an acute infectious process, and is lost with progression to late CD (Kugathasan, Saubermann et al. 2007). There are several further observations that implicate Th1 cells as being major mediators of the pathogenesis of CD:
- Macrophages in CD produce increased amounts of IL-12 (Monteleone, Biancone et al. 1997).
- Nuclear extracts from T-cells obtained from inflamed CD tissue contain high levels of Th1 associated transcription factors (STAT4 and T-bet) (Neurath, Weigmann et al. 2002).
- Enhanced production of IL-18 (reinforces Th1 cell polarisation) in actively inflamed CD mucosa (Monteleone, Trapasso et al. 1999).
- T-cells isolated from areas of active CD have high levels of the IL12Rβ2 chain (found with Th1 cells) (Parrello, Monteleone et al. 2000).
- T-cells isolated from areas of active CD express increased amounts of IFNγ and reduced amounts of IL-4 (Th2 cytokine) compared to healthy controls (Fuss, Neurath et al. 1996).

However, it has now become clear that the Th17 pathway also plays a significant role in CD (Annunziato, Cosmi et al. 2007). While Th1 and Th2 differentiation depends on presence of their cytokines for differentiation (IFNγ and IL-4 respectively), polarisation of Th17 cells does not require IL-17. Murine Th17 cells originate from naïve CD4+ T-cells in the presence of IL-6 and TGFβ. Their development is then increased by IL-21 and IL-23 (Bettelli, Carrier et al. 2006). IL-12 and IL-23 share the same p40 subunit, and therefore antibodies have been developed to target the IL-12/IL-23p40 subunit in the hope of down-regulating both Th1 and Th17 pathways simultaneously.

Following contact with foreign antigens, APCs release cytokines resulting in differentiation of T-cells. In addition, pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β, which are predominately released from DCs and macrophages, have a direct role leading to intestinal inflammation in CD. TNFα is produced by both Th1 and Th17 cells in CD (Abraham and Cho 2009).
5.2.4 Hypothesis and Aims

Hypothesis

We hypothesised that, when cultured \textit{in vitro}, IFX would modulate the phenotype and cytokine production of LDC, in a dose-dependent manner.

Aims

- To identify changes in LDC phenotype following culture with IFX, including:
  - Homing marker expression
  - Activation marker expression
  - Maturation marker expression

- To identify changes in ongoing LDC cytokine production following culture with IFX.

- To investigate changes in the cytokine content of the cell-free supernatant solution following culture with IFX.
5.3 Methods

Patients were recruited from IBD outpatient clinics and the endoscopy unit at St Mark’s Hospital between October 2011 and June 2012. Patients had active ileocolonic CD (HBI $\geq 7$), confirmed by endoscopic with or without radiological studies within 6 months or recruitment. Healthy control subjects were recruited in parallel to patient recruitment.

All patients were recruited directly by the candidate to ensure standardisation of patient selection. A Microsoft Excel database was created containing phenotypical data for each patient recruited, including the following parameters:

- Age
- Gender
- Number of years since diagnosis with CD
- Montreal classification of CD
- Disease activity (measured using Harvey Bradshaw Index, HBI)
- Current medications
- Date of most recent endoscopic examination
- Date of most recent radiological examination

Inclusion and exclusion criteria were applied to patient recruitment (outlined in section 2.2.2), to ensure an accuracy of data obtained.

5.3.1 Enrichment of LDCs and preparation of IFX

LDCs, enriched for DCs, were isolated on a Nycoprep gradient from non-adherent cells after overnight culture of PBMCs (described in section 2.3.2).

IFX was prepared from a stock solution at 10mg/ml concentration. Aliquots of the desired test concentrations (1μg/ml, 10μg/ml and 100μg/ml) were made by
dilution of the stock with complete medium. LDCs were then cultured for a further 24 hours in 1ml of complete medium (0.5x10^6 cells/ml) with either a test concentration of IFX or in basal conditions (complete medium alone).

Following culture, cell expression of homing, maturation and activation markers was determined by monoclonal antibody labeling and flow cytometry.

5.3.2 Quantification of ongoing intracellular cytokine production

To quantify ongoing cytokine production by LDCs, cells were incubated for 4 hours with or without monensin. Monensin is a protein transport inhibitor that blocks secretion of proteins by cells via the Golgi apparatus, thereby causing an accumulation of cytokines at the endoplasmic reticulum or Golgi (figure 40). Cytokines being synthesised by LDCs incubated with monensin over the 4-hour period accumulate within the cell, whereas those cells incubated in basal conditions do not retain cytokines. Following the 4 hour culture period, LDCs were fixed and permeabilised to allow for intracellular cytokine staining and flow cytometry. LDCs cultured without monensin were taken as 'control' samples and subtracted from the LDCs cultured with monensin, to give an overall accurate picture of ongoing cytokine production by LDCs over the 4-hour period.

Figure 40. Molecular structure of monensin. Monensin is an inhibitor of intracellular protein transport and was used to allow quantification of ongoing cytokine production by LDCs.
Although technically challenging and time consuming, the major advantage of this method is that it allows quantification of intracellular cytokine production in the absence of external stimuli, which may bias their cytokine profile.

5.3.3 Quantification of cell-free supernatant cytokine profile

The BD CBA Human Th1/Th2/Th17 Cytokine Kit uses bead array technology to simultaneously detect and quantify the concentration (in pg/ml) of multiple cytokine proteins in cell-free supernatant samples.

Seven bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for their specific cytokine (IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ and IL-17a). The seven bead populations are then mixed together to form a bead array, which is analysed in the APC channel of the 6-colour flow cytometer.

5.3.4 Statistical analysis

Statistical analysis of differences between LDCs from patients with active CD compared to HC was performed using unpaired t-test. Analysis of the effect of IFX on LDC phenotype and cytokine profile was performed using and one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison test.

P values of <0.05 were considered statistically significant in all cases.
5.4 Results

5.4.1 Patient characteristics

Five patients with active ileocolonic CD, who met the inclusion and exclusion criteria previously outlined, were identified and recruited to provide blood samples for the study. Six healthy control subjects were recruited. Demographical and clinical data is shown in table 14.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Gender</th>
<th>Age</th>
<th>Years since diagnosis</th>
<th>CD location</th>
<th>HBI</th>
<th>Current medications</th>
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<td>8</td>
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<td>Ileocolonic</td>
<td>12</td>
<td>AZA</td>
</tr>
<tr>
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<td>31</td>
<td>4</td>
<td>Ileocolonic</td>
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<td>AZA</td>
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<td>Ileocolonic</td>
<td>10</td>
<td>AZA</td>
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<td>47</td>
<td>-</td>
<td>HC</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 14. Patient and healthy control characteristics and demographical data.

HC = healthy control, 6-MP = 6-Mercaptopurine, AZA = Azathioprine
5.4.2 Identification and purification of LDCs from blood

Human blood low density mononuclear cells were identified at multi-colour flow cytometry using forward scatter (FSC) and side scatter (SSC) characteristics, as shown in figure 41. This figure also shows the efficacy of Nycoprep in the LDC enrichment process, by selecting the low density cells and excluding the smaller and denser lymphocyte population. Cells falling within the LDC region (region R1) are taken as the antigen presenting cells (APCs).

Figure 41. Nycoprep gradient in the enrichment of LDC population. A) Pre-Nycoprep histogram showing APC population (LDCs) in region R1 and lymphocyte population in region R2. These regions contain 8.7% and 79.5% of events respectively. B) Post-Nycoprep histogram in which the proportion of events within the APC region (R1) has increased to 85.7% and events in the lymphocyte region (R2) has fallen to 1.9%.
LDCs have previously been shown to comprise predominately DCs, but also contain other types of APC as well. Figure 42 below shows HLA DR and CD14 staining of the LDC population. As expected, >90% of cells express HLA DR in both HC and CD, but we also found significant CD14 expression. Despite this, the LDC population shares several properties with DCs including the presence of veiled exteriors (Knight, Farrant et al. 1986) and the capacity to stimulate naïve (CD4+CD45Ra+) T-cells which is not true for monocytes.

Culturing LDCs with IFX did not affect HLA DR or CD14 expression.

Figure 42. HLA DR and CD14 expression on circulating LDCs in HC and patients with active CD.

HC = healthy control, CD = active ileocolonic CD, ns = not significant
Statistical analysis comparing HC and CD groups performed using unpaired t-test. Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.
5.4.3 Activation and maturation markers

To examine the effect of IFX culture on pattern recognition receptors on circulating blood LDCs, we investigated TLR2 and TLR4 expression (figure 43). Although there were no statistically significant differences in TLR2 and 4 expression between HC and patients with active CD, there was a trend towards higher TLR expression in the active CD state. There also appeared to be a stepwise reduction in TLR2 and 4 expression in CD with increasing doses of IFX. However, this did not reach statistical significance.

![Figure 43. TLR2 and 4 expression by circulating LDC in HC and patients with active ileocolonic CD and effect of IFX culture on expression.](image)

HC = healthy control, CD = active ileocolonic CD, ns = not significant

Statistical analysis comparing HC and CD groups performed using unpaired t-test.
Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.

HLA DR expression on APCs (including LDCs) correlates with the cell activation potential. Over 90% of the LDC population express HLA DR, so in order to detect changes in HLA DR expression in HC and CD and the effect of IFX, we assessed the intensity ratio of staining on HLA DR+ cells. There were no differences seen in the intensity ratio of HLA DR expression between HC and CD, or with the addition of IFX to the cell culture (figure 44). Similarly, there were no changes in the expression of the co-stimulatory molecules CD40 and CD80.
5.4.4 Homing marker expression

In order to investigate the expression of gut-homing markers on circulating LDCs and the effect of IFX culture, CCR9 and β7 were examined (figure 45).

The expression of the β7 integrin was increased on LDCs isolated from patients with active ileocolonic CD compared to HC (p=0.032). This correlates with findings in the first results chapter, where β7 expression was increased on circulating DCs from patients with active colonic CD compared to HC. A reduction
in β7 expression was seen on CD LDCs cultured with the greatest concentration of IFX (p<0.05). There were no differences in the intensity ratio of β7 expression.

There was a trend towards greater expression of CCR9 on LDCs from patients with active CD compared to HC, but this did not reach statistical significance. Following culture with IFX, the intensity ratio of CCR9 expression was reduced in both the 10μg/ml (p<0.05) and 100μg/ml (p<0.01) concentrations.

**Figure 45.** Expression of gut homing markers on circulating LDCs in HC and patients with active ileocolonic CD and effect of IFX culture on expression. A) β7 expression on LDCs in terms of percentage expression and intensity ratio of expression. B) CCR9 expression on LDCs in terms of percentage expression and intensity ratio of expression.

*HC = healthy control, CD = active ileocolonic CD, ns = not significant
In basal conditions:  * = p<0.05
In IFX conditions:  * = p<0.05 when compared to basal condition
  ** = p<0.01 when compared to basal condition

Statistical analysis comparing HC and CD groups performed using unpaired t-test. Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.
The expression of skin-homing markers on LDCs, and effect of IFX on this expression, was examined by investigating CCR4, CCR8 and CCR10 chemokine receptors (figure 46). We found relatively high expression of all 3 skin homing markers on circulating LDCs. There were no differences in expression between HC and CD and no differences seen following culture with IFX.

Figure 46. Expression of skin homing markers on circulating LDCs in HC and patients with active ileocolonic CD and effect of IFX culture on expression.

HC = healthy control, CD = active ileocolonic CD, ns = not significant

Statistical analysis comparing HC and CD groups performed using unpaired t-test.
Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.
The final two homing markers examined were CCR7 (lymph node homing) and CCR5 (directs homing to sites of inflammation), shown in figure 47. There were no differences in expression of these markers in LDCs from active CD or HC or following culture with IFX.

Figure 47. Expression of CCR7 and CCR5 on circulating LDCs in HC and patients with active ileocolonic CD and effect of IFX culture on expression.

HC = healthy control, CD = active ileocolonic CD, ns = not significant

Statistical analysis comparing HC and CD groups performed using unpaired t-test. Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.
5.4.5 **Ongoing intracellular cytokine production**

Due to limitations in the number of LDCs available for experimental work, six pathologically relevant cytokines were selected for the study (figure 48).

Circulating LDCs isolated from patients with active ileocolonic CD had reduced ongoing production of TNFα (p=0.0043) and IL-6 (p=0.0066) compared to HC in basal conditions. There were no changes in the ongoing production of these cytokines when IFX was introduced into the cell culture.

The production of the Th1-differentiating cytokine, IL-12, was increased in LDCs from HC following the addition of IFX to the cell culture (1μg/ml IFX, p<0.01; 10μg/ml, p<0.01; 100μg/ml, p<0.05). There were no changes in LDCs isolated from patients with active CD.

There were no statistically significant differences in production of TGFβ, IL-10 or IL-15 between HC and CD or following the addition to IFX to the cell culture.
Figure 48. Cytokine production by LDCs from HC and patients with active CD and the effect of IFX.

HC = healthy control, CD = active ileocolonic CD, ns = not significant

In basal conditions:  * = p<0.05, ** = p<0.01

In IFX conditions:  * = p<0.05 when compared to basal condition
                                ** = p<0.01 when compared to basal condition

Statistical analysis comparing HC and CD groups performed using unpaired t-test. Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.
5.4.6 Cytokine profile in cell-free supernatant

In addition to assessing ongoing LDC intracellular cytokine production, we also quantified the presence of cytokines in the cell-free supernatant following cell culture with and without IFX. In order to do this, we used a Th1/Th2/Th17 Multiplex kit, which included TNFα, IFNγ, IL-2, IL-4, IL-6, IL-10 and IL-17a (figure 49).

![Cytokine profile graphs](image)

**Figure 49.** Cytokine concentrations in cell-free supernatant samples following culture of LDCs with and without IFX.

Detection limits (pg/ml): IL-2=6.0, TNFα=5.2, IL-6=4.8, IFNγ=4.1, IL17a=3.0, IL-4=6.6, IL-10=3.9

HC = healthy control, CD = active ileocolonic CD, ns = not significant

Statistical analysis comparing HC and CD groups performed using unpaired t-test.
Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.
We had previously found ongoing TNFα and IL-6 production by LDCs to be lower when isolated from patients with active CD compared to HC (figure 48). However, when the same cytokines were studied in the cell-free culture supernatants we found significantly increased cytokine levels in the LDC cultures obtained from patients with active ileocolonic CD compared to HC (p=0.031 and p=0.033 respectively). Having identified these differences between LDCs from HC and CD, we moved onto examine the effect of IFX on LDC cytokine profiles in HC and CD.

The concentration of free TNFα is reduced with increasing concentration of IFX in the cell culture (1μg/ml p<0.05 with p<0.01 with 10 and 100μg/ml concentrations of IFX). A similar trend towards reduced concentration of free IL-6 in the supernatant with increasing amounts of IFX added is also seen, but this did not reach statistical significance.

There were no differences seen in concentrations of IL-4, IL-10 or IFNγ when comparing CD to HC or with the addition of IFX to the cell culture. However, there was a reduction in free IL-2 concentration in the cell culture samples from HC LDCs at the highest concentration of IFX (p<0.05).

5.4.7 Correlation studies

We set out to examine whether there was any correlation between LDC properties (including surface marker expression, ongoing cytokine production and cytokine secretion) and the demographical and/or clinical data collected on the patients and HC.

Surface marker correlation data in patients with active ileocolonic CD

We found a positive correlation between the IR of HLA DR expression on circulating LDCs and percentage expression of β7 with disease severity (measured by the HBI) in patients with active ileocolonic CD. Full correlation data is shown in table 15.
<table>
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<th>Clinical parameter</th>
<th>Age</th>
<th>Years since diagnosis</th>
<th>HBI</th>
<th>Gender:</th>
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<td>Homing marker</td>
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<td></td>
<td></td>
<td>% expression (mean ± 5D)</td>
</tr>
<tr>
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<td>male; female; &lt;sup&gt;p value&lt;/sup&gt;</td>
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<td>r = +0.24&lt;sub&gt;ns&lt;/sub&gt;</td>
<td></td>
<td>r = +0.14&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r = -0.27&lt;sub&gt;ns&lt;/sub&gt;</td>
<td></td>
<td>34.8±29.3; 23.9±21.3; &lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD40</td>
<td></td>
<td>r = -0.32&lt;sub&gt;ns&lt;/sub&gt;</td>
<td></td>
<td>r = -0.08&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r = +0.14&lt;sub&gt;ns&lt;/sub&gt;</td>
<td></td>
<td>68.9±13.7; 72.7±3.3; &lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD80</td>
<td></td>
<td>r = +0.21&lt;sub&gt;ns&lt;/sub&gt;</td>
<td></td>
<td>r = -0.60&lt;sub&gt;ns&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>r = +0.59&lt;sub&gt;ns&lt;/sub&gt;</td>
<td></td>
<td>13.4±1.9; 30.0±13.0; &lt;sup&gt;ns&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 15. Correlation studies between low density cell surface marker expression and clinical parameters in patients with active ileocolonic CD. Statistical analysis of age, years since diagnosis and HBI was performed using Pearsons correlation. Correlations in gender were performed using unpaired t-test.
Surface marker correlation data in healthy control subjects

No correlations were seen between surface marker expression and the age and gender of HC subjects, as shown in table 16.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age</th>
<th>Gender: % expression (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homing marker</td>
<td>r value x value</td>
<td>male; female; p value</td>
</tr>
<tr>
<td>HLA DR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- IR</td>
<td>r = -0.59</td>
<td>31.2±11.6; 33.2±26.0; ns</td>
</tr>
<tr>
<td>Beta7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = +0.01</td>
<td>53.0±6.1; 48.7±9.3; ns</td>
</tr>
<tr>
<td>- IR</td>
<td>r = -0.54</td>
<td>1.8±0.3; 2.1±0.4; ns</td>
</tr>
<tr>
<td>CCR9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = -0.66</td>
<td>69.4±5.9; 63.1±12.2; ns</td>
</tr>
<tr>
<td>- IR</td>
<td>r = -0.32</td>
<td>4.9±0.7; 5.2±0.8; ns</td>
</tr>
<tr>
<td>CCR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = -0.63</td>
<td>61.2±2.0; 53.7±2.5; ns</td>
</tr>
<tr>
<td>CCR8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = +0.11</td>
<td>85.6±3.5; 63.9±26.8; ns</td>
</tr>
<tr>
<td>CCR10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = -0.36</td>
<td>47.4±10.0; 25.0±4.7; ns</td>
</tr>
<tr>
<td>CCR5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = +0.52</td>
<td>24.0±16.1; 54.8±5.6; ns</td>
</tr>
<tr>
<td>CCR7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = -0.02</td>
<td>59.4±6.9; 63.2±8.6; ns</td>
</tr>
<tr>
<td>TLR2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>TLR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CD40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = -0.37</td>
<td>70.9±7.9; 71.6±7.0; ns</td>
</tr>
<tr>
<td>CD80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- %</td>
<td>r = +0.66</td>
<td>35.9±20.2; 56.4±18.5; ns</td>
</tr>
</tbody>
</table>

Table 16. Correlation studies between low density cell surface marker expression and clinical parameters in healthy control subjects. Statistical analysis of age was performed using Pearsons correlation. Correlations in gender were performed using unpaired t-test.

n/a = expression of surface marker falls below the detection limit in >80% of data points.
Ongoing intracellular cytokine production correlation data in patients with active ileocolonic CD

We found that the amount of ongoing production of IL-6 by LDCs positively correlated with disease severity and HBI in patients with active CD. There was a negative correlation between ongoing production of TGFβ and the age of CD patients recruited (table 17).

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age</th>
<th>Years since diagnosis</th>
<th>HBI</th>
<th>Gender: % expression (mean ± SD)</th>
<th>male; female; p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine ↓</td>
<td>(r value ± p value)</td>
<td>(r value ± p value)</td>
<td>(r value ± p value)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>r = -0.70 **</td>
<td>r = -0.51 **</td>
<td>r = +0.53 **</td>
<td>62.1±2.2; 65.3±3.0; ns</td>
<td></td>
</tr>
<tr>
<td>TGFβ</td>
<td>r = -0.94 **ns</td>
<td>r = -0.67 **</td>
<td>r = +0.27 **</td>
<td>55.6±8.3; 56.5±6.1; ns</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>r = -0.33 **</td>
<td>r = -0.38 **</td>
<td>r = +0.89 **0.04</td>
<td>62.6±2.1; 60.9±4.2; ns</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>r = +0.37 **</td>
<td>r = +0.57 **</td>
<td>r = -0.37 **</td>
<td>52.9±31.0; 69.0±4.8; ns</td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>r = -0.67 **</td>
<td>r = -0.30 **</td>
<td>r = -0.41 **</td>
<td>57.3±4.9; 66.8±15.1; ns</td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td>r = +0.12 **</td>
<td>r = -0.36 **</td>
<td>r = -0.24 **</td>
<td>68.9±11.0; 71.3±7.0; ns</td>
<td></td>
</tr>
</tbody>
</table>

Table 17. Correlation studies between ongoing intracellular cytokine production by low density cells and clinical parameters in patients with active ileocolonic CD. Statistical analysis of age, years since diagnosis and HBI was performed using Pearson’s correlation. Correlations in gender were performed using unpaired t-test.
Ongoing intracellular cytokine production correlation data in healthy control subjects

No correlations were seen between ongoing cytokine production by circulating LDCs from HC subjects and age and gender of those subjects, as displayed in table 18.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age</th>
<th>Gender:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine</td>
<td>(r value ± value)</td>
<td>% expression (mean ± SD)</td>
</tr>
<tr>
<td>TNFα</td>
<td>r = -0.13</td>
<td>83.1±2.8; 72.6±6.9; ns</td>
</tr>
<tr>
<td>TGFβ</td>
<td>r = -0.03</td>
<td>52.7±9.3; 56.2±3.1; ns</td>
</tr>
<tr>
<td>IL-6</td>
<td>r = +0.04</td>
<td>83.4±4.5; 75.4±3.6; ns</td>
</tr>
<tr>
<td>IL-10</td>
<td>r = +0.67</td>
<td>74.5±5.0; 81.3±2.7; ns</td>
</tr>
<tr>
<td>IL-12</td>
<td>r = -0.48</td>
<td>50.7±10.4; 49.3±22.7; ns</td>
</tr>
<tr>
<td>IL-15</td>
<td>r = +0.09</td>
<td>82.3±3.7; 72.6±7.5; ns</td>
</tr>
</tbody>
</table>

Table 18. Correlation studies between ongoing intracellular cytokine production by low density cells and clinical parameters in healthy control subjects. Statistical analysis of age was performed using Pearson's correlation. Correlations in gender were performed using unpaired t-test.
Cell-free supernatant cytokine concentration correlation data in patients with active ileocolonic CD

There were no correlations between the cell-free cytokine concentrations following culture of LDCs from patients with active CD and patient demographical and clinical parameters, as shown in table 19. The concentration of three of the studied cytokines fell below detection limits (IFNγ, IL-4 and IL-17a) and therefore correlation analysis on these cytokines was not performed.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age</th>
<th>Years since diagnosis</th>
<th>HBI</th>
<th>Gender: concentration, pg/ml (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine ↓</td>
<td>(r value ± p value)</td>
<td>(r value ± p value)</td>
<td>(r value ± p value)</td>
<td>male; female; p value</td>
</tr>
<tr>
<td>TNFα</td>
<td>r = +0.17 ns</td>
<td>r = +0.23 ns</td>
<td>r = -0.07 ns</td>
<td>12.9±1.2; 14.3±4.6; ns</td>
</tr>
<tr>
<td>IFNγ</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>IL-2</td>
<td>r = +0.34 ns</td>
<td>r = +0.76 ns</td>
<td>r = +0.51 ns</td>
<td>6.7±0.7; 20.4±13.3; ns</td>
</tr>
<tr>
<td>IL-4</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>IL-6</td>
<td>r = +0.38 ns</td>
<td>r = +0.45 ns</td>
<td>r = +0.27 ns</td>
<td>39.1±2.4; 28.9±10.1; ns</td>
</tr>
<tr>
<td>IL-10</td>
<td>r = +0.30 ns</td>
<td>r = -0.84 ns</td>
<td>r = +0.44 ns</td>
<td>6.4±0.3; 7.6±1.2; ns</td>
</tr>
<tr>
<td>IL-17a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 19. Correlation studies between cell-free supernatant cytokine concentration and clinical parameters in patients with active ileocolonic Crohn’s disease. Statistical analysis of age, years since diagnosis and HBI was performed using Pearson's correlation. Correlations in gender were performed using unpaired t-test. n/a = Cytokine concentration falls below the detection limit in >80% of data points.
Cell-free supernatant cytokine concentration correlation data in healthy control subjects

There were no correlations between the cell-free cytokine concentrations following culture of LDCs from HC and age/gender of HC, as shown in table 20. Again, the concentration of IL-4 and IL-17a fell below detection limits and therefore correlation analysis on these cytokines was not performed.

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Age</th>
<th>Gender: concentration, pg/ml (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(r value ( ^{2\text{metry}} ))</td>
<td>male; female; p value</td>
</tr>
<tr>
<td>Cytokine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>( r = +0.40^{\text{ns}} )</td>
<td>7.4±1.3; 6.7±1.5; ns</td>
</tr>
<tr>
<td>IFNγ</td>
<td>( r = -0.40^{\text{ns}} )</td>
<td>4.7±0.3; 6.0±1.9; ns</td>
</tr>
<tr>
<td>IL-2</td>
<td>( r = +0.60^{\text{ns}} )</td>
<td>18.6±6.7; 30.1±22.3; ns</td>
</tr>
<tr>
<td>IL-4</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>IL-6</td>
<td>( r = -0.19^{\text{ns}} )</td>
<td>15.8±4.3; 8.8±1.1; ns</td>
</tr>
<tr>
<td>IL-10</td>
<td>( r = -0.47^{\text{ns}} )</td>
<td>10.6±2.3; 10.4±3.4; ns</td>
</tr>
<tr>
<td>IL-17a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 20. Correlation studies between cell-free supernatant cytokine concentration and clinical parameters in healthy control subjects. Statistical analysis of age, years since diagnosis and HBI was performed using Pearsons correlation. Correlations in gender were performed using unpaired t-test. 

\( \text{n/a = Cytokine concentration falls below the detection limit in >80\% of data points.} \)
5.4.8 Summary of results

Differences between LDCs from patients with active CD compared to HC:
- Increased $\beta 7$ expression on LDCs from patients with active CD
- Reduced ongoing production of TNF$\alpha$ and IL-6 in LDCs isolated from patients with active CD, but increased levels of the same cytokines (TNF$\alpha$ and IL-6) in the cell-free culture supernatant.

Effect of IFX on LDC phenotype:
- Reduced gut-homing affinity of LDCs from patients with active ileocolonic CD when cultured with IFX, as shown by reduction in $\beta 7$ expression and reduction in intensity ratio of CCR9 on positive cells.
- Trend towards reduced TLR2 and 4 expression on LDCs cultured with IFX in a dose-dependent fashion (p=ns).

Effect of IFX on ongoing cytokine production and secretion:
- Increased production of IL-12 by LDC (HC) cultured with IFX.
- Reduction of TNF$\alpha$ (CD and HC) and IL-2 (HC) in the cell-free supernatant when cultured with IFX. Possible trend towards reduced IL-6 (CD) in supernatant with IFX (p=ns).

Correlation studies
- $\beta 7$ expression, IR of HLA DR and ongoing production of IL-6 on LDCs from patients with active ileocolonic CD all display positive correlation with disease severity (as measured by HBI).
- Negative correlation between ongoing production of TGF$\beta$ and the age of patients with active CD.
5.5 Discussion

The study of human DCs is difficult due to low numbers of DCs in human samples and the absence of a single marker on DCs to allow for identification. In order to study the effect of IFX on the phenotype and cytokine profile of circulating DCs in CD and HC in vitro, our first challenge was to obtain a cell population which would accurately represent DCs in human blood. As previously discussed, our laboratory does not have access to a flow sorter and magnetic sorting was not possible due to lack of optimisation of the methods involved in this process. Other studies on blood DCs have utilised MoDCs, however this approach to study homing marker expression would yield inaccurate results, due to homing marker depletion that occurs during the 5-day culture required to produce MoDC (Bernardo, Mann et al. 2013).

LDCs are highly enriched for DCs, with the majority of the cell population displaying DC phenotype (HLA DR+, CD11c+, CD83+, CD86+) and morphology at both optical and electron microscopy (Knight, Farrant et al. 1986). Isolating LDCs from human blood does involve a period of culture (24 hours) and therefore LDCs are more mature than DCs which have been identified as part of a PBMC population. Despite this, the LDC population enables us to perform in vitro experiments with a physiologically representative DC model.

Differences between LDC phenotype and cytokine profile from patients active CD and HC

LDCs from patients with active CD had elevated β7 expression compared to HC. In these experiments, positive expression of β7 is taken to represent α4β7 integrin expression on cells, although this has already been identified as a limitation in the methodology (section 1.2.11). There was also a trend towards increased CCR9 expression in CD, but this did not reach statistical significance. This is consistent with the results from chapter 3, in which circulating DCs isolated from patients with active colonic CD had increased β7 expression (figure
those from patients with active SB CD had increased CCR9 expression (figure 23, section 3.4.3.4) compared to HC. This indicates that the DC enrichment protocol has worked as the phenotype of LDCs from patients with active CD appears to mirror the phenotype of fresh circulating DCs.

The patients with active CD recruited for this part of the study all had ileocolonic disease, hence up regulation of both β7 and CCR9. Unfortunately, the relative anatomical burden of disease (small bowel versus colonic) in each subject at the time of recruitment was not measurable. It is possible that this patient cohort had increased colonic inflammation compared to SB inflammation, which may account for the relatively increased ratio of β7:CCR9 expression compared to HC, but this is impossible to know.

There was a trend towards increased expression of both TLR2 and TLR4 on LDCs from patients with active CD compared to HC. This did not reach statistical significance, although this is likely due to small sample size. The upregulation of TLR expression in active CD has previously been demonstrated in intestinal DC isolated from patients with active CD (Hart, Al-Hassi et al. 2005), likely leading to increased recognition of bacterial products and an enhanced response to them. Conversely, CD80 expression and the IR of HLA DR appeared to be reduced on LDCs from patients with active CD compared to HC (p=ns). Although surprising, this finding may be a reflection on the active recruitment of more mature circulating LDCs to the intestinal sites of inflammation in CD, leaving the immature LDCs in the circulation. This hypothesis could be further tested by performing the same phenotypic studies on paired blood and tissue (gut) samples from patients with active CD.

Upregulation of TLR expression on LDCs in CD may be secondary to the presence of inflammatory factors in the diseased state. Studies have shown that the presence of pro-inflammatory cytokines such as TNFα, IL-6 and IFNγ can cause increased expression of TLR2 and 4 (Martins, Kallas et al. 2003). In addition, bacterial products may act directly via TLRs to upregulate further TLR
expression on DCs, as previously demonstrated in patients with UC (Hausmann, Kiessling et al. 2002). Increased levels of intestinal permeability in CD may lead to greater exposure of DCs to microbial products and the subsequent bacterially-driven increased TLR expression.

LDCs isolated from patients with active CD had reduced ongoing production of TNFα and IL-6 compared to those from HC, but that the levels of the same two cytokines were increased in the cell-free supernatant. These two cytokines are integral in the pathogenesis of CD (Th1 and Th17 pathways respectively). The relationship between these two cytokines in terms of ongoing production versus content in the supernatant is an interesting finding and may be the result of two possible explanations.

- Firstly, the method in which ongoing production of the cytokines is quantified may be relevant. The monensin method is a way of assessing ongoing intracellular cytokine production in the absence of external stimuli. The benefit of this is that no additional stimuli or molecules need to be added to the cells in order to obtain results, and therefore the results are not affected by any other extrinsic factor(s). However, the ongoing production of cytokines is measured after 24 hours of incubation, and therefore you obtain a 4 hour ‘snapshot’ of intracellular cytokine production after a substantial period of cell culture. The cells have been producing cytokines throughout that whole period, and this likely explains the elevated levels of TNFα and IL-6 in the CD supernatants. As a result of the culture period, it may be that the stimulating factors resulting in the pro-inflammatory LDC activity are reducing and therefore, the ongoing production of these cytokines is subsequently falling. Future experiments may address this hypothesis by repeated assessments of ongoing intracellular cytokine production and cell-free supernatant cytokine concentrations at multiple time points during the cell culture process (for example 2hrs, 4hrs, 6hrs etc until 24hrs post-initiation of cell culture).
• Another possibility may be a negative feedback mechanism whereby the elevated extracellular levels of TNFα and IL-6 are exerting a negative feedback on their own ongoing intracellular production. One study has shown that NKT-produced Th1/Th2 cytokine balance to be maintained under the control of a negative feedback loop through DCs, with Th1 cytokine-pretreated DCs mainly inducing Th2 cytokine production and Th2 cytokine-pretreated DCs inducing Th1 cytokine production by stimulated NKT-cells (Onoe, Yanagawa et al. 2007). This type of negative feedback loop may be amplified in *in vitro* conditions due to the physical restrictions placed on the cells (ie the cells remain in 1ml volume throughout the culture period). Further work would be necessary to ascertain if this is the case.

**Effect of IFX on LDC phenotype**

Following 24 hours of *in vitro* LDC culture with the various test doses of IFX, there were significant changes in the phenotype of the LDCs. There was a stepwise reduction in expression of β7 as well as a similar reduction in the intensity ratio of CCR9 expression on positive cells. Changes in β7 expression and CCR9 IR were only seen in LDC from patients with active CD, apart from the maximum concentration of IFX, where we saw reduced CCR9 IR in LDCs from HC as well. This effect was not seen in the previous chapter when culturing PBMCs with IFX and assessing homing marker expression on a gated (HLA DR+, lineage cocktail-) DC population, and may be a reflection on LDCs comprising a different population of APCs than DCs isolated as part of PBMC population.

The significance of this change in homing marker expression is a reduced gut-homing affinity of LDCs from patients with active CD following culture with IFX, and the impression that the phenotype of these LDCs is becoming more ‘normal’, ie returning to a phenotype similar to that of LDCs from HC. Directing the pro-inflammatory LDCs away from the gut may represent one mechanism of action of
IFX, albeit the effect was only studied in *in vitro* conditions in these experiments. It remains unclear if this effect is a direct result of the action of IFX on LDCs themselves or an indirect action via other molecules, such as cytokines. This hypothesis is further supported by our observation that the ongoing intracellular production of cytokines by LDCs and the cytokine milieu in cell-free supernatant samples were altered following culture with IFX.

**Effect of IFX on LDC cytokine profile**

There was increased ongoing production of IL-12 by LDCs from HC subjects following the addition of IFX to the culture (not observed with LDCs from active CD patients). It seems strange that the addition of IFX would cause greater production of IL-12 (a Th1 cytokine) by LDCs. However, there is ample evidence that TNFα blockade can cause upregulation of other pro-inflammatory pathways and cytokines in the literature. Notley et al. examined the effect of TNFα blockade on Th1 and Th17 cells in mice with collagen-induced arthritis (Notley, Inglis et al. 2008). The TNFα blockade was achieved using murine p75 TNFR-Fc or anti-mouse TNFα monoclonal antibodies. The authors found expanded populations of both Th1 and Th17 cells in the periphery, but also an improvement in joint inflammation. Additional experiments demonstrated that, while the numbers of Th1 and Th17 cells had increased, these were located mainly in the lymph nodes and did not accumulate in the joints, hence the improvement in arthritis. This may explain, to some extent, upregulation of IL-12 by LDCs following TNFα blockade.

In the cell-free CD LDC culture supernatant, we found a stepwise reduction in free TNFα with increasing doses of IFX (figure 49, section 5.4.6). Studies in CD have shown an increased number of lamina propria sTNF-producing cells and high concentrations of sTNF can be detected in the stool of patients with active inflammatory responses (Braegger, Nicholls et al. 1992; Breese and MacDonald 1995). As with the other commercially available anti-TNFα antagonists, IFX is
known to effectively neutralise the bioactivity of sTNF. In light of the fact that we have shown there is no effect of IFX on ongoing intracellular TNFα production by LDCs \emph{in vitro}, direct neutralisation of free TNFα in the cell-free supernatant by IFX is the likely explanation for this finding, and also explains the dose-dependent effect observed.

There were lower levels of TNFα seen in the cell-free supernatant from HC LDCs. And a further reduction in TNFα was observed following culture with IFX at the higher concentrations (\(p<0.05\)). Data suggests that there are differences in the sTNF neutralisation potency of IFX depending on the concentration of sTNF present, which might have important consequences \emph{in vivo}. At high concentrations of sTNF (>2ng/ml, as measured in inflamed tissues in CD) (Van Deventer 1997), the potency of IFX was high (comparable with adalimumab and etanercept). However, at low sTNF concentrations (=0.1ng/ml), the potency of IFX falls to levels equivalent to adalimumab, but 20 fold less potency than etanercept. This may explain the differences in reduction of TNFα with IFX between the active CD group and HC seen in the cell free supernatants.

The concentration of IL-2 in the cell-free supernatant was also reduced in both CD and HC groups following culture with IFX in a dose-dependent way, although statistical significance was only achieved at the highest concentration of IFX in the HC group. Studies have shown that anti TNFα therapy has a broad impact on the immune system that exceeds the blockade of TNFα itself. One study, using CD45RB high T-cell transfer model of colitis, demonstrated that – in addition to inducing a marked improvement in colonic inflammation – anti-TNFα therapy down regulated IL-2 and IFNγ secretion by lamina propria T-cells, as well as significantly decreasing expression of IL-23p19 and IL-17 in the inflamed colon (Liu, Jiu et al. 2007).

Early production of IL-2 by LDCs is essential for the development of an IFNγ-secreting Th1 response and therefore very relevant in the pathogenesis of CD. The reduction in concentration of IL-2 that we observed in the cell-free
supernatant may be the result of ‘reverse signaling’ of IFX on LDCs, whereby binding of IFX to tmTNF causes suppression of pro-inflammatory cytokine production (in addition to reduced cell activation and upregulation of adhesion molecules). Further studies are required to investigate whether this is the case.

Correlation studies

We identified positive correlations between the intensity ratio of HLA DR expression, percentage expression of β7 and ongoing production of IL-6 by LDCs with disease severity (HBI). Greater IR of HLA DR and production of IL-6 implies LDCs have greater immunogenic potential and therefore are likely to lead to greater T-cell stimulation in more severe disease. Higher expression of β7 in severe disease implies a greater gut-homing potential of LDCs. The HBI is predominately a measure of luminal disease activity in CD, and therefore these two findings appear to match the clinical patient parameters. Indeed, we have also shown in this chapter that percentage expression of β7 on LDCs and the quantity of IL-6 in culture supernatants are reduced following the addition of IFX to cell cultures in a dose-dependent manner. This adds further weight to the importance of these molecules in CD.

Previous studies have shown that age-related changes in the immune system contribute to increased susceptibility of elderly people to infection, vaccine failure and possibly autoimmunity and cancer (Pawelec 1999; Targonski, Jacobson et al. 2007). The effect of ageing on DC function specifically is still not fully understood. While in vitro generated MoDCs from elderly subjects are functionally intact regarding differentiation and maturation (Steger, Maczek et al. 1996), they have been shown to have reduced ability for micropinocytosis and phagocytosis of apoptotic cells when compared to those from younger subjects (Agrawal, Agrawal et al. 2007). These two functional deficits are likely to contribute to reduced antigen uptake, and may also affect antigen processing and presentation by DCs, leading to an overall reduction in effective T-cells responses.
in ageing (Weiskopf, Weinberger et al. 2009). Furthermore, CD40 and CD40L expression (co-stimulatory molecule and ligand, essential for efficient T-cell stimulation by DCs) are reduced in ageing and therefore the CD40-CD40L pathway becomes dysfunctional with age (Fernandez-Gutierrez, Jover et al. 1999). We found a negative correlation between CD40 expression on LDCs and age, but this was non-significant.

Finally, our data revealed a negative correlation between ongoing production of TGFβ by LDCs and age in patients with active CD. This correlation was not seen in the HC cohort. This raises the possibility of the phenomenon of immunosenescence affecting patients with CD to a greater extent than HC due to the disease state. Another possibility is that LDCs producing greater quantities of TGFβ have already been recruited to the inflamed tissue in patients with active CD. It is likely that greater patient numbers in future studies may reveal further correlations between LDC markers and cytokine production with age.
Chapter 6

The effect of infliximab pre-treated human blood-enriched dendritic cells from patients with active Crohn’s disease and healthy controls on subsequent human T-lymphocyte phenotype and cytokine production
6.1 Abstract

Introduction
DCs play a key role in discriminating between commensal microorganisms and potentially harmful pathogens. Expression of surface markers and cytokine production by DCs at the time of antigen presentation control T-cell differentiation, cytokine profile and homing properties imprinted on stimulated T-cells. This process defines the type of immune response that occurs (regulatory/inflammatory) and its anatomical location. In CD, dysregulation of the immune response to gut microbiota and aberrant immune cell trafficking play a central role in disease pathogenesis. IFX is a widely used and effective treatment for CD, but its precise mechanism of action is unclear.

In this study, we investigated the effect of IFX pre-treated blood-enriched DCs, isolated from patients with active Crohn's disease and healthy control subjects, on human T-cell proliferation, phenotype and cytokine content.

Methods
LDCs, which are enriched for DCs, were obtained following Ficoll and Nycoprep gradient separation of fresh blood obtained from patients with active ileocolonic CD (HBI≥7) and HC. LDCs were cultured (0.5x10^6 cells/ml) with IFX (1μg/ml, 10μg/ml, 100μg/ml & basal) for 24 hours.

T-cells were enriched from allogeneic HC blood by negative immunomagnetic bead separation, selecting CD3+ cells, and labelled with CFSE. LDCs were added to T-cells in complete medium (400,000cells/ml) at basal, 1%, 2% and 3% concentrations and incubated for 5 days.

Following incubation, T-cell proliferation, expression of β7 & CLA surface homing markers and intracellular cytokine content of stimulated T-cells (TNFα, TGFβ, IFNγ, IL-10, IL-15 and IL-17) was quantified by flow cytometry on
stimulated T-cells. Unpaired t-test, one-way and two-way ANOVA statistical analysis with Dunnett’s multiple comparisons tests were applied.

**Results**

In basal conditions, LDCs from HC and CD patients did not differ in their stimulatory capacity for allogeneic T-cells or in the cytokine profile acquired by T-cells. However, T-cells stimulated by LDCs from CD patients increased β7 intensity ratio (p<0.05).

Following culture with IFX, LDCs decreased their stimulatory capacity in a dose-dependent, stepwise fashion in both HC and CD (p<0.001 in both conditions).

Culture with IFX did not have any effect on the acquired homing profile of stimulated T-cells, although these T-cells had a trend (p=ns) towards lower TNFα and higher IL-17 content when stimulated by LDCs from CD patients.

**Conclusions**

The marked reduction in the ability of LDC to stimulate T-cells following culture with IFX represents one plausible explanation for the efficacy of anti-TNFα therapies in the treatment of CD. This effect was dose-dependent (within our range of test concentrations) suggesting that higher doses of IFX further reduce T-cell stimulation and may provide one explanation of the clinical benefits of dose escalation in refractory CD.

*This abstract was presented at BSG and ECCO conferences 2013 and subsequently published in Gut and JCC journals with the following references. Permissions to reprint abstract in Appendix, section 10.2 and 10.3 respectively.*

The effect of infliximab pre-treated human blood-enriched dendritic cells from patients with active Crohn’s disease and healthy controls on subsequent human T-lymphocyte phenotype and cytokine production in vitro

**Peake ST**, Bernardo D, Mann ER, Landy J, Al-Hassi HO, Knight SC, Hart AL

*Gut* 2013; 62: A253-A254

*JCC* 2013; 7(1): S150
6.2 Introduction

Intestinal DCs are constantly sampling the luminal microenvironment of the gut for antigenic material. Once an antigen is detected, it is processed within the endoplasmic reticulum of the DC to form a small peptide that is subsequently presented on MHC class II molecules to lymphocytes in the mesenteric lymph nodes. In order to migrate to the draining mesenteric lymph nodes, DCs undergo a process of maturation as previously described in section 1.2.6.

Circulating naïve T-cells express high levels of L-selectin and CCR7 that allow them to enter secondary lymphoid tissue through interactions with their ligands (L-selectin ligand and CCL21 respectively) on endothelial vessels (Cyster 1999). The end result of this process is a high concentration of naïve T-cells in lymphoid tissue, awaiting presentation of antigenic material by DCs.

These interactions between DCs and lymphocytes regulate several aspects of the immune response. DCs have several roles in this respect; to activate and imprint antigen specificity on the lymphocyte and also to direct lymphocytes to the site where the antigen is most likely to be encountered by (tissue-specificity). Lymphocyte priming by intestinal DCs in mucosal tissue increases the expression of the two main gut-homing markers, α4β7 and CCR9 (Stagg, Kamm et al. 2002; Mora, Bono et al. 2003). Expression of α4β7 and CCR9 by mucosal lymphocytes also requires the presence of retinoic acid.

Furthermore, DCs determine the nature of the immune response. Early production of IL-2 is essential for the development of an IFNγ-secreting Th1 response, whereas production of IL-1, IL-6 and IL-23 will drive a Th17 response. Antigen presentation taking place in the presence of IL-10 or TGFβ will lead to the induction of regulatory T-cells with an anti-inflammatory phenotype. Retinoic acid – in addition to being required for imprinting of gut-homing receptors of lymphocytes – can also synergise with TGFβ to induce T-cells with a regulatory phenotype and gut-homing properties (Coombes, Siddiqui et al. 2007).
The process of lymphocyte priming in lymphoid tissue includes changes in lymphocyte homing phenotype. Activated effector lymphocytes down-regulate CCR7 and L-selectin expression and up-regulate markers required for migration to target tissues.

### 6.2.1 Hypothesis and Aims

**Hypothesis**

We hypothesised that LDCs pulsed with IFX would have a reduced T-cell stimulatory capacity, and that the reduction in stimulation would correlate with the concentration of IFX used during LDC culture. Furthermore, we hypothesised that there would be dysregulated T-cell phenotype and cytokine content following stimulation by IFX-pre-treated LDCs.

**Aims**

- To identify changes in the stimulatory capacity of LDCs following pre-treatment with IFX.

- To identify changes in T-cell phenotype and cytokine content following stimulation by IFX-pre-treated LDCs.
6.3 Methods

6.3.1 Isolation of allogeneic T-cells

In order to assess the stimulatory capacity of IFX-pre-treated LDCs and the effect of these cells on T-cell phenotype and cytokine profile, LDCs (the ‘stimulators’) were incubated with allogeneic T-cells (the ‘responders’) in a mixed leukocyte reaction (MLR).

PBMCs were obtained from a healthy control, as previously described in section 2.3.1, and re-suspended in 0.5ml of MiniMACS buffer (sterile PBS containing 0.5% BSA and 2mM EDTA). The cells were then labelled with anti-CD14, anti-HLA DR and anti-CD19 immunomagnetic beads (30μl each) on ice for 20 minutes.

Cells were then washed twice with miniMACs buffer and passed through the magnetic cell sorting system (VarioMACSTM Separation System, Miltenyi Biotech, Bisley, UK), followed by 4ml of miniMACs buffer at 4°C. The resultant solution is depleted of CD19+, CD14+ and HLA DR+ cells. In order to increase T-cell purity, a new depletion column was setup and the T-cell solution was run through the column for a second time.

The final solution has a CD3+ T-cell purity of >98% (Bernardo, Al-Hassi et al. 2012). The cells were washed twice in cold PBS at 4°C and re-suspended in 3ml PBS. The enriched T-cell solution is then labelled with carboxyfluorescin diacetate succinimidyl ester (CFSE) to permit quantification of T-cell proliferation following the MLR process.

A trypan blue cell count was then performed to determine the T-cell concentration. Once the cell count was known, the solution was further diluted with complete medium, to give a final concentration of 4 million T-cells/ml.
6.3.2 Mixed leukocyte reaction

0.5x10^6 stimulator cells (LDCs) per ml were cultured with the different test concentrations of IFX in 1ml complete medium for 24 hours. LDCs in complete medium only served as a control. Following the 24-hour culture period, the stimulator cells were washed twice in complete medium to remove any traces of IFX from the solution.

The CFSE-labelled responder T-cells were plated out in a 96-well round bottom plate at a concentration of 400,000 cells in 200μL complete medium. The stimulator cells were added at 1% (4000 LDCs), 2% (8000 LDCs) and 3% (12,000 LDCs) concentrations and each well was made up to 200μl total volume with complete medium. Negative control tubes contained T-cells with 200μL of complete medium, without LDCs.

After 5 days culture at 37°c, 5% CO₂ and high humidity, cells were recovered and T-cell proliferation, phenotype and cytokine content was analysed by flow cytometry.
6.3.3 Quantification of T-cell proliferation

Prior to the MLR process, all responder T-cells were labelled with CFSE (figure 50). Dividing T-cells were identified by the dilution in CFSE staining intensity, therefore allowing for quantification of T-cell proliferation.

**Figure 50.** Quantification of T-cell proliferation. A) Two-parameter FSC:SSC histogram with region R1 identifying all viable PBMCs negative for HLA DR, CD14 and CD19 expression. B) One-parameter histogram gated on region R1 identifying all CD3+ cells (region R2). C) A one-parameter histogram gated on regions 1 and 2 identifying cells that are negative for CFSE staining and therefore have undergone cell division following stimulation by LDCs (region R3).

6.3.4 Statistical analysis

T-cell proliferation was analysed using two-way analysis of variance (2-way ANOVA) with Dunnett’s multiple comparison test.

Differences in T-cell phenotype and cytokine profile between HC and CD was analysed using unpaired t-test. The effect of IFX on phenotype and cytokine profile was analysed using one-way ANOVA with Dunnett’s multiple comparison test.

P values of <0.05 were considered statistically significant in all cases.
6.4 Results

6.4.1 Patient characteristics

Samples from healthy control subjects (n=4) and patients with active ileocolonic CD (HBI ≥ 7) (n=3) were obtained. These were the same individuals as used in the previous chapter. Demographic and clinical data is shown in table 21.

<table>
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<tr>
<th>Case number</th>
<th>Gender</th>
<th>Age</th>
<th>Years since diagnosis</th>
<th>CD location</th>
<th>HBI</th>
<th>Current medications</th>
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<td>-</td>
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</tr>
</tbody>
</table>

Table 21. Patient characteristics and demographical data.

HC = Healthy control subject, 6-MP = 6-Mercapopurine, AZA = Azathioprine

Inclusion / exclusion criteria were applied for all patient recruitment, as outlined in section 2.2.2.

6.4.2 T-cell stimulation

LDCs from HC and patients with active CD stimulated and led to proliferation of the allogeneic responder T-cells in the MLR experiment (figure 51). In all conditions, the percentage of proliferating T-cells increases as the concentration of stimulating LDCs added increases.
There is a stepwise reduction in the stimulatory capacity of LDCs with increasing concentrations of IFX added to the LDC culture. This trend is apparent in both HC and active CD conditions. Statistical significance was achieved at several points regarding this trend, as shown below in figure 51.

Figure 51. Stimulatory capacity of LDCs following pre-treatment with IFX in HC and active CD. Concentrations of IFX used in LDC culture shown in coloured key.

A) LDCs isolated from HC (n=4).

- 1μg/ml IFX: p=ns
- 10μg/ml: p<0.05 at 2% LDC; p<0.01 at 3% LDC
- 100μg/ml: p<0.05 at 1% LDC; p=ns at 2% LDC; p<0.001 at 3% LDC

B) LDCs isolated from patients with active ileocolonic CD (n=3).

- 1μg/ml and 10μg/ml IFX: p=ns
- 100μg/ml: p<0.05 at 1% LDC; p=ns at 2% LDC; p<0.001 at 3% LDC

Statistical analysis performed using two-way ANOVA with Dunnett’s multiple comparison test.
There was no statistically significantly difference in T-cell proliferation between LDCs from HC compared to those from patients with active CD. Figure 51 appears to show increased T-cell proliferation with LDCs from HC compared to CD, however this appearance is the result of one outlying LDC sample in the HC cohort which did indeed show a very high stimulatory capacity. This may be the result of a wide difference in HLA profile of this HC subject and the donor of the allogeneic T-cells in this particular case, which has led to increased T-cell stimulation.

6.4.3 Phenotype of stimulated T-cells

To examine the phenotype of stimulated T-cells, we investigated the surface expression of β7 (gut-homing) and CLA (skin-homing) markers on proliferating T-cells, as identified by negative CFSE expression following LDC stimulation (figure 52).

We found a high percentage expression of β7 on proliferating T-cells following MLR (46.7±9.8% in HC; 63.7±14.0% CD; 55.8±7.4% overall). There was a trend towards higher expression of β7 on T-cells stimulated by LDCs from patients with active CD compared to HC, but this did not reach statistical significance (p=0.08). However, the intensity ratio of β7 expression on positive cells was significantly greater on T-cells following stimulation by LDCs from patients with active CD compared to HC (p<0.05). These findings correlate with those from the previous chapter, where the same LDCs isolated from patients with active CD had significantly greater β7 expression than those from HC. Pre-treating LDCs with IFX prior to MLR did not affect β7 expression on proliferating T-cells.

CLA expression on proliferating T-cells following LDC stimulation ranged from 20.7±4.4% in HC to 35.3±11.0% in CD with overall mean expression being 26.9±5.6%. There were no differences in CLA expression between T-cells
stimulated by LDCs from patients with active CD compared to HC. Pre-treating LDCs with IFX prior to MLR stimulation also did not affect CLA expression.

Figure 52. T-cell phenotype following stimulation. A) Percentage expression and intensity ratio (IR) of β7 expression on proliferating T-cells stimulated by IFX-pre-treated LDCs from patients with active CD and HC. B) Percentage expression and IR of CLA expression on proliferating T-cells stimulated by IFX-pre-treated LDCs from patients with active CD and HC.

HC = healthy control, CD = active ileocolonic CD, ns = not significant, * = p<0.05

Statistical analysis comparing HC and CD groups performed using unpaired t-test. Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.
6.4.4 Cytokine profile of stimulated T-cells

Following MLR stimulation, we assessed the intracellular cytokine content of proliferating T-cells (figure 53). There was a trend towards reduced TNFα and increased IL-17 in T-cells stimulated by LDCs from patients with active CD compared to HC, but this did not reach statistical significance (p=0.10 and 0.07 respectively). There were no differences when analysing the effect of IFX-pre-treatment of LDCs on the intracellular cytokine content of stimulated T-cells.

![Graphs showing cytokine content of T-cells stimulated by LDCs from patients with active ileocolonic CD and HC in addition to the effect of IFX-pre-treatment of LDCs prior to T-cell stimulation.](image)

**Figure 53. Intracellular cytokine content of proliferating T-cells.** Graphs showing cytokine content of T-cells stimulated by LDCs from patients with active ileocolonic CD and HC in addition to the effect of IFX-pre-treatment of LDCs prior to T-cell stimulation.  

*HC = healthy control, CD = active ileocolonic CD, ns = not significant*  

**Statistical analysis comparing HC and CD groups performed using unpaired t-test. Analysis of effects of IFX performed using one-way ANOVA with Dunnett’s multiple comparison test.**

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6.4.5 Summary of results

Differences between CD and HC states

- Increased intensity ratio of β7 expression on positive T-cells stimulated by LDCs from patients with active CD compared to HC.

- Trend towards reduced TNFα and increased IL-17 content of T-cells primed by LDCs from patients with active CD compared to HC (p=ns).

Effect of IFX-pre-treatment on stimulatory capacity and T-cell phenotype and intracellular cytokine content

- Dose-dependent reduction in T-cell proliferation when stimulated by IFX-pre-treated LDCs from both patients with active CD and HC.

- No effect of IFX-pre-treatment on phenotype or intracellular cytokine content of stimulated T-cells.
6.5 Discussion

Differences between T-cells stimulated from LDCs from CD and HC states

There was increased intensity ratio of β7 expression on proliferating T-cells that had been stimulated by LDCs from patients with active CD compared to HC. In the previous chapter, we found elevated β7 expression on the same CD LDCs that we used for stimulation in this chapter (figure 45, section 5.4.4). In these experiments, positive expression of β7 is taken to represent α4β7 integrin expression on cells, although this has already been identified as a limitation in the methodology (section 1.2.11).

Previous studies designed to investigate the ability of DCs to imprint gut-homing properties on T-cells used murine DC from the mesenteric lymph nodes or Peyer’s patches (with gut-homing phenotype) to stimulate T-cells from different sources. The results showed that α4β7 expression was induced on the T-cells, thereby directing them back to intestinal tissue (Stagg, Kamm et al. 2002; Mora, Bono et al. 2003). Conversely, T-cell stimulation by DCs from other tissues resulted in the expression of skin-homing molecules on the T-cells (Mora, Cheng et al. 2005). Our results indicate that, in CD, there is greater β7 expression on circulating LDCs and this leads to greater β7 expression on stimulated T-cells. The α4β7 : MAdCAM-1 axis has been repeatedly shown to be central in development and persistence of chronic intestinal inflammation in CD. One recent study demonstrated overexpression of MAdCAM-1 in the gut endothelium in patients with active CD. However, 10 weeks following treatment with IFX, patients who responded to therapy had significantly decreased expression of MAdCAM-1 in the gut, and a corresponding increase in circulating β7+ T-cells (Biancheri, Di Sabatino et al. 2013). This study suggests that IFX itself is able to exert an inhibitory effect on T-cell recruitment in the gut, and this may contribute to reducing chronic inflammation in active CD.
There was a trend towards a reduction in TNFα and increased IL-17 content in proliferating T-cells that had been stimulated by LDCs from patients with active CD compared to HC, albeit these results did not reach statistical significance. In the previous chapter, we observed a reduction in ongoing production of TNFα by LDCs from patients with active CD (figure 48, section 5.4.5), and this effect appears to have been passed on to the stimulated T-cells. We did not assess the concentration of TNFα in the cell-free supernatant in this part of the study.

Elevated IL-17 content in T-cells that have been stimulated by LDCs from CD patients supports the involvement of the Th17 pathway in the pathogenesis of CD. Although IL-17 is not required for Th17 pathway polarisation (this process occurs in the presence of IL-6 and TGFβ and amplified by IL-21 and IL-23), Th17 cells are characterised by increased expression of IL-17a and IL-17F (Wilson, Boniface et al. 2007). Studies have consistently shown that IL-17 is highly expressed in the mucosa and serum of patients with active CD (Fujino, Andoh et al. 2003), therefore supporting their role in CD pathogenesis. Furthermore, the receptors for IL-17 are expressed on intestinal epithelial cells (Awane, Andres et al. 1999), and Th17 cells are known to have a significant action on the epithelial barrier in CD.

**Effect of IFX-pre-treated LDCs on T-cell stimulation, phenotype and ongoing cytokine production**

As expected, there was a clear correlation between the quantity of LDCs present in the culture and T-cell proliferation. The greater the number of LDCs (stimulator cells), the greater the amount of T-cell (responder cell) proliferation that occurs. IFX was shown to have a marked effect on the T-cell stimulatory capacity of LDCs. This was observed with both LDCs from HC subjects and those with active ileocolonic CD.
IFX reduces the stimulatory capacity of LDCs from HC and patients with active ileocolonic CD in a dose-dependent stepwise manner. This pattern achieved statistical significance at several concentrations in both conditions. The mechanism by which IFX exerts this action is unclear, especially as we have previously shown no changes in activation markers or co-stimulatory markers on LDCs following the addition of IFX to in vitro cell culture. The reduction in stimulatory capacity may occur as a result of alterations in the cytokine milieu produced by LDCs in which antigen presentation is taking place. In the previous chapter we observed that cell-free culture supernatants from CD have significantly higher concentrations of pro-inflammatory cytokines such as TNFα and IL-6 when compared to HC supernatants (figure 49, section 5.4.6). Furthermore, cytokines have previously been shown to be the most important factor affecting the induction of effector functions in T-cells (de Jong, Smits et al. 2005).

Clinically, patients treated with IFX may ‘lose response’ to the drug during the maintenance treatment phase. Looking across the trials of anti-TNFα therapy use in CD, the cumulative loss of response after 12 months ranges between 23-46% for both IFX and ADA (Ben-Horin and Chowers 2011). The disease process can often be ‘recaptured’ by increasing the amount if IFX given, either by reducing the interval between scheduled doses or increasing the amount of the drug given at each interval (Katz, Gisbert et al. 2012) thereby increasing the serum concentration of IFX. The reduction in the stimulatory capacity of LDCs with increasing doses of IFX that we have observed in vitro may go some way towards explaining why such dose-escalation works in vivo. Further studies are needed to identify the mechanism of reduced LDC stimulatory capacity with IFX.

Finally, there were no changes in T-cell phenotype or intracellular T-cell cytokine content following culture with IFX-pre-treated LDCs. This was a surprising finding, especially in light of the changes observed on LDCs following IFX culture shown previously in chapter 5. IFX has been shown to induce clinical remission and achieve immune tolerance in patients with active CD in vivo.
Immune tolerance can be achieved by passive or active mechanisms. Passive immune tolerance occurs by reduction in stimulatory capacity of DCs, whereas active immune tolerance is achieved by induction of a generation of T-cells with regulatory phenotype. Based on these results, it seems likely that IFX induces regulatory properties in a passive way.
Chapter 7

Discussion
7.1 General discussion

DC phenotype in CD

The first line of investigation was to examine the phenotype of circulating DCs in patients with CD. CD is a heterogeneous condition in which inflammation can occur at any point along the GIT from mouth to anus, or in extra-intestinal sites. We know that DCs express tissue-specific homing markers (Mann, Bernardo et al. 2012), and have the capacity to imprint these homing markers on effector T-cells. Therefore, we hypothesised that DCs were dictating the location of inflammation in patients with active CD, and hence their homing phenotype would be altered in patients with different anatomical locations of CD inflammation.

Circulating DCs obtained from patients with isolated active colonic CD showed up regulation of β7 expression with low levels of CCR9 expression. This gives rise to an overall increased gut-homing potential but reduced small bowel homing, therefore these DCs are programmed to migrate to the colon. The ligand for β7 (Mad-CAM-1) is expressed on intestinal lamina propria high endothelial venules, and it's expression increases significantly in the presence of active intestinal CD (Arihiro, Ohtani et al. 2002). Conversely, DCs obtained from patients with active SB CD, had high expression of CCR9 and low expression of CLA (leading to increased single positive β7 expression). Murine studies have shown that CCR9 expression confers SB homing properties on DCs (Hart, Ng et al. 2010) and the corresponding ligand (CCL25/TECK) is highly expressed on epithelial cells in the SB but almost absent in the colon (Papadakis, Prehn et al. 2000). Furthermore, we found a strong correlation between CCR9 expression on mDCs and disease severity in patients with active SB CD. DCs isolated from patients with skin-related EIMs had increased CCR10 and reduced CCR4 expression, but no change in the gut-homing markers (β7 and CCR9) compared to HC.
These results appear to indicate that the homing profile on circulating DCs does correlate with the anatomical location of inflammation in CD. Exactly how this occurs remains unclear. The expression of homing markers on DCs is a dynamic process and studies have previously identified the importance of constituents of the local microenvironment in determining DC homing phenotype (Mann, Bernardo et al. 2012; Bernardo, Mann et al. 2013). It may be that inflamed tissue in patients with CD secretes factors into the microenvironment which modulates DC homing profile, thereby ‘attracting’ them to their own site of inflammation.

There were no differences between the phenotype of circulating DCs when comparing healthy controls and patients with ileocolonic CD that had been inactive for ≥3 months. Although patients with active ileocolonic CD were not studied in this section, it seems likely that they would have a dysregulated DC phenotype based on the above observations. This suggests that when patients with CD enter clinical remission and the burden of inflammation falls, the DCs phenotypically return to a ‘resting state’, which mirrors that of the healthy individuals.

**Effect of IFX on DC phenotype**

Having revealed that the phenotype of circulating DCs correlates with the anatomical location of active CD, we moved on to investigate the effect of IFX on the phenotype of peripheral blood mononuclear cells. The PBMC population was chosen (as opposed to solely a DC population) to investigate whether IFX modulated homing marker expression on other immune cells in addition to DCs. In these *in vitro* studies, we used a physiological concentration of IFX (100µg/ml) which is similar to the concentration found *in vivo* when patients are treated with IFX in clinical practice (Ordas, Mould et al. 2012).

Following culture with IFX, we found increased expression of CCR7 on circulating monocytes. The ligands for CCR7 (CCL19 and CCL21) are expressed in
lymph nodes and therefore monocytes appear to up regulate their lymph node homing capacity, where they are able to perform antigen presentation to effector T-cells. If these cells expressed a tolerogenic phenotype then this may indicate one potential way in which IFX works.

T-cells appeared to inherit a skin-homing phenotype following culture with IFX (reduced β7 and CCR7 expression and increased CCR4 expression). This novel finding may represent a previously undescribed mechanism by which paradoxical inflammation (PI) occurs, when patients with active CD are treated with anti-TNFα therapies in vivo and develop cutaneous psoriatic or eczematous inflammation. This exciting finding warrants further investigation by recruiting and examining immune cell homing phenotype in patients who develop PI after IFX treatment (with a matched control group). The reported incidence of PI in patients with IBD is 5-9% (Fidder, Schnitzler et al. 2009; Rahier, Buche et al. 2010) and therefore recruiting enough patients to find changes in immune cell phenotype would be challenging, especially when applying exclusion criteria for the study.

There were no changes seen on DC phenotype following culture with IFX. However, these experiments were carried out in the setting of freshly isolated PBMCs and therefore we moved onto examining the effects of IFX on enriched human DCs.

Effect of IFX on blood-enriched DCs

Most studies to date investigating human DCs have used monocyte-derived DCs (MoDC). As previously discussed, DCs produced by this method cannot be used to study homing markers, due to the gradual loss of homing marker expression on cells during the 5 day cell preparation and culture phase (figure 39, section 5.2.1), likely due to the lack of ongoing exposure to tissue microenvironment required to condition DCs (Bernardo, Mann et al. 2013). Our laboratory does not
have access to a flow sorter and magnetic sorting was ruled out by lack of optimisation of the techniques involved.

Therefore, we have used low density cells (LDCs) as our in vitro model of circulating human DCs. Cell analysis has shown the isolated LDC population to be highly enriched for DCs with a high surface expression of HLA DR, CD11c, CD83 and CD86 (Knight, Farrant et al. 1986). Previous work from our laboratory has also shown that the enriched DC population isolated by this method is 98-100% positive for HLA DR expression and has morphological features of DC at both optical and electron microscopy (Knight, Farrant et al. 1986; Holden, Bedford et al. 2008). The use of LDCs as a model of circulating DCs is further supported by our results which showed elevated β7 expression and a trend towards elevated CCR9 expression on LDCs from patients with active ileocolonic CD when compared to HC. These findings are mirrored by the previous work when we found up regulation of β7 and CCR9 on DCs from patients with active colonic and SB CD respectively, when we examined DC phenotype as part of PBMCs (figures 20, section 3.4.3.1 and figure 23, section 3.4.3.4, respectively).

We found further differences between LDCs from patients with active ileocolonic CD and HC in terms of cytokine profiles. The ongoing intracellular production of TNFα and IL-6 was reduced in LDCs from patients with CD, however the concentration of the same two cytokines was increased in the cell-free culture supernatant. This may be the result of the experimental methodology we used to quantify ongoing cytokine production, in which we measured cytokine production over a 4 hour period following 24 hours of cell culture. This process means we are only seeing a ‘snapshot’ of cytokine production within this time frame. Another possibility is the existence of a negative feedback mechanism whereby the high levels of extracellular TNFα and IL-6 that have been produced and accumulated during the cell culture phase exert a negative feedback message, thus reducing intracellular production. A similar system has previously been observed with NKT cells and Th1/Th2 cytokine balance (Onoe, Yanagawa et al. 2007), but further studies would be needed to confirm this finding in LDCs.
Following culture with IFX, we found that the elevated levels of TNFα, IL-6 and IL-2 in the culture supernatant were significantly reduced. These 3 cytokines are integrally involved in the development of Th1 and Th17 responses that characterise CD. The reduction in the concentration of these cytokines following culture with IFX is likely to be one of the ways in which IFX works in patients with active CD.

We also found changes in LDC phenotype following IFX culture. The most significant of these was a reduction in gut-homing affinity, as shown by down regulation of β7 expression and a reduction in the intensity ratio of CCR9 expression on positive cells following the IFX culture period. As previously hypothesised, directing immune cells away from the gut may be one way in which IFX works in patients with active luminal inflammation. Interestingly, there was also a trend towards down regulation of TLR2 and TLR4 expression on LDCs, but this did not reach statistical significance. TLRs are used by DCs to sense microbes and pathogens which then lead to maturation of the DC. In health, TLR2 and 4 expression by human colonic DCs is low (Hart, Al-Hassi et al. 2005) and this contributes to their tolerogenic function and prevents unnecessary activation of the immune system to commensal microbiota. In IBD, expression of TLR2 and 4 by colonic DC is up regulated (Hart, Al-Hassi et al. 2005) and this is likely to drive dysregulated immune response to bacterial components of the gut lumen. Down regulation of TLR2 and 4 on LDCs following culture with IFX may again be another mechanism by which IFX exerts its action.

Overall, the main effects of IFX on LDCs appear to be two-fold. Firstly, there is a reduction of pro-inflammatory Th1/Th17 cytokines in the cell-free culture supernatant and secondly there is a reduced affinity of LDCs for gut homing. The importance of these two factors in the disease process in patients with active CD was further reinforced when analysing the patient correlation data. We identified that both β7 expression and the ongoing production of IL-6 by LDCs positively correlate with disease severity. This overall gives the impression that
β7 expression and IL-6 production are of particular importance when examining the effect of IFX on LDCs and this warrants further investigation in the future.

**Effect of IFX pre-treated LDCs on T-lymphocytes**

Having proved that human enriched blood DCs are modulated *in vitro* by IFX, we wanted to investigate the functional consequences of this. In order to study this, we examined the stimulatory capacity of IFX pre-treated LDCs, and the phenotype and cytokine profile of stimulated effector T-cells *in vitro*.

A major finding arising from this section of the project was the marked reduction in LDC stimulatory capacity following culture with IFX. This effect was dose-dependent and was observed following culture with LDCs from both patients with active CD and HC subjects. Interestingly, we did not observe any changes in activation or co-stimulatory markers on the LDCs that may lead to this reduction in stimulatory capacity. It seems likely that this may – in part – be due to the cytokine milieu in which antigen presentation is occurring. We have shown that cell-free supernatant when LDCs have been cultured with IFX contains significantly less TNFα and IL-2 with a trend towards lower IL-6 concentration (figure 49, section 5.4.6). It is possible that the reduced levels of these pro-inflammatory cytokines in the milieu are responsible for the reduction in T-cell stimulation seen with IFX-pre-treated LDCs, although further studies would be needed to confirm this.

The stimulating LDCs from patients with active CD have previously been shown to have higher expression of the β7 integrin compared to those from HC, and this appears to have been transferred to the responding T-cells, which had higher β7 intensity ratio than T-cells stimulated by LDCs from HC. It therefore seems that LDCs from patients with active ileocolonic CD have an enhanced capacity to imprint gut-homing onto responding T-cells. If this effect also occurs *in vivo*, it may highlight one method by which LDCs are triggering gut inflammation.
7.2 Experimental limitations

As mentioned during this thesis, one clear methodological limitation is the assumption that β7 expression on immune cells is equivalent to α4β7 expression and therefore represents a gut-homing phenotype. A tiny proportion of circulating lymphocytes (2%) express αεβ7 and therefore will also stain positive for β7 expression. The ligand for αεβ7 is e-cadherin and this is expressed on epithelial surfaces such as the gut, but also including the lungs and skin, hence the cells have a different homing phenotype. However, in view of the scarcity of circulating lymphocyte αεβ7 expression, cells which express β7 in the experimental work in this thesis are assumed to have gut-homing properties.

When recruiting patients with CD for inclusion in the studies, we included patients taking a stable dose of azathioprine. Previous work carried out in our laboratory has shown that concurrent use of azathioprine does not affect or alter homing marker expression on immune cells. Furthermore, thiopurine therapy is widely used in patients with CD, and exclusion of these patients would have significantly hindered recruitment to the studies. However, recent studies have identified Vδ2 T-cells to play a role in the pathogenesis of CD, by secreting pro-inflammatory cytokines and stimulating enhanced IFNδ by intestinal CD4+ T-cells (McCarthy, Bashir et al. 2013). Further work by the same group has shown that these cells are ablated in the blood and tissue from CD patients who are receiving azathioprine, and post-treatment Vδ2 T-cell recovery correlates with time when the drug is withdrawn (McCarthy, Hedin et al. 2015). In view of this, it may be that azathioprine is a confounding factor, and its effects on immune cells would need to be established in future studies.

Another potential methodological limitation relates to the control groups used when studying effects of IFX. When performing further work examining the effect of anti-TNFα therapy in vitro, it would be sensible to add in a further control group to allow for non-specific effects of the antibody therapy, using a control human IgG.

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7.3 The future of targeted biological therapy for CD

Current evidence suggests that a dysregulation of mucosal immunity in the gut of patients with CD leads to an overproduction of pro-inflammatory cytokines and trafficking of effector leukocytes to the bowel, resulting in gut inflammation. The pivotal role of cytokines in this process has led to therapies designed to target specific molecules or steps in the inflammatory cascade, for example anti TNFα therapies. To date, Infliximab and Adalimumab are the only biological agents licensed to treat CD in Europe. Although often efficacious, these therapies have clinical drawbacks, such as primary and secondary non-response, the development of neutralising antibodies or side effects and complications associated with therapy. Golimumab (another human monoclonal antibody that targets the TNFα cytokine) has been shown to bind TNFα to a higher affinity than IFX or ADA (Shealy, Cai et al. 2010) and is currently in Phase III placebo-controlled trials for treatment of moderate to severely active UC patients.

Increasing knowledge of the pathogenesis of IBD and the mechanisms, pathways and molecules involved in the inflammatory process has led to the identification of new potential targets for therapy. These new biological agents are at various stages of clinical trials and include:

- Antibodies targeting integrins
  - Natalizumab (anti-α4)
  - Etrolizumab (anti-β7)
  - Vedolizumab (anti-α4β7)

- Compounds targeting integrin receptors
  - PF-00547659 (anti-MAdCAM)
  - Alicaforsen (anti-ICAM-1)

- Compounds targeting chemokine receptors
  - Traficet-EN (anti-CCR9)
• Inhibitors of T-cell activation
  o IDEC-131 (anti-CD40L)
  o cM-T412 (anti-CD4)
  o Visilizumab (anti-CD3)

• Inhibitors of T-cell polarization
  o Fontolizumab (anti-IFNγ)
  o ABT-874/J695 (anti-IL12)
  o Basiliximab / Daclizumab (anti-IL2)

Targeting integrins, receptors and cytokines is not an exact science and can be associated with serious complications. Natalizumab (anti-α4 integrin) showed efficacy in moderate to severe CD by blocking gut-homing (Targan, Feagan et al. 2007) as well as in the treatment of multiple sclerosis by blocking the brain homing VLA-4 (α4β1) (Miller, Khan et al. 2003). However, the program had to be halted by the manufacturers when there were several cases of re-activation of latent JC virus and progressive multifocal leukoencephalopathy (PML). Natalizumab inhibits the shared α4 integrin component of both α4β7 and α4β1, and this is likely to have a much broader impact on the immune system, including impairment of lymphocyte trafficking leading to reactivation of viruses when dysregulated immune cell homing is no longer tissue-specific, but overlaps with other organs. PML was also reported in patients treated for psoriasis with Efalizumab (an adhesion molecule targeting αLβ2), and was subsequently withdrawn from the market (Kothary, Diak et al. 2011).

At the time of submitting this thesis (2015), anti-integrin therapy (Vedolizumab) is being increasingly used in clinical practice in both anti-TNFα naïve patients and those that have failed conventional biological treatment. Vedolizumab (anti-α4β7 integrin) has shown to be efficacious in clinical trials for UC (Feagan, Rutgeerts et al. 2013) and CD (Sandborn, Feagan et al. 2013). A major theme that has run through this thesis is the importance of β7 expression on immune cells
in CD. This feature has been consistent in all results chapters, both in terms of anatomical location of inflammation and correlation with disease severity. The effectiveness of anti-α4β7 integrin therapy in CD further underlines the relevance of β7 expression on immune cells in CD and it is clear that targeting this integrin is going to be a major development in the medical treatment of CD in the future.

In this way, an improved understanding of homing markers and integrins in CD has the potential to lead to unique and personalised therapy. For example, we have shown that patients with isolated active SB CD have dysregulated expression of CCR9 on circulating immune cells, while the same cells from patients with isolated active colonic CD preferentially express β7. Therefore, it may be that patients with isolated SB CD can be treated with anti-CCR9 therapy (Vercirnon, also known as Traficet-EN or CCX282) while those with colonic disease receive Vedolizumab. This form of personalised therapy may be more effective with fewer side effects, as well as being more cost-efficient for the health service.
7.4 Future directions for this research

This work has identified some interesting differences in homing marker expression on circulating DCs in different phenotypes of CD. There appears to be a specific homing marker profile which is unique to the anatomical location of inflammation in patients with CD. There are also correlations between the quantity of expression of some homing markers and disease severity, for example CCR9 expression on circulating mDCs positively correlates with HBI in patients with active SB CD (table 8, section 3.4.5). These findings may have a significant impact on our understanding of the inflammatory process in CD and furthermore may represent important areas of study when looking at targets for new therapeutic agents for CD in the future. It is clear that DCs play a fundamental role in directing migration of immune cells to target tissues in CD, although there is still some uncertainty about how exactly DCs know where to migrate to.

The answer to this may lie in the constituents of the microenvironment in which DC conditioning occurs. It is known that IL-6 modulates DC homing profile and stimulatory capacity in UC (Bernardo, Vallejo-Diez et al. 2012), and a variety of other cytokines and vitamins have also been shown to affect immune cell homing and phenotype (eg retinoic acid, TGFβ and vitamin D, described in section 1.2.11). We found significantly elevated levels of IL-6 in LDC culture supernatants from patients with active CD compared to HC as well as a correlation between IL-6 production by LDCs and disease severity. In light of these findings, future work should examine the role of plasma constituents in the development of DC homing profiles. One method of studying this would be to assess the cytokine and vitamin concentrations in plasma from patients with CD and then use the plasma to condition DCs isolated from a HC. The homing profile of these conditioned DCs could be compared to those conditioned with plasma obtained from a separate HC and potential differences between the two DC homing profiles identified. If this process were to identify soluble factors responsible for aberrant immune cell homing profiles, it may be possible to block
the factors responsible and prevent pathological alterations to homing profiles. This approach may represent a future direction for the development of targeted therapy in CD.

We have identified several effects of IFX on the homing and cytokine profiles of circulating LDCs. Although widely used in clinical practice, the precise mechanism of action of IFX remains unclear and there is little published literature on its effect on human DCs. We found that LDCs have a reduced gut-homing affinity (reduced β7 and CCR9 expression) and reduced levels of the pro-inflammatory cytokines TNFα and IL-2 in the supernatant following culture with IFX. There were no changes in surface expression of ‘activation markers’ such as HLA DR, CD80 and CD40 on LDCs. Despite this, we found that IFX pre-treated LDCs have a significantly reduced T-cell stimulatory capacity which is dose-dependent. Taken together, these findings reinforce previous observations that cytokine profile is the most important factor effecting the induction of T-cell effector function (de Jong, Smits et al. 2005). Further work is needed to examine and identify the molecular and cellular mechanisms through which IFX is exerting its effect on immune cells.

The work carried out in this thesis was performed using circulating human DCs and blood-enriched DCs in vitro. This approach has several advantages when setting out to study DC homing marker expression. For example, there are high numbers of immune cells in fresh blood, thereby providing ample cell numbers to test a wide range of homing markers and cytokines. Previous work has shown that intestinal DCs only compromise a very small proportion (0.5-1%) of viable cells obtained from fresh human gut biopsies [Dr David Bernardo, personal communication]. In view of the limited cell numbers obtainable for experimental work, we felt it would have been useful to have already identified markers of interest to study from blood work. Our original study design was to identify patients with active CD involving distal colon, who were being worked up for treatment with IFX. In addition to acquiring blood samples, we planned to perform a flexible sigmoidoscopy to obtain fresh colonic biopsies from inflamed
areas of mucosa. DCs would be isolated and phenotyped from both blood and intestinal mucosa. The process would have been repeated after the 8 week IFX induction course had been administered.

Unfortunately, we found it difficult to recruit patients for a longitudinal in vivo study, as described above, for a number of reasons. Concurrent medications was an issue, as patients had often had their immunomodulator therapy increased recently (within 3 months) prior to starting biological therapy or were taking corticosteroids, thereby being excluded from the study. Another problem was lack of distal colonic inflammation which we could sample with flexible sigmoidoscopy (ie those patients with isolated SB or ileocaecal CD would be excluded). Only two patients were recruited over a 6 month period. Blood and gut samples were taken and analysed on both, but unfortunately one patient withdrew consent for the second round of sample acquisition and the other was prescribed a course of corticosteroids before the 8 week IFX induction period had finished. The natural next step would be to confirm our findings using fresh intestinal DCs isolated from patients with active CD and HC in a prospective, longitudinal study providing paired data for analysis. In particular, it would be interesting to correlate changes in DC phenotype following IFX therapy to particular cohorts of patients, such as primary non-responders, patients with EIMs or those that develop paradoxical inflammation as a consequence of IFX therapy.

Finally, all the work on the effect of anti-TNFα therapy on immune cells in this thesis was carried out using IFX. Future work may investigate effects of other anti-TNFα therapies on immune cells in the same setting, in particular ADA and CZP, both of which are widely used for the treatment of CD, albeit the latter is not yet licensed in the UK. Studies have shown the IFX, ADA and CZP share several mechanisms of action (neutralisation of TNFα and upregulation of Treg population). However, they have different molecular structure and this can result in functional variations. For example, CZP lacks an Fc region and therefore does not induce cytotoxicity of tmTNF-bearing cells by antibody- or
complement-dependent cell cytotoxicity, whereas IFX and ADA were shown to be very effective in this manner (Scallon, Cai et al. 2002). The differential effect of these therapies on DCs has not previously been studied and may be of interest in the future.

In conclusion, we found that homing marker expression on circulating human DCs correlates well with anatomical location of inflammation in patients with CD. In vitro culture with IFX effects homing marker expression, cytokine profiles and the stimulatory capacity of LDCs and this is likely to represent one mechanism by which anti-TNFα therapies work in vivo.
Chapter 8

Publications
Publications arising from this work

Review article: Mechanisms of action of anti-tumor necrosis factor alpha therapies in Crohn's disease
Peake ST, Bernardo D, Mann ER, Al-Hassi HO, Knight SC, Hart AL
Inflamm Bowel Dis 2013; 19(7): 1546-1555

Infliximab induces a dysregulated tissue-homing profile on human T-lymphocytes in vitro: A novel mechanism for paradoxical inflammation?
Peake ST, Bernardo D, Mann ER, Al-Hassi HO, Knight SC, Hart AL
J Crohns Colitis 2013; 7(9): 765-767

Homing marker expression on circulating dendritic cells correlates with different phenotypes of Crohn's disease
Peake ST, Bernardo D, Knight SC, Hart AL
J Crohns Colitis 2013; 7(7):594-596

Intestinal dendritic cells: Their role in intestinal inflammation, manipulation by the gut microbiota and differences between mice and men
Mann ER, Landy J, Bernardo D, Peake ST, Hart AL, Al-Hassi HO, Knight SC
ImmunoLett 2013; 150(1-2): 30-40

Skin- and gut-homing molecules in human circulating gamma delta T-cells and their dysregulation in inflammatory bowel disease
Mann ER, McCarthy NE, Peake ST, Milestone AN, Al-Hassi HO, Bernardo D, Tee CT, Landy J, Pitcher MC, Cochrane SA, Hart AL, Stagg AJ, Knight SC

Other publications

Compartment-specific immunity in the human gut: properties and functions of dendritic cells in the colon versus the ileum
Gut 2016; 65(2): 256-270

A new instrumental platform for Trans-Anal Submucosal Endoscopic Resection (TASER)
Gut 2015; 64(12): 1844-1846

Human gut dendritic cells drive aberrant gut-specific t-cell responses in ulcerative colitis, characterized by increased IL-4 production and loss of IL-22 and IFNγ
Mann ER, Bernardo D, Ng SC, Rigby RJ, Al-Hassi HO, Landy J, Peake ST, Spranger H, English NR, Thomas LV, Stagg AJ, Knight SC, Hart AL
Inflamm Bowel Dis 2014; 20(12): 2299-307
Altered human gut dendritic cell properties in ulcerative colitis are reversed by Lactobacillus plantarum extracellular encrypted peptide STp
*Mol Nutr Food Res* 2014; 59(5): 1132-43

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Tsiamoulos ZP, *Peake ST*, Nickerson C, Rutter MD, Saunders BP
*Colorectal Dis* 2014; 16(1): 70-71

Dysregulated circulating dendritic cell function in ulcerative colitis is partially restored by probiotic strain Lactobacillus Casei ShirotA
*Mediators Inflamm* 2013; article ID 573576

Oral tacrolimus as maintenance therapy for refractory ulcerative colitis – an analysis of outcomes in two London tertiary centres
Landy J, Wahed M, *Peake ST*, Hussein M, Ng SC, Lindsay JO, Hart AL
*JCC* 2013; 7(11): 516-521

Lost therapeutic potential of monocyte-derived dendritic cells through lost tissue homing, stable restoration of gut specificity with retinoic acid

Endoscopic mucosal ablation: a novel technique for a giant nonampullary duodenal adenoma
Tsiamoulos ZP, *Peake ST*, Bourikas LA, Saunders BP
*Endoscopy* 2013; 45(2): 12-13

Commentary: Predicting response to ciclosporin in acute severe ulcerative colitis
*Peake ST*, Hart AL
*Aliment Pharmacol Ther* 2012; 36:1095-6

IL-6 promotes immune responses in human ulcerative colitis and induces a skin-homing phenotype in the dendritic cells and T-cells they stimulate
*Eur J Immunol* 2012; 42(5):1337-53
T-cell proliferation and forkhead box P3 expression in human T cells are dependent on T-cell density: physics of a confined space?
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Patient preference in choosing biological therapy in Crohn's disease
Peake ST, Landy J, Hussein M, Tee CT, O'Connor M, Tyrrell T, Akbar A, Hart AL
*Inflamm Bowel Dis* 2011;17(7):E79

Review article: The management of acute severe ulcerative colitis
Peake ST, Landy J, Hart AL
*Foundation Years Medical Journal* 2011; 5(8):35-40

Vulval Crohn's disease: a tertiary centre experience of 23 patients
Landy J, Peake ST, Akbar A, Hart AL
*Inflamm Bowel Dis* 2011;17(7):E77

H1N1 vaccines in a large observational cohort of patients with inflammatory bowel disease treated with immunomodulators and biologics
*Gut* 2011; 60(4):456-462

**Book Chapters**

Inflammatory Bowel Disease and Fertility, Pregnancy and Breast Feeding
Peake ST, Hart AL
*Inflammatory Bowel Disease – an Evidence-based Practical Guide*
TFM Publishing Ltd, Editors: Hart AL and Ng SC

Localised Ileocaecal Crohn’s Disease
Peake ST, Akbar A, Bashir G, Warusavitane J
*Inflammatory Bowel Disease – an Evidence-based Practical Guide*
TFM Publishing Ltd, Editors: Hart AL and Ng SC
Abstracts (selection)

Unravelling the mechanism of action of infliximab in Crohn’s disease and healthy controls following in vitro culture with blood-enriched dendritic cells
Peake ST, Bernardo D, Mann ER, Landy J, Al-Hassi HO, Knight SC, Hart AL

The effect of infliximab pre-treated human blood-enriched dendritic cells from patients with active Crohn’s disease and healthy controls on subsequent human T-lymphocyte phenotype and cytokine production in vitro
Peake ST, Bernardo D, Mann ER, Landy J, Al-Hassi HO, Knight SC, Hart AL

Altered homing molecule expression on blood dendritic cells in different phenotypes of Crohn’s disease
Peake ST, Bernardo D, Mann ER, Al-Hassi HO, Landy J, Knight SC, Hart AL
Gut 2012; 61(S3): A390

Shorter infliximab administration infusion times are safe and well tolerated in Crohn’s disease
Peake ST, Landy J, Tyrrell T, O’Connor M, Middleton H, Bernardo D, Hart AL

Anti TNF-alpha induces a dysregulated tissue-homing profile on human immune cells in-vitro
Peake ST, Bernardo D, Mann E, Al-Hassi HO, Landy J, Tee CT, Knight SC, Hart AL
JCC 2012; 6(1):S15

Vaccination Against Opportunistic Infections in patients with Inflammatory Bowel Disease on Immunomodulator Therapy
Peake ST, Tee CT, Landy J, Arebi N

Endoscopic resection of giant sessile colonic polyps: techniques and outcomes
Tsiamoulos ZP, Peake ST, Suzuki N, Bourikas LA, Warusavitarne J, Saunders BP
Gut 2013; 62(S1): A239 and GIE 2013; 77(5): AB542

Dendritic cell characteristics in pouchitis
Landy J, Al-Hassi HO, Mann ER, Peake ST, Ciclitira PJ, Nicholls J, Clark SK, Knight SC, Hart AL
Gut 2013; 62(S1): A161

A prospective controlled pilot study of faecal microbiota transplantation for chronic refractory pouchitis
Landy J, Al-Hassi HO, Mann ER, Peake ST, McLaughlin SD, Perry-Woodford ZL, Ciclitira PJ, Nicholls J, Clark SK, Knight SC, Hart AL
Gut 2013; 62(S1): A162
Phenotype of CD11c-CD123+ lamina propria dendritic cells in the ileum of ulcerative colitis patients and healthy controls
Landy J, Ronde E, **Peake ST**, Al-Hassi H, English N, Mann E, Ciclitira PJ, Clark SK, Knight SC, Hart AL
*Gut* 2012; 61(S3): A154

Dysregulated dendritic cell function in ulcerative colitis is partially restored by Lactobacillus Plantarum extracellular encrypted peptide
Mann ER, Bernardo D, Sanchez B, Al-Hassi HO, **Peake ST**, Landy J, Urdaci M, Margolles A, Knight SC
*Gut* 2012; 61(S3): A59

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Landy J, Al-Hassi H, Ronde E, **Peake ST**, English N, Mann E, Ciclitira PJ, Clark SK, Knight SC, Hart AL
*Gut* 2012; 61(S3): A154

Inappropriate inflammatory responses in the ileum of ulcerative colitis patients
Landy J, Al-Hassi HO, **Peake ST**, English N, Ciclitira PJ, Nicholls RJ, Clark SK, Knight SC, Hart AL
*Gut* 2012; 61(S3): A222

Dysregulation of human dendritic cell function in ulcerative colitis
Mann ER, Bernardo D, **Peake ST**, Vallejo-Diez S, Al-Hassi HO, Martinez-Abad B, Montalvillo E, Tee CT, Daulatzai N, Hart AL, Nunez H, Fernandez Salazar L, Garrote JA, Arranz E, Knight SC
*JCC* 2012; 6(1):S37

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Tee CT, **Peake ST**, Bernardo D, Mann E, Landy J, Daulatzai N, Wallis K, Gabe SM, Knight SC, Al-Hassi HO
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Landy J, **Peake ST**, Akbar A, Hart A

Oral Tacrolimus in Refractory Inflammatory Bowel Disease – Longterm Treatment Outcomes and Follow Up in a UK cohort
Landy J, **Peake ST**, Karim N, Ikin N, Ng S, Akbar A, Hart A
*Gut* 2011; 60: A207
Chapter 9

References


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Chapter 10

Appendix
10.1 Permission from *Inflammatory Bowel Disease* journal to reprint published manuscript.

This manuscript has been used in section 1.3 (Anti tumour necrosis factor alpha therapies for Crohn’s disease).
10.2 Permission from *Gut* journal to reprint published abstracts.

These previously-published abstracts appear at the start of chapters 3, 5 and 6 (sections 3.1, 5.1 and 6.1 respectively).

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Altered homing molecule expression on blood dendritic cells in different phenotypes of Crohn's disease
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*Gut* 2012; 61(S3):A390

Unravelling the mechanisms of action of infliximab in Crohn's disease and healthy controls following in vitro culture with blood-enriched dendritic cells
Peake ST, Bernardo D, Mann ER, Landy J, Al-Hassi HO, Knight SC, Hart AL
*Gut* 2013; 62:A254

The effect of infliximab pre-treated human blood-enriched dendritic cells from patients with active Crohn's disease and healthy controls on subsequent human T-lymphocyte phenotype and cytokine production in vitro
Peake ST, Bernardo D, Mann ER, Landy J, Al-Hassi HO, Knight SC, Hart AL
*Gut* 2013; 62:A253-4

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These previously-published abstracts appear at the start of chapters 4, 5 and 6 (sections 4.1, 5.1 and 6.1).