A thesis presented for the degree of Doctorate of Philosophy

The Tolerogenic Modulation of Dendritic cells 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

“She had the same lone thoughts and wanderings
The quest for hidden knowledge
And a mind to comprehend the universe”
George Gordon Byron,
Manfred, II, ii, 109-111

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Abstract

Crohn's disease is a chronic relapsing remitting inflammatory disease of the gut. The aetiology is multifactorial, and incompletely understood. The immune system plays a key role as evidenced by animal and human studies, as well as the predominance of immune-targeting treatments amongst the Crohn's disease therapeutic arsenal. Defects in the adaptive immune system in Crohn's have been well described, and there is an increasing body of evidence that the innate immune system, including dendritic cells (DCs) may be involved as well. Vitamin D is of interest as a potential adjunctive therapy in IBD, and its potential mechanisms of action are incompletely understood.

In this thesis, we utilised flow cytometry, ELISA, electron microscopy and PCR to explore the interactions between DCs, anti-TNF treatment and vitamin D in Crohn's disease. We found abnormalities in circulating DCs in Crohn's disease, especially in the expression of homing markers, and in the production of inflammatory cytokines. Expression of homing markers were discriminatory for both location of disease and degree of inflammation. There were abnormalities in the less well studied plasmacytoid DC (pDC) population. Abnormalities in intracellular DC production of inflammatory cytokines were mirrored by serum cytokine concentrations.

The phenotype and function of circulating DCs was seen to alter after *ex vivo* treatment with anti-TNF. Anti-TNF therapy was associated with a switch away from gut-homing circulating DC phenotype towards skin homing, and with a decrease in on-going DC production of intracellular cytokines such as TNFα and IL-6. These findings may provide further mechanisms of action of

anti-TNF treatment as well as an explanation for its high rates of dermatological complications. Vitamin D was seen to alter DC homing profile in both *in vitro* and *ex vivo* studies to non-skin homing, and in addition augmented down-regulation of TNFα production by DCs. This could have potential clinical applications.

Glossary of abbreviations

CCR	C-C chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous leucocyte antigen
DCs	Dendritic cells
EIM	Extra-intestinal manifestation
ELISA	Enzyme-linked immunosorbent assay
Fc (region)	Fragment crystallizable (region)
HC	Healthy control
IFN	Interferon
IL	Interleukin
ILT	Immunoglobulin-like transcript
MS	Mouse
NF	Nuclear factor
RT	Rat
RT-qPCR	Realtime quantitative polymerase chain reaction
TGF	Transforming Growth Factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor

Dedication

In loving memory of my late father Dr Michael Steven Hendy. You have always been an inspiration to me, and this thesis, which interested you so much, is dedicated in your honour.

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Chapter 1 - Introduction

1.1 Introduction to Crohn's Disease

A complex inflammatory disease where the understanding of underlying immunological aberrations has led to significant advances in management.

1.1.1 Epidemiology of Crohn's disease

Crohn's disease, a chronic transmural granulomatous inflammatory bowel disease (IBD), is a relapsing remitting condition (Hendy and Hart, 2013). When symptomatic, it is characterised by abdominal pain, diarrhoea, rectal bleeding, fever and weight loss. Any part of the alimentary tract may be affected from mouth to anus. Constitutional symptoms include fatigue, malaise, anorexia, lethargy and nausea. The condition has many associated extra-intestinal manifestations, particularly affecting the eyes, joints and skin and which occur in up to 40% of patients (Bernstein *et al.*, 2001). It can impact severely on the quality of life of sufferers who must contend not only with the unpredictability of a multitude of symptoms but also with the consequences of 'living with the disease' which may result in psychological sequelae of helplessness, social isolation, anger and depression (Knowles and Mikocka-Walus, 2015).

Chronic intestinal inflammation is a risk factor for developing colorectal cancer (CRC). A significant number of patients with IBD (5-10%) will develop CRC after 20 years and this risk increases further with greater duration of disease (12-30% after 30 years)(Ekbom *et al.*, 1990).

Predisposing factors for development of CRC include extensive disease, concomitant primary sclerosing cholangitis, a family history of colonic polyps or colonic cancer and early onset disease.

The prevalence of Crohn's disease in the UK is around 145 per 100,000 population (NICE CKS, 2015; Group, 2017). With the UK population in 2016 of 65.6 million this equates to approximately 95,000 Crohn's sufferers.

1.1.2 Aetiology of Crohn's disease

The disease has a complex multifactorial aetiology. It is thought that chronic inflammation develops because of an excessive and dysregulated immune response to intestinal microbiota in genetically susceptible individuals. Whilst the disease has become far more prevalent over the last century, human genetics haven't altered significantly, and this implicates environmental factors such as smoking, diet, infections, stress, geography and migration in the development of inflammation in those with a genetic predisposition. It is plausible that these aetiological factors are interconnected, as migrants whose risk alters are exposed to different climate, diet, sanitation and medications compared with their country of origin.

1.1.2.1 The mucosal immune system and its dysfunction in IBD

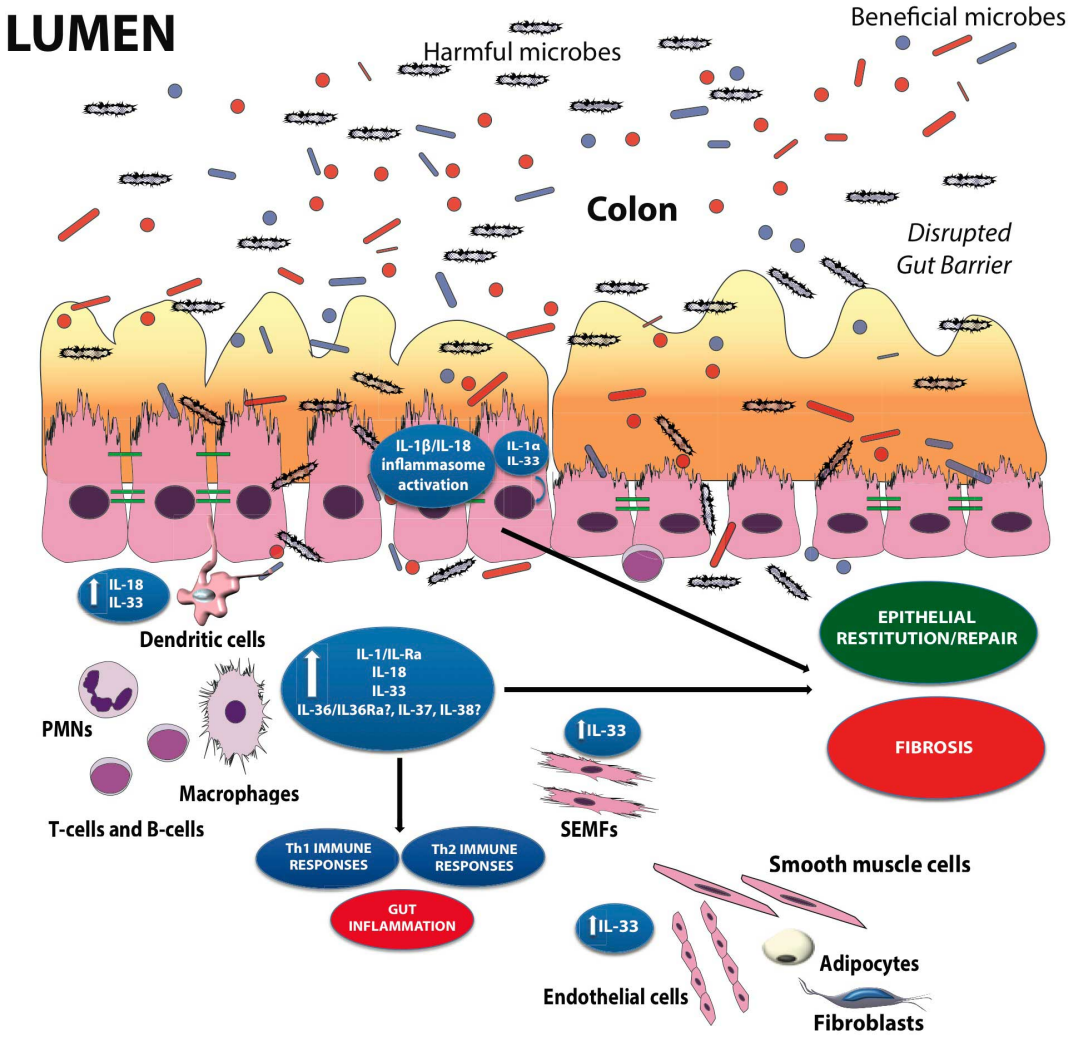


Figure 1.1: Cartoon schematic of the mucosal immune system featuring both innate and adaptive components (Lopetuso, 2014).

The immune system in the human gut walks the tightrope between excessive activity which would lead to chronic inflammation, and inadequate surveillance which might allow pathogenic infiltration and infection or development of neoplasia and cancer. To achieve the correct balance, the mucosal immune system is in a 'tolerogenic' state in comparison with other tissue or

circulatory sites (Magrone and Jirillo, 2013) (figure 1.1). Although the inflammatory bowel diseases have traditionally been thought of as diseases of the adaptive immune system, with Crohn's disease being a T-helper cell type 1 mediated process, and ulcerative colitis (UC) being a T-helper cell type 2 mediated disease, in fact abnormalities have been discovered in almost every niche of the immune system (Shih, Targan and McGovern, 2008; Wallace *et al.*, 2014). The main driver of immune dysfunction in IBD are aberrant T-cell responses, characterised by excessive production of pro-inflammatory cytokines (or more correctly, an overall imbalance between production of pro- and anti-inflammatory cytokines), including TNFα and IL-6. Knowledge of the immune pathways which underlie IBD have led to the development of a number of efficacious therapies, targeting cells, pathways or individual cytokines.

1.1.2.1.1 The innate immune system in IBD

The innate immune system is characterised by rapid speed of onset, lack of specificity and lack of memory. In the innate immune system in IBD, there are abnormalities of the physical barrier (mucosal epithelial barrier, mucous layer, microbial sensing, anti-microbial polypeptide (AMP) production), autophagy and cellular components of the innate system (neutrophils, dendritic cells (DCs), natural killer (NK) cells, eosinophils, and basophils).

Intestinal mucous layer. The intestinal mucosa is protected by a layer of mucous which is produced by the goblet cells of the epithelium. The mucous layer is firmly adherent and acts as a physical barrier to bacteria. The mucous layer contains mucin and antimicrobial peptides, including cathelicidin related peptides, human lysozyme and Human β-defensin (Dupont *et al.*, 2014). IBD susceptibility is conferred by mutations to the *MUC2* gene, an important gene for

mucin production. Furthermore, MUC 2 deficient mice develop spontaneous colitis (Van der Sluis *et al.*, 2006). The profile of AMPs, produced by Paneth cells, in the intestinal mucous of patients with IBD also differs from healthy controls (HCs) (Wehkamp *et al.*, 2003, 2005; Arijs *et al.*, 2009).

Epithelial cell barrier. The epithelial cell barrier is formed by tight junctions which occur between enterocytes, effective at preventing ingress of most substances. Defects of epithelial integrity may contribute to the pathogenesis of IBD by allowing microbes and other antigens to cross the epithelium and reach the immune cell rich lamina propria. Intestinal permeability is increased in IBD and the epithelium is characterised by epithelial breaks and reduction in tight junction strands (Bruewer, Samarin and Nusrat, 2006; Teshima, Dieleman and Meddings, 2012; Landy *et al.*, 2016). The inflammatory cytokine milieu in IBD also promotes alterations in the tight junctions (Heller *et al.*, 2005). In addition, defects of the *OCTN1* gene, whose protein product is an important epithelial transmembrane transport protein, confer an increased risk of Crohn's disease (Peltekova *et al.*, 2004).

Microbial sensing. Another function of the intestinal epithelium is to allow bi-directional communication between the immune cells in the lamina propria and the antigenic content of the bowel lumen. Enterocytes and gut immune cells both express pattern recognition receptors (PRRs) which recognize and bind to pathogen associated molecular patterns (PAMPs) on luminal microbes. The Toll-like receptor (TLR) class is an important such example, and defects in several TLRs (including TLR8 and TLR9) confer increased risk of developing both UC and Crohn's disease (Saruta *et al.*, 2009; O'Hara *et al.*, 2012).

Autophagy. Autophagy, or 'self-eating', is a process of lysosomal degradation of intracellular organelles and extracellular debris. Autophagy is important in maintaining cellular homeostasis, particularly after inflammation or infection. Autophagy is impaired in IBD, and mutations to two important autophagy genes (*ATG16L1* and *IRGM*) confer some of the highest relative risk of developing Crohn's disease (Hampe *et al.*, 2007; Levine, Mizushima and Virgin, 2011).

Natural killer cells and Innate lymphoid cells. NK cells are a component of the innate immune system with cytotoxic abilities. Until recently they were thought to be the only innate cell derived from lymphoid progenitors, but other innate lymphoid cells (ILCs) have now been recognised. Currently there are three groups of ILCs, differentiated by their expressed receptors. ILCs secrete cytokines and are thought of as the complementary innate cell to the T-helper cells of the adaptive immune system. ILCs are found in high concentrations in the lamina propria of patients with Crohn's disease, and murine models have demonstrated that ILCs (in particular Group 3) are required for the development of colitis (Buonocore *et al.*, 2010; Geremia *et al.*, 2011; Eken *et al.*, 2014).

Antigen presenting cells. Mononuclear phagocytes (especially macrophages and DCs) span both innate (with their secretion of cytokines) and adaptive (being the foremost antigen presenting cells (APCs)) immune systems. These cells are dealt with in some depth later in this chapter (Section 1.2). B cells, whilst antigen presenting, are included in the adaptive system.

1.1.2.1.2 The adaptive immune system and IBD

In contrast with the innate immune system, the adaptive system is highly specific and confers long-lasting 'memory'. The main components of the adaptive immune system are T cells and B

cells. T helper (Th) cells, also called CD4 cells, augment the activity of many other immune cell groups by releasing a variety of cytokines (figure 1.2). T helper cells assist with the function of B cell antibody production; activation and growth of $CD8^+$ cytotoxic T cells; and the phagocytic activity of cells such as macrophages. In the adaptive immune system, there are abnormalities in number and function of Th1, Th2, Th17, Th22 and T regulatory (Treg) cell groups, as well as B cell aberrations (Sun *et al.*, 2015). Other important components of the adaptive immune system in the gut are adhesion molecules, such as integrins and selectins, which allow tissue ingress of activated T cells, and their ligands in endothelial tissues.

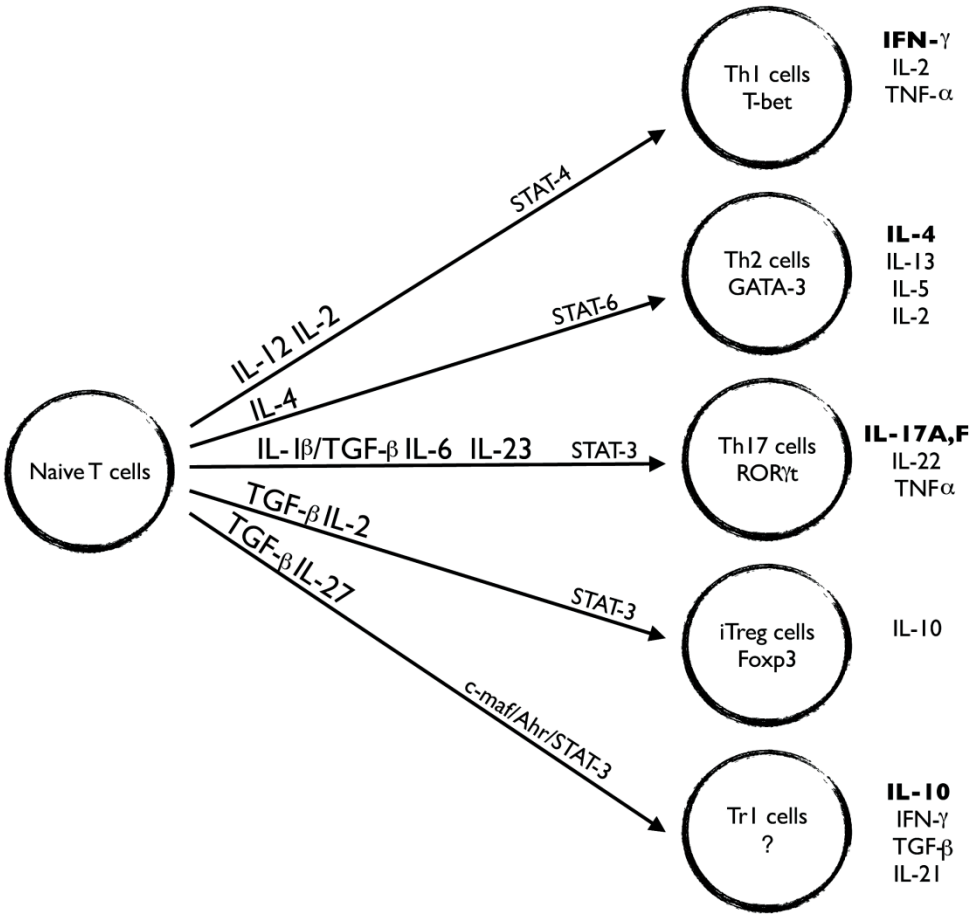


Figure 1.2: Schematic of the T helper cell differentiation pathway from Naive T cell to a range of functional Th cells with different cytokine outputs (Gagliani and Huber, 2012).

TH1 cells. Th1 cells predominantly secrete IFN-γ (a potent initiator of macrophage intracellular killing, with the priority function of protecting the host from intracellular pathogens) and IL-2. The Th1 population of cells has long been associated with Crohn's disease, due to the high levels of IFN-γ and IL-12 seen in this condition (de Souza and Fiocchi, 2016).

TH2 cells. Th2 cells are characterised by IL-5 and IL-13 production and are historically associated with the development of UC.

Th17 cells. Th17 cells and their cytokine products (including IL-17, IL-21, IL-22) are crucial mediators in both UC and Crohn's disease (Gálvez, 2014)**.** These cells are found in high concentrations in inflamed mucosal tissue in IBD, and GWAS studies confirm an association between mutations in Th17 related genes, such as *JAK2*, *STAT3* and *IL-23R*, and IBD (Fujino *et al.*, 2003; Thompson and Lees, 2011; Weaver *et al.*, 2013). Some studies have suggested a tissue protective effect of the Th17 cytokines whilst the majority support a pathogenic effect, and the effect, pathogenic or not, may be dictated by the predominant cytokine (Ogawa *et al.*, 2004; Yang *et al.*, 2008). There is some plasticity in function of Th17 cells and they can, in some conditions, produce Th1 and Th2 associated cytokines (Annunziato *et al.*, 2007). In fact, it is the interplay between the Th1/Th2/Th17/Treg populations which controls inflammation in IBD.

Tregs. The role of the Treg is particularly crucial in the gut where tolerance to self-antigens, and non-pathogenic antigens (e.g. foodstuffs, commensal organisms) is essential. Tregs are $CD4^+$ $FOXP3^+$ $CD25^+$ and can be found in low concentrations in healthy gut mucosa, and raised levels in IBD mucosa. Tregs generally secrete anti-inflammatory cytokines such as IL-10 and TGF-β.

Adoptive transfer of Tregs is a successful cure for colitis in murine models (Mottet, Uhlig and Powrie, 2003).

Humoral immunity. Whilst no antibodies with pathogenic potential in IBD have been identified, a number of immunoglobulins are significantly associated with IBD. Anti-neutrophil cytoplasmic antibodies (ANCA) are present in the sera of 50-90% of patients with UC, whilst anti-*saccharomyces cerevisiae* ASCA is prevalent in the sera of patients with Crohn's disease (McKenzie *et al.*, 1990; Duerr *et al.*, 1991; Quinton *et al.*, 1998). Interestingly, the number of bacteria to which one has antibodies and the titers can predict disease course severity (Mow *et al.*, 2004). Work from our laboratory (under review) has shown enhanced B cell memory responses to commensals in IBD. These facts support the relevance of the microbiota in developing IBD.

1.1.2.2 Genetic susceptibility and IBD

Genetic susceptibility in IBD includes racial and familial factors as well as scores of susceptibility loci. Up to 20% of patients with Crohn's disease have a first degree relative with the disease (Russell and Satsangi, 2004). Should both parents suffer Crohn's disease the offspring will have a 36% whole life risk of developing it (Noble and Arnott, 2008). Studies on identical twins have shown that should one twin develop Crohn's disease the other has a life-time one in three risk of developing the disease (Noomen, Hommes and Fidder, 2009). In the USA the disease is more common in certain ethnic groups such as Ashkenazi Jews, African Americans and Hispanics (Nguyen *et al.*, 2006). Immigrants from a low incidence area for IBD develop higher incidence levels once living in an area of greater prevalence (Li *et al.*, 2011).

Before genome-wide association studies (GWAS) only a few gene variations, including nucleotide-binding oligomerization domain containing protein 2 (NOD2) were recognised as increasing susceptibility to Crohn's disease. The advent of GWAS has seen this number increase to more than 200, with a majority of loci being shared by the two diseases (Jostins *et al.*, 2012; Liu *et al.*, 2015). The technique compares the genome of individuals in populations with and without (controls) Crohn's disease. On its simplest level the technique will identify single nucleotide polymorphisms (SNPs), whereby either cytosine, guanine, adenine or thymine is replaced by one of the other 3 nucleotides. Interestingly, in Crohn's disease identification of the gene SNPs has outstripped functional identification of the underlying pathophysiology. Conversely this has led to intensification of basic research into the interplay between metabolic pathways, particularly about cytokine/cellular interactions. One method to achieve this is via mapping of the protein-protein interactions between the GWAS variants to reconstruct functional pathways. The network shown below (fig. 1.3) highlights many of the molecules currently under research scrutiny, as well as several IBD therapeutic targets.

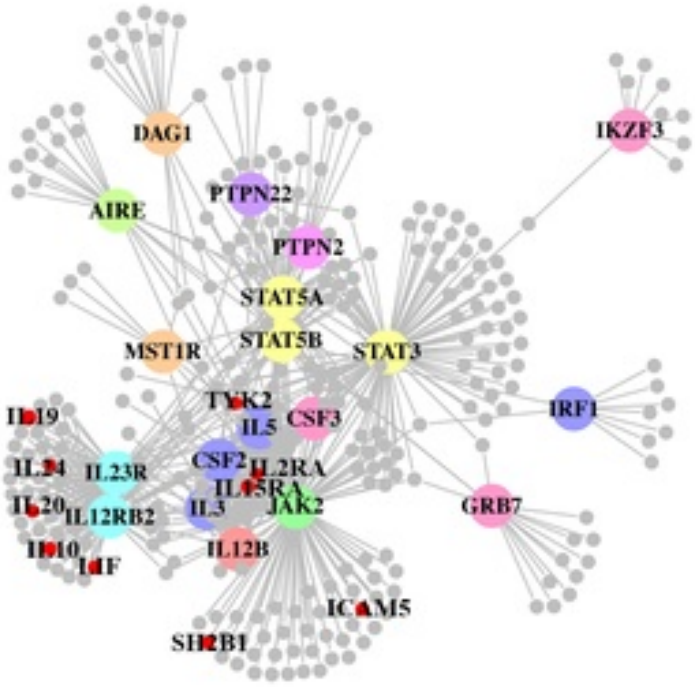


Figure 1.3: GWAS variant network mapping, via protein-protein interaction, to develop a functional construct (Rossin *et al.*, 2011).

In Crohn's disease, certain SNPs have been identified as being predisposing. One domain which codes for NOD2 codes for enzymes which have, at one end, two caspase activation and recruitment domains (CARD's). Caspase has a role in programmed cell death (PCD), so it is not difficult to appreciate how a SNP may lead to a lack of PCD of aberrant cell lines and the precipitation of inflammation (Strober and Watanabe, 2011).

1.1.2.3 Smoking and Crohn's disease

Crohn's disease is associated with tobacco smoking, which predisposes to development of the disease and worsens the clinical course. Indeed, smoking cessation is regarded as a primary

therapy to positively influence the course of illness (Johnson, Cosnes and Mansfield, 2005; Ananthakrishnan, 2015).

1.1.2.4 Microbiological factors and Crohn's disease

Microbiological factors have been implicated in the aetiopathogenesis of Crohn's disease. It is unclear, yet, whether this involvement is a single causative pathobiont (although this seems unlikely), a general dysbiosis with shifts in the $\alpha-$ or $\beta-$ diversity of different populations or a functional change in the microbiota (with the organisms remaining the same, but the chemical products of the microbiota changing). In general, studies have shown increased representation of facultative anaerobes (especially *Escherichia Coli* and other species from the Proteobacteria phylum) and decreased representation of obligate anaerobes (especially *Faecalibacterium prausnitzii* from the Firmicutes phylum) in IBD. Evidence supporting aberrant microbiota as being causative for IBD include the fact that germ free animal models do not develop IBD whilst IBD can be induced in animal models (with intact immunity) by bowel inoculation with colitogenic gut microbiota (Rutgeerts *et al.*, 1984; Sartor, 1998).

Bacteria postulated to be causative for Crohn's disease and under recent and on-going investigation include adherent invasive *E. coli* and mycobacteria such as *Mycobacterium tuberculosis* and *Mycobacterium avium* subspecies *paratuberculosis* (Darfeuille-Michaud *et al.*, 1998, 2004; Nazareth *et al.*, 2015; Seo *et al.*, 2017). The latter has been shown to maintain chronic intestinal inflammation in many animal species, including primates.

Yersinia enterocolitica, *Campylobacter sp*, Salmonella *sp*, Shigella *sp*, *E. coli* and other infections may mimic Crohn's disease both clinically and histopathologically. A diagnosis of Crohn's disease must therefore be made cautiously in the setting of a first attack of colitis. Clinical histology, natural disease history and the presence or absence of any pathogen may help in cementing the diagnosis (De Hertogh *et al.*, 2008). Several authors have demonstrated that both α- and $\beta-$ diversity of the human gut microbiota is reduced in Crohn's disease, with the largest study published by Gevers (Gevers *et al.*, 2014).

Studies of the virome have yielded evidence for implication in Crohn's disease both at pathogenic and geographical levels. Crohn's disease patients, when compared with non-Crohn's disease controls from the same geographical area, show increased viral load and diversity. The bacteriophage *caudovirales* predominated in patients with Crohn's disease and appears to be disease specific (Norman *et al.*, 2015).

1.1.2.5 Diet and IBD

Diet has been implicated in Crohn's disease in several studies which have suggested a causal relationship, but the data are generally quite weak. Increased risk has been demonstrated with sugars, artificial sweeteners (Sakamoto *et al.*, 2005; Amre *et al.*, 2007), total saturated fat (Amre *et al.*, 2007) and animal protein (Jantchou *et al.*, 2010). In addition, a high-fibre intake may be protective against developing Crohn's disease with a risk reduction of 30% (Jantchou *et al.*, 2010). Breast fed infants show some protection against developing Crohn's (Klement *et al.*, 2004).

Stratification following meta-analysis of several studies indicated that breast feeding may provide some protection against development of early onset Crohn's disease (Barclay *et al.*, 2009).

Perhaps the strongest evidence for diet as an aetiological factor in Crohn's disease is the fact that exclusive enteral nutrition (EEN) is a successful treatment for inducing remission in the paediatric cohort (clinical remission rates of up to 85%) and, to some degree, in the adult population (Heuschkel *et al.*, 2000; Grover, Muir and Lewindon, 2014). Indeed, EEN has equal efficacy to steroids in inducing remission for Crohn's disease in children, and without the growth limiting side effects of corticosteroids. More recent work has shown that a Crohn's disease exclusion diet, in conjunction with partial (non-exclusive) enteral nutrition is as effective as inducing remission, and is better tolerated, than EEN alone (Levine *et al.*, 2019).

1.1.2.6 Pollution and IBD

Age-related specific effects are indicated, with children and young adults living in car exhaust polluted urban areas (exposed to high nitrogen dioxide levels) having a high incidence of Crohn's disease, whilst conversely and perhaps contradictorily middle-aged adults living in the same environs having a lower incidence (Kaplan *et al.*, 2010b). Crohn's flare ups are more common in English counties with higher ambient air pollution (Ananthakrishnan *et al.*, 2011). The mechanisms underlying this association is unclear although animals fed an urban pollution diet show pro-inflammatory gut mucosal changes and changes in the composition of the microbiome (Kaplan *et al.*, 2010a).

1.1.2.7 Stress and emotional disorders

Results of studies linking stress to Crohn's disease are inconclusive, as it is difficult to prove causality (e.g. is a patient depressed because they have symptoms related to Crohn's disease, or are their abdominal symptoms more severe because the depression renders them more sensitive to normal stimuli). It is known, however, that patients with IBD are more likely to experience emotional disorders, and conversely that depression and anxiety may influence the course and severity of the IBD (Sgambato *et al.*, 2017). Furthermore, failure of coping strategies in both chronic and acute stress will cause patients with IBD to be more likely to suffer early relapse of their disease (Mawdsley and Rampton, 2005). Animal studies have indicated that stress may lead to pro-inflammatory gut changes by increasing intestinal permeability thus reducing the inherent barrier and allowing micro-organisms to gain a foothold (Söderholm *et al.*, 2002).

1.1.2.8 Medications and risk of Crohn's disease

a) **NSAID** therapy has been linked to Crohn's disease in terms of both incidence and disease relapse (Takeuchi *et al.*, 2006; Ananthakrishnan, 2012). The Nurses' Health Study (with a cohort of 76,000 women) found an absolute correlation between development of IBD and those subjects who ingestion NSAIDs for more than 15 days per month. In a study involving patients with Crohn's disease in remission, NSAIDs were associated with a 15-30% risk of relapse within 9 days of ingestion. Mechanistically it is thought that NSAIDs predispose to development of Crohn's disease by inhibiting cyclo-oxygenase which in turn reduces prostaglandin levels resulting in lower anti-inflammatory cytokines (Berg *et al.*, 2002).

b) Several studies have linked Crohn's disease to **antibiotic** therapy (Hildebrand *et al.*, 2008; Hviid, Svanström and Frisch, 2011). This also applies to antibiotics given during the first year of life (Shaw, Blanchard and Bernstein, 2010a). The exact mechanisms are not known.

c) Meta-analysis has shown a direct correlation between the length of taking the **oral contraceptive** and the development of Crohn's disease and further, that discontinuing such therapy leads to long term remission (Timmer, Sutherland and Martin, 1998). Although the mechanism is unknown, changes in intestinal permeability and microbiota constitution due to endogenous hormone levels have been proposed as explanations (Khalili, 2016). In a prospective trial there was a three-fold risk of relapse in those who continued on the oral contraceptive with risk amplification in those who continued to smoke tobacco (Shaw, Blanchard and Bernstein, 2010).

1.1.2.9 Vitamin D as an aetiological factor in Crohn's disease

Vitamin D is discussed in depth later in this chapter (Section 1.3) and so will be dealt with only briefly here. There are indications that vitamin D supplementation may play a pivotal role in reducing Crohn's disease relapse in those with deficiency, although results were not entirely conclusive (Miheller *et al.*, 2009; S. P. Jørgensen *et al.*, 2010; Yang *et al.*, 2013). Further randomised interventional trials with vitamin D supplementation are required. One problem facing researchers is to know how much vitamin D to give. The Institute of Medicine and the Endocrine Practice Guidelines Committee recommend that, in those at risk of deficiency, a maintenance dose of 1000 IU daily be given (Ross *et al.*, 2011). However, in those with

documented deficiency 2000 IU daily be given for 6 weeks followed by a maintenance dose if risk factors are established. Pappa et al investigated the optimal level of vitamin D therapy (at three dose levels) in patients with Crohn's disease aged 5-21 years who were vitamin D deplete (Pappa *et al.*, 2011). The results showed that 50,000 IU per week of vitamin D2 for 6-weeks and 2000 IU of vitamin D3 daily were superior to vitamin D2 2000 IU daily. Furthermore, the regime using D2 was superior to the vitamin D3 regime in achieving blood levels of greater 32 ng/mL. Many questions remain to be answered including: Does low serum levels of vitamin D predispose to Crohn's disease? What is the role of vitamin D in prevention of disease activity and prevention of relapse?

1.1.3 Clinical presentation and disease classification of Crohn's disease

The clinical presentation of Crohn's disease is largely governed by the site/s of involvement and the behaviour of the disease. The most common site for Crohn's inflammation is ileocolonic, with isolated terminal ileal, and perianal being other common affected sites (Farmer, Hawk and Turnbull, 1975). Less commonly, patients may have pan-enteric small bowel disease or upper gastrointestinal disease. Crohn's is frequently inflammatory in nature early in its disease course, with fewer patients having stricturing or penetrating disease at the time of diagnosis. However, as the interval since diagnosis increases so the risk of developing deep ulcers, strictures and fistulae increases (Gupta *et al.*, 2010) (figure 1.4). Thus, inflammatory (rather than stricturing or fistulising) small bowel Crohn's disease may present with abdominal pain and diarrhoea; symptoms consistent with subacute small bowel obstruction; or with malabsorptive sequelae.

Colonic Crohn's disease often has a similar clinical presentation to UC, with diarrhoea and rectal bleeding predominating. Perianal disease may present with debilitating perineal pain, malodorous discharge or frank perianal sepsis. For those who have developed stricturing disease of the small bowel or colon; abdominal pain, distension, constipation, bloating and vomiting will predominate. The symptoms of fistulising luminal Crohn's disease will relate to the anatomical location of the fistula. Common sites are entero-peritoneal, entero-enteric, entero-cystic, and entero-cutaneous. In addition to gastro-intestinal disease, patients with IBD may develop complications at extra-intestinal sites (see 1.1.3.1).

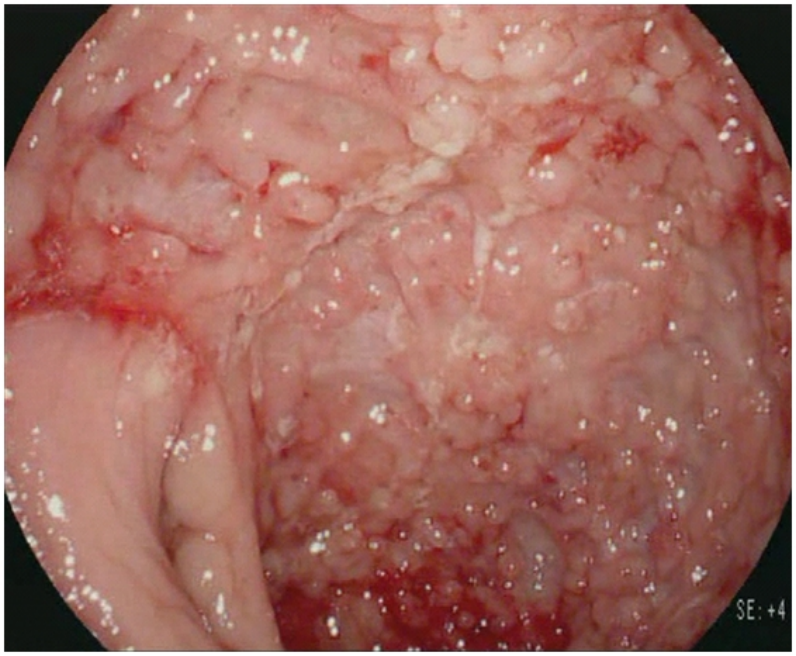


Figure 1.4: High-definition endoscopic images demonstrating typical Crohn's disease inflammation and ulceration with frank bleeding (Neumann *et al.*, 2012).

For the purposes of this thesis, clinical descriptors are based upon anatomical and symptomatic classifications, namely the Montreal Classification and the Harvey-Bradshaw Index (HBI). This is

because accurate anatomical classification of disease and precise symptom scales have become increasingly important to clinicians. They allow for standardisation of practice, prognostic assessment and meaningful counselling.

1.1.3.1 Montreal Classification

The Montreal Classification is a standardised methodology for defining the age at diagnosis (A), disease location (L) and pathological behaviour (B) of Crohn's disease (Silverberg, Satsangi, Ahmad, I. D. Arnott, *et al.*, 2005). It was published as "the Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology", and was an update to replace the original consensus from Vienna (Gasche *et al.*, 2000).

Montreal Classification of Crohn's disease	
Age at diagnosis	A1: Less than 16 years A2: Between 17 and 40 A3: Over 40 years
Location of disease	L1: Isolated ileal disease L2: Isolated colonic disease L3: Ileocolonic disease L4: Proximal disease*
Behaviour of disease	B1: Luminal/ inflammatory B2: Stricturing B3: Penetrating/ fistulising P: Perianal disease**

Table 1.1: The Montreal Classification of Crohn's disease (Silverberg, Satsangi, Ahmad, I. D. R. Arnott, *et al.*, 2005). **L4 disease may occur in isolation or in conjunction with any of L1-L3 disease. ** Perianal disease may occur in isolation or in conjunction with any of B1-B3 disease.*

Should the Montreal classification be combined with a measure of a disease activity classification (e.g. Harvey-Bradshaw Index, below), then a robust assessment of the patient's status at that time is achieved.

1.1.3.2 Harvey-Bradshaw Index

The Harvey-Bradshaw index (HBI) is a disease activity assessment score commonly used in clinical practice (Harvey and Bradshaw, 1980). The HBI (see Table 1.2 below) was chosen as a simple to use and well validated scoring system for assessing Crohn's disease activity. The first three parameters were assessed over the 24 hours prior to study attendance, with abdominal mass and complications assessed by the physician during the study visit.

Harvey-Bradshaw Index

Symptom/ sign	Severity	Score
General well being	Well	0
	Slightly poor	1
	Poor	2
	Very poor	3
	Extremely poor	4
Abdominal pain	None	0
	Mild	1
	Moderate	2
	severe	3
Diarrhoea		1 for each liquid stool per day
Abdominal mass	None	0
	Dubious	1

	Definite	2
	Definite with tenderness	3
Complications	None	Score 1 for each item except for “None”
	Arthralgia	
	Uveitis	
	Erythema	
	nodosum	
	Aphthous ulcers	
	Pyoderma gangrenosum	
	Anal fissure	
	New fistula	
	Abscess	

Table 1.2: The Harvey-Bradshaw disease severity index. HBI <5 remission; 5-7 mild disease; 8-16 moderate disease; >16 severe disease.

For drug therapy research purposes, the Crohn's Disease Activity Index (CDAI) takes elements of the HBI and adds further parameters (Haematocrit, percentage deviation from standard weight, concomitant use of opiate or other anti-diarrhoeal agent). The CDAI was not used in our study.

1.1.3.3 Extra-intestinal complications of Crohn’s disease

Most body systems can be affected by IBD, with the skin, joints and eyes being most commonly involved (Vavricka *et al.*, 2015; Hendy, P 2017). The list below is not exhaustive:

Body system	Disease(s)
Dermatological	Pyoderma gangrenosum, erythema nodosum, buccal aphthous ulceration
Rheumatological	Axial arthritides (ankylosing spondylitis and sacroileitis)
	Type I polyarthritis - Involvement of <5 large joints; usually associated with active disease
	Type II polyarthritis - >5 small joints are involved; not associated with active disease and tend to be chronic
Ocular	Episcleritis, scleritis and uveitis
Haematological	Iron deficiency anaemia
Renal	Oxalate stones, amyloid deposition, tubulointerstitial nephritis, glomerulonephritis
Hepatobiliary	Primary sclerosing cholangitis, IBD therapy-induced hepatotoxicity, primary biliary cirrhosis, chronic active hepatitis, cholelithiasis
Respiratory	Bronchiectasis, bronchiolitis obliterans organizing pneumonia (BOOP), fibrosing alveolitis, pulmonary embolism, IBD therapy-induced lung disease
Nutritional deficiencies	Secondary to: malabsorption, intestinal protein loss, extensive ileal resection

Table 1.3: Extra-intestinal manifestations of inflammatory bowel disease, by body system.

1.1.4 Investigations for Crohn's disease used in this thesis

There are many investigations used in making the diagnosis of Crohn's disease and to aid prognostication and disease severity assessment, but here we shall just review the two clinical laboratory investigations used in this thesis; c-reactive protein (CRP) and faecal calprotectin.

1.1.4.1 CRP

C-reactive protein is a pentameric acute phase protein produced by hepatocytes at low levels during health, but at high levels during inflammation. CRP levels correlate strongly with other disease activity markers in Crohn's disease, unlike in UC where the CRP response is more modest (Vermeire, Van Assche and Rutgeerts, 2004). The highest correlation in Crohn's disease occurs with isolated colonic and ileocolonic disease, and a weaker correlation occurs with isolated ileal disease. Whilst CRP is a relatively sensitive marker for inflammation in Crohn's disease it is not specific and will be raised with other causes of systemic inflammation including infections and malignancy and indeed CRP has been used in staging multiple myeloma (Bataille *et al.*, 1992).

1.1.4.2 Faecal calprotectin

Faecal calprotectin (FC) is a neutrophil derived calcium and zinc binding protein found in stool, with high sensitivity and specificity for gastrointestinal mucosal inflammation. Calprotectin comprises 60% of neutrophil cytosolic protein content, and therefore faecal calprotectin levels are directly proportional to neutrophil migration to the tissue of the gut. As a result, faecal calprotectin is more sensitive than CRP in both Crohn's disease and UC (Chang, Malter and

Hudesman, 2015). Whilst sensitive and specific for gut inflammation, faecal calprotectin is not specific for IBD, and indeed can be raised with gastrointestinal infection, neoplasia and the use of non-steroidal anti-inflammatory medication (NSAID).

The faecal calprotectin value cut-off for normal, non-inflamed colon has yet to be definitively established, and probably varies depending upon the clinical situation (e.g. differentiation of IBD from IBS may have a different FC cut-off from the cut-off between active and inactive disease states in Crohn's disease). Bringing the cut-off lower will improve sensitivity but reduce specificity, whilst increasing the cut-off value will increase specificity for detection of inflammation, but reduce sensitivity. In practice, a value of >50 $mg.ml^{-1}$ would necessitate endoscopic evaluation in a patient whose symptoms cannot be distinguished between IBD and IBS. For those patients with a known diagnosis of IBD, higher levels are used as a cut-off to indicate active disease, and faecal calprotectin <150 $mg.ml^{-1}$ has been demonstrated to have negative predictive value 97% and positive predictive value 71% for active inflammation (Naismith *et al.*, 2014).

1.1.5 Medical therapies to treat Crohn's disease

Current therapies to treat Crohn's disease are not universally effective, and are frequently discontinued due to an inadequate primary response, a secondary loss of response, or due to excessive side effects. Given the narrow range of therapeutic options available, it is important to optimize these drugs, both in terms of searching for possible treatment adjuncts as well as with accurate prognostication to allow suitable patient selection. Vitamin D has been postulated as a possible therapeutic adjunct in IBD.

Current therapies may be considered under four headings of symptomatic, therapeutic non-biologic, biologic and psychological. The last is of increasing importance; with some evidence that cognitive behavioural therapy (CBT), hypnotherapy and mindfulness may all have a role in managing IBD. CBT has been shown to improve quality of life scores and coping skills for patients with IBD (Knowles, Monshat and Castle, 2013), whilst hypnotherapy is associated with reduction of inflammatory markers (Mawdsley and Rampton, 2005). There are some data supporting the use of mindfulness in IBD, with improvement in quality of life, anxiety and depression (Neilson *et al.*, 2015).

Symptomatic therapies relate to control of abdominal pain and diarrhoea; include analgesia and anti-diarrhoeal classes, and will not be discussed further here.

Therapeutic, non-biologic medications comprise of well-established treatments which are known for their efficacy and whose side effects are well described. Steroids, 5-ASA, azathioprine, methotrexate, ciclosporin, metronidazole and ciprofloxacin are all used with varying strengths of evidence base. Biologic agents include anti-TNF (adalimumab, certolizumab, infliximab (IFX), golimumab), anti-IL-12/23 (ustekinumab) and anti-integrin (vedolizumab, natalizumab, etrolizumab) monoclonal antibody therapies (see 3.2). Newer biological therapies targeting other components of the inflammatory cascade are nearing clinical use. Biological drugs are increasingly being used in a top down regimen whereby they are introduced early for moderate to severe disease (HBI 9/10->16) with the aim of top down therapy preventing the pathway of inflammatory to stenotic or fistulising disease (Rogler, 2013).

1.1.5.1 Anti-TNF therapy mechanisms of action

The anti-TNF drug infliximab is studied in this thesis, and hence receives extra scrutiny here. The mechanism of action of anti-TNF drugs is incompletely understood (Levin, Wildenberg and van den Brink, 2016). In order to gain a greater understanding one must first review the biology of the target cytokine, which is more than just a pro-inflammatory protein. TNF is produced as a membrane bound protein (mTNF). Soluble TNF (sTNF) is produced when mTNF is enzymatically cleaved by TNF-alpha converting enzyme (TACE). TNF expresses two receptors (TNF receptor I and II, or TNFRI and TNFRII) and the receptors activate different signaling pathways (Kalliolias and Ivashkiv, 2016). TNFRI mainly binds to sTNF whilst TNFRII binds mTNF. TNFRI may activate both pro- (caspase-8 dependent cell death signalling) and anti- (NF-$\kappa\beta$) inflammatory pathways, and it is the balance between the two which dictates the cellular response. TNFRII, however, does not induce cell death signal pathways, only cell survival and inflammation pathways.

TNF, therefore, is not only responsible for inflammation but also for cellular migration, proliferation and death. Further evidence of TNF being more than just an inflammatory cytokine is given by studies demonstrating wound healing induction by TNF (Dubé, Punit and Polk, 2015), and murine models where IL-10 colitis is aggravated by the inability to produce TNF (Hale and Greer, 2012).

The first drug to successfully antagonize TNF was infliximab, a chimeric (mouse and man), monoclonal IgG1 antibody and this was followed by development of two further structurally similar drugs (adalimumab and golimumab). Targeting soluble TNF has also been successful in rheumatology, with soluble TNFRI receptor, onercept, and TNFRII-Fc fusion protein, etanercept,

both showing efficacy. A further anti-TNF drug, certolizumab (a pegylated anti-TNF F2 fragment), attempted to reduce immunogenic side effects by removing interaction with the Fc receptor (involved with binding to B cells, and hence antibody formation).

Interestingly, there are differences in clinical outcome between the different anti-TNF medications. Not only is etanercept ineffective at treating IBD (Sandborn *et al.*, 2001), but it may also induce the development of IBD (predominantly Crohn's disease) when used to treat rheumatic disease (Toussirot *et al.*, 2012; O'Toole, Lucci and Korzenik, 2016). That all of the above anti-TNF medications are successful in treating rheumatoid arthritis, but etanercept, golimumab and onercept are ineffective at treating Crohn's disease, points to additional mechanisms, beyond neutralization of sTNF, involved in the efficacy of anti-TNF therapies in Crohn's disease.

Two significant and plausible pathways by which anti-TNF drugs may work have been postulated as induction of lamina propria T cell apoptosis (Ten Hove *et al.*, 2002) and Fc region-dependent induction of M2 macrophages (Vos *et al.*, 2011). The apoptosis of inflammatory cells such as T cells and neutrophils in the lamina propria is an important step in the process of resolution of inflammation in IBD. Two separate studies demonstrated that treatment with infliximab is associated with induction of apoptosis of lamina propria inflammatory cells within 24 hours, and that this process continues up to four weeks (Van den Brande *et al.*, 2003; Atreya, Zimmer, Bartsch, Waldner, Atreya, Neumann, Hildner, Hoffman, Kiesslich, Rink, Rau, Rosejohn, *et al.*, 2011). Extent of apoptosis (as measured by uptake of technetium-labelled annexin V) correlated with improvement in CDAI. Interestingly, several studies have demonstrated that infliximab, adalimumab and certolizumab all bind to activated T cells, but that etanercept does not, supporting T cell apoptosis as a mechanism of drug action, and potentially explaining the

difference in efficacy of these different anti-TNF therapies. Since dendritic cells can shape T cell homing, phenotype and population expansion, they may be relevant in this suggested mechanism. Indeed, blockade of TNF (using a p55 sTNF receptor) during the maturation of DCs (from blood monocytes) induces an immature DC phenotype with altered chemokine expression (Van Lieshout *et al.*, 2005).

The second postulated mechanism of induction of M2 macrophages, described above, is specific for anti-TNFs that contain an Fc region, such as infliximab, adalimumab, but not certolizumab. M2 macrophages (see appendix 8.2) are regulatory and key to wound healing. Increases in M2 macrophages were seen in the mucosa of those subjects who responded to infliximab, but not those who didn't (Vos *et al.*, 2012). Certolizumab, with no Fc region, does has a poor rate of mucosal healing compared with the infliximab and adalimumab. Furthermore, murine transfer model studies demonstrate that anti-TNF effects are dependent on Fc binding (McRae *et al.*, 2016).

1.1.5.2 Cutaneous side effects of anti-TNF therapy

As with any drug, the list of potential side effects of anti-TNF agents is long. Here, special attention is paid to cutaneous 'paradoxical' manifestations (CPM), and the possible mechanisms behind them, as it is of particular relevance to the thesis.

Cutaneous side effects of anti-TNF therapy are many and common, occurring in up to one third of patients (Pellicer *et al.*, 2012). Whilst infusion reactions and injection site inflammation and infections might be obvious, more surprising is the occurrence of paradoxical psoriasis, especially

given that anti-TNF is a licensed treatment for psoriasis. Other skin complaints associated with anti-TNFs are: skin cancers, cutaneous lupus and lupus like syndrome, skin vasculitides.

The side effect of new onset psoriasis and worsening of pre-existing psoriasis (including pustular and palmoplantar subtypes) is integral to the pathoimmunological changes as described in Chapter 3 of this thesis. It has been shown previously that infliximab can alter the homing marker expression of circulating T cells, thus the study of impact of infliximab upon DC homing marker expression is of interest (Peake, Bernardo, Mann, *et al.*, 2013a).

Psoriaform lesions are the most common presentation of CPM and occur more commonly in females more than males. Most patients are able to continue with anti-TNF treatment by using standard topical therapies, but those with more severe CPMs are required to discontinue the drug (Sridhar *et al.*, 2018).

Occurrence of CPMs is not associated with infliximab trough levels (Coutzac *et al.*, 2015), and whilst rheumatological paradoxical manifestations are associated with antinuclear antibodies, the same is not the case for CPMs. The occurrence of paradoxical psoriasis is class dependent, rather than drug dependent, occurring with all anti-TNFs, and Just over half of patients who develop psoriaform skin reaction with one anti-TNF will have a recurrence when treated with a second anti-TNF (Cullen *et al.*, 2011).

Conversely, anti-TNFs may successfully treat skin EIMs related to IBD, with erythema nodosum and pyoderma gangrenosum both responding well (and usually with complete resolution) to infliximab (Regueiro *et al.*, 2003).

Mechanisms of paradoxical skin reactions are poorly understood. Since there are numerous different reactions it would seem likely that there are a number of different processes underlying each. Here the focus is on mechanism of paradoxical psoriasis. Whereas classical psoriasis is driven by TNF/IL-23/Th17 pathways, paradoxical psoriasis is postulated to be secondary to the action of plasmacytoid DCs, possibly manifested through excessive on-going production of Type 1 IFN (Mylonas and Conrad, 2018). This is similar to lupus, which is also characterised by high concentrations of Type I IFN (Ytterberg and Schnitzer, 1982). Blockade of TNF increases the ability of pDC to produce IFN. pDC are seen in excess in the dermis of psoriasis, and blocking the production IFN by pDC prevented development of psoriasis in a murine model, but these data are for classical psoriasis, and similar data are lacking for paradoxical psoriasis. However, high levels of IFN are found in lesional skin in paradoxical psoriasis, and not found in healthy skin of the same patient, or indeed in lesional skin of patients with non-anti-TNF related dermatological conditions (Seneschal *et al.*, 2009).

Unlike in classical psoriasis, the process appears to be independent of T cell infiltration (Conrad *et al.*, 2018). This is relevant, because the disease does not recur after discontinuation of the drug, unlike T cell mediated classical psoriasis, where immune memory exerts an influence.

1.1.5.3 Other side effects of anti-TNF therapy

I) Crohn's disease patients should be screened for latent *M. tuberculosis* prior to biologic therapy. If found to be positive, then anti-tuberculous chemotherapy should be commenced prior to the introduction of anti-TNF therapy. Screening should include a chest radiograph, an IGRA

(interferon gamma release assay) blood test and a tuberculin skin test. Treatment, when indicated, should be with isoniazid monotherapy for 6 months or rifampicin and isoniazid dual therapy for 3 months. The drug side effects should be emphasized, specifically fulminant hepatic failure.

II) Serious and potentially life-threatening disease include lymphoma, leukaemia, neutropenia, agranulocytosis, ITP, TTP, erythema multiforme, Stevens Johnson syndrome, toxic epidermal necrolysis and acute liver failure.

III) It is also important for the patient to undergo appropriate annual vaccinations including influenza and pneumococcal vaccines.

1.2 Introduction to Dendritic Cells

An antigen presenting cell with innate functions which acts as the master controller of the adaptive immune system.

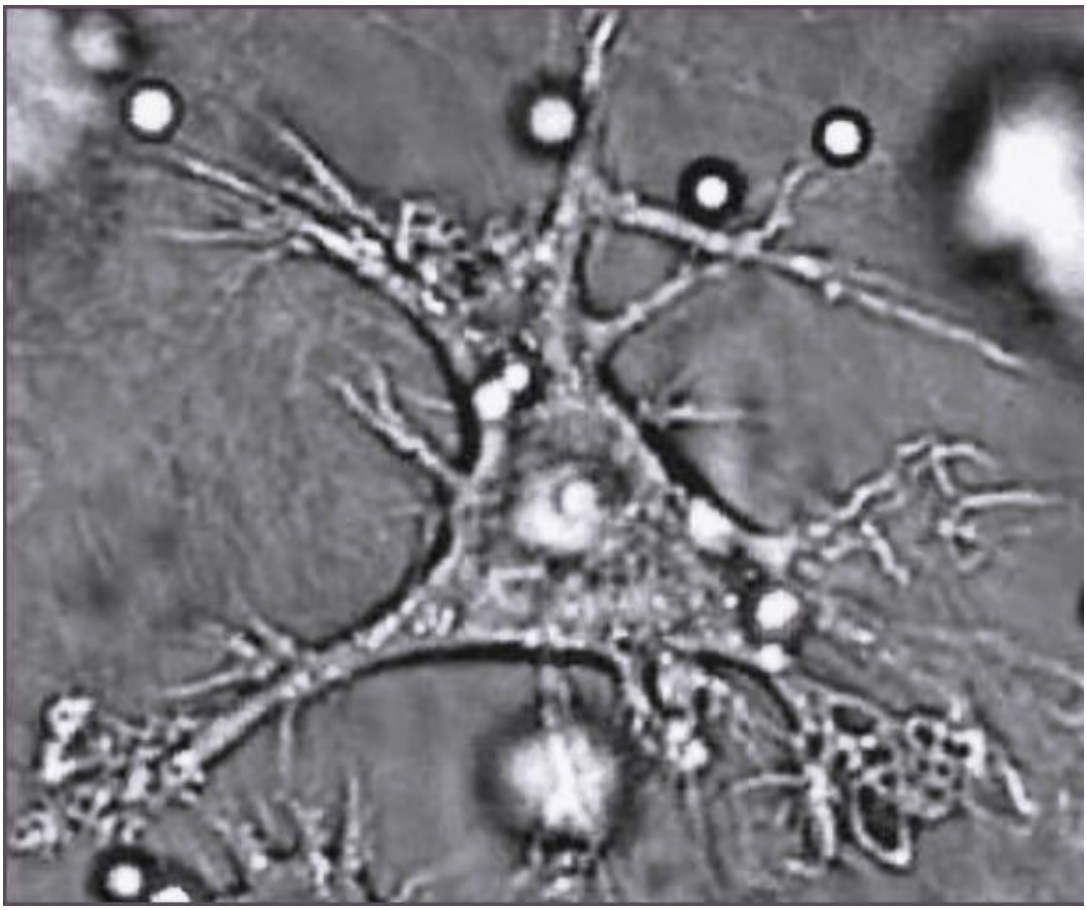


Figure 1.5: Electron microscopy image of a dendritic cell interacting with its surroundings. The numerous veiled projections on the surface of the dendritic cells are clearly visible (Behnsen *et al.*, 2007).

1.2.1 Characterisation of dendritic cells

Dendritic cells (figure 1.5) are the most potent of antigen presenting cells and can promote either immunity or tolerance through local actions (innate response) and through the stimulation of T cells (adaptive immunity). With their unique ability to stimulate naive T cells, and thus direct T cell response to either immunity or tolerance, DCs have been implicated in the pathogenesis of Crohn's disease (known to be associated with an exaggerated T cell response).

Dendritic cells are bone marrow derived immune cells present primarily in lymphoid tissue and in circulation. Under inflammatory conditions DCs may also arise from blood monocyte precursors. DCs play a key role in both initiating and regulating the immune response. The principal role of the DC is as an APC, but DCs have other non-APC mechanisms of action such as the production of cytokines.

There is no single marker whose presence identifies DCs. Instead DCs are usually identified by expression of HLA-DR (MHC-II molecule) and the absence of markers expressed by other leucocytes termed 'lineage cocktail' (Cluster of differentiation (CD) 3, CD14, CD16, CD19 and CD34 see Table 1.4 below) which excludes cells with similar morphology including T cells, B cells, NK cells, haematopoetic stem cells, monocytes and macrophages (Merad *et al.*, 2013; Mann, Bernardo, English, Landy, Al-Hassi, Peake, *et al.*, 2016).

Cocktail of lineage markers to aid identification of dendritic cells

Surface marker	Cell type stained
CD3	T lymphocyte
CD14	Macrophage, monocyte, neutrophils, eosinophils
CD16	Natural killer (NK) lymphocytes, macrophages, cultured monocytes, and neutrophils
CD19	B lymphocytes
CD34	Haematopoietic stem cell

Table 1.4: cell surface markers included in the lineage exclusion to identify DCs, and the cell type which they are intended to exclude.

Various DC subsets have been identified in humans, defined by their surface receptor profile. Different terminologies are utilised when describing DCs, with nomenclature differing between murine and human models and also by tissue type under study (Mann *et al.*, 2013). Myeloid DCs (mDC [also known as classical DC]) and plasmacytoid DCs (pDC) constitute the most significant subsets. Myeloid DC are $CD11c^{+}CD123^{-}$, whilst pDC are characterised by a $CD11c^{-}CD123^{+}BDCA2^{+}$ profile. mDC can be further subdivided into cross-presenting (BDCA-3+) or direct-presenting (BDCA-1+). The functional properties of DCs depend upon the state of maturity of the DC (immature, activated, tolerogenic etc.) as well as upon which subset it belongs to. Circulating $CD11c^{+}$ mDC are immature with low expression of co-stimulatory molecules and a low capacity

to stimulate a T cell response. Upon stimulation by a bacterial product, mDC become activated with ability to present antigen associated with MHC-I or MHC-II molecules (signal 1) and will upregulate co-stimulatory molecules like CD80/CD86 (signal 2). Together these signals stimulate antigen-specific T cell proliferation. Immature and semi-mature DC (lack of signal 2), on the other hand, may stimulate T cell anergy or tolerance. Dendritic cells are found in many tissues types and in the circulation. In this introduction, the focus will be on circulating DCs, as these were studied in this thesis, and on intestinal DCs, as these are highly relevant to Crohn's disease and have a significant body of literature (Davies and Abreu, 2015).

1.2.2 Dendritic cells and intestinal inflammation

In the gut, the mucosal immune system is exposed to a significant antigenic load, in the form of ingested food and the resident microbiota. The balance between immune tolerance, (allowing non-reaction to commensal organisms and food) and immunogenic inflammatory response (facilitating the killing of pathogens and prevention of cancer) is crucial to maintaining health. Antigen presenting cells, including DCs, macrophages and intestinal epithelial cells 'sample' the gut antigenic load with pattern recognition receptors (PRRs) which recognise and bind to the well conserved pathogen-associated molecular patterns (PAMPs) (Abraham and Medzhitov, 2011). Dendritic cells act as a bridge between the innate and adaptive immune systems in the gut. They are responsible for stimulating production of many proteins involved in immune defence (innate immunity) and, upon presentation of antigen, can also facilitate either a regulatory or an inflammatory T cell response (adaptive immunity), and hence are considered to be important in the development of intestinal inflammation.

1.2.3 Modulation of dendritic cell activity

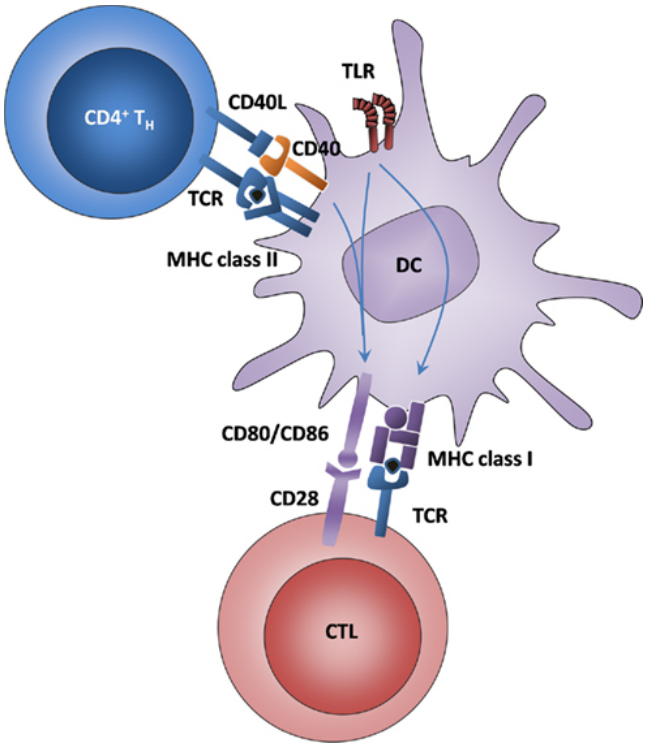


Figure 1.6: A mature dendritic cell expressing the markers required for effective interaction with naïve T cells, including MHCII, PRR and costimulatory molecules (Thaiss *et al.*, 2011).

Dendritic cells are thought of as the "master controller" cell of the immune system (figure 1.6), and their functional activity (and hence that of the cells which they direct) is modulated in four main ways in health, and these are true of circulating as well as tissue DCs:

i) **Microbial sensing.** Level of expression of PRRs such as toll-like receptors (TLRs) affects the ability of DCs to 'sample' gut microbes. Thus, down regulation of PRRs will promote a tolerogenic immune response. This will affect both innate and adaptive immune responses.

ii) **Activation markers.** Expression of co-stimulatory molecules such as CD40, CD80, and CD86 occurs upon DC activation and promotes downstream Th1/Th17 responses, known to be

mechanistic for the development of inflammation in Crohn's disease. Reduced expression of these co-stimulatory markers may correlate with reduced levels of inflammation (Steinman, Hawiger and Nussenzweig, 2003; Hart *et al.*, 2005a). Expression of Immunoglobulin-like transcript 3 (ILT3) on DCs has the opposite effect to the above activation markers, being an inhibitory receptor associated with immune inactivity (Cella *et al.*, 1997).

iii) **Homing markers.** Expression of homing surface markers can direct the DCs to specific tissues. The integrin β7 is associated with gut homing; C-C chemokine receptor 9 (CCR9) is associated with small bowel homing; CCR7 directs DCs to lymph tissue; whilst CCR4, CCR10 and cutaneous lymphocyte-associated antigen (CLA) are skin homing. In IBD, directing DCs away from the gut may help decrease the gut inflammatory response (PA Hendy *et al.*, 2016). Indeed, directing lymphocytes away from the gut is a major therapeutic mechanism of the anti-integrin class of drugs, including vedolizumab which interferes with the interaction between the mucosal madCAM receptor and the α4β7 receptor on the lymphocyte.

iv) **Cytokine profile.** The cytokine profile of DCs can dictate downstream immune responses. TNFα, IL-1β, IL-6, IL-12 or IL-23 are some of the key DC-derived cytokines which can promote pro-inflammatory Th1 and Th17 responses; whilst IL-10 may predispose to a modulatory Treg response.

1.2.4 Intestinal dendritic cells

The largest body of knowledge about intestinal DCs (GI-DCs) is from murine studies. GI-DCs direct development of gut-homing regulatory T cells and B cells in a process dependent on retinoic acid (RA) and the cytokine TGFβ. Dietary vitamin A is converted into RA via the RALDH-2 enzyme. The RALDH-2 enzyme is present on GI-DCs, but not on DCs from other tissue types, thus GI-DCs are innately more tolerogenic than DCs in other tissues (Persson *et al.*, 2013). There are subpopulations of GI-DCs, and the $CD103^+$ GI-DCs, found in both mice and men, are particularly tolerogenic, carrying both the RALDH-2 enzyme and αVβ8, the integrin required for activation of TGFβ. In mice, there are further subpopulations denoted by expression or absence of other surface proteins, such as CD11b; hence $CD103^+CD11b^+$ GI-DCs have different properties to $CD103^+CD11b^-$ GI-DCs, with the former being regarded as the more tolerogenic. There are differences in nomenclature and markers between mice and humans, but the human DC which corresponds to the tolerogenic murine $CD103^+CD11b^+$ GI-DCs is the CD103+SIRPa+ GI-DC (Watchmaker *et al.*, 2014).

Human GI-DCs in health are characterised by a regulatory phenotype with reduced microbial sensing capacity; reduced expression of HLA-DRII and surface co-stimulatory molecules; increased phagocytic properties and production of anti-inflammatory cytokines such as IL-10 (Hart *et al.*, 2005b; Mann, Bernardo, English, Landy, Al-Hassi, Peake, *et al.*, 2016). These adaptations bestow a decreased stimulatory capacity upon the GI-DCs, as compared with DCs from other tissues, a significant advantage in the most antigen-rich environment in the body – higher stimulatory capacity here would result in continuous inflammatory responses to food and non-pathogenic microbes.

Human GI-DCs vary in number, phenotype and function depending upon certain demographic characteristics of the individual, including age (Orsini *et al.*, 2012). The location in the GI tract also plays a role, as colonic DC are more tolerogenic than their terminal ileal counterparts and, furthermore, distal colonic DCs have greater numbers of CD103+SIRPa+ GI-DCs (regulatory in nature) than their proximal colonic equivalents (Mowat and Agace, 2014; Mann, Bernardo, English, Landy, Al-Hassi, Peake, *et al.*, 2016).

1.2.5 Circulating dendritic cells

Circulating DCs are immunologically immature, with high phagocytic capacity, high expression of PRRs (to enable PAMP recognition), and low T cell activation potential with low expression of co-stimulatory surface molecules (CD40, CD80 and CD86) (Villadangos and Schnorrer, 2007). Upon stimulation in peripheral tissues, DCs mature with internalization of PRRs, migration to the lymph nodes and increased expression of MHC and the co-stimulatory molecules, allowing for interaction with lymphocytes. Factors that can stimulate immature DCs and promote maturation include cytokines, PAMPs and DAMPs (Guermonprez *et al.*, 2002).

The two main DC subsets in circulation are mDC ($CD11c^+$) and pDC ($CD123^+$) of which mDC are the better studied subset, but even the lesser known pDC have some abnormalities in IBD.

1.2.6 Dendritic cell aberrancy in IBD

In IBD there may be an aberrant DC expression of any of the above markers (PRRs, co-stimulatory molecules, homing receptors and cytokines). There is also a fall in circulating DC numbers and an associated rise in GI-DC numbers (Baumgart *et al.*, 2005a).

i) Microbial sensing. Intestinal DCs exhibit an increased expression of the PRRs TLR2 and TLR4 in IBD (both UC and Crohn's disease) as compared with the expression in healthy controls (HC)(Hart *et al.*, 2005a). The increased expression of PRRs likely increases the ability of DCs to recognise or respond to microbial components (PAMPS) (either increasing affinity for the component, or allowing interaction with previously unrecognised components) and hence promote an immunogenic response.

ii) Activation markers. The profile of co-stimulatory receptor expression also differs between HCs and patients with IBD. Expression of CD40 is elevated in inflamed tissue, but not in non-inflamed tissue, in Crohn's disease. Expression of CD40 falls to normal levels after treatment with anti-TNF drugs. Because low expression of co-stimulatory receptors is associated with immune tolerance and high expression is associated with immunity this is another potential mechanism for the loss of control of intestinal homeostasis in IBD. CD40 is also found to be raised on circulating DCs in blood of patients with UC and Crohn's disease (Vuckovic *et al.*, 2001; Ma and Clark, 2009).

iii) Homing markers. Dysregulated homing marker expression is witnessed on T cells in IBD. Circulating γδ T cells express high levels of gut homing marker (CCR9) in IBD, whilst patients with IBD and its cutaneous manifestations express high levels of skin homing molecule (CLA) (E. R. Mann *et al.*, 2012). Homing receptor expression is also aberrant in the blood DC population in

IBD, with increased CCR9 and decreased CLA expression in active isolated small bowel Crohn's disease and increased β7 (gut homing) and decreased CCR9 in active isolated large bowel Crohn's disease (Peake, Bernardo, Knight, *et al.*, 2013).

iv) Cytokine profile. This is covered in Section 1.2.7 (next).

1.2.7 Cytokines, their interactions with dendritic cells and their role in IBD

Cytokines are small (up to 20Da) proteins which are synthesised within a variety of cells and which, when secreted, modulate the activity of other cells (figure 1.7). By this means of cell signalling, immune cells (T and B lymphocytes, macrophages, dendritic cells and mast cells) can modify their responses. GWAS studies have identified several IBD susceptibility loci containing genes which code for cytokines or proteins involved in cytokine signalling (Chen and Sundrud, 2016). IL-10 and IL-2 KO mice both develop spontaneous colitis. Treatment with blockade of pro-inflammatory cytokines has successfully translated from the animal laboratories to the IBD clinic (unlike therapy with anti-inflammatory cytokines, such as IL-10, IL-11 and IFNβ, which has been a disappointment). Cytokines may also play a prominent role in the development of stricturing and fistulising disease as well as extra-intestinal manifestations of IBD. As cytokines may circulate in the serum it seems reasonable to hypothesise that a circulating inflammatory load may trigger inflammatory sequelae at sites distant to the gut. This thesis studies the following cytokines, all previously linked to IBD: IL-1β, IL-6, IL-10, IL-12, IL-23, IFNα, TGFβ, TNFα. Some of these are discussed in further detail in Section 4.2

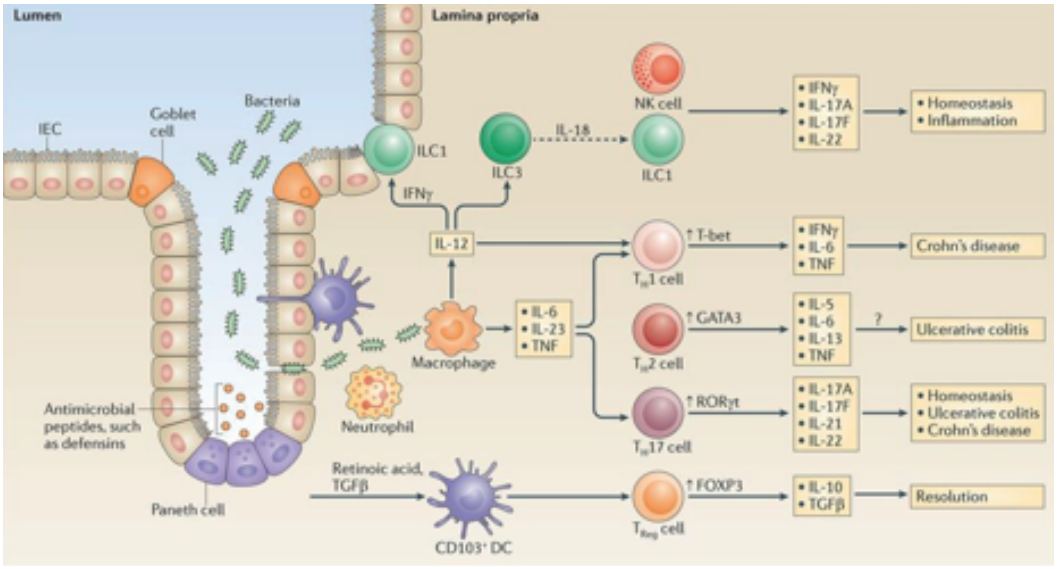


Figure 1.7: Production and effects of cytokines in health and in IBD (Neurath, 2014).

Discordant cell-cell communication via cytokine release is likely to be causal in the pathogenesis of Crohn's disease. As an example of this; DCs and macrophages produce excessive IL-23. Downstream effector cytokines of IL-23 include IFN-α, IL-17A/F, IL-22 and GMCSF, all of which have been implicated in the genesis of IBD (Strober and Fuss, 2011). The inflammation in Crohn's disease is known to be driven by the Th1/ Th17 pathway, and the levels of the inflammatory cytokines IL-6, IL-12, IL-17, IL-23 and TNF-α found in intestinal mucosa are all raised.

1.2.8 Difficulties with the study of dendritic cells

There are many difficulties in studying DCs. They are a relatively rare cell type (comprising roughly 1% of peripheral blood mononuclear cells) so numbers in any given sample are correspondingly low (Dauer *et al.*, 2003). In addition, there is no single receptor unique to dendritic cells to allow for easy identification, thus the methods used to identify them differ in the literature. DCs are

heterogeneous across various tissues, for example circulating, gut, lymphatic and skin resident dendritic cells all have different phenotypes. The majority of studies on DCs are performed in mouse models and although there are parallels between mouse and human, the many differences make it difficult to draw any firm conclusions relevant to humans based solely on murine systems (Mann *et al.*, 2013). Finally, within human research the majority of studies have used laboratory-generated monocyte-derived dendritic cells, whilst far fewer (including our studies here) examine fresh DCs labelled within the peripheral blood mononuclear cell (PBMC) population and there are protean and significant differences between the two types of DC.

1.3 Introduction to Vitamin D

There is mounting evidence that vitamin D deficiency in Crohn's disease is prevalent and associated with worse outcomes. Vitamin D supplementation has yet to be shown to be efficacious in modulating Crohn's disease.

1.3.1 What is vitamin D?

The effects of vitamin D on bone metabolism are well known (and not covered in any detail in this thesis). It increases intestinal absorption of calcium, phosphate and magnesium ions. It promotes mineral deposition in the skeleton and it inhibits calcium excretion in the urine. The main source of pro-vitamin D in humans is from the synthesis of cholecalciferol (from cholesterol) in the skin (figure 1.8). Subsequently a two-stage hydroxylation occurs, firstly in the liver and then in the kidneys, to produce $1,25(OH)_2D$ which is the active form of vitamin D (also known as calcitriol). Vitamin D and parathyroid hormone (PTH) form a tightly controlled feedback loop and together are the major regulators of calcium and phosphate homeostasis. PTH is a major stimulator of vitamin D in the kidneys, whilst vitamin D is inhibitory for PTH secretion.

Sources of vitamin D2 include mushrooms and fortified beverages. Sources of vitamin D3 include fish liver oils and fatty fish including mackerel, salmon, sardines and tuna. The immunological role of vitamin D is less well appreciated than its role in bone metabolism.

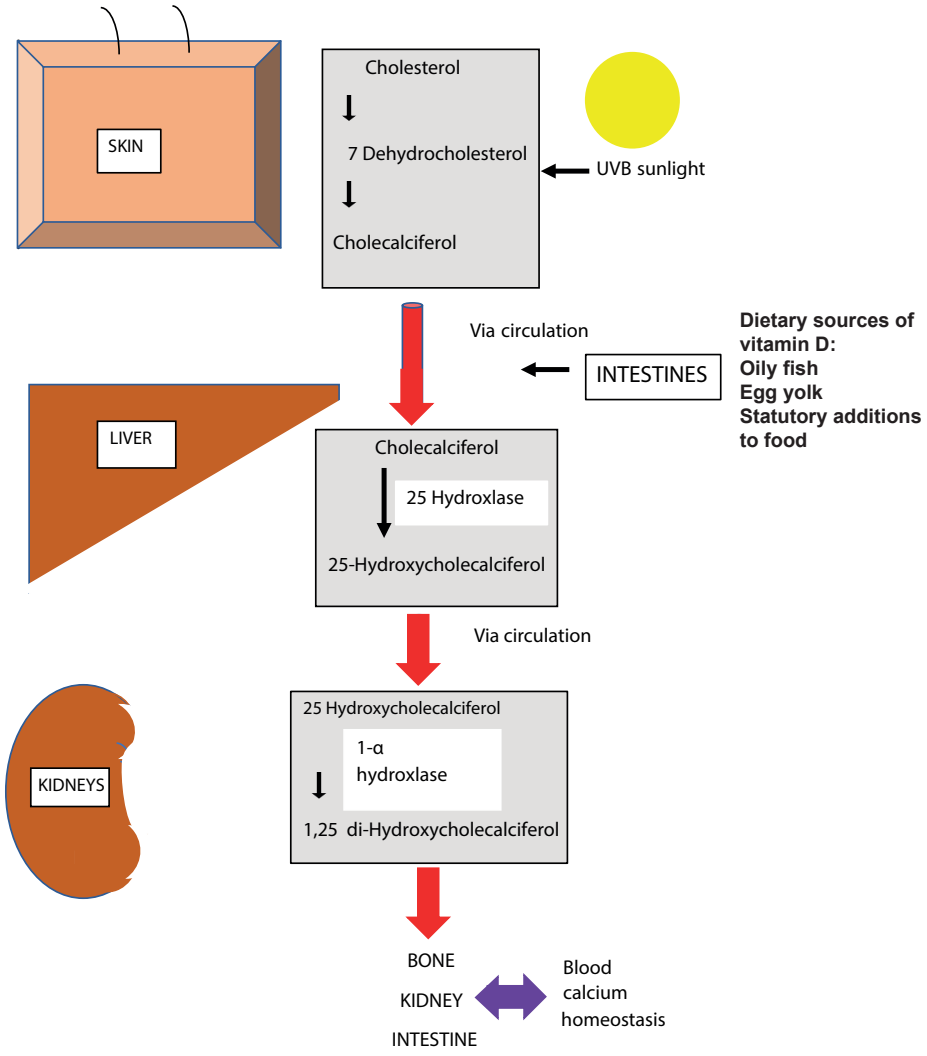


Figure 1.8: The synthesis of vitamin D from cholesterol in the skin.

1.3.1.1 What is the optimal serum concentration of vitamin D in humans?

Vitamin D has several different functions in human physiology, including; bone health; calcium metabolism and immunological roles. There are a few potential ways of measuring body stores of vitamin D, including quantifying D2, D3 and total (D2 + D3) levels (Holick, 2009). In addition, both hydroxy- and dihydroxy- vitamin D may be measured. Hydroxy-vitamin D levels are the only

levels that are monitored in clinical practice; 25(OH)D has a half-life of 2-3 weeks, whilst the half-life of $1,25(OH)_2D$ is only 4-6 hours. In addition, serum $1,25(OH)_2D$ circulates at a thousand-fold lower levels than 25(OH)D and even these low levels are extremely transient and prone to greater flux – they will rise in response to activation by PTH; thus a patient with low vitamin D stores may well have a normal or raised $1,25(OH)_2D$ level.

The laboratory "normal range" has changed with time. Originally, normal levels were often judged by taking several hundred healthy control volunteers and using mean +/- 2SD of their results as the normal range. Of course, now we know that a significant proportion of the general population in Europe and the USA are vitamin D deficient (Holick, 2006). If 25(OH)D levels are plotted as a function of PTH levels, the higher the vitamin D level the greater the suppression of PTH, up to a level of 30-40ng/mL (70-90nmol/L) where the PTH level plateaus (Chapuy *et al.*, 1997). Vitamin D provocation testing has suggested that levels of <20ng/mL (<50nmol/L) should be considered deficient (Heaney *et al.*, 2003). Today some laboratories take >50nmol/L (>20ng/mL) as normal, whilst others (including the laboratory at London Northwest Healthcare Trust) take >75nmol/L (>30ng/mL) as normal. Indeed, different academic societies propose different reference ranges for vitamin D. The Institute of Medicine (IOM) in its 2011 update suggested that 40nmol/L serum 25(OH)D level would ensure that 50% of the population would meet its vitamin D requirement in terms of bone health, whilst if the level was set at 50nmol/L that figure would change to 97.5% (Ross *et al.*, 2011). In conflict with this, the Endocrinology Society has proposed that levels under 50nmol/L are deficient and optimal levels are >75nmol/L (Holick *et al.*, 2011).

Interestingly, median 25(OH)D levels in hunters and farmers in equatorial Africa (the "cradle" from which mankind started its journey to inhabit the globe) are 115nmol/L (Luxwolda *et al.*, 2012). These subjects spend more time outdoors and wear less clothing than the Western population. This suggests that Western practices of spending large periods of time in doors, and covering a large percentage of the body in clothing, have dramatically altered the median serum levels of vitamin D. It may be that the higher range is our "evolutionary target" vitamin D level, but it is worth bearing in mind that this may not be the optimal target now, as millennia have passed and dietary and physiological adaptations may (or may not) render such high target levels less safe for high latitude Caucasians.

1.3.1.2 Mechanism of genomic effects of vitamin D

Most currently described intracellular actions of vitamin D occur through genomic regulation via the intranuclear vitamin D receptor (VDR). The VDR is a member of a superfamily of nuclear hormone receptors which includes other receptors implicated in IBD (peroxisome proliferator-activated receptors (PPAR) and the glucocorticoid receptor; involved in the therapeutic actions of 5-aminosalicylates and corticosteroids respectively). Immune cells, including DCs, T cells and monocytes express VDR in their nuclei. Upon binding of the active form of vitamin D, $1,25(OH)_2D$, the VDR acts as a heterodimer with the retinoid X receptor (RXR) to regulate downstream vitamin D target genes. The heterodimer interacts with vitamin D-responsive elements (VDREs) within the promoter region of target genes, with consequent up- or down-regulation of transcription of a wide range of genes (Rachez and Freedman, 2000; Christakos *et al.*, 2003; DeLuca, 2004). Importantly, there are several IBD susceptibility loci which locate within VDR genes, including:

Fok1, Bsm1, Taq1, Apa1, Cdx2, poly (A), and Bgl1 (Simmons *et al.*, 2000; Dresner-Pollak *et al.*, 2004; Jostins *et al.*, 2012; Xue *et al.*, 2013). Both DCs and T cells express the necessary enzymatic machinery (CYP27A1) to synthesise $1,25(OH)_2D$ from its precursor 25(OH)D, thus they have a degree of autonomy to self-regulate nuclear transcription. VDR expression, in terms of both mRNA and protein, is significantly reduced in IBD (Abreu *et al.*, 2004; Wada *et al.*, 2009).

1.3.2 Vitamin D and its relevance to IBD and other chronic conditions

Vitamin D has been implicated in the pathogenesis of IBD through evidence from epidemiological studies, GWAS and animal models of intestinal inflammation. *In vitro* studies, including in IBD, have shown that treatment with vitamin D may have an immunomodulatory effect. Vitamin D has recently been introduced as a therapeutic adjunct to standard therapy in the treatment of other diseases including *Mycobacterium tuberculosis* and multiple sclerosis.

1.3.2.1 Epidemiological factors implicating vitamin D in IBD

Vitamin D deficiency is more common in patients with Crohn's disease than in healthy controls, and is present in those with newly diagnosed disease (Leslie *et al.*, 2008). The authors of the study concluded that low vitamin D levels present at the time of diagnosis suggest a causative link, rather than merely an association caused by the legion of compounding factors mentioned below. The aetiology of hypovitaminosis D in the IBD cohort is multifactorial and includes: malabsorption due to mucosal inflammation, surgical bowel resection and bile salt abnormalities;

reduced sunlight exposure; diet; reduced physical activity levels; a systemic inflammatory state; and, putatively, a causal role in the development of IBD (Garg *et al.*, 2012). The incidence of IBD is more common in cold northern climes than their sunny southern counterparts, but proving a causal relationship with retrospective data has been difficult (Sonnenberg, McCarty and Jacobsen, 1991; Loftus and Sandborn, 2002). To that end, two prospective studies have linked Crohn's disease with sunlight exposure as a proxy for vitamin D levels. A French cohort of 91,870 women with 123 incident cases of IBD showed a significant inverse relationship between sunlight exposure and Crohn's disease (but not UC) (Jantchou *et al.*, 2014). Review of data from the Nurses' Health Study 1&2 in America has shown that incidence of both Crohn's disease and UC increases with latitude (Khalili *et al.*, 2012) and similar findings have also been demonstrated in Europe (Shivananda *et al.*, 1996).

There has traditionally been a high incidence of IBD in the developed West and a low incidence in developing nations. Migrants from South Asian countries to the UK develop an increased risk of developing IBD up to above that of the UK indigenous Caucasian population (Probert *et al.*, 1992). It is unclear whether this is related to sunlight exposure or to other environmental factors such as lifestyle changes (e.g. diet, alcohol, medications, smoking tobacco) or alterations in the intestinal microbiota. Dark skinned patients with IBD in the UK have significantly lower vitamin D levels than their Caucasian counterparts (Chatu *et al.*, 2013).

There is some evidence that being born in the Winter or Spring months predisposes to IBD (both Crohn's and UC) (Jung *et al.*, 2013; Souradet Y. Shaw *et al.*, 2014). There is contradictory evidence as to whether disease flares occur more frequently in the colder seasons (Aratari *et al.*, 2006; Souradet Y Shaw *et al.*, 2014).

1.3.2.2 IBD disease activity and vitamin D

There are several predictors of low vitamin D levels in Crohn's disease, namely: duration of disease; level of disease activity; ileal disease; and previous bowel surgery (Tajika *et al.*, 2004). Again, it is not clear if the low vitamin D levels are causally linked with, or a consequence of, the above associations. Ananthakrishnan *et al* published a large (n = 72,719) prospective dataset which showed that hypovitaminosis D was most often present at the time of diagnosis of IBD (Ananthakrishnan *et al.*, 2012). They argue that this suggests that vitamin D may be a contributor to disease pathogenesis rather than just a corollary (this author disagrees with their conclusions, as the long lag between symptom onset and diagnosis could easily allow normal levels to drop due to the long list of factors above). Higher pre-diagnosis predicted levels of vitamin D assessed by serum 25(OH)D levels were associated with a significant reduction in risk of incident Crohn's disease and the median interval between assessment of 25(OH)D level and diagnosis of Crohn's disease was 10 years.

Low vitamin D levels are negatively correlated with Harvey Bradshaw Index and increase after infliximab treatment. Vitamin D receptor (VDR) gene expression in $CD4^+$ T cells is increased in active disease and lower in quiescent disease.

1.3.2.3 Immunomodulatory effects of vitamin D

As discussed in 1.1.2.1.1, the innate and adaptive immune system are both dysfunctional in IBD. (Geremia *et al.*, 2014). T helper cells, the mucosal epithelial barrier, microbial sensing, production of antimicrobial polypeptides and autophagy all play a role in IBD; and all may be modified by calcitriol.

Vitamin D's endocrine role in the control of calcium homeostasis is long established, but the immunoregulatory functions are also well recognised now. Vitamin D has been shown to be involved in many aspects of the gut immune regulation; from the maintenance of the epithelial barrier, to the innate and the adaptive immune systems.

Vitamin D receptor binding sites have been identified in genes associated with Crohn's disease (including the NOD2 gene).

1.3.2.3.1 Animal models of intestinal inflammation and vitamin D

Numerous animal model studies have implicated vitamin D in the pathogenesis of intestinal inflammation, and furthermore have shown that vitamin D supplementation can ameliorate these effects. Animals that lack the VDR or the enzyme required for the synthesis of the active form of vitamin D (CYP27A1) are more susceptible to development of intestinal inflammation (Froicu *et al.*, 2003). In another study, VDR knock-out (KO) mice demonstrated intestinal inflammation, dysbiosis (mirroring IBD in humans, with increased Proteobacteriae and Bacteriodetes and fewer Firmicutes) and a reduction in tolerogenic DCs when compared with their wild-type litter-mates (Ooi *et al.*, 2013). The dysbiosis and intestinal inflammation were reversed with vitamin D treatment. A Clcn5 (a Cl^-/H^+ ion transporter protein) knock out (KO)/ dextran sulphate sodium (DSS) model of murine colitis has also been ameliorated by a vitamin D high diet (Alex *et al.*, 2010) (figure 1.9).

Interleukin 10 (IL-10) knock-out mice fed a vitamin D deficient diet develop an accelerated form of enterocolitis whilst those with a vitamin D replete diet do not. Supplementation of vitamin D

in the dietary deficiency group with intestinal inflammation will reverse the enterocolitis (Cantorna *et al.*, 2000). In an immunodeficiency induced model of murine colitis, combination treatment of transplanted DCs with dexamethasone plus vitamin D promoted a tolerogenic DC response and an effective reduction in intestinal inflammation, more so than either treatment alone (Pedersen *et al.*, 2004, 2009). This supports the hypothesis that vitamin D may act as an effective treatment adjunct by optimising existing therapies, rather than as an effective solo treatment.

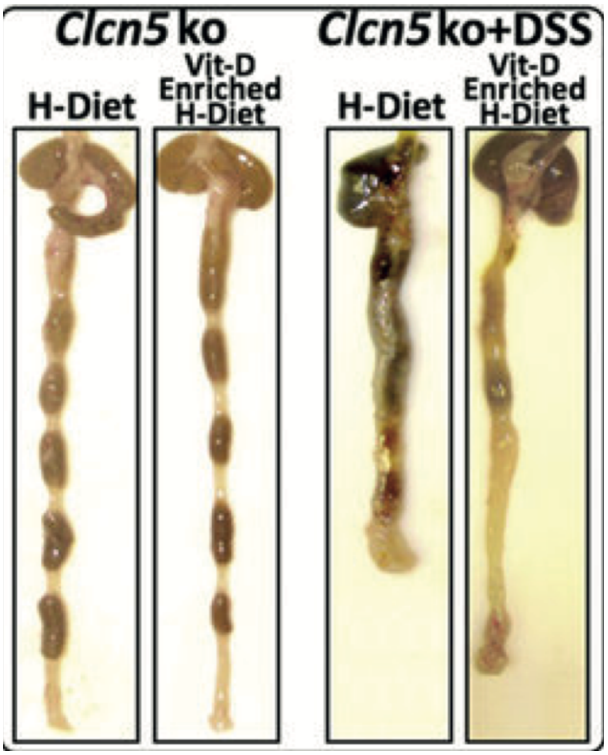


Figure 1.9: Photographs demonstrating loss of gut length in DSS and Clcn5ko induced colitis, but preservation of length with vitamin D high diet (Alex *et al.*, 2010).

1.3.2.3.2 Vitamin D and other chronic diseases

In *Mycobacterium tuberculosis* (*Mtb*), hypovitaminosis D is linked to treatment resistance and vitamin D supplementation can induce macrophage antimicrobial activity against *Mtb*. Moreover, transcriptome analysis has linked the VDR to lipid metabolism in tuberculosis (TB); with vitamin D treatment of TB infected macrophages abrogating the infection induced accretion of lipid droplets necessary for intracellular *Mtb* growth (Salamon *et al.*, 2014). There is an increasing trend of respiratory physicians ensuring adequate vitamin D in patients who are being treated with anti-tuberculosis therapy to optimise treatment of TB.

1.3.2.3.3 Vitamin D and the mucosal epithelial barrier

Reduced intestinal permeability, secondary to defects in the epithelial intercellular junctions (both tight junctions and adherens junctions), may predispose to inflammation, by allowing translocation of microbial material. Claudin-2, a tight junction protein, has been implicated in the pathogenesis of Crohn's disease. Expression of this protein is controlled by VDR binding of the PTPN2 gene (incidentally this is also a Crohn's disease susceptibility locus), and in this way vitamin D may impact upon tight junction integrity and the permeability of the intestinal epithelium (Ramagopalan *et al.*, 2010). A 2013 study has suggested that gut epithelial VDR signalling may inhibit inflammation by protecting the mucosal epithelial barrier, independent of non-epithelial immune VDR actions (Liu *et al.*, 2013).

1.3.2.3.4 Vitamin D and microbial sensing

Cells of the innate immune system, including DCs, macrophages and epithelial cells all 'sample' luminal microbes by binding to the PAMPs via their own PRRs. The NOD2 receptor is one such microbial sensing receptor, recognising the bacterial breakdown product muramyl dipeptide (MDP). The NOD2 receptor is mainly expressed on APCs and epithelial cells. The gene encoding the receptor, also known as *NOD2*, was the first mutant allele discovered to confer risk of Crohn's disease (homozygosity confers a 17 fold relative risk) (Economou *et al.*, 2004; Thia *et al.*, 2008; Verway, Behr and White, 2010). Vitamin D induces *NOD2* gene expression and its downstream functions and thus likely carries an important role in mucosal microbial sensing.

1.3.2.3.5 Vitamin D and antimicrobial polypeptide production

The production of antimicrobial polypeptides at the mucosa provides defence against bacteria, viruses and fungi (Komatsuzawa *et al.*, 2006). Antimicrobial polypeptides have direct antimicrobial effects, but are also involved in chemotaxis, cytokine production, cell proliferation and vascular permeability (Schwalfenberg, 2011). Vitamin D is a potent stimulator of antimicrobial polypeptides including cathelicidin and defensin hBD2, and binds to and activates the gene segments (*CAMP* gene for cathelicidin, which is regulated by the vitamin D receptor) encoding their transcription when exposed to bacterial breakdown products (Liu *et al.*, 2006; Bhan *et al.*, 2011).

1.3.2.3.6 Vitamin D and autophagy

Autophagy is a conserved homeostatic process used by cells to "clean up" their own components, get rid of unwanted constituents and recycle others. More recently it's been described to be involved in intracellular pathogen eradication. Thus, its importance in both homeostasis and host protection are likely to be relevant to IBD pathogenesis. For instance, inadequate recycling or clean-up or apoptotic cells via autophagy could contribute to inflammation irrespective of microbial involvement. It is a highly-conserved process and involves the envelopment of pathogens by a double membraned vesicle. Autophagy is known to be activated by stimulated TLRs, which in turn can be modulated by vitamin D. Two genes coding for autophagy, ATG16L1 and IRGM, have been implicated in Crohn's disease risk through GWAS. Importantly, mutations in the *NOD2* gene also induce altered autophagy, another mechanistic association linking vitamin D and autophagy (Cabezón and Benítez-Ribas, 2013a).

1.3.2.3.7 Vitamin D and dendritic cells

As described above (see 1.2.1), the phenotype of an activated DC can direct it towards either immunity or tolerance, however in IBD the activation of DCs is aberrant and they are predisposed to immunogenicity, hence the chronic inflammation (Hendy *et al.*, 2016). The phenotype and function of DCs may be modulated by culturing in the presence of various substances including: vitamin D, dexamethasone and sirolimus (sirolimus is more commonly used in the transplant population, but has been shown to be effective in IBD (Naranjo-Gómez *et al.*, 2011; Mutalib *et al.*, 2014)). Addition of dexamethasone to immature DCs increases DC IL-10 production and decreases DC production of IL-12p70, IL-23 and TNFα (Naranjo-Gómez *et al.*, 2011). Tolerogenic DCs produced by culture with vitamin D did not promote a Th1 profile; there was increased

production of IL-10, and production of the inflammatory cytokines IL-23 and IFNα remained low despite stimulation with LPS (Pedersen *et al.*, 2009). In 2014 Bartels et al published an *ex vivo* study demonstrating that the maturation of monocyte derived DCs, including expression of CD80 and production of various cytokines (IL-10, IL-1β, and IL-6), is reduced in patients with Crohn's disease who respond to supplementation with vitamin D3 (Lars E Bartels *et al.*, 2014a).

1.3.2.3.8 The adaptive immune system and vitamin D

The adaptive immune system is highly specific, adaptable and confers long lasting immunity. Naïve T cells may differentiate into Th1, Th2, Th17 or Treg cells depending upon the cytokine milieu to which they are exposed. It has long been thought that aberrant Th1 pathways (induced by IL-12 and producing high amounts of IFNγ and TNFα) were involved in the pathogenesis of Crohn's disease, whilst Th2 pathways (induced by IL-4 and releasing high amounts of IL-13) were the driver of UC. In one study, colonic biopsies from inflamed and non-inflamed segments of UC patients were cultured and used to condition human blood enriched DCs from HCs. Supernatant cytokine levels were measured, and suggested a mixed cytokine profile, rather than Th1 as expected, with IL-6 predominant (Bernardo, Vallejo-Díez, Elizabeth R Mann, *et al.*, 2012a). Interestingly, DCs cultured with supernatant from inflamed, but not non-inflamed, tissue acquired a pro-inflammatory phenotype. The more recently described Th17 cells are a third T helper cell subset implicated in IBD. They are induced by IL-6 and TGF-β, with IL-23 having a role in the maintenance and population expansion, and are characterised by the secretion of IL-17A and IL-21 (Tesmer *et al.*, 2008). Regulatory T cells, defined by the expression of FOXP3 and $CD25^{Hi}$, are capable of suppressing T helper cell proliferation *in vivo.*

In VDR KO mice, T cells are more likely to be characterised by an activated phenotype and ready development into Th17 cells (with associated over production of IL-17), and are less likely to mature as Tregs (Bruce *et al.*, 2011). In human *in vitro* studies, vitamin D generates "tolerogenic" dendritic cells (tol-DC) characterised by increased production of the anti-inflammatory cytokine IL-10; decreased production of the pro-inflammatory IL-12 and TNF-α; down-regulation of co-stimulatory molecules CD80, CD83 and CD86; and diminished capacity to activate naïve T cells (Piemonti *et al.*, 2000; Lars E Bartels *et al.*, 2013a; Nikolic and Roep, 2013). T cell proliferation is markedly decreased when naive Th cells are exposed to tol-DCs, and this holds true for tol-DCs produced by culture with vitamin D, dexamethasone or sirolimus. In addition, T cells stimulated by tol-DC, as opposed to mature DCs, produce significantly less IFNγ. However, the picture is not completely clear, as patients treated with vitamin D have also been noted to have increased IL-6 levels (a pro-inflammatory cytokine) and an expansion of $CD4^+$ T cells.

1.3.2.4 Clinical trials of vitamin D in Crohn's disease

The clinical evidence for use of vitamin D in IBD is rather weaker. Vitamin D reduces faecal calprotectin levels and may increase the numbers of enterobacteriaceae, a beneficial microbe, in patients with UC (Garg *et al.*, 2018). Two clinical trials have studied vitamin D supplementation as a treatment for Crohn's disease. A Scandinavian double blinded, placebo randomised control trial (RCT) randomised 46 patients into daily 1200IU vitamin D for 12 months, with 48 patients given placebo. The study and placebo groups had similar baseline characteristics (including similar vitamin D levels, which were non-deficient). There was a non-significant reduction in number of flares (29 v 13; p= 0.06), the primary end point (S P Jørgensen *et al.*, 2010). The study

was underpowered, for several reasons: 12 subjects were excluded from analysis (unexpected disease activity as measured by CRP); and the study number was calculated using an expected reduction in flares of 50%, far higher than reductions seen even with biological drugs in IBD RCTs.

A 2015 non-blinded RCT in Iran treated 108 IBD (either Crohn's disease or UC) patients with 12 weeks of high dose vitamin D (50,000IU weekly) or with nothing (in the control group). The treatment group had a lower starting vitamin D concentration and a higher finishing concentration than the control group. The study found that vitamin D treatment caused a non-significant reduction in TNFα levels from mean 33.3pg/ml to mean 26.64pg/ml (p=0.07), and that this effect correlated with clinical disease activity index (Dadaei *et al.*, 2015). An unpublished open label study (ClinicalTrials.gov identifier NCT00742781) of daily vitamin D, up to 5000IU, performed as a proof of feasibility study, showed efficacy in raising vitamin D levels and reducing disease activity indices; but the study was small and with no placebo control.

Three recent studies of vitamin D in IBD were terminated early, due to difficulties recruiting and due to change in study protocol before commencement of the study (ClinicalTrials.gov Identifier: NCT03053414, NCT02208310 and NCT00621257). A number of clinical trials are currently studying vitamin D in IBD, but none are randomised, placebo controlled trials.

There are no published clinical trials which assess whether optimisation of vitamin D levels as an adjunct to usual treatment can be an effective therapy in IBD.

1.4 Hypothesis and Aims

1.4.1 Hypothesis

We hypothesised that abnormalities in the circulating dendritic cell population contribute to the inflammatory burden in luminal Crohn's disease and furthermore that modulation of the phenotype and function of dendritic cells by correction of low vitamin D may be beneficial as a treatment adjunct (alongside infliximab).

1.4.2 Aims by chapter

Chapter 3

- To characterise circulating dendritic cell phenotype, with markers of homing, microbial sensing, and maturity all to be studied.
- To assess the impact of circulating dendritic cell phenotype upon Crohn's disease location and vice-versa.
- To assess the impact of circulating dendritic cell phenotype upon Crohn's disease activity, and vice versa.
- To compare the surface marker phenotype of the two main circulating dendritic cell subtypes (mDC and pDC) in Crohn's disease.

Chapter 4

- To characterise function (on-going intracellular cytokine production) of circulating dendritic cells in Crohn's disease and in health.
- To compare the different roles of the two main circulating dendritic cell subtypes (mDC and pDC) in Crohn's disease.
- To quantify the serum cytokine profile, using ELISA, and compare with the cytokine profile produced by circulating DCs.

Chapter 5

- To study the *in vitro* effects of vitamin D upon DC homing marker expression.
- To study the *ex vivo* effects of infliximab upon DC phenotype, especially homing marker expression.
- To determine whether high dose IM vitamin D, as a treatment adjunct, can induce a tolerogenic dendritic cell phenotype and function in active Crohn's disease above that of the standard treatment (infliximab) alone.

Chapter 6

- To assess the effects of anti-TNF treatment upon the function (on-going intracellular cytokine production) of circulating DCs in Crohn's disease.

- To assess the effects of adjuvant treatment with vitamin D (alongside anti-TNF treatment) upon the function (on-going intracellular cytokine production) of circulating DCs in Crohn's disease.
- To assess whether change in vitamin D concentration correlates with any decrease in pro-inflammatory cytokines (if witnessed).
- To quantify the serum cytokine profile (using ELISA), in subjects before and after treatment, and compare with the cytokine profile produced by circulating DCs.

Chapter 2 - Materials and Methods

2.1 Ethical Approval

Ethical approval for this study was obtained from Brent Research and Ethics Committee.

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

All subjects recruited (including patients and healthy control volunteers) to the study were provided with information sheets and informed, written consent was obtained before enrolment.

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

2.2 Materials

2.2.1 Human blood samples

Whole venous blood was collected from patients with Crohn's disease and healthy subjects into heparinized vacutainers (Becton Dickinson, USA). Fresh blood samples were processed within five minutes of venepuncture.

2.2.2 Culture Medium

Dutch modification of Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Aldrich) was used for cell suspension and dilution during whole blood processing. The modification incorporates low sodium bicarbonate for gas exchange and HEPES (hydroxyethyl-piperazine ethane sulphonic acid) which acts as a buffer.

2.2.3 Complete Medium

Complete Medium comprises of RPMI 1640 (Dutch modification) supplemented with 2mM L-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin, 50µg/mL gentamicin and 10% heat inactivated foetal calf serum (FCS, Harlan Laboratories). Cells were suspended in complete medium for washing and culture.

2.2.4 Buffers

2.2.4.1 FACS (Fluorescence associated cell sorting) buffer

To prepare cells for surface antibody staining, FACS buffer was used for washing and subsequent re-suspension. FACS buffer was phosphate buffered saline (PBS) augmented with FCS (2%), sodium azide 0.02% (Sigma, UK) and EDTA 1mM (Sigma, UK). The latter was used to prevent aggregation of cells and clogging.

2.2.5 Reagents

2.2.5.1 Ficoll-paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

Ficoll is a highly-branched polysaccharide with high molecular mass. It is hydrophilic and readily dissolves in water. With centrifugation, it creates a density gradient which is exploited to isolate PBMC's from fresh venous blood samples.

2.2.5.2 Flow-count Fluorospheres

Flow-count™ Fluorospheres (Beckman Coulter, Buckinghamshire, UK) have uniform size and fluorescence intensity (each fluorosphere contains a dye which has a fluorescent emission range of 525 nm to 700 nm when excited at 488 nm). The assayed concentration of fluorospheres is known for each vial. By adding a known volume of fluorospheres to an equivalent volume of cells, the absolute count of cells in a specimen can be determined using the following formula:

Absolute Count (cells/µl) = (Total number of cells counted/Total number of Fluorospheres) x Flow-Count Fluorospheres assayed concentration.

2.2.5.3 Foetal calf serum (FCS)

FCS (TCS Cellworks, Buckingham, UK) was added to human cell culture media as a source of growth factors. FCS was also used to block non-specific binding during monoclonal antibody labelling. It is the most widely used serum-supplement for the *in vitro* cell culture of eukaryotic cells.

2.2.5.4 Leucoperm A

Leucoperm A (Serotec, UK) is a fixative agent containing 5-10% paraformaldehyde (polyoxymethylene) and <0.1% methanol and is used in this study for intracellular cytokine staining of DCs.

2.2.5.5 Leucoperm B

Leucoperm B Is a membrane permeabilisation reagent allowing pore size to increase to a level where antibodies can access the intracellular milieu allowing for cytokine staining of DCs by flow cytometry.

2.2.5.6 Monensin

Monensin is an ionophore (a polyether able to transport cations across cell membranes) which selectively affects the Golgi apparatus, leading to trapping of newly synthesized proteins. Monensin, for scientific research and agricultural use, is derived from the fermentation of the bacterium *Streptomyces cinnamonensis.* A stock solution of Monensin (Sigma, UK), at a concentration of 3mM, was prepared using ethanol and stored at -70°c. Further dilution of the Monensin stock solution to 3 µM was undertaken prior to its addition to cell cultures.

2.2.5.7 Paraformaldehyde (PFA)

Paraformaldehyde 4% premade solution (BDH chemicals, UK) was diluted in saline (0.85%) to make a 1% stock solution. This was added directly to antibody-labelled cells as a fixative reagent (thus preventing cellular degradation).

2.2.5.8 Trypan blue

Trypan blue (Sigma, Poole, UK) was used at a working concentration of 0.4% for live cell counts.

It is a diazo dye (a derivative of diazene, HN=NH) which selectively diffuses into dead or dying cells with compromised cell membranes. Conversely, Trypan blue does not permeate viable cells with intact membranes. The number of live cells in a study sample were counted using a haemocytometer slide under a light microscope.

2.2.5.9 LIVE-DEAD™ Fixable Near-IR Dead Cell Stain Kit

In the majority of FACS experiments in this study, no viability marker other than Trypan Blue was used. As a six colour FACS machine was used for most of the experiments, the priority was to identify and subtype DCs, and study two antibodies of interest per tube; this excluded the use of a viability marker. For the last few experiments, after the FACS machine was upgraded to eight colour capacity, LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation (Invitrogen/Thermo Fischer Scientific) was used to identify viable cells by FACS. The stain is an amine reactive dye and enables discrimination of live and dead cells by fluorescence intensity of dye.

2.2.5.10 LPS

Lipopolysaccharide (LPS) (Invivogen) is the major component of the outer membrane of Gram negative bacteria. It stimulates immune cells when bound by PRRs, and *e coli* LPS was used as a positive control in some experiments here (2µL/2ml).

2.2.5.11 Vitamin D3

1mM 1,25$(OH)_2$D3 Calcitriol (Tocris) was made into a working stock of 100µMolar by dilution of 5µL vitamin D with 45µL of complete medium. Further dilutions with complete medium were performed to achieve the physiological range of concentrations used in Chapter 4 (range 100nM–1nM).

The laboratory test used to measure serum 25(OH)D levels was liquid chromatography tandem mass spectrometry of 25(OH)D2/D3.

2.2.6 Antibodies

Fluorochrome-conjugated antibodies for specific cell markers were used, along with their matched isotype-controls. Table 2.1 (below) lists the antibodies and isotype-controls used together with their clone, conjugate and manufacturer, whilst Table 2.2 highlights the biological function of certain proteins which play a key role in this study.

Antibody Specificity	**Clone**	**Conjugate**	**Manufacturer**
β7	FIB504	PE	BD
CCR7	150503	PE	R&D
CCR9	248621	PE	R&D
CD3	UCHT1	PE-Cy5	BD
CD11c	3.9	FITC	eBioscience

CD11c	Bu15	PE-Cy5	Biolegend
CD14	61D3	PE-Cy5	Serotec
CD16	3G8	PE-Cy5	BD
CD19	HIB19	PE-Cy5	BD
CD34	581	PE-Cy5	BD
CD40	LOB7/6	PE	Serotec
CD80	L307.4	FITC	BD
CD86	BU63	FITC	Serotec
CD123	6H6	APC-Cy7	eBioscience
CLA	HECA-452	FITC	BD
HLA-DR	G46-6	APC	BD
IL-1β	B-A15	FITC	eBioscience
IL-6	MQ2-13A5	PE	eBioscience
IL-10	BT-10	FITC	eBioscience
IL-12 (p40/70)	C11.5	FITC	BD
IL-23 (p19)	727753	PE	R&D
IFN-α	LT27:295	FITC	Miltenyi
MSγ1	X40	FITC	BD
MSγ2A	G155-178	PE	BD
RTGM	R4-22	FITC	BD
RTγ2A	R35-95	PE	BD
TLR2	TLR2.3	FITC	Serotec
TLR4	HTA125	FITC	Serotec
TGFβ	35409	PE	R&D
TNFα	cA2	PE	Miltenyi

Table 2.1: Antibodies for flow cytometry including antibody specificity, clone, conjugate and manufacturer.

Antibody	Biological function
CD40	Maturation marker
CD80	Maturation marker
CD86	Maturation marker
ILT3	Inhibitory receptor/marker of immaturity
CLA	Homing marker (to skin)
β7	Homing marker (to gut)
CCR7	Homing marker (to lymph)
CCR9	Homing marker (to small bowel)
TLR2	Pattern recognition receptor
TLR4	Pattern recognition receptor

Table 2.2: Principle biological function of the antibodies under study (excluding cytokines, which are discussed in 1.2.7).

2.2.7 Dendritic cell negative enrichment kit

Dendritic cells were enriched from PBMC using the EasySep™ Human Pan-DC Pre-enrichment kit (STEMCELL Technologies, Vancouver, Canada) according to the kit instructions. Purity of live HLA-DR^+ Lineage $(CD3/CD14/CD16/CD19/CD34)^-$ cells was between 40-80%.

2.2.8 Enzyme linked immunosorbent assay (ELISA) kit

The DuoSet® ELISA Development System (R&D Systems, Abingdon, UK) was used to quantify both human IL-6 and human TNFα proteins in sera. The system included:

- 96 well Greiner ELISA flat-bottomed plates (Scientific Laboratory Supplies Ltd) and parafilm (VWR International)
- PBS: 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.4mM KH_2PO_4
- Wash buffer: 0.05% Tween® 20 in PBS
- Reagent diluent: 1% BSA in PBS
- Substrate solution: 1:1 mixture of colour reagent A (H_2O_2) and colour Reagent B (tetramethylbenzidine, Thermofischer Scientific).
- Stop solution: Sulfuric Acid Solution 2N H_2SO_4 (Sigma Aldrich)

2.2.9 PCR kit

- Qiagen RNEasy mini kit (includes the buffers RDD, RDE and RW1. These are proprietary buffers and the manufacturer limits details on their ingredients).
- RNase-free DNase set (Qiagen).
- TRIzol (TRI solution).
- Chloroform.
- AMV First-Strand cDNA synthesis kit (Roche).
- First Strand Hot Start PCR kit (Roche).

2.3 Methods

Flow cytometry, ELISA and electron microscopy (EM) were used to interrogate circulating DCs for phenotype and function. *Post-hoc* analysis of the flow cytometry histograms gave information about monocytes (see Supplemental Data in Appendix for monocyte results)

Laboratory, analytical and statistical methodology will be presented here in full, together with those aspects of trial design common to all the results chapters. Full details of patient selection and trial design will be detailed for each study in its relevant chapter (from 3-6). Of note, some subjects were used for several studies, and this is detailed in the methodology for each chapter.

Chapter 3 presents flow cytometry and EM data demonstrating the differences between phenotype (cell surface markers) of circulating DCs from healthy subjects and from subjects with Crohn's disease.

Chapter 4 presents flow cytometry and ELISA data demonstrating differences between function (measured as on-going intracellular cytokine production) of circulating DCs from healthy subjects and from subjects with Crohn's disease.

Chapter 5 presents flow cytometry data demonstrating the effect of different therapies (namely vitamin D and infliximab) upon the phenotype (cell surface markers) of circulating DCs in both *in-vitro* and *ex-vivo* studies of patients with Crohn's disease.

Chapter 6 presents flow cytometry and ELISA data demonstrating the effect of different therapies (namely vitamin D and infliximab) upon the function of circulating DCs in patients with Crohn's disease.

2.3.1 Data collection

2.3.1.1 Patient and disease related data

The following data were collected for all subjects recruited to the experiments presented in this thesis:

- Demographic data (age, sex, race)
- Disease characteristics (Montreal classification; presence or absence of stoma and perianal disease)
- Past and current medical therapy
- Evidence of disease activity (see below)
- Disease activity score (HBI see 1.1.3)

2.3.1.2 Assessment of disease activity for study entry

All subjects with Crohn's disease recruited to the study had active disease (this was an inclusion criterion). The following clinical and biochemical assessment tools were used to gauge evidence of inflammatory activity, required for entrance into the study. Subjects were required to have one or more modality, below, as positive for evidence of inflammation. More than one assessment tool was not required e.g. study entry with a normal Harvey Bradshaw Index (HBI) score was acceptable if there was another assessment tool showing evidence of inflammation.

- HBI

- CRP
- Faecal calprotectin
- Endoscopic disease activity
- Radiological disease activity

In addition to use for study entry, the following modalities of disease activity assessment were recorded for the purpose of correlation between study results and level of inflammation: HBI, CRP and faecal calprotectin. These three were chosen as the least subjective and best validated of the five tools listed above.

2.3.2 Experimental methodology – flow cytometry

A schematic for the sample collection and processing is shown below (Figure 2.1)

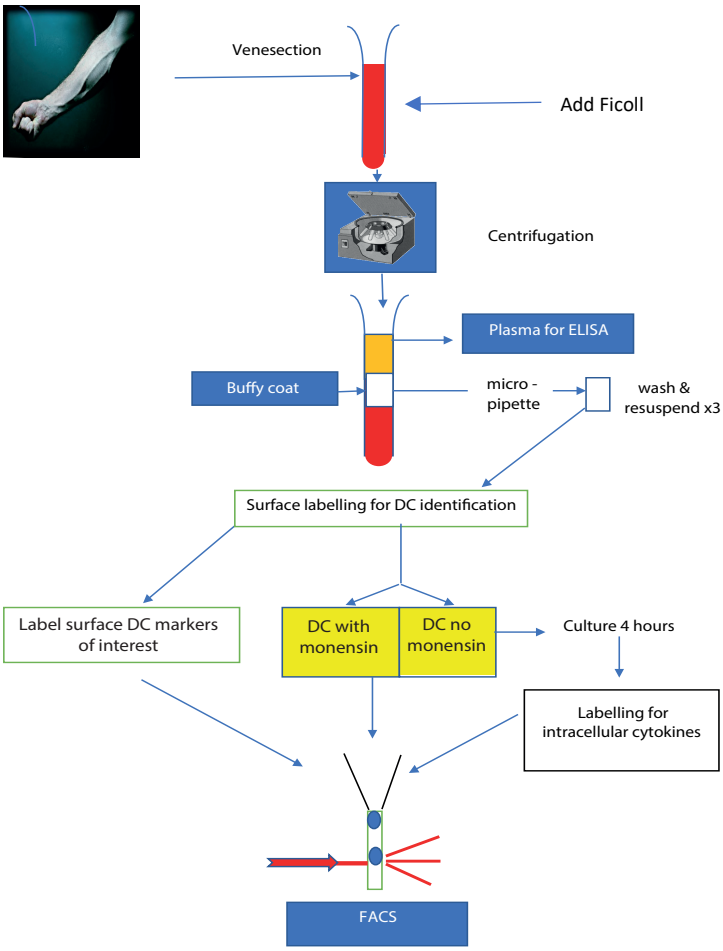


Figure 2.1: Flow diagram of experimental procedure from venesection to flow cytometry.

2.3.2.1 Isolation of PBMCs

Human peripheral venous blood was layered over Ficoll-Paque in 50ml conical tubes and centrifuged at 650g for 30 minutes with the brake off. This resulted in the blood being separated into three layers. Within the buffy coat were the PBMCs. The buffy coat was micro-pipetted off and washed with culture medium before centrifugation at 650g for 5min. The remaining pellet, containing PBMCs, was again washed and centrifuged (with the supernatant again discarded) before final resuspension in culture medium.

PBMCs were subsequently used for the following: - (A) Direct antibody labelling of cells for phenotypic analysis of DC- (B) Negative enrichment of DC for electron microscopy - (C) qPCR quantification of cytokine receptor expression.

The PBMCs were studied as fresh, rather than cryopreserved samples. Although the exigence of processing fresh samples adds difficulty to the experimental process, fresh PBMCs give an improved immunogenic assay response in comparison to cryopreserved samples and hence this was chosen (Weinberg *et al.*, 2009).

2.3.2.2 Trypan blue viability assessment and cell count

Known volumes of cells, medium and trypan blue dye were added to a mixing well at the ratio 1:3:1 (cells: medium: dye) to enable calculation of the viable cell count. 10µL of the cell/ dye solution was pipetted onto a haemocytometer slide and viable cells were enumerated under a light microscope. Dead or dying cells take up the blue dye which is easily visible under the light microscope. The number of viable cells mL^{-1} was determined by calculating the mean viable cell count in the four quadrants of the haemocytometer slide, multiplied by 5 (dilution factor of the cell mix [cells, Trypan blue, medium]), further multiplied by 10^4 (volume conversion factor required because volumes used are µl whilst cell counts are presented as cells/mL).

2.3.2.3 Antibody labelling – surface receptor and intracellular staining

PBMCs were labelled with monoclonal antibodies at predetermined optimal concentrations. Table 2.1 above shows the specificity, conjugate, flourochrome and supplier for each antibody, and table 2.2 demonstrates the principle biological function of the markers under study.

Cells were labelled with markers allowing identification of the dendritic cell population (figure 2.3) as well as antibody of the surface marker of interest or appropriate isotype-matched control antibodies in FACS buffer (PBS supplemented with 5% FCS, 1mM EDTA and 3mM sodium azide). Cells were then fixed with 1% paraformaldehyde, and stored at 4°C before acquisition on a flow cytometer (storage for no longer than 48 hours). For assessment of ongoing intracellular cytokine production, PBMC were first surface labelled and then cultured without stimulation for 4 hours (at 37°C, 5% CO_2) in either the presence or absence of 2 μM monensin, a Golgi apparatus transport inhibitor (Biolegend). In tubes without monensin there was an efflux of produced cytokines, whereas cytokines were retained within the Golgi apparatus in the monensin treated tube of interest. Thus, on-going real time intracellular cytokine production from within the dendritic cell population could be measured. Cells were then fixed with Leucoperm A (AbD Serotec, Kidlington, United Kingdom) and permeabilised with Leucoperm B (AbD Serotec) before intracellular antibody labelling (IL-1ß, IL6, IL10, IL12, IL23, IFNα, TGFβ, TNFα).

2.3.2.4 Flow cytometry

Labelled samples were acquired on a six-colour fluorescence activated cell sorting (FACS) Canto-II flow cytometer (BD Biosciences) using FACS Diva software and results analysed using WinList™ software (Verity, Topsham, ME).

Flow cytometry is a potent method for simultaneously studying large numbers (thousands per second) of cells within a biological sample. The process of running the sample through the flow cytometer to gain data is known as acquisition. Acquisition involves passing the cells in single file in front of a laser beam. The light scattered through a cell, also known as “forward scatter” gives information about the size of the cell, with larger cells giving greater forward scatter. Light refracted by the cell in a direction outside of the original light path is known as “side scatter” and gives information about the density, or granularity, of the cell. This enables the identification of cells based upon their physical properties.

In addition to information gleaned by light scatter, cells were labelled with monoclonal antibodies specific to receptors of interest and conjugated to fluorescent markers (fluorochromes), which are excited by light at a specific wavelength compatible with different lasers. The fluorochromes then emit light (fluoresce) at different wavelengths, which can be detected and quantification of each fluorochrome, and ergo its conjugated marker, can be determined for each cell “acquired” by the FACS machine. In this study, the FACS Canto used had a red (633-647nm) and a blue (488nm) laser and was capable of analysing panels consisting of 6 fluorochromes, hence it was possible to assess up to six markers of interest in each test tube of cells. In later experiments the flow cytometer was upgraded to include a violet (405-407nm) laser, and an extra two fluorochromes were utilised. In this body of research, the fluorescent-

conjugated antibody staining was used to identify dendritic cell surface receptors of interest, and to assess on-going real-time intracellular dendritic cell cytokine production.

Channel	Fluorochrome	Excitation laser line (nm)	Emission wavelength (nm)	Laser
1	FITC	488	519	Blue
2	PE	488	578	Blue
3	PE-Cy5	488	667	Blue
4	PE-Cy7	488	774	Blue
5	APC	633	660	Red
6	APC-Cy7	633	774	Red
7	BV421	405	421	Violet
8	BV605	405	605	Violet

Table 2.3: Six flourochromes (1-6) were used extensively in this study. Excitation laser line, emission wavelength and colour distinguished the different fluorochromes. An extra two colours were used in later experiments once a third laser was added to the FACS II Canto flow cytometer.

2.3.2.5 Compensation

Each fluorochrome has excitation and emission spectra. The former is a range of light wavelengths that donate energy to a fluorochrome, causing it to emit energy, as light, in another range of wavelengths, the emission spectrum.

Within a flow cytometer, bandpass filters select the appropriate spectra of excitation and emission wavelengths. Emission spectra may overlap, and when this happens fluorescence from

multiple fluorochromes may be detected. Fluorescence compensation is used to correct for this spectral overlap. This makes certain that the light detected does indeed originate from the fluorochrome under study.

Given that cells from different samples differ in constitution and emit varying degrees of background auto-fluorescence it is necessary to perform compensation separately for every separate experiment. Listmode data files are the raw flow cytometry data files which list values of the scatter and fluorescence parameters for each event, in the order in which the events passed through the laser. FACS Diva software (BD Biosciences) compensation of the listmode data was performed prior to offline analysis with WinList^{TM} software (Verity, Topsham, ME, US).

For compensation, seven compensation tubes containing PBMCs in FACS buffer suspension were made. Six of the tubes were each labelled with a different fluorochrome-conjugated antibody (FITC, PE, PECy5, PE-CY7, APC, and APC-Cy7), one for each tube under study. BD Compensation beads were added to each of the seven tubes. Initially, the one unlabelled PBMC compensation tube was used to set the current and voltage on the machine, dictated by the samples innate autofluoresence. An FSC:SSC plot was generated on the BD software and was adjusted using FSC gain and range and SSC voltage. Adjustments were made until the cell population of interest was centred, thus eliminating a significant amount of cellular debris. The fluorochrome labelled compensation tubes were then analysed and compensation similarly adjusted for each fluorochrome. Samples were then acquired and saved as listmode data files.

2.3.2.6 Identification of cell populations including PBMC, DCs and monocytes

The buffy coat which is collected following centrifugation of whole blood contains the peripheral blood mononuclear cell (PBMC) population. PBMCs are blood cells with a single round nucleus, and there are several cell types, including lymphocytes, monocytes, macrophages, NK cells, DCs and macrophages. DCs comprise around 1% of the PBMC population, therefore measures must be taken to identify this 1% from within the buffy coat.

Whilst most other cell lines can be positively identified by staining with a lineage-defining marker (e.g. CD3 for T lymphocytes), there is no single marker identifying DCs. A double gating strategy is used to identify DCs and has been published previously by our laboratory (Bernardo *et al.*, 2016). Firstly, a forward versus side scatter plot of the PBMCs is used. Visual analysis of the histogram, (showing size against granularity – a useful first step for identifying different cell types) will show not only where the debris lies, but also where the lymphocyte and APC populations lie. Gating is performed on the live cell population, thereby excluding cellular debris, erythrocytes and platelets (figures 2.2 and 2.3A).

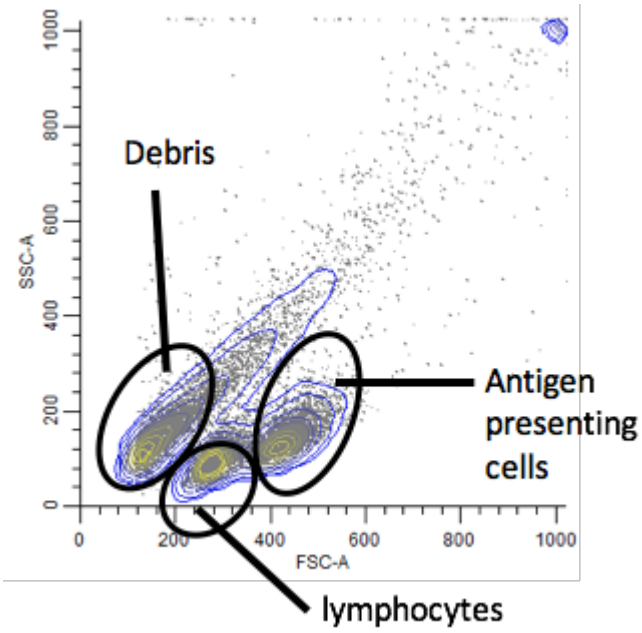


Figure 2.2: Using flow cytometry to identify cell populations. 2-dimensional (2-D) histogram demonstrating FSC (cell size) on the x-axis and SSC (representing cellular granularity) on the y-axis.

Within this gated population, a further histogram (forward scatter-area v forward scatter-height) is then constructed to exclude doublet cells, which can bias results unless removed. The DC population is then identified as positive for expression of HLA-DR, on the X axis, and negative for expression of the DC lineage cocktail (CD3, CD14, CD16, CD19 and CD34 see table 2.4 below) on the y axis as shown in figure 2.3C below. DC subtypes were identified as myeloid (mDC, $CD11c^{+}CD123^{-}$) or plasmacytoid (pDC, $CD11c^{-}CD123^{+}$) (D in figure 2.3).

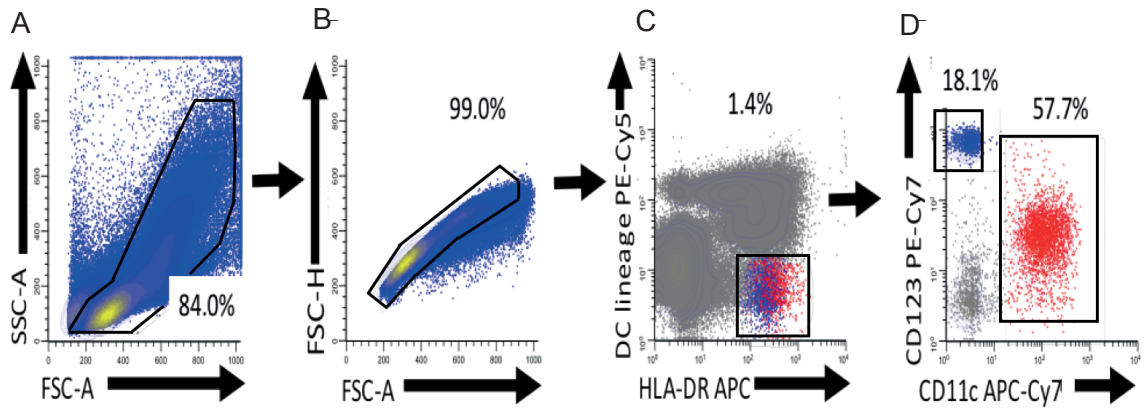


Figure 2.3: Example of gating technique used to identify dendritic cells. A) Forward (FSC-A) and side (SSC-A) scatter. B) Doublet discrimination. C) Dendritic cells were identified as HLA-DR +ve and DC lineage -ve. D) Dendritic cells were further subtyped as myeloid (CD11c+CD123-) and plasmacytoid (CD11c-CD123+).

Lineage marker	Cell line excluded
CD3	T lymphocytes
CD14	Monocytes, macrophages, eosinophils, neutrophils
CD16	NK cells, macrophages, cultured monocytes, neutrophils
CD19	B lymphocytes
CD34	Haematopoietic stem/ progenitor cells

Table 2.4: The dendritic cell lineage markers used in this study and the immune cell type which they identify, negative selection of which will highlight the DC population.

2.3.2.7 Gating and analysis to establish presence of markers of interest

There are several techniques for analysing the markers of interest following flow cytometry, all with different advantages and disadvantages. In the interest of consistency, in this thesis the following techniques were used throughout:

For cell surface marker assessment - region gating for percentage of cells staining positive (over and above that of the isotype control).

For intracellular cytokine production – SEDmax subtraction of the control tube from the tube of interest.

Both methods have been used, in isolation, to present data in published articles. The methods and strengths of these techniques are discussed below.

2.3.2.7.1 Region gating and percentage positive – for cell surface marker analysis

The proportion of each DC subset expressing surface markers of interest was determined by comparing fluorescence intensity to that of an isotype-matched antibody control. This could be done in a 1-D histogram for one marker, or for two markers (in the same tube) shown in 2-D, as below (figure 2.4). With the 2-D histograms it is possible to identify not just the presence of each marker, but also the percentage of cells which are "double positive" i.e. express both markers. This is particularly relevant for homing markers, as high expression of one marker may be less important if there are high expression of other homing markers, whereas tissue specificity is conferred when there is high expression of one homing marker, and lack of expression of other

markers. This method of analysis has been used recently to publish flow cytometry data (Bernardo *et al.*, 2016).

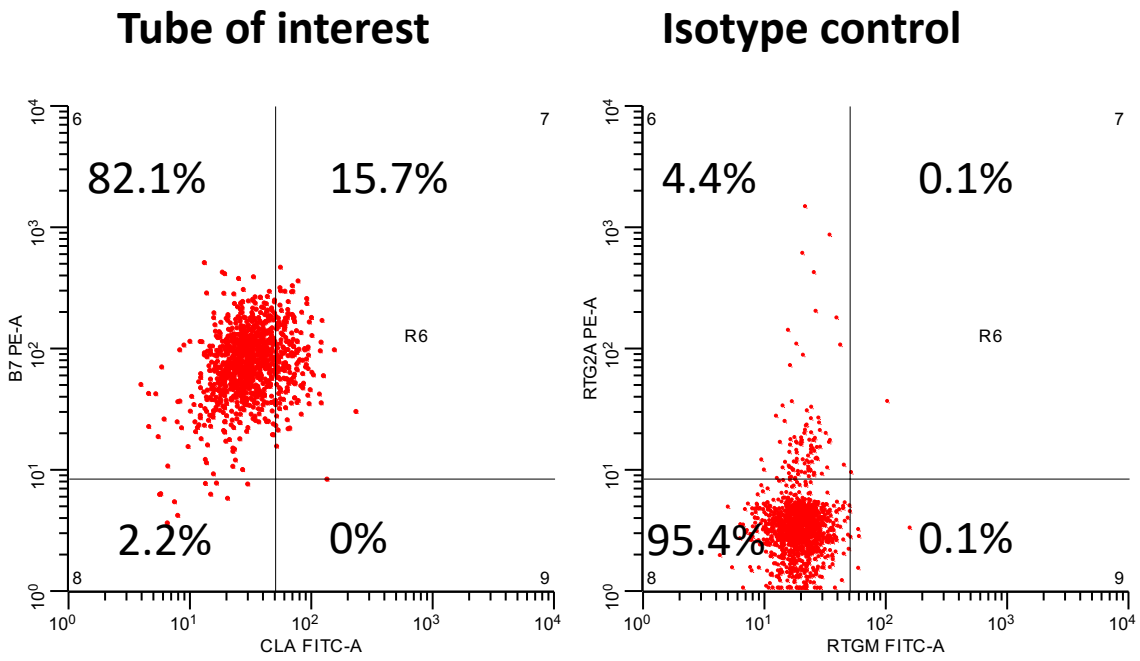


Figure 2.4: As an example of calculating the percentage of cells expressing the marker of interest: 2-D histogram of expression of CLA and β7 in the tube of interest, calculated by gating against RTGM and RT2GA respectively in the isotype control.

2.3.2.7.2 SEDmax subtract technique – for intracellular cytokine production analysis

Although regional gating is the standard of practice for identification of cell surface antibodies, it may be less accurate when performing quantitative analysis such as with intracellular cytokine analysis. This is because the subtraction technique avoids the occurence of spectral overlap between marker of interest and isotype control which may occur with regional gating.

The proportion of cells producing intracellular cytokines was calculated by superenhanced Dmax (SED_{max}) normalised subtraction of a non-monensin control histogram from the monensin-treated tube of interest histogram (figure 2.5), and the results reported as percentage positive values (this technique has been published previously(Bernardo, Vallejo-Díez, Elizabeth R. Mann, *et al.*, 2012)). The intensity ratio (IR) is another measure which is generated by the subtraction method (IR denotes the amount of marker that is expressed on positive cells). An IR > 1.3 is accepted as a reasonable threshold in intracellular staining, and was used in this study to help confirm validity of results. IRs are higher for surface staining techniques, where an IR > 2 might be expected. Another advantage of the SED_{max} technique is that it is more sensitive for small shifts in populations, as occur with intracellular staining, that might be missed using regional gating techniques.

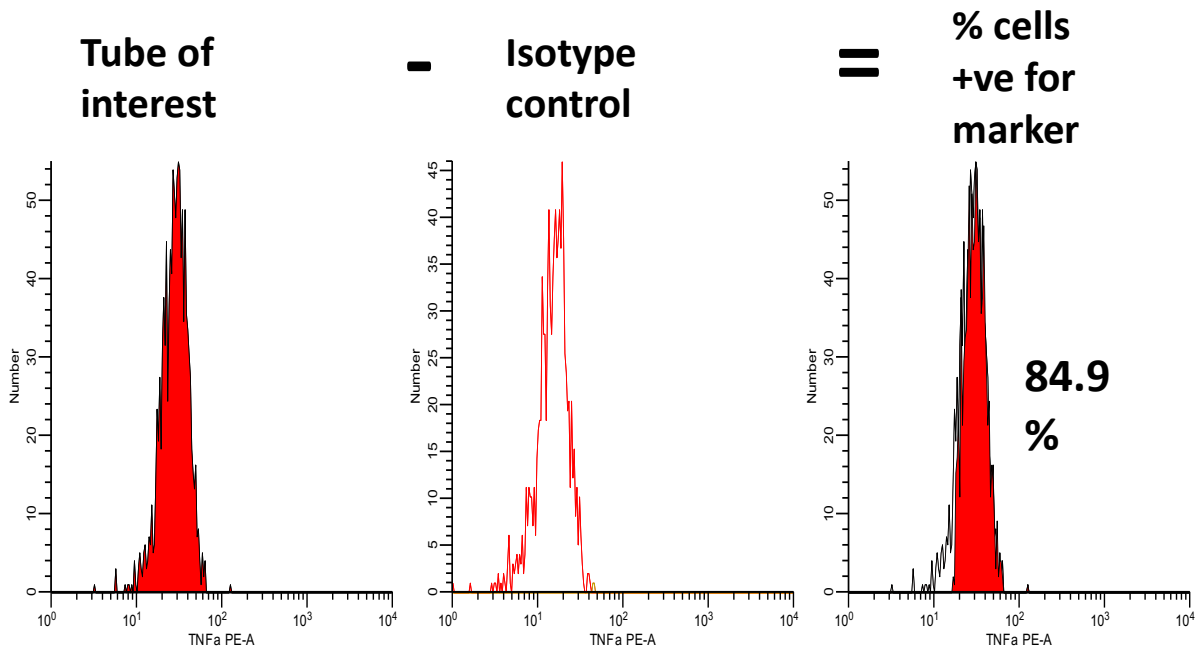


Figure 2.5: Demonstrating SED_{max} subtraction of isotype control tube of interest.

2.3.2.8 Back-gating

The practice of 'back-gating' is useful for identifying the population of interest within the main population, and for confirming that the correct gating strategy has been used. Usually it is clear where the region should be drawn, but on occasion it is less clear, and in these cases drawing the parameter around the population of interest can help define the gating strategy. An example of backgating is seen in figure 2.3 above, with image D showing gating on mDC (red) and pDC (blue), and these colours back-gated into image C, confirming that these two DC subpopulations lie in the expected position within the mononuclear cell population in terms of HLA-DR^{Hi} and lineage cocktail negative expression.

2.3.2.9 Gating strategy to identify phagocytic monocytes

Separate, pilot, experiments were performed on human PBMCs from HCs to identify the monocyte population. In these pilot studies, phagocytic monocytes were identified using phenotype (size and granularity) and CD64+ and CD14+CD16+ staining. In addition to expressing CD64, macrophages may be: CD14+CD16- (classical monocytes); CD14+CD16+ (intermediate) or CD14-CD16+ (non-classical monocytes). Monocytes (and macrophages) are usually HLA-DR low or medium and rarely high (distinguishing them from DC which are HLA-DR^{Hi}) and are larger than lymphocytes.

The flow cytometry software (Winlist) setting to identify monocytes were taken from these separate pilot experiments and applied to the data plots previously used to identify the DC population. In addition to application of these settings, the monocytes were confirmed as: live

cells, CD45+, larger than lymphocytes, medium-low HLA-DR expression, low CD11c expression and negative CD123 (figure 2.6). Hence, a post-hoc identification of the monocyte population could be achieved.

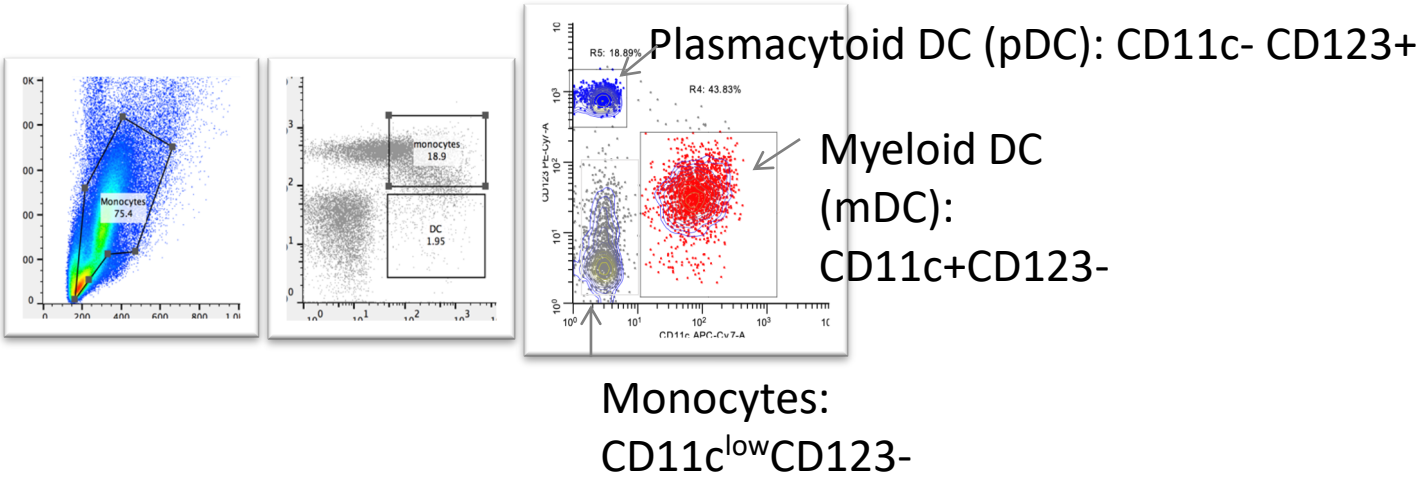


Figure 2.6: Gating strategy for putative monocytes as described in text.

Most of the Winlist gating for monocytes was carried out by P Bhat. All other aspects of the study (design, recruitment, sample acquisition, laboratory protocol, FACS acquisition) were carried out by Philip Hendy.

2.3.2.10 Quantification of cell counts and cellular events

During the acquisition stage of flow cytometry, acquisition of Flow-Count fluorospheres was also performed, to enable absolute cell counts to be generated. A FL1:SSC (fluorescence 1; side scatter) histogram plot was created, gating on which allowed identification of fluorescent counting beads. The absolute cell count was 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 were entered into the equation below

with the final cell count expressed as number of cells per µL of blood:

$$\text{Number of cells (µL)} = \frac{\text{Number of cells in gated region}}{\text{Number of beads in gated region x 5}} \times \text{Bead concentration (µL)}$$

Due to technical difficulties, absolute cells counts were only calculated for a limited number of experiments. This, unfortunately, meant that no accurate data for absolute cell counts could be calculated overall for the study population, but for the experiments in which cell counting was successful, it was possible to ascertain that cell counts did not differ from expected levels. In addition, a proxy was used to enable relative quantification of cell counts: the percentage of DCs, mDCs and pDCs within the PBMC population were calculated for healthy controls and for subjects.

2.3.2.11 Power calculations

As with other basic science studies, it is challenging to provide accurate powering calculations. Unlike with clinical studies where there is likely to be a hard-primary end-point, such as number of patients in remission at time-point X; the end-point with this study is less well defined. Alterations in immunogenicity may take the form of several different phenotypic or functional changes and knowing which marker to use as the end-point, and how much to expect it to alter by (if the null hypothesis is to be ignored) in order to perform a formal power calculation is too challenging to give accurate results.

The numbers chosen here for the different studies in this thesis were based on those required to achieve statistically significant results in similar, published studies. Given the enormous processing power of the flow cytometry technique (where thousands of cellular events are captured each second), relatively low numbers of subjects may be required.

2.3.2.12 Statistical analysis

The Kolmogorov-Smirnov test was applied by the Winlist software to assess whether two histograms in a pair are significantly different. Further sstatistical evaluations were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Paired t-testing was used to compare paired samples, unpaired t-testing was used to compare unpaired samples, and ANOVA was used for comparison between multiple groups. Results were considered statistically significant if $p<0.05$. Pearson's rank correlation coefficient test was applied for correlation of subject demographic data with laboratory data.

The application of Bonferroni correction in this study was carefully considered but not used, for several reasons. Although multiple markers were under review, these were testing several different hypotheses (e.g. hypothesis 1 about homing marker upregulation, and hypothesis 2 about changes in cytokine production following treatment). When several markers were testing the same hypothesis (e.g. CD40, CD80, CD86, ILT3 to test DC maturity) these were more limited in comparison number (and certainly below the 20 comparisons needed to generate a significant result by chance). In addition, a maximum of two markers were tested per tube of cells; it has previously been argued that this method compares only two markers per experiment and thus

obviates the need for correction (although the author of this thesis thinks that this is a weaker argument). Some sources recommend presenting all results, rather than just interesting and positive results, instead of performing correction for multiple comparisons; and that is the method opted for in this thesis.

2.3.2.13 Negative selection cell purification for electron microscopy

Dendritic cells were enriched from within the PBMC population using the EasySep™ negative selection kit manual protocol (STEMCELL Technologies, Vancouver, Canada). 50mls blood was taken from the study subject and PMBCs were harvested as described above on a Ficoll density gradient. PBMCS were placed in 5mL round bottom tubes designed to fit into the EASYSep™ purple magnet. The EasySep™ Human Pan DC Pre-Enrichment Cocktail (containing monoclonal antibodies bound in bispecific tetrameric antibody complexes (TAC) which are directed against the following antigen: CD3, CD9, CD14, CD16, CD19, CD34, CD56, CD66b, glycophorin A) was then added at 50µL/mL cells and was incubated for 30 minutes after thorough mixing. This step allows labelling of the unwanted (non-DC) cells with the TAC. EasySep™ D Magnetic Particles (dextran-coated particles which are recognized by the TAC) were vortexed until there were no visible aggregates and then added at 100 µL/mL cells, mixed and incubated for 10 minutes at room temperature. This step allows for binding of the TAC to the dextran-coated magnetic particles. EasySep Buffer or FACS buffer was added to bring the tube volume to 2.5mL and the contents were gently mixed with a pipette and the tube was placed into the magnet and left untouched for 5 minutes. The magnet and tube were then lifted and inverted and the solution, containing the enriched DC population, was poured into a new 5mL tube whilst the magnetically labelled

unwanted cells remained in the original tube within the magnet. A small volume of cells was then labelled with antibodies for flow cytometric determination of the percentage purity whilst the majority of the enriched DC population then entered the EM protocol.

2.3.3 Experimental Methodology - Transmission Electron microscopy

Dendritic cells were enriched from within the PBMC population using the EasySep™ negative selection kit (STEMCELL technologies, Vancouver, Canada) methods previously described (Ueno *et al.*, 2010). The purified cell population of at least 200,000 cells (77% DC purity) was labelled with biotinylated anti-CLA antibody (Miltenyi Biotech, USA) and subsequently 10nm goat anti-biotin gold (BBInternational, Cardiff,Uk). A gold alone control was also prepared.

Electron microscopy was performed and morphological identification of mDC and pDC was performed, as previously published, and qualitative analysis of CLA expression was undertaken(Patterson *et al.*, 1991; Robinson *et al.*, 1999).

The cells were fixed in 3% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.4 at room temperature for at least 30minutes and then at $4^{O}C$ until processed further. The cells were washed in the 0.1 M pH 7.4 sodium phosphate buffer and embedded as a pellet in low gelling temperature agarose (Sigma). The cells were given two further washes with the buffer and post fixed in 1% osmium tetroxide in 0.1% pH 7.4 sodium phosphate buffer for 1 hour.

After thorough washing with water to remove the phosphate and keeping in water overnight, the cells were block stained in 2% uranyl acetate for 2-4 hours.

The cells were washed with distilled water and dehydrated using an acetone gradient. They were gradually infiltrated with araldite resin. After at least 2 changes of araldite over 4-8 hours, the cells were embedded in the araldite resin and cured for 18 hours at 65^{o}C. The blocks of cells were sectioned using a Reichert –Jung Ultracut E microtome. The ultrathin sections (100 nm thick) were collected on formvar /carbon 200 mesh copper grids. (Agar Scientific). They were stained with Reynold`s lead citrate. The grids of sections were viewed using a Jeol JEM- 1200 EX electron microscope. At least 100 cells were counted in the sample. The cells were identified as previously published and the 10nm gold grains on the cell surfaces were counted for qualitative analysis of CLA expression (Patterson *et al.*, 1991; Robinson *et al.*, 1999). The cells were scored for the presence of rough endoplasmic reticulum (RER) as plasmacytoid dendritic cells contain large amounts of RER. Cells that contained multiple segments of RER, stretching up to a third of the cytoplasm, were given a score of 1+. Cells that contained multiple segments of RER, stretching up to two thirds of the cytoplasm, were given a score of 2+ and cells that contained multiple segments of RER, stretching over two thirds of the cytoplasm, were given a score of 3+. The cells with an RER score of 2+ or more were identified as plasmacytoid dendritic cells. *Transmission electron microscopy was performed by Nicholas English; study design, recruitment, sample acquisition and DC purification performed by Philip Hendy.*

2.3.4 Experimental Methodology - Enzyme Linked Immunosorbent Assay (ELISA)

The following procedures were performed on saved frozen serum for each subject and control in the study. The pro-inflammatory cytokines IL-6 and TNFα were studied, having given interesting results in the DC analysis by flow cytometry.

The capture antibody, diluted to working concentration in PBS (4µg/ml), was used in 100µl aliquots to coat each well in the 96 well plate. The plate was sealed and allowed to incubate overnight at room temperature. Next, each well was aspirated and washed with the wash buffer (0.05% Tween® 20 in PBS). This was repeated to a total of three times. Each well plate was then blocked by the addition of 100µL of reagent diluent (1% BSA in PBS), incubated at room temperature for 1 hour. The reagent diluent was then removed by addition and aspiration of the wash buffer as in the previous step.

Next, 100µL of either sample, standard or diluent (all in duplicate) were added to each well and incubated at room temperature for 2 hours. The plate was washed, as previously, and then 100µL of detection antibody (diluted in reagent diluent) was added to each well and incubated for 2 hours. Following a further wash, 100µL of Streptavidin-HRP was added to each well and incubated for 20 minutes in the dark. The wells were washed, and then 100µL of substrate solution (1:1 mixture of colour reagent A (H_2O_2) and colour Reagent B (tetramethylbenzidine)) were added and incubated for 20 minutes in the dark at room temperature. 50µL of stop solution (Sulfuric Acid Solution 2N H_2SO_4) was added to each well and the plate was placed into a microplate reader (Infinite F50, Tecan) set to 540nm.

Duplicate readings for standard, control and samples were averaged and the average zero standard optical density (O.D) was subtracted. Microsoft excel was used to generate a four-parameter logistic curve fit (figure 2.7).

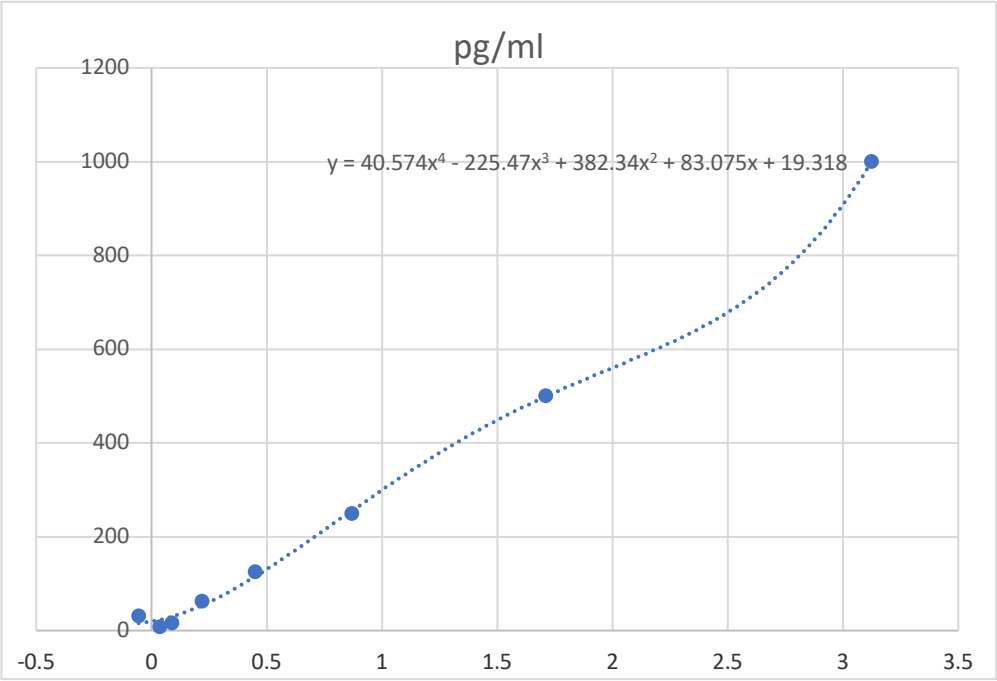


Figure 2.7: An example of the four-parametric logistic curve fit used to calculate values generated from ELISA testing in our study.

2.3.5 Experimental Methodology – Real time qualitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed upon thawed harvested cells, to help detect and quantify mRNA of TNFα, IL-6 and IL-10 in the study population.

1a) Preparation of total RNA from harvested cells.

The sample was completely lysed. This required mixing with Trizol-0.75 mL using a pipette and incubating each lysate for 5 minutes at room temperature. The process was repeated several times to ensure complete dissociation of nucleoprotein complexes. This was followed by centrifugation of lysate at 12000 g for 10 minutes at 4^{o} C. The supernatant was transferred to a fresh tube, 0.2 mL of chloroform was added and the tubes were then hand-shaken vigorously for 15 seconds. This was followed by incubation at room temperature for 3 minutes. Following centrifugation (12000 g for 15 minutes at 4 ^{o} C) the RNA containing upper aqueous phase was collected into a fresh tube.

An equal volume of alcohol (70% proof) was added to the aqueous phase i.e. a final 35% ethanol solution of total RNA. 700 µL of the sample were transferred to an RNeasy spin column placed in a 2mL collection tube. This was followed by centrifugation for 15 secs at 8000g.

1b) DNase digestion (on-column) with RNase free DNase Set.

DNase I stock solution was first prepared (prior to using the RNase-Free Set). Lyophilised DNase (1500 Kunitz units) was dissolved in 550 µL of the RNase free water provided. RNase free water was injected into the vial using an RNase free needle and syringe. Mixing was achieved by gently inverting the vial. 350 µL of buffer RW1 (Qiagen, Netherlands) were added to the RNeasy spin column. To wash the spin column, membrane centrifugation was at 8000g for 15 seconds, and the flow through was discarded. 10µL of DNase I stock solution was added to buffer RDD (Qiagen). The tube was gently (to avoid denaturation of DNase) inverted to achieve mixing and brief centrifugation was used to collect any residual liquid from the sides of the tube. 350 µL of buffer RW1 was added to the RNeasy spin column. Centrifugation was again at 8000g for 15

seconds. The flow-through was discarded and 500µL of buffer RPE (Qiagen) was added to the RNeasy spin column. Centrifugation was at 8000g for 15 seconds, thus allowing the spin column membrane to be washed with the flow through discarded. 500 µL of buffer RPE was added to the RNeasy spin column. To prevent downstream interference from residual alcohol centrifugation was at 8000g for 2min thus allowing the spin column membrane dry.

The RNeasy spin column was placed in a new 1.5mL collection tube and 30-50mL of RNase-free water was added to the spin column membrane. This was followed by centrifugation for 1minute at 80000g to elute the RNA.

2) DNA isolation using Reverse Transcription with cloned AMV First-Strand cDNA Synthesis kit

NB All reagents were kept on ice following thawing to prevent RNases degrading mRNA. Gloves were always used (sweat contains RNases) and 'PCR' tips were used on pipettes. The procedures below were all performed in the pre-PCR room and a laboratory coat (in that room) was kept separately.

Five 0.5mL flip top tubes were labelled for the reactions (2 for each mRNA sample, 1 for reverse transcription (RT) +ve and 1 for RT-ve). The tubes were kept on ice in a tray. Into each tube was pipetted 9µL of the appropriate mRNA, 2µL of dNTP mx and 1µL of oligo dt Primer.

Tubes were securely capped and then incubated in a water bath for 5 minutes at 65^{o} C. Reagents were vortexed before use (especially the 5xcDNA Synthesis buffer)

RT -ve Samples

The following agents were added:4µL 5xcDNA Synthesis buffer; 1µL 0.1M DTT; 3µL DEPC treated water.

RT +ve Samples

The following agents were added:4µL 5xcDNA Synthesis buffer; 1µL o.1M DTT; 1µL RNase OUT (40µ/µL); 1µL DEPC Treated water; 1µL Cloned AMV Reverse Transcriptase (15U/µL).

After adding all reagents, the tubes were pulse spun on the microcentrifuge. Following vortex, a further pulse spin was given. For the reverse transcription reaction, the tubes were incubated for 1 hour at 50^{o} C followed by 5 minutes at 85^{o} C. The heating at 85^{o} C inactivates the reverse transcriptase so that the cDNA product was not converted back to RNA.

3) Quantitative real-time PCR on the Roche Light Cycler using Fast-Start DNA Master SYBR Green Kit (Relative quantification with respect to a housekeeping gene)

The relative expression of the gene under study (TNFα, IL-6 or IL-10) relative to the housekeeping gene (ubcH5B) was performed, following optimised conditions. Magnesium chloride concentration and calibration curves were done to allow Relative Quantification software compensate. The samples were thawed to room temperature and then underwent vortex and pulse spin on the micro-centrifuge. cDNA samples and the appropriate RT-ve controls samples were diluted with water to give a sufficient volume so that 6µL could be added to each appropriate LightCycler capillary. 10µL of LightCycler Fast Start Enzyme were pipetted into a tube of the LightCycler Fast Start.

A master mix, on ice, for the test gene and housekeeping gene was prepared. The following were added to the LightCycler so that each contained a final volume of 20µL:

Water 6.4µL; magnesium chloride 1.6µL; forward primer 2.0µL; reverse primer 2.0µL; LightCycler FastStart Reaction Mix 2.0µL (Containing the enzyme).

The LightCycler capillaries were arranged in a cooled adaptor box with each sample in triplicate. Thus, the test gene triplicates results are recorded alongside those of the housekeeping gene. 14µL of the appropriate master mix were pipetted into the appropriate LightCycler capillaries. 6µL of the appropriate cDNA samples (dilutes to 1 in 20 in water) were pipetted into the LightCycler capillaries and then capped. RT-ve control was used for each combination of samples and test gene or housekeeping gene. The capillaries were transferred into the carousel and centrifuged in the LightCycler before being placed in the LightCycler.

PCR was performed under the following conditions:

95^{o} C 10 seconds; 60^{o} C 5 seconds 45 cycles; 72^{o} C 8 seconds.

Following this a melt curve was produced by heating from 65^{o} C to 95^{o}C over 15 minutes. A single peak in the melt curve showed that fluorescence of the exponential curve which the LightCycler used to determine the crossing point is due to one appropriate PCR product. The relative quantification software was used to quantify the expression of each test gene relative to the housekeeping gene for each sample. This was done with reference to a calibration curve for each gene so that the efficiency of the reaction could be taken into account by the software.

2.3.5.1 Statistical analysis of PCR data

Analysis of each sample was performed in triplicate to prove methodological validity. For each set of triplicate data, data points with <10% discrepancy were included, and those with >10% discrepancy were excluded. The mean of results (either two or three values) was recorded for each triplicate set. For samples where none of the replicate samples were within 10% values, the sample was excluded from analysis.

Chapter 3 - The Phenotype of Circulating Dendritic Cells in Crohn's Disease

3.1 Abstract

Introduction

Crohn's disease is characterised by an exaggerated immune response to mucosal antigen. Dendritic cells (DCs) are the primary antigen presenting cells and may promote either tolerogenic or inflammatory T cell responses to mucosal antigens. A range of cell surface markers (receptors) modulate the ability of DCs to identify and present antigen, and to elicit lymphocyte responses. We characterised phenotype of circulating DC subsets from patients with Crohn's disease and from healthy controls.

Methods

DCs within peripheral blood mononuclear cells from adults with active luminal Crohn's disease and from healthy controls (HCs) were characterised using flow cytometry. DCs were identified as HLA-DR$^+$ and negative for markers of other cell lineages (CD3, CD14, CD16, CD19, CD34). The two DC subsets, myeloid DCs (mDC, CD11c$^+$CD123$^-$) and plasmacytoid DCs (pDC, CD11c$^-$CD123$^+$) were identified and assessed for phenotype (homing markers, maturation status, and pattern recognition receptors) by surface staining. Electron microscopy was performed on a purified DC population (purification by negative selection) labelled to identify homing markers.

Results

In patients with Crohn's disease (n = 19), a greater proportion of circulating myeloid DCs expressed a gut-homing profile ($CLA^{-}\beta7^{+}$, p = 0.0011) compared to controls (n = 12) where most myeloid DCs were not tissue-specific ($CLA^{+}\beta7^{+}$, p = 0.0038). This loss of non-tissue specificity in circulating mDCs in Crohn's occurred as a result of down-regulation of skin homing receptor CLA (p = 0.0112).

Differences in expression of homing markers on circulating DCs in Crohn's disease were seen to associate with disease severity and disease location. Higher expression of gut homing marker ($\beta7$) on circulating mDCs was associated with a faecal calprotectin of >150μg/g (p = 0.0075). Elevated expression of gut/small bowel homing marker CCR9 was associated with greater extent of disease (ileocaecal, rather than isolated ileal,) in both DC subsets (mDC p =0.0366; pDC p =0.0190). Similarly, higher $\beta7$ expression on pDC was associated with ileocaecal rather than isolated ileal disease (p = 0.0396).

Expression of CD40, CD80, CD86, TLR2 and TLR4 on DCs were not different between Crohn's disease patients and controls for either DC subset.

In both healthy controls and subjects with Crohn's disease there were significant differences in expression of markers of homing, microbial sensing, and activation between the two circulating DC subsets, mDCs and pDCs.

Conclusion

The “gut-specific” phenotype of circulating mDC in Crohn’s disease may contribute to mucosal inflammation in Crohn’s disease. The abnormalities in homing marker expression on circulating pDC suggest that they may be a cell of interest in Crohn’s disease. Differences between surface marker expression on mDCs and pDCs suggest that they should be studied as separate entities rather than a single population. That severity and distribution of inflammation in Crohn’s disease can be inferred from the phenotype of circulating DCs puts them forward as a potential non-invasive “window to the gut”.

3.2 Introduction

Gastrointestinal manifestations of chronic inflammation can result in stricturing and fistulising disease and the development of colorectal cancer. Extra-intestinal manifestations can be significant and include skin, joint and eye disease.

In peripheral blood in health, DCs express multiple homing markers including both skin and gut specific markers but on reaching peripheral tissue they lose this generality and become tissue-specific. Dendritic cells in tissues then patrol areas of potential pathogenic invasion, such as gut and skin, and sample environmental pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) with their veiled protrusions (Elizabeth R Mann *et al.*, 2012; Peake, Bernardo, Mann, *et al.*, 2013a). They process and present antigen to lymphocytes and promote either tolerogenic (promoting non-responsiveness) and immunogenic lymphocyte responses. DCs identify and interact with PAMPs and DAMPs via their own pattern recognition receptors (PRRs), including toll-like receptors (TLRs). Upon antigenic encounter and acquisition, DCs mature (expressing co-stimulatory markers including CD40, CD80, CD86) and migrate to local lymphoid tissue where they present antigen to, and prime, T-cells. In addition, DCs can imprint homing markers onto lymphocytes during cognate interactions directing them to skin, gut, lymph and other tissues and determining lymphocyte cytokine production. Dendritic cells are producers of, and influenced by, cytokines including TNFα and IL-6 (Stagg, Kamm and Knight, 2002; Mora *et al.*, 2003; Blanco *et al.*, 2008).

Dendritic cells are heterogeneous, with their function and phenotype varying between tissues (Banchereau *et al.*, 2000). Gut DCs are tolerogenic in health, showing reduced expression of

major histocompatibility complex-II (MHC-II), PRR and co-stimulatory molecules, enabling mucosal tolerance to the constant antigenic load (Scott, Aumeunier and Mowat, 2011). There are two main circulating phenotypically distinct DC subsets, namely myeloid (mDC) and plasmacytoid (pDC). Differences in genetic lineage and expression of phenotype markers such as PRRs and maturity markers between mDC and pDC suggest that these subtypes should be studied separately rather than as a single DC population, but many studies to date do not make this distinction. The pDC subset has been less extensively studied than the mDC, but is generally viewed as tolerogenic with anti-viral properties (Swiecki and Colonna, 2015). Recent data have highlighted abnormalities of the virome in patients with IBD, thus making the 'anti-viral' pDC of potential interest in the pathophysiology of Crohn's disease (Norman *et al.*, 2015).

It is clear from past work that dendritic cells are abnormal in Crohn's disease, although causality is difficult to prove (Stagg *et al.*, 2003). In IBD, colonic DCs are increased in numbers, and show increased MHC-II, PRR and co-stimulatory molecule expression and production of pro-inflammatory cytokines (Hart *et al.*, 2005a; Verstege *et al.*, 2007). Expression of co-stimulatory molecule CD40 on lamina propria DCs is raised in inflamed Crohn's mucosa compared to inactive Crohn's disease and healthy controls (HC), and is reduced following anti-TNFα therapy (Stagg *et al.*, 2000).

DCs in health do not enter the efferent lymph from lymph nodes so are not believed to recirculate, so it was not expected to find blood DCs mirroring gut tissue specificities (Fossum, 1988a). We considered whether there are specific changes in the peripheral DC populations in Crohn's disease that may render them a suitable future therapeutic target. Extracting peripheral blood from patients naïve to biologic agents but with active disease on stable treatment, and

matched controls, we examined tissue homing, microbial sensing and maturation marker expression on the major DC subgroups, mDC and pDC.

3.3 Hypothesis and Aims

3.3.1 Hypothesis

We hypothesised that abnormalities in phenotype (expression of a range of cell surface markers) of circulating dendritic cells contribute to the inflammatory burden in luminal Crohn's disease.

3.3.2 Aims

- To characterise circulating dendritic cell phenotype, with markers of homing, microbial sensing, and maturity all to be studied.
- To assess the impact of circulating dendritic cell phenotype upon Crohn's disease location and vice-versa.
- To assess the impact of circulating dendritic cell phenotype upon Crohn's disease activity, and vice versa.
- To compare the surface marker phenotype of the two main circulating dendritic cell subtypes (mDC and pDC) in Crohn's disease.

3.4 Methods

3.4.1 Patient selection and recruitment

Patients and controls were recruited according to the criteria outlined in Table 3.1 below. Recruited patients had an established diagnosis of Crohn's disease. All patients had active luminal disease (as evidenced by: raised disease activity indices, blood or stool inflammatory markers or typical radiological or endoscopic findings) at the time of recruitment from the IBD outpatient clinic. As both corticosteroids and immunomodulators can alter DC biology, patients were on a stable dose of immunomodulator (stable for at least 3 months), and not treated with corticosteroids in the 6 months prior to the study (Aldinucci *et al.*, 2010; Ng *et al.*, 2010). Subjects were naïve to biological therapy.

Inclusion and exclusion criteria		
Crohn's disease inclusion criteria	**Crohn's disease exclusion criteria**	**Healthy controls inclusion criteria**
Age 18-80	Corticosteroid therapy within 6 months	Age 18-80
Willing to provide their informed consent to join the study	Vitamin D therapy within 6 months	Willing to provide their informed consent to join the study

Documented diagnosis of Crohn's disease, with diagnosis confirmed by histology	Biological therapy ever	No known diagnosis of Crohn's disease
Active luminal disease	Local or systemic sepsis	No known chronic inflammatory condition
Stable immunomodulator therapy for 3 months	Co-existing inflammatory condition (e.g. tuberculosis, sarcoid, diverticulitis)	No current significant gastrointestinal symptoms
	Current or past medical history of cancer	

Table 3.1: Inclusion and exclusion criteria for recruitment to the study for patients with Crohn's disease, and inclusion criteria for healthy control subjects.

Clinical and demographic data of the patients recruited to the studies in Chapters 3 & 4 is shown below in Table 3.2. Most patients had clinical and biochemical evidence of active disease. Patient 6, with normal HBI and faecal calprotectin, had radiological evidence of active disease.

It is worth noting that many of the patients with Crohn's disease who were studied against healthy controls here in Chapters 3 & 4, were also included in the interventional studies in Chapters 5 & 6.

Subject ID	Age (years)	Gender	Montreal Classification	Race	HBI	Stoma	Current IM	Faecal Calprotectin (ug/g)	Vitamin D level (nmol/L)
CD 1	22	M	A2L3B3p	Asian	9	N	No	-	18
CD 2	36	M	A2L3B3	White	10	N	No	573	13
CD 3	31	M	A2L3B2	White	4	N	AZA	1100	50
CD 4	19	F	A2L3B3p	White	n/a	Y	AZA	84.1	26
CD 5	28	M	A2L1B3	White	0	N	No	989	35
CD 6	34	F	A2L1B1p	White	0	N	AZA	5.3	68
CD 7	47	F	A2L3B1p	Asian	n/a	Y	No	604	63
CD 8	19	F	A2L1B1	White	0	N	AZA	439	35
CD 9	43	M	A2L3B2p	White	0	N	No	191	39
CD 10	24	M	A1L3B1	White	5	N	6MP	628	51
CD 11	18	M	A1L3B1	White	1	N	6MP	2000	57
CD 12	21	F	A3L3B3p	Asian	N/a	Y	AZA	10.9	35
CD 13	47	M	A2L3B2	White	1	N	AZA	187	57
CD 14	50	M	A2L3B1p	White	3	N	AZA	202	55
CD 15	49	F	A2L3B1	White	7	N	AZA	609	32
CD 16	19	F	A1L2B1	white	7	N	AZA	2000	25
CD 17	41	M	A2L3B1	Asian	3	N	AZA	-	51
CD 18	34	M	A2L1B1	White	3	N	AZA	143	
CD 19	25	F	A2L3B1	White	4	N	AZA	1600	

Mean values	31.9	58% M 42% F	-	79% W 21% A	4	16N 3Y	-	669	

Table 3.2: Clinical and demographic data for subjects with Crohn's disease included in studies in Chapters 3 & 4.

Age and sex matched healthy adult volunteers from the hospital were recruited as controls. The healthy volunteers had no known gastrointestinal or chronic inflammatory diagnosis, and were symptom free at the point of recruitment. Demographic data of the HCs recruited to the studies in Chapter 3 & 4 is shown below in Table 3.3.

Subject ID	**Age**	**Gender**	**Race**
HC 1	32	M	White
HC 2	40	F	White
HC 3	33	M	Asian
HC 4	34	F	White
HC 5	33	M	Asian
HC 6	25	F	White
HC 7	36	M	Asian
HC 8	32	M	White
HC 9	32	M	Asian
HC 10	45	F	White
HC 11	35	F	White

HC 12	34	M	Asian
Mean	34.25	7M (58%) 5F (42%)	7W (58%) 5A (42%)

Table 3.3: Healthy control demographic characteristics for healthy control subjects recruited to studies in Chapters 3 & 4.

3.4.2 Patient and disease related data

Patient and disease related data were collected as per methods 2.31.

3.4.3 The Montreal classification

In this study, the Montreal Classification (see Table 1.1 in 1.13) was used to record the phenotype and behaviour of Crohn's disease for each subject. All phenotypes of disease were accepted into the study, although in practice the majority had ileal or ileocolonic disease.

3.4.4 Harvey–Bradshaw Index

The HBI (see Table 1.2 in 1.1.3) was chosen as a simple to use and well validated scoring system for assessing Crohn's disease activity. The first three parameters were assessed over the 24 hours prior to study attendance, with abdominal mass and complications assessed by the physician during the study visit.

3.4.5 Experimental methodology

Detailed methodology is in Chapter 2, but in short, blood was taken from subjects and controls, and fresh peripheral blood mononuclear cells (PBMCs) were isolated for DC flow cytometry characterisation and, separately, DC enrichment (by negative selection) for transmission electron microscopy (EM) analysis.

3.4.6 Statistics

Unpaired t-test was utilised to compare healthy control with disease subject data. For the sub-analysis comparison of results by location or by inflammation score, the Mann-Whitney test was used. P values of <0.05 were considered statistically significant.

3.5 Results

3.5.1 Baseline demographic and clinical characteristics of study patients

According to recruitment criteria, patients had active luminal disease (mean faecal calprotectin 669ug/g) and were immunomodulator experienced but naïve to treatment with biological agents and clear of steroids for 6 months. There were no significant differences in baseline characteristics (including age, gender or ethnicity) between patients with Crohn's disease and HCs. The average age of Crohn's patients was 31.9 years (range 19-50, median 31 years), and 58% of patients were male (Table 3.2 below). Healthy controls were comparable with average age 34.2 years (range 25-36, median 33 years), and 58% male (Table 3.3 below). Of the 19 patients with Crohn's disease, the disease location was ileocolonic in 14, isolated ileal in four and isolated colonic in one. Five patients had a history of fistulising disease, three of stricturing disease and three of the patients had a stoma. Most patients were symptomatically stable with HBI <5, but active disease as evidenced by high faecal calprotectin levels, (14 patients had levels >100, and 11 patients had levels over 200). Five patients were on no immunomodulator treatment, while 12 were on azathioprine and two were on 6-mercaptopurine.

3.5.2 Homing markers

3.5.2.1 Gut-homing marker specificity on circulating dendritic cells is up-regulated in Crohn's disease

Dendritic cells in the peripheral circulation are bone-marrow-derived and directed to tissue by specific homing markers. We measured expression of both the gut homing marker, β7, and the skin homing marker, cutaneous leukocyte antigen (CLA), on mDC and pDC. Examining DCs expressing gut-homing receptor alone (i.e. in the absence of expression of skin-homing receptor), $CLA^{-}\beta7^{+}$ (gut homing) mDC were present at a significantly higher frequency in the circulation in Crohn's disease (35% v 8%, Crohn's v HC, p=0.0011) with a similar trend in pDC (figure 3.1 A-E). Skin homing marker expression was less frequent in the mDC population in Crohn's disease when compared to healthy controls (figure 3.1 F), and non-tissue specific mDC, $CLA^{+}\beta7^{+}$, positive for expression of both skin and gut homing markers, were less common in patients with Crohn's disease than controls (56% v 82%, Crohn's v HC, p=0.0038) (figure 3.1 H).

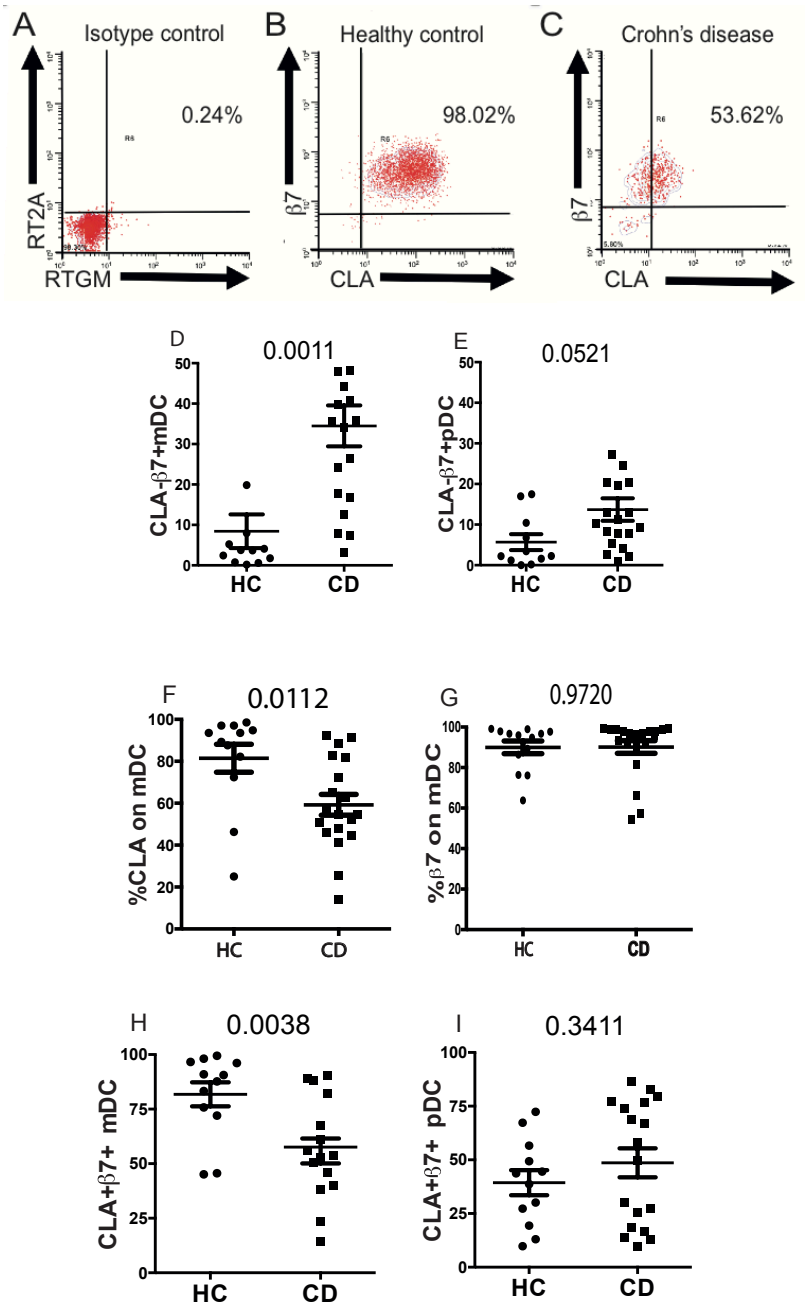


Figure 3.1: Comparison of expression of homing markers in healthy controls (HC) and Crohn's disease (CD) subjects. (A) shows the matched isotype control; whilst (B)&(C) demonstrate skin homing (CLA, x-axis) and gut homing (β7, y-axis) mDC in a healthy control (B) and a subject with Crohn's disease (C). The double staining population is shown as a percentage. (D) & (E) demonstrate whole study population data comparing healthy subjects with Crohn's disease subjects for: gut homing phenotype in (D) mDC and (E) pDC. (F) and (G) show population data for healthy controls and Crohn's disease for total expression of CLA and β7 respectively. (H) and (I) show $CLA^{+}\beta7^{+}$ "double positive" expression in healthy controls in mDC and pDC respectively.

3.5.2.2 Lymphoid tissue homing marker CCR7 expression

Upon acquisition of antigen, DCs upregulate CCR7 which enables binding to the CCL19 and CCL21 ligands and migration into the local lymph nodes, where they encounter naïve T cells and other activated DCs. CCR7 was expressed only on a low percentage of mDC in both health and Crohn's disease. A greater percentage of pDC expressed CCR7 but again with no significant difference between health and Crohn's disease (figure 3.2).

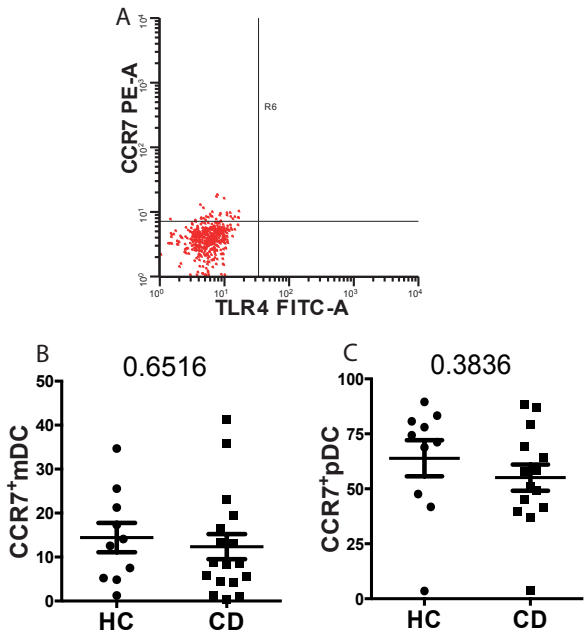


Figure 3.2: (A) Histogram showing low CCR7 expression on mDC in Crohn's disease. (B) and (C) demonstrate population cumulative graphs of CCR7⁺ expressing cells on healthy controls (HC) and Crohn's disease (CD) on mDC and pDC respectively.

3.5.2.3 Small bowel/ gut homing marker expression

Likewise, we investigated whether these cells have the potential to home to small intestine more frequently in Crohn's disease by examining expression of CCR9, important particularly for pDC migration (Wendland *et al.*, 2007; Mann, Bernardo, English, Landy, Al-Hassi, Peake, *et al.*, 2016).

CCR9 was expressed on less than 5% of circulating DCs in both HC and in Crohn's disease in both the mDC and pDC subpopulations (figure 3.3).

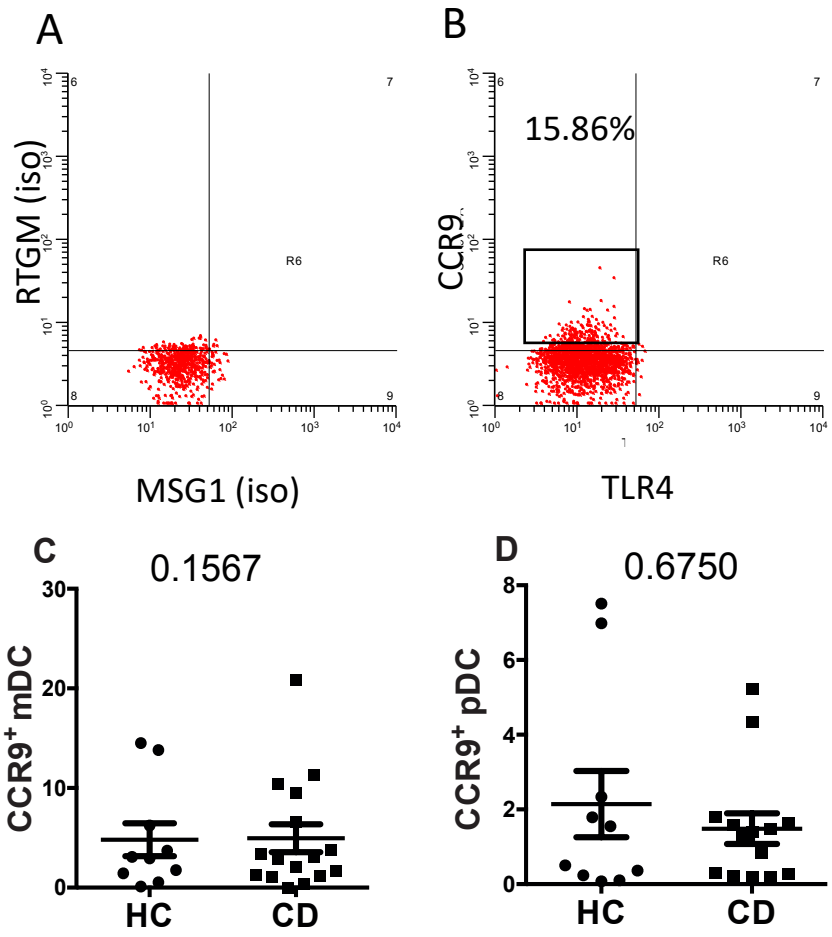


Figure 3.3: (A) 2-D histogram showing the isotype gating together with (B) low expression of CCR9 in mDC in a patient with Crohn's disease (CD). (C) and (D) plot the population data characteristics for CCR9 expression for healthy controls (HC) against Crohn's disease for mDC (C) and pDC (D). [Data for TLR4 shown in (B) discussed later in 3.5.3.3]

3.5.2.4 Profile of homing marker expression varies with intestinal disease distribution

Crohn's disease is a heterogenous condition with a varied presentation of both disease distribution and severity (see Table 3.1 for subject characteristics in this study). We examined whether distribution of disease in the intestine was associated with differences in homing marker expression in circulating mDC and pDC (figures 3.4 – 3.7). In this cohort, all but one patient had either isolated ileal disease (L1) or ileocolonic disease (L3).

3.5.2.5 Gut homing marker (β7) expression on DCs varies with disease location

β7 expression on circulating DCs was seen to alter with distribution of the disease. Whilst close to 100% of mDC expressed β7 in both L1 and L3 disease, significantly fewer pDCs expressed β7 in isolated ileal disease (37% v 70%, L1 v L3, p=0.0396) than in ileocolonic (figure 3.4).

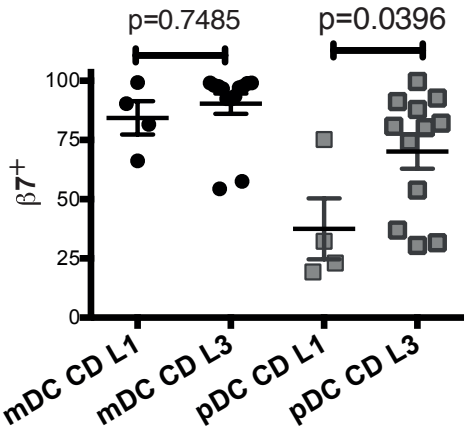


Figure 3.4: *Comparison of Crohn's disease location (as per Montreal classification) with expression of homing markers demonstrating isolated ileal (Montreal L1) v ileocolonic disease (Montreal L3) for expression of ß7⁺ (gut homing) DCs. Mann-Whitney test used.*

3.5.2.6 Skin homing marker (CLA) expression on DCs varies with disease location

CLA, skin homing, expression on pDC was high in all disease distributions, but expression of CLA on mDC was significantly greater in ileocolonic than isolated ileal disease (69% v 46% , L3 v L1, p=0.0101) (figure 3.5).

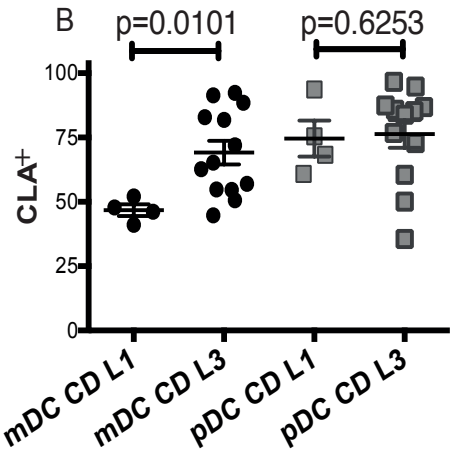


Figure 3.5: Comparison of Crohn's disease location (as per Montreal classification) with expression of homing markers demonstrating isolated ileal (Montreal L1) v ileocolonic disease (Montreal L3) for expression of skin homing (CLA^+) DCs. Mann-Whitney test used.

3.5.2.7 Lymphoid tissue homing marker (CCR7) expression on DCs does not vary with disease location

Expression of the lymphoid marker, CCR7, was not significantly affected by disease distribution, but there was a trend toward higher frequency of expression on mDCs and pDCs (figure 3.6).

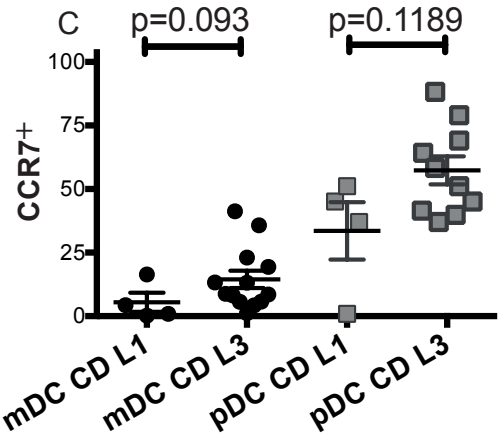


Figure 3.6: Comparison of Crohn's disease location (as per Montreal classification) with expression of homing markers demonstrating isolated ileal (Montreal L1) v ileocolonic disease (Montreal L3) for expression lymphoid tissue homing ($CCR7^+$) DCs. Mann-Whitney test used.

3.5.2.8 Small bowel/ gut homing marker (CCR9) expression on DCs varies with disease location

Expression of the small intestine homing marker, CCR9, was significantly higher in ileocolonic disease than in isolated ileal disease in both circulating DC subsets, although figures were low in all groups (for mDC: 7% v 1% , L3 v L1, p=0.0366; for pDC: 1% v 5%, L3 v L1, p=0.0190) (figure 3.7).

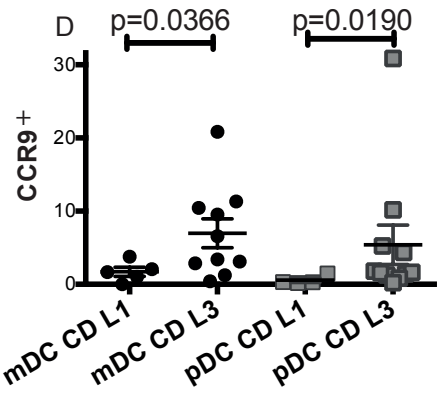


Figure 3.7: Comparison of Crohn's disease location (as per Montreal classification) with expression of homing markers, demonstrating isolated ileal (Montreal L1) v ileocolonic disease (Montreal L3) for expression small bowel homing ($CCR9^+$) DCs. Mann-Whitney test used.

3.5.2.9 Homing marker expression varies with disease activity

We investigated whether the degree of intestinal inflammation was associated with homing marker expression on circulating DCs (figures 3.8-3.12). The CRP was almost universally within normal range, and therefore faecal calprotectin was used for this analysis. The cut-off for normal,

non-inflamed colon was taken as 150µg/g (shown to have negative predictive value 97% and positive predictive value 71%(Pavlidis, Chedgy and Tibble, 2013).)

3.5.2.10 Gut homing marker (β7) expression increases with increased disease activity

Calprotectin values over 150µg/g were associated with increased frequency of mDC expressing β7 (91% v 67% , f calpro high v f calpro low, p=0.0075). There was no discrimination of faecal calprotectin value (high or low) by frequency of pDC expressing β7 (figure 3.8). It is noteworthy that the number of patients in the <150µg/g group (n=4) was lower than in the >150µg/g group (n=13).

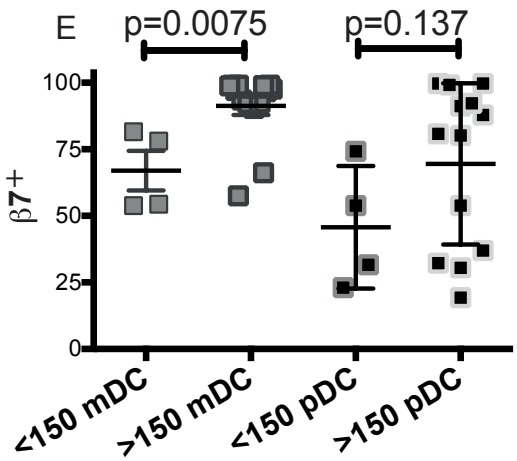


Figure 3.8: *Comparison of Crohn's disease severity (assessed by faecal calprotectin value) with expression of homing markers demonstrating faecal calprotectin high (>150µg/g) versus faecal calprotectin low (<150µg/g) for gut homing DC ($\beta7^+$). Mann-Whitney test used.*

3.5.2.11 Skin homing marker (CLA) expression does not alter with disease activity

Skin homing marker (CLA) appeared unaffected by mucosal inflammation in both the mDC and pDC subsets, with CLA expression not discriminating between faecal calprotectin high or low values (figure 3.9).

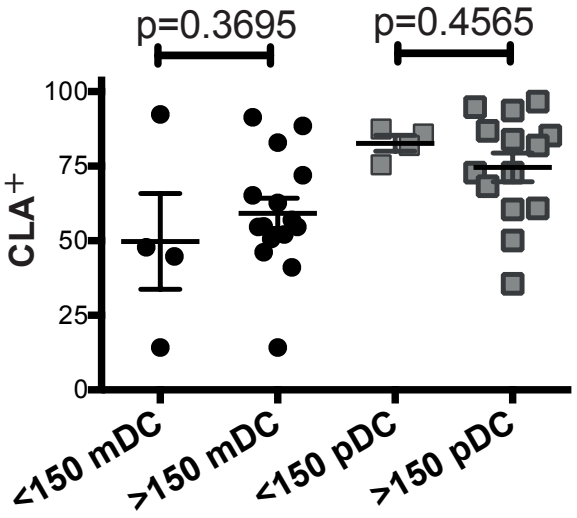


Figure 3.9: Comparison of Crohn's disease severity (assessed by faecal calprotectin value) with expression of homing markers demonstrating faecal calprotectin high (>150μg/g) with faecal calprotectin low (<150μg/g) for skin homing (CLA^+) mDC and pDC. Mann-Whitney test used.

3.5.2.12 Lymphoid tissue homing marker (CCR7) expression does not have an effect on disease activity

Lymphoid tissue homing (CCR7) appeared unaffected by mucosal inflammation in both the mDC and pDC subsets (figure 3.10), with no correlation of faecal calprotectin value with DC homing marker expression.

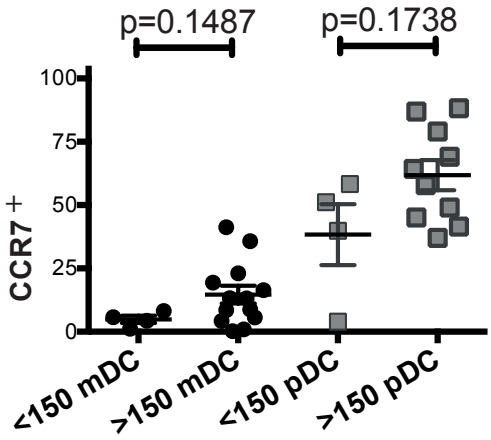


Figure 3.10: Comparison of disease severity (assessed by faecal calprotectin value) with expression of homing markers demonstrating faecal calprotectin high (>150μg/g) and faecal calprotectin low (<150μg/g) for lymphoid tissue (CCR7⁺) homing mDC and pDC. Mann-Whitney test used.

3.5.2.13 Expression of small bowel/ gut homing marker (CCR9) expression does not have an effect on disease activity

Small bowel tissue homing marker expression (CCR9) did not vary by mucosal inflammation in either the mDC and pDC subsets, with no correlation of faecal calprotectin value with DC homing marker expression (figure 3.11).

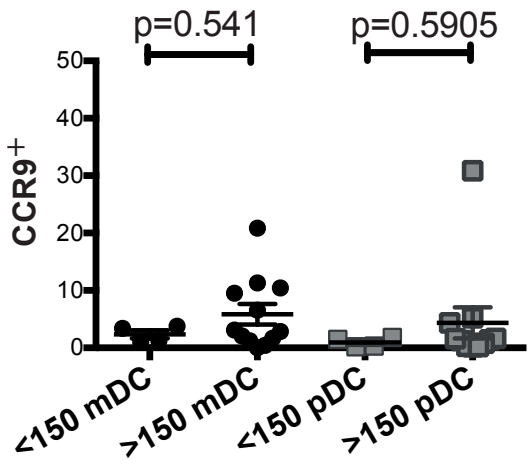


Figure 3.11: Comparison of disease severity (assessed by faecal calprotectin value) with expression of homing markers demonstrating faecal calprotectin high (>150μg/g) and faecal calprotectin low (<150μg/g) for gut/ small bowel (CCR9$^+$) homing mDC and pDC. Mann-Whitney test used.

3.5.2.14 In Crohn's disease, mDC express gut homing markers and pDC express skin and lymphoid homing markers

The data described above suggest that homing marker phenotype on circulating DC subtypes may be associated with disease distribution and/ or the quantity of inflamed mucosa and support a functional role for these cells in the mucosal inflammatory process. However, the function and relative importance of pDC and mDC was not clear. In Crohn's disease, circulating DCs that are not gut-homing may represent a distinct functional subset involved in the extra-intestinal manifestations of Crohn's disease. We compared expression of different tissue homing markers in circulating mDC and pDC compartments of patients with Crohn's disease.

Frequency of expression of skin homing marker, CLA, was lower on mDC than on pDC (4% v 26%, mDC v pDC, $p<0.0001$) (figure 3.12 A). Fig A is from a subject with Crohn's, but similar results were seen in the healthy controls. The dramatic difference in expression of CLA by pDC suggested a distinct cellular functional phenotype which we examined further (see 3.5.2.7).

Gut homing marker β7 was more frequently expressed on mDC than pDC, consistent with our earlier findings in comparison to HCs. Lymph node homing marker CCR7 was expressed significantly more frequently on pDC than mDC and this held true in both healthy controls and subjects with Crohn's disease. There was a trend toward increased frequency of circulating mDC expressing small bowel homing receptor CCR9 ($p=0.053$ figure 3.12 D) but expression was too low overall for this to be of any great interest.

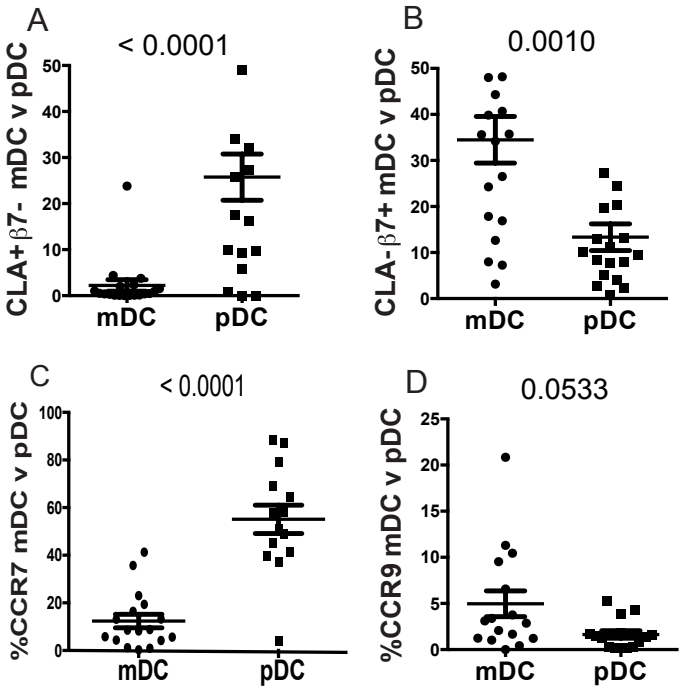


Figure 3.12: Flow cytometric comparison of homing marker expression on DC subtypes. (A) Comparison of skin homing ($CLA^+ß7^-$) mDC and pDC. (B) Comparison of gut homing (CLA^- $ß7^+$) mDC and pDC. (C) Comparison of lymph homing ($CCR7^+$) mDC and pDC. (D) Comparison of small bowel homing ($CCR9^+$) mDC and pDC.

3.5.2.15 Expression of CLA on circulating DCs differs with different DC subtypes and DC maturity, assessed by transmission electron microscopy

Having established with flow cytometry that CLA is expressed on circulating pDC at higher frequencies than on mDC, and that levels on mDC vary with disease distribution, we examined the expression of CLA on the surface of DCs, by transmission EM experiments of purified cells. Identification of $CD123^{+}$ pDC was confirmed in separate experiments by isolation of immunofluorescence labelled (CD123) enriched cells and showed characteristic prominent rough endoplasmic reticulum (figure 3.13).

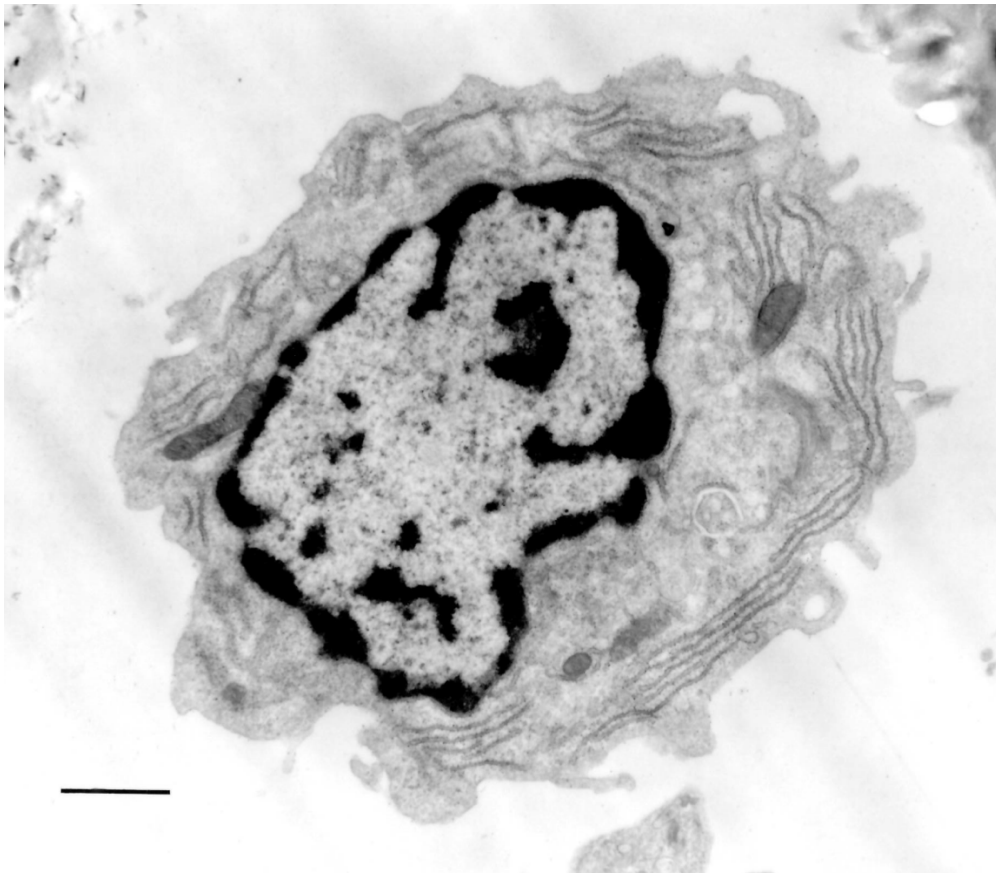


Figure 3.13: Transmission electron micrograph Identifying a plasmacytoid DC, with characteristic shorter veils than mDC, and extensive rough endoplasmic reticulum.

Anti-CLA gold labelling revealed CLA staining on the surface of >96% of putative pDC (figure 3.14).

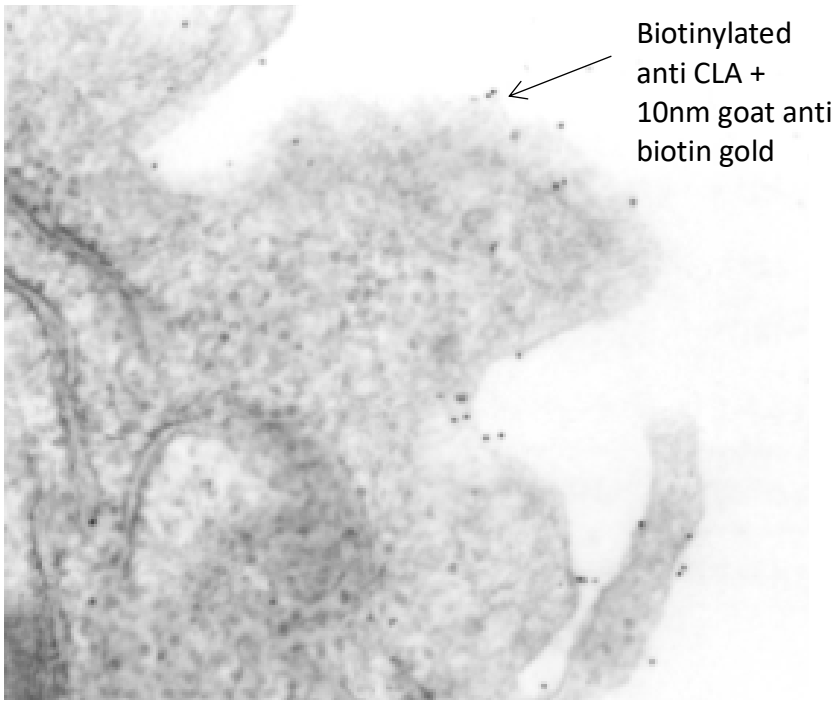


Figure 3.14: Transmission electron micrograph of plasmacytoid DC, demonstrating heavy staining with biotinylated anti-CLA$^+$ 10nm goat anti-biotin gold.

In these experiments, up to 70% of mDC of mature phenotype with more cytoplasm and longer veils also expressed CLA (figure 3.15), whereas it was not expressed on less mature mDC (figure 3.16).

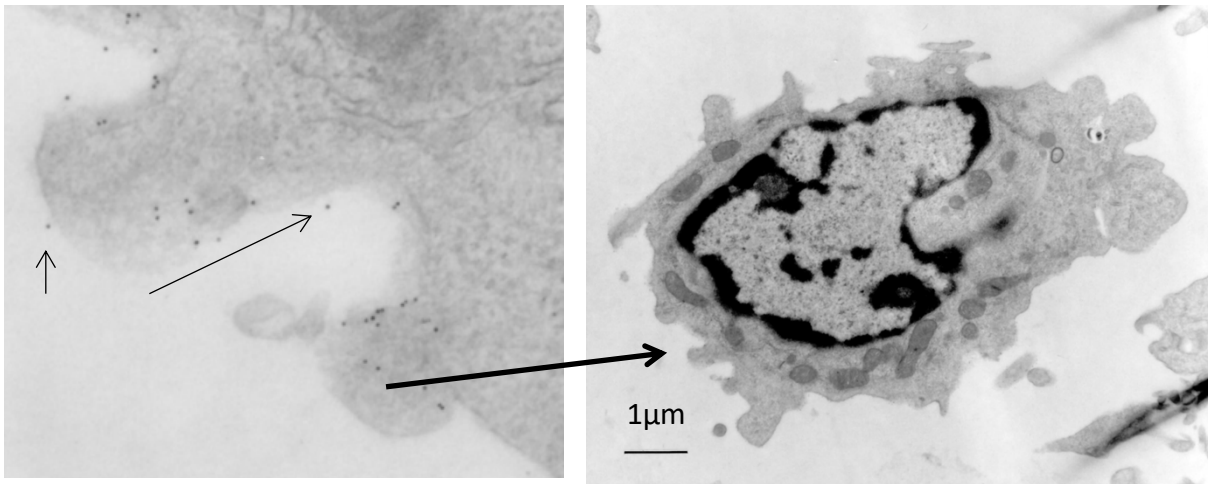


Figure 3.15: Transmission electron micrograph of a maturing mDC, demonstrating staining with biotinylated anti-CLA+ 10nm goat anti-biotin gold.

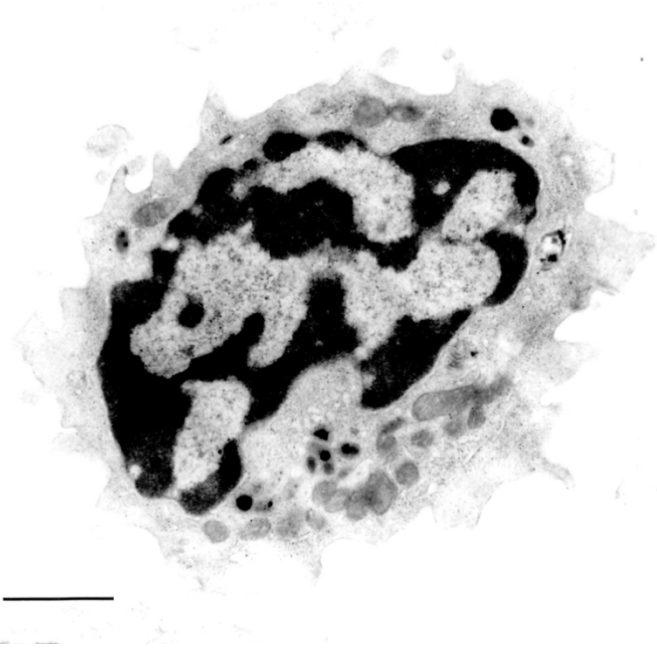


Figure 3.16: Transmission electron micrograph of a very immature mDC, demonstrating absent staining with biotinylated anti-CLA+ 10nm goat anti-biotin gold.

These data suggest that CLA expression is ubiquitous on pDC but is gained on mDC during or after maturation rather than early in development in the bone marrow. These data, taken together with the flow cytometry data, suggest that circulating mDC have the capability of expressing CLA but express less in Crohn's disease, leaving the DCs with a preferentially gut homing phenotype.

3.5.3 Maturation and activation markers

Changes to maturation and activation markers of circulating DCs were not observed in Crohn's disease

Having demonstrated that homing marker expression may alter during DC maturation, we examined expression of several maturation and functional markers on both circulating mDC and pDC in Crohn's disease. These markers included, MHCII, ILT3, TLR2, TLR4, CD40, CD80, CD86 (see 1.2.3).

3.5.3.1 MHC-II expression

MHC-II expression, known to be down-regulated on tolerogenic DCs, did not differ between DC subtypes and was not up-regulated in Crohn's disease compared with controls (figure 3.17).

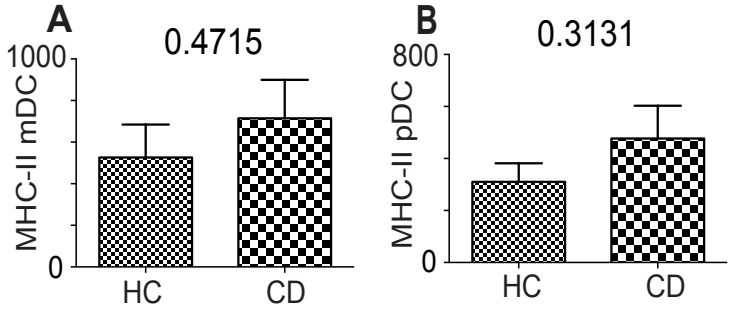


Figure 3.17: Expression of MHCII in healthy controls (HC) and subjects with Crohn's disease (CD) on (A) mDC and (B) pDC.

3.5.3.2 ILT3 expression

Tolerogenic DCs also express high levels of ILT3, which is down-regulated when the cells become activated in an inflammatory environment. In patients with Crohn's disease, there were high levels of ILT3 expression in circulating DCs, prior to reaching the gut, also in keeping with a tolerogenic phenotype (figure 3.18).

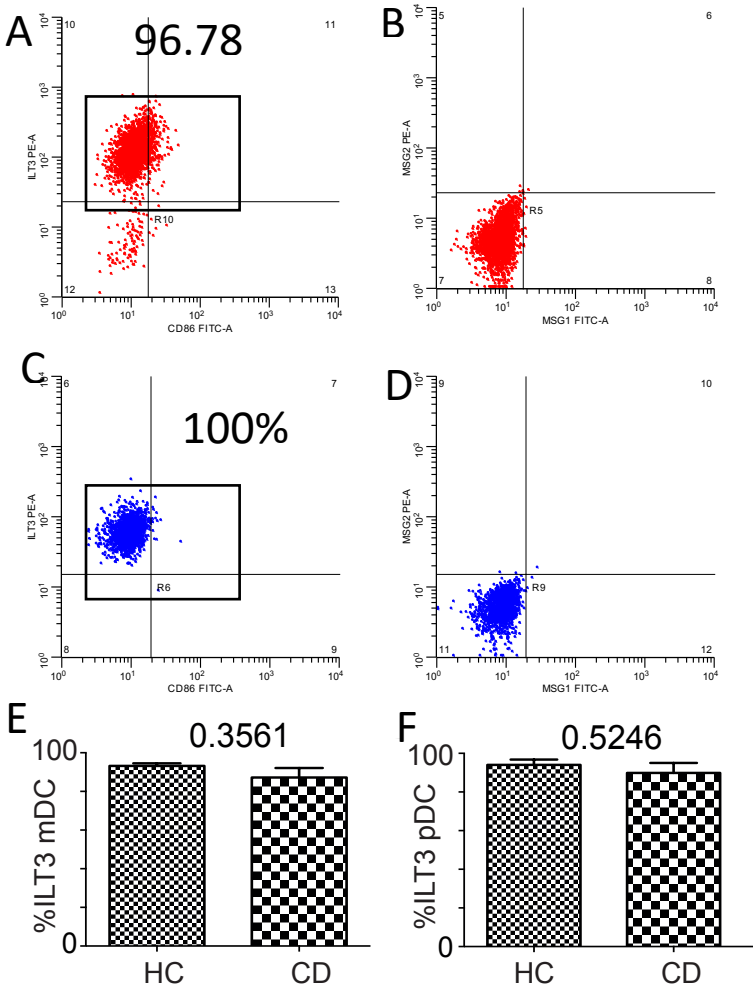


Figure 3.18: Expression of ILT3 in a healthy control (HC) and a subject with Crohn's disease (CD) on (A) mDC (plotted against isotype control (B)) and (C) pDC (plotted against isotype control (D)). (E) and (F) show population data for ILT3 expression on mDC and pDC respectively.

3.5.3.3 Expression of the TLR pattern recognition receptors

Bacterial antigens likely play an important role in influencing the inflammatory environment of Crohn's disease. Dendritic cells sense these antigens via PRR, resulting in cell activation and subsequent antigen presentation to T cells. Myeloid DCs express TLR 1-6, 8 and 10 whilst pDC, classically virus-stimulated cells, express TLR 7, 9 and 10. In these circulating cells, prior to contact with the mucosal environment, there was a trend towards increased TLR2 in Crohn's disease, but overall, there was no significant up-regulation in the TLRs on mDC (figures 3.19 and 3.20). Plasmacytoid DCs did not express TLR2 or TLR4, a concept well documented in the resting state (Kaisho and Akira, 2006; Weber *et al.*, 2015).

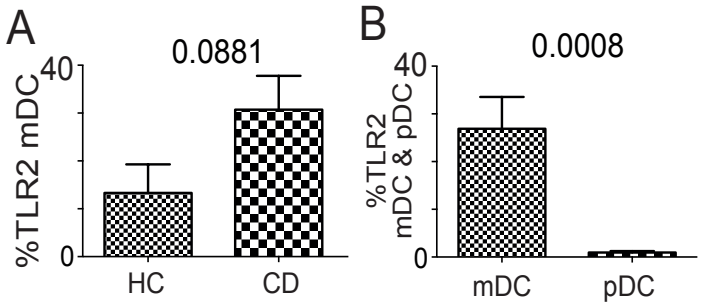


Figure 3.19: Expression of pattern recognition receptor TLR2 in (A) mDC of healthy controls (HC) and subjects with Crohn's disease (CD); and (B) mDC v pDC in CD.

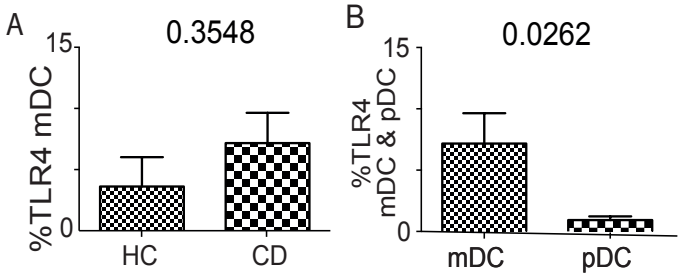


Figure 3.20: Expression of pattern recognition receptor TLR4 in healthy controls (HC) and subjects with Crohn's disease (CD) on (A) mDC and (B) mDC V pDC.

3.5.3.4 Co-stimulatory molecule expression

Both mDC and pDC express maturation markers CD40, CD80 and CD86 upon exposure to ligands and subsequent activation (Ma and Clark, 2009). These markers were not up-regulated in Crohn's disease compared with HCs, suggesting that DCs in peripheral circulation were not pre-activated (figure 3.21).

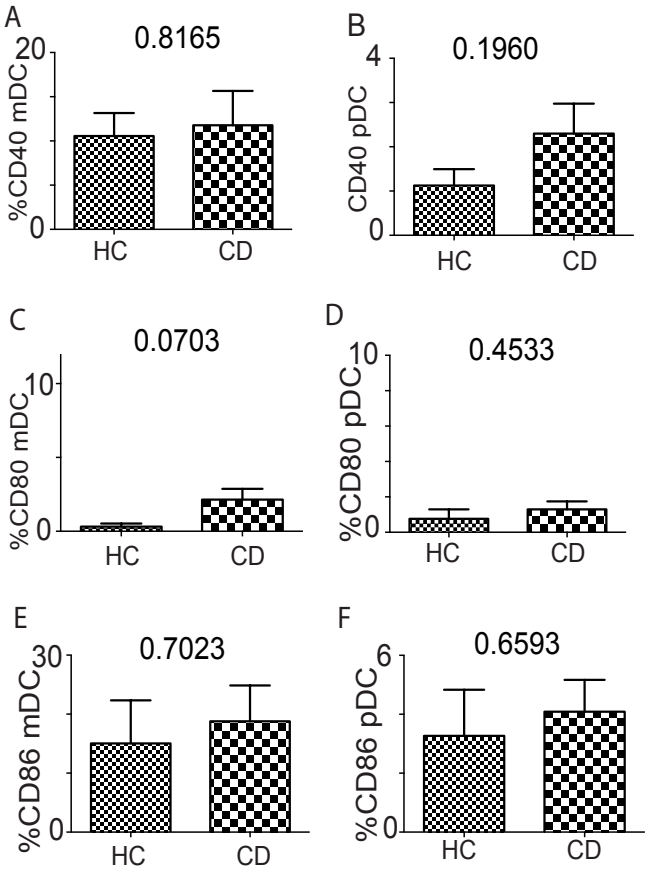


Figure 3.21: Expression of maturity/ costimulatory marker CD40 in healthy controls (HC) and subjects with Crohn's disease (CD) on (A) mDC and (B) pDC. Expression of CD80 on (C) mDC and (D) pDC. Expression of CD86 on (E) mDC and (F) pDC.

Next, a comparison of DC subtype (mDC versus pDC) maturation marker expression in Crohn's and HCs was performed. CD40 is a co-stimulatory molecule for TNFα, a key pro-inflammatory

cytokine in active Crohn's disease. There was a greater expression of CD40 on mDC than pDC (figure 3.22) in disease and controls.

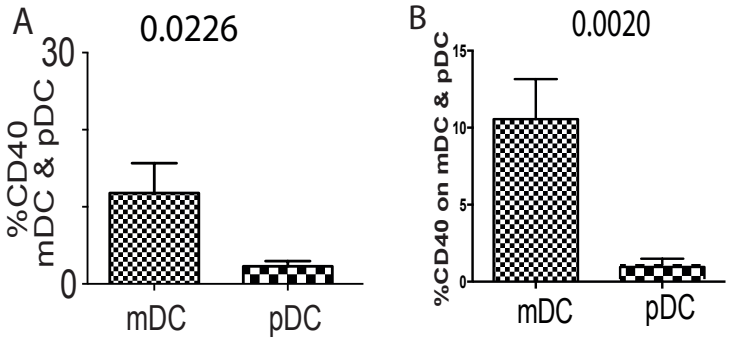


Figure 3.22: Expression of maturity/ costimulatory marker CD40 in mDC and pDC in (A) Crohn's disease and (B) healthy control.

CD80 and CD86 are co-stimulatory molecules determining interactions between DCs and T cells. In patients with Crohn's disease, CD86 (but not CD80) was preferentially up-regulated on mDC (figure 3.23).

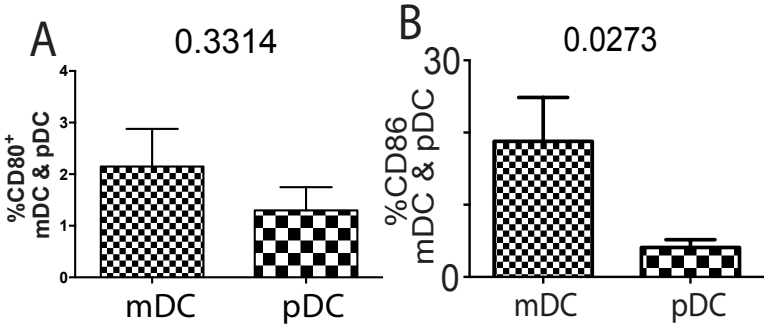


Figure 3.23: Expression of maturity/ costimulatory markers (A) CD80 and (B) CD86 in mDC and pDC.

3.5.4 Quantification of cell populations

There were no significant differences between healthy controls and subjects with Crohn's disease for DCs as a proportion of the PBMC population (Figure 3.24).

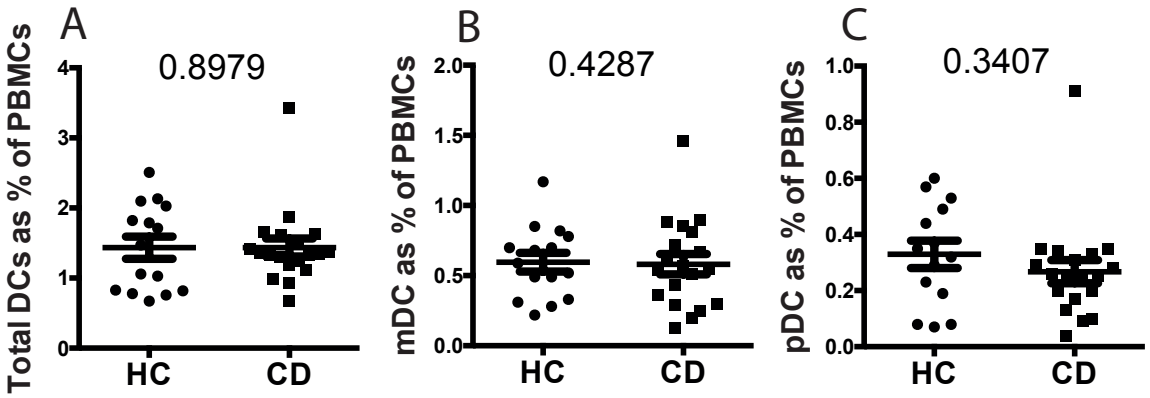


Figure 3.24: (A) total (B) myeloid and (C) plasmacytoid DCs as a percentage of the PBMC population in healthy controls (HC) and subjects with Crohn's disease (CD).

3.6 Summary of results for Chapter 3

- In Crohn's disease, there is abnormal homing marker expression on circulating DCs, both mDC and pDC.
- mDC lose skin homing marker CLA and thus become "tissue specific" to gut homing ($\beta7$) phenotype, with a similar trend in pDC.
- Abnormalities in homing marker expression on circulating DCs vary according to disease location and activity:
    - More extensive disease (L3 compared to L1) is associated with greater $\beta7$, CLA and CCR9 antibody expression on DCs.
    - Active disease is associated with greater expression of gut homing marker $\beta7$ than non-active disease.
- There are significant differences between the mDC and pDC population for expression of homing and maturation status markers.
- Electron microscopy confirmed the flow cytometry findings of differential expression of CLA in circulating DC subsets.

3.7 Discussion

3.7.1 Abnormal circulating DC populations

In this chapter, circulating dendritic cells are demonstrated to have aberrant expression of gut and skin homing markers in Crohn's disease which may focus immune activity to the gut. The abnormalities in DC homing seen in Crohn's disease are likely to be of some importance, because expression of tissue specific homing markers can direct DCs to, or away from, sites of potential inflammation. If the changes in phenotype of circulatory DCs seen here in Crohn's were merely due to movement of DCs from the blood into the gut tissue, leaving a distorted population in the blood, one would expect to find lower, and not higher, expression of gut homing DCs in circulation. Not only are DCs able to exert local effects through cytokine production, but they can stimulate adaptive immune system responses and, furthermore, DCs can imprint these homing markers on T-cells, the key immune cell driver of inflammation in IBD. Here, then, we see changes in homing marker expression on circulating DCs which may be transferred to T cells.

Circulating DCs have historically been thought of as a bone-marrow derived, naïve population which benignly circulate until called into action in tissue. There is some evidence that, in IBD at least, these circulating cells may have a somewhat more active phenotype than previously thought (Vuckovic *et al.*, 2001; Baumgart *et al.*, 2005b). Here, abnormalities seen in peripheral circulating DCs, before they are exposed to the modulatory effects of local tissue microenvironments, support the hypothesis that primary DC abnormalities contribute to the pathogenesis of inflammation in IBD, rather than occur as a response to it. There is evidence that DCs do not re-circulate once they enter tissue (Fossum, 1988b; Yrlid *et al.*, 2006), a rare exception

being a report of the appearance of DCs in efferent lymph during carcinogenesis in sheep (Ragg *et al.*, 1994). Thus, circulating DCs can be thought of largely as naïve of tissue exposure. This concept is supported from studies of a murine colitis model; innate DC homing abnormalities in bone marrow derived monocytes (with no local tissue experience) lead to greater numbers of these cells with inflammatory phenotypes migrating to the gut (Peña *et al.*, 2009). Additionally, the idea that circulating DCs are inherently abnormal and possibly pathogenic in IBD is supported by genome wide association studies which found polymorphisms in loci associated with DCs (such as *LY75* and *CCL20*) in Crohn's disease patients and not in healthy subjects (Strisciuglio *et al.*, 2013; Liu *et al.*, 2015).

3.7.2 Alterations in gut and skin homing marker expression

Gut specific circulating DCs bind to MAdCAM-1, the cognate ligand of α4ß7, which is present in the bowel mucosa, allowing access to the gut mucosal tissue (indeed T cells bind to the same ligand). Hence a primary abnormality in circulating DCs might contribute to the excess concentration of DCs seen in the lamina propria of inflamed IBD tissue. We show a significantly greater number of circulating mDC with gut homing phenotype $CLA^{-}ß7^{+}$ in Crohn's disease than in HCs (and a similar trend for pDC). Most previously published studies of DC populations in Crohn's disease were performed using monocyte-derived DCs which are developed from circulating monocytes *in vitro* using cell culture factors. These cells lose expression of homing receptors during the culturing process (Bernardo *et al.*, 2013), a problem avoided here by studying fresh PBMCs. In this study, greater gut-homing marker expression by mDC is associated

with more severe inflammation (as evidenced by f calpro >150µg/g), suggesting modulation by primary changes in the bone marrow-derived DC population which may be driving the inflammation. It also raises the possibility of developing serum DC β7 level testing as a non-invasive biomarker for disease activity assessment, although as DCs are a small component of PBMCs this may be impractical. On direct comparison of the two circulating DC subtypes, mDCs are seen here to express a gut homing ($CLA^-\beta7^+$) phenotype, while pDCs express a skin (CLA^+) and lymph ($CCR7^+$) homing phenotype. These differences are seen in both health and Crohn's disease, suggesting different physiological roles and target tissues for these DC subtypes. Perhaps pDC have more of a skin regulatory role than mDC; certainly high numbers of pDC are seen in the dermis of patients with psoriasis, and far higher numbers of mDC than pDC are found in colonic mucosa in both health and Crohn's disease (Wlloenberg A, Wagner M, 2002; Ráki *et al.*, 2013). In agreement with this hypothesis, in this chapter we demonstrated transmission electron microscopy results of purified DCs stained for CLA which revealed that CLA was ubiquitous on the pDC, absent from immature mDC and present on mature mDC.

3.7.3 CCR7

The chemokine receptor, CCR7, enables DCs to home to lymphoid tissue, a process which occurs both in the steady state and during inflammation. The role of CCR7 expression in influencing the behaviour of peripheral DCs prior to migration to tissue in active Crohn's disease was unknown. In this study, there was no difference in the expression of CCR7 between HCs and subjects with Crohn's disease, suggesting the ability of the DCs to migrate to tissue was not affected by gut

inflammation. Additionally, mDC expressed much lower levels of CCR7 than pDC in both HCs and subjects with Crohn's disease ($p<0.0001$). CCR7 allows circulating pDC direct access to the high endothelial venules (HEV) of lymph tissue, so unlike mDC they do not require activation by antigenic contact with their TLRs to access lymph tissue (Seth *et al.*, 2011). This idea supports the view that pDC, unlike mDC, may enter the lymph nodes directly without activation within the tissues.

3.7.4 Differences in mDC and pDC phenotype

The technique of identifying DCs by staining from within the PBMC population allowed a direct comparison of cell surface receptor expression between mDC and pDC. It is well known that mDC and pDC express different PRRs for microbial sensing: TLR2 and TLR4 are present only on mDC whilst pDC express TLR7 and TLR9 (Kaisho and Akira, 2006; Thompson *et al.*, 2011). However, the relationship between homing expression and DC subtype was not known.

Plasmacytoid DCs have historically been regarded as a minor DC population with primarily tolerogenic and anti-viral roles, but the data in this chapter both challenges those paradigms and hints further at the virome or viral antigenic mimicry as possible causative factors in Crohn's disease and psoriasis (Norman *et al.*, 2015). This study demonstrates that circulating pDC express greater gut homing specificity and increased IL-6 production in Crohn's disease, suggesting that inflammatory pDC may migrate to the gut and stimulate on-going inflammation. In keeping with this concept, pDC have recently been identified as having a specific immune surveillance role in the colon (Toivonen *et al.*, 2016). The results are in concordance with a paper showing pDC in IBD

are mature, abnormally distributed and producing excess pro-inflammatory cytokines (D C Baumgart *et al.*, 2011); however, homing marker expression was not recorded in the published study. Similarly, pDC up-regulate β7 in response to HIV infection and correspondingly migrate to the colon, with migration halted by blockade of the integrin α4β7 (Kwa *et al.*, 2011).

β7 exists in two forms, as αεβ7 and α4β7. In this study, an antibody to the β7 moiety was used, rather than to the α4β7 moiety. Previous work in our laboratory has demonstrated that whilst αε (also called CD103) is prevalent in the colon, it is not seen in the circulation, therefore β7 levels in the circulation are a *de facto* surrogate for α4β7.

3.7.5 CCR9

Although circulating DC CCR9 expression was not altered in Crohn's disease, expression levels on both mDC and pDC varied with differing disease distribution, with ileocolonic disease having greater representation than isolated ileal. Traditionally, CCR9 has been regarded as a "small bowel" homing marker, with minimal colonic expression. However, a recent study has shown that colonic lymphocytes express CCR9 in active colitis (95% expression in patients refractory to therapy) but not in healthy controls (5% expression of CCR9). Although pDC are scarcely seen in the colonic mucosa of HCs, they are seen (but in small numbers) in active IBD and in high numbers in inflamed psoriatic skin (Hart *et al.*, 2005a; Ng *et al.*, 2009; Afzali *et al.*, 2014). Perhaps future trials should tailor anti-CCR9 therapy to those patients with heavy CCR9 expression in the intestine, small or large, to improve therapeutic response rates.

3.7.6 Clinical relevance

Dendritic cells express tissue homing markers and can imprint them on T cells (Worbs, Hammerschmidt and Förster, 2016). Targeting homing markers on T cells has led to the development of efficacious therapies for multiple sclerosis and IBD. In IBD, natalizumab (a humanized monoclonal antibody against the cell adhesion molecule α4) was found to be effective, but was withdrawn from practice in Europe due to its association with progressive multifocal encephalopathy (Gordon *et al.*, 2001; Bartt, 2006). Vedolizumab (an α4ß7 blocker) is effective in both Crohn's disease and UC (Feagan *et al.*, 2013; William J Sandborn *et al.*, 2013) and etrolizumab (binds β7, those blockading both α4β7 and αEβ7) is currently in a phase 3 trial. Promising Phase 2 data for a CCR9 antagonist were followed by a negative Phase 3 study. In our study, unlike β7, CCR9 is not increased in Crohn's disease, and levels do not associate with more active disease, perhaps contributing to the efficacy of vedolizumab and the lack of efficacy of the CCR9 antagonist. Previous, unpublished, work from our laboratory has shown raised CCR9 on circulating DCs in some untreated patients with Crohn's disease as well as in children refractory to enteral nutrition.

3.8 Discussion summary points – Chapter 3

- By studying "fresh" circulating DCs (i.e. DCs identified from within the whole PBMC population by staining), the significant drop-off in homing receptor expression (as seen when using cultured Mo-DCs) is avoided, and accurate recordings of homing marker expression on circulating DCs can be made.
- Circulating mDC lose their "non-tissue specific" phenotype ($CLA^+\beta7^+$) in Crohn's disease, by down-regulation of CLA, leaving them with a "gut-specific" phenotype ($CLA^-\beta7^+$). This may be one mechanism of promotion of mucosal inflammation in Crohn's disease, as DCs drive the T cell response which characterises IBD.
- In Crohn's disease, circulating pDC express a gut homing phenotype (in comparison to HCs), which suggests that pDC may be involved in the initiation or maintenance of mucosal inflammation in Crohn's disease.
- The differences shown here between mDC and pDC in terms of expression of several surface markers suggests that they should preferably be studied as separate entities in future research.
- The fact that severity and distribution of inflammation in Crohn's disease can be inferred from the phenotype of circulating DCs puts them forward as a potential non-invasive "window to the gut", although the small number of cells and laborious laboratory work may render this unachievable.

Chapter 4 - The Function of Circulating Dendritic Cells in Crohn's Disease

4.1 Abstract

Introduction

Crohn's disease is characterised by an exaggerated immune response to mucosal antigen. Dendritic cells (DCs) are the primary antigen presenting cells and may promote either tolerogenic or inflammatory T cell responses to mucosal antigens. We assessed function of circulating dendritic cells by measuring ongoing cytokine production by circulating DC subsets from patients with Crohn's disease and comparing to healthy controls.

Methods

DCs within peripheral blood mononuclear cells from adults with active luminal Crohn's disease or from healthy controls (HCs) were characterised using flow cytometry. DCs were identified as discussed previously and DC subtypes, mDC and pDC, were studied for on-going intracellular cytokine production by intracellular staining. ELISA quantification of several serum cytokines was also performed.

Results

Production of pro-inflammatory cytokines by circulating DCs was up-regulated in Crohn's disease (n = 19) as compared with health (n = 12), with myeloid DCs producing higher levels of TNF-α (p = 0.0042) and plasmacytoid DCs producing higher levels of IL-6 (p = 0.013). ELISA confirmed that the increased DC production of TNFα and IL-6 were mirrored by increased concentrations in serum.

Expression of IFN-α, Il-1β, IL-10, Il-12 p40/70 (can bind to p40 monomer and p70 heterodimer, but not p35 monomer), IL-23 p19, and TGFβ on DCs were not different between Crohn's disease patients and controls for either DC subset.

Conclusion

Circulating DCs are functionally abnormal in Crohn's disease. Increased basal (unstimulated) production of two pro-inflammatory cytokines which are key to Crohn's disease (TNFα and IL-6) by circulating DCs further supports the argument that abnormalities in DCs are contributory to disease pathogenesis. Abnormalities in pDC function further implicate this cell group in Crohn's disease.

Verification of cytokine abnormalities by ELISA quantification of serum cytokine levels demonstrates that changes in cytokine production by DCs (a relatively rare cell type) are mirrored by changes in circulatory levels.

On-going production of the other examined cytokines was not different between Crohn's disease and controls, which might be truly reflective, or may be due to a combination of effects of past or current immunomodulator use or need for greater patient numbers.

4.2 Introduction

Having seen in Chapter 3 that circulating DCs have abnormal surface marker phenotype, here we look at function, studying on-going intracellular production of cytokines by circulating DCs. Cytokines, their interaction with (and production by) DCs and their importance in IBD are discussed in some detail in the Introduction chapter (1.2.7). Here, we pay particular attention to key individual cytokines, namely: IL-6, IL-10 and TNFα (the cytokines studied the deepest in this thesis) as well as IFNα, IL-12 and IL-23:

IL-10 is a pleiotropic anti-inflammatory cytokine, possibly the most important in the human response in IBD (Asadullah, Sterry and Volk, 2003; Glocker *et al.*, 2011). IL-10 gene KO mice develop spontaneous colitis, and mutations in the IL-10 gene predispose to very early onset IBD (Strober, Fuss and Blumberg, 2002). IL-10 is produced by $CD4^+$ Th2 cells, Th1 cells (in some inflammatory settings), Tregs ($CD4^+CD25^+FOXP3^+$), Tr1 cells, macrophages, monocytes, DCs, and mast cells. IL-10 exerts its downstream effects in several ways. Firstly, it indirectly interferes with factor nuclear factor-κB (NFκB) signalling, which is involved in effector actions of several pro-inflammatory cytokines (Wang *et al.*, 1995; Schottelius *et al.*, 1999). IL-10 also down-regulates expression of MHCII and costimulatory molecules (CD40 CD80) on APCs (both DCs and macrophage/ monocytes) resulting in decreased stimulation of T cells (Albright, Sartor and Tonkonogy, 2009). A number of trials have assessed the efficacy of IL-10 in treating Crohn's disease, with variable results; a Cochrane analysis of three of these studies concluded that IL-10 does not provide a benefit for the treatment of active Crohn's disease (van Deventer, Elson and Fedorak, 1997; Fedorak *et al.*, 2000; Colombel *et al.*, 2001; Buruiana, Solà and Alonso-Coello, 2010).

Secretion of IL-6, an inflammatory response mediator, by lamina propria T lymphocytes and macrophages is increased in IBD. IL-6 binds to the soluble IL-6 receptor (sIL-6R) and this complex can bind to the gp130 receptor which is present on several target cells. Hence, IL-6 may interact with many cells, including DCs, macrophages and T lymphocytes where it stimulates inflammatory responses. IL-6 can increase the resistance of mucosal T cells to apoptosis and, in combination with TGFβ, it promotes Th17 differentiation (Atreya *et al.*, 2000). IL-6 levels are raised in both UC and Crohn's disease. IL-6 blockade has been studied in animal models as well as human trials and although it is in the armamentarium for rheumatoid arthritis, it has yet to be shown to successfully treat IBD (Atreya *et al.*, 2000; Yamamoto *et al.*, 2000; Ito *et al.*, 2004).

TNFα is involved in acute phase and systemic inflammation and anti-TNF therapy has been a mainstay of therapy for moderate to severe IBD over the past couple of decades. TNF is produced in the form of a transmembrane protein, from which soluble TNF is released via enzymatic cleavage by TNF-converting enzyme (TACE). Macrophages, adipocytes, fibroblasts and T cells may all synthesise TNF, and this production is heightened in IBD (Atreya, Zimmer, Bartsch, Waldner, Atreya, Neumann, Hildner, Hoffman, Kiesslich, Rink, Rau, Rose–John, *et al.*, 2011). TNF may exert its downstream effects by binding to its receptors, TNFR1 and TNFR2, and subsequent intracellular activation of the transcription factor NFκB, or by activation of other pathways including receptor-interacting protein kinase 1 (RIPk1) or caspase 3 protein, amongst others (Di Sabatino *et al.*, 2007; Günther *et al.*, 2011). Cells that may be influenced by TNF include endothelial cells, macrophages, Paneth cells, myofibroblasts and effector T cells.

IFNα, the principle cytokine associated with pDCs, has been previously investigated (unsuccessfully) as a potential therapy for Crohn's disease (Gasché *et al.*, 1995). A study published

by Baumgart et al has shown that IFNα levels may be reduced in Crohn's disease and it was hypothesised that this may be contributory to the disease state (as IFNα has some regulatory effects including the induction of T cell tolerance and reduction of IL-6 production) (Steinman *et al.*, 2000; D. C. Baumgart *et al.*, 2011).

There is strong evidence that IL-12 (along with TNFα and IFNγ) is crucial to the Th1 pathway in the development of Crohn's disease (Trinchieri, 1994). The importance of the inflammatory cytokines IL-12 and IL-23 in IBD has been put into relief by the efficacy of the monoclonal antibody ustekinumab in treating the disease (Mannon *et al.*, 2004; Sandborn *et al.*, 2012). Genes for both IL-12R and IL-23R are within IBD susceptibility loci. IL-12 is expressed and actively released by intestinal LPMCs and intestinal DCs and this is upregulated in Crohn's disease (Monteleone *et al.*, 1997; Ng *et al.*, 2011). Production of IL-12 and IL-23 has not been studied in circulating pDCs previously. IL-23p19 expression in IBD mucosa is increased and promotes IEL and LPMC inflammatory responses (and Th17 differentiation) (Liu *et al.*, 2011).

4.3 Hypothesis and Aims

4.3.1 Hypothesis

We hypothesised that abnormalities in on-going cytokine production by circulating dendritic cells contribute to the inflammatory burden in luminal Crohn's disease.

4.3.2 Aims

- To characterise function (on-going intracellular cytokine production) of circulating dendritic cells in Crohn's disease and in health.
- To compare the different roles of the two main circulating dendritic cell subtypes (mDC and pDC) in Crohn's disease.
- To quantify the serum cytokine profile, using ELISA, and compare with the cytokine profile produced by circulating DCs.

4.4 Methods

Patient selection and recruitment is covered in Section 3.4, with Table 3.2 showing patient clinical and demographic data, and Table 3.3 showing HC demographic data. Experimental protocol is covered in detail in Section 2.3, but in short: fresh PBMCs were isolated from the peripheral blood of patients with Crohn's disease and from healthy controls. Surface staining was then performed to allow identification of DCs and DC subtypes, and thereafter intracellular staining (ICS) for cytokines was performed. Flow cytometry was completed, SEDmax subtraction technique was utilised on Winlist software (as discussed in 2.3.2.7.1), with subtraction of the non-monensin control events from the monensin tube of interest allowing real-time cytokine quantification.

Although ICS is both technically taxing and time consuming, it allows real time quantification of cytokine production and assessment of the cell type and subtype which is producing them; neither of which can be done with other experimental techniques.

Serum was stored (frozen) for ELISA quantification of cytokines which was performed as a batch upon the thawed serum samples after recruitment and sample acquisition was completed.

4.5 Results

For baseline demographic and disease characteristics of the patient in the study see 3.6.

4.5.1 Cytokine production

Another important marker of inflammatory DC response is their ability to produce cytokines. This has not only local, but systemic effects as the cytokine milieu produced by DCs will influence the differentiation of T cells. We examined DC production of TNFα and IL-6, which have been heavily implicated in the pathogenesis of Crohn's disease. Dendritic cells were isolated and cultured either with or without monensin, in the absence of stimulation, for 4 hours before staining for intracellular cytokines and examination by flow cytometry.

4.5.1.1 TNFα production – assessed by flow cytometry and ELISA

Low production of TNFα was seen in the HC population (figure 4.1).

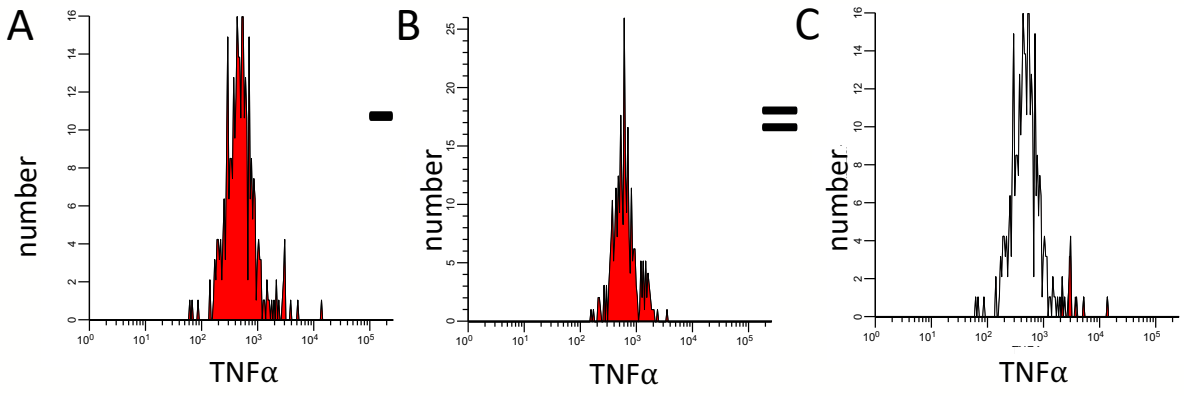


Figure 4.1: *(C) Histogram demonstrating low TNFα staining in the mDC of a healthy control calculated by the subtraction of (B) the non-monensin control from (A) the pre-subtraction 'monensin TNFα value'.*

TNFα production by circulating DCs was higher in subjects with Crohn's disease than in HCs, and this increase reached significance in the mDC population (figure 4.2).

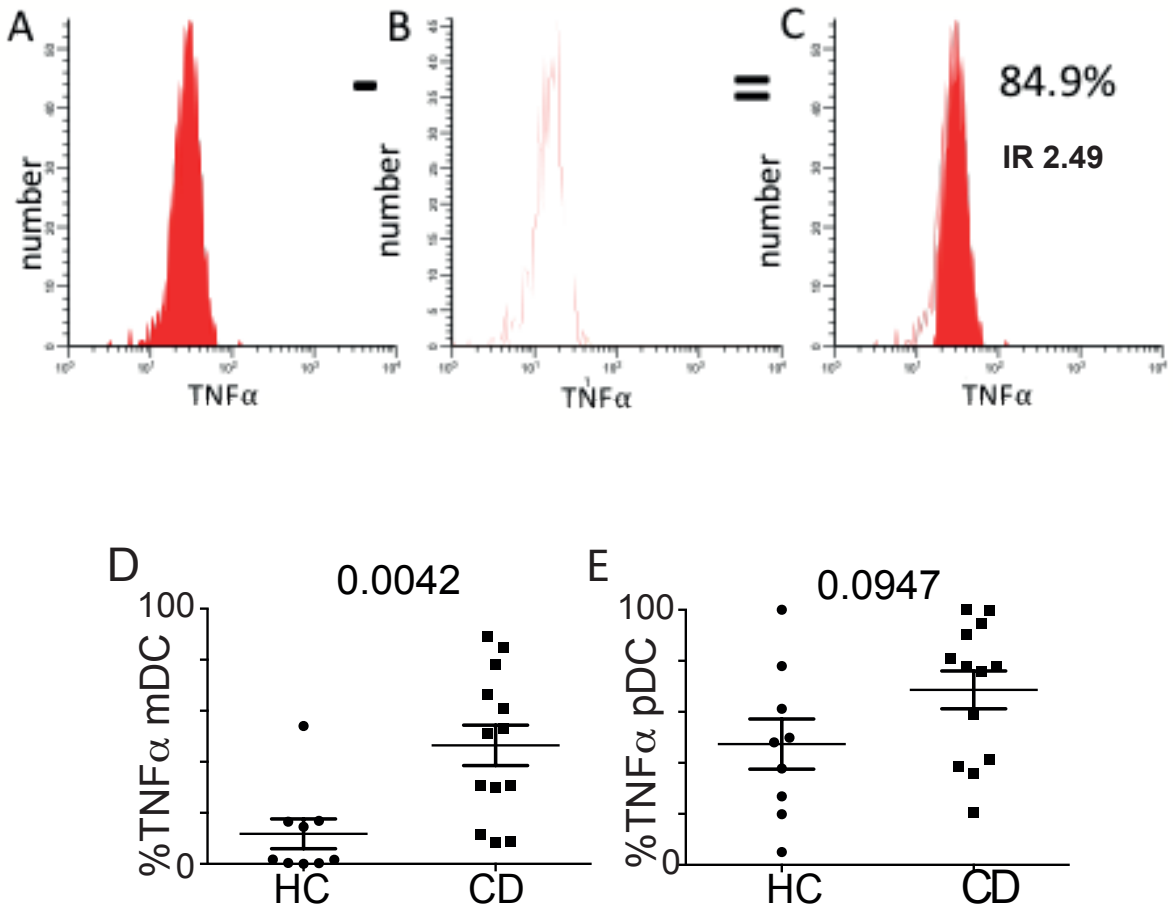


Figure 4.2: *(C) Histogram demonstrating TNFα staining in the mDC of a subject with Crohn's disease calculated by the SEDmax subtraction of (B) the isotype control from (A) the pre-subtraction TNFα value. (D) and (E) demonstrate population data for TNFα staining comparing healthy controls (HC) and subjects with Crohn's disease (CD) on mDC and pDC respectively. Unpaired t-test was used.*

On comparison of TNFα production between the DC subtypes in Crohn's disease, there was no significant difference. ELISA demonstrated higher serum concentration of TNFα in Crohn's disease than in HCs, although this failed to reach statistical significance (figure 4.3).

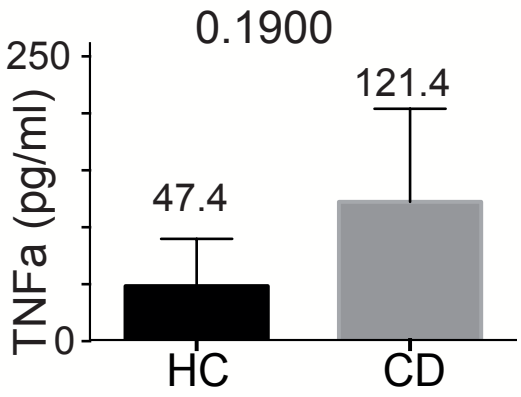


Figure 4.3: Graph representing whole population ELISA results for concentration of TNFα (units pg/ml) in serum of healthy controls (HC) and subjects with Crohn's disease (CD).

4.5.1.2 IL-6 production assessed by flow cytometry and ELISA

We next measured ongoing expression of the pro-inflammatory cytokine IL-6. In Crohn's disease, basal IL-6 production by pDC was elevated compared with HCs, (figure 4.4), while there was a non-significant trend to increased production by mDC (figure 4.4B).

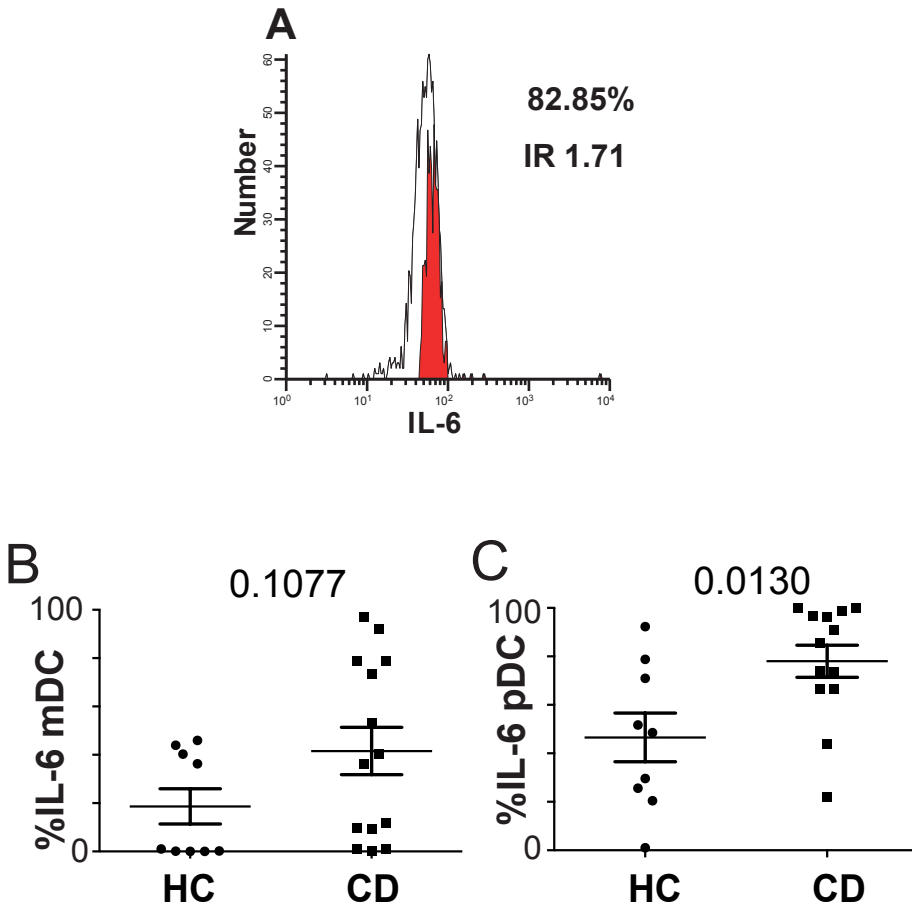


Figure 4.4: (A) SEDmax subtraction histogram of IL-6 staining on pDC in a subject with Crohn's disease. (B) and (C) show population data comparing IL-6 staining on health controls (HCs) and subjects with Crohn's disease (CD) on mDC and pDC respectively. Unpaired t-test was used.

ELISA confirmed that serum IL-6 concentration was higher in Crohn's disease than in HCs (figure 4.5).

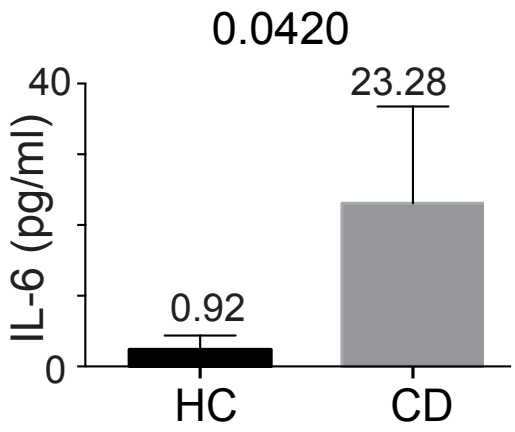


Figure 4.5: Graph representing cumulative population ELISA result for concentration of IL-6 (units pg/ml) in serum of healthy controls (HC) and subjects with Crohn's disease (CD).

These findings show heightened pro-inflammatory cytokine responses by both circulating DC subsets in Crohn's disease, alongside raised serum cytokine concentrations. These data suggest differential roles for mDC and pDC in both health and disease settings with differing production of TNFα and IL-6 by the subgroups in health, and differential upregulation in Crohn's disease.

4.5.2 Production of key cytokines by circulating DCs does not vary with location of disease

As with comparison of disease location to expression of homing markers in Chapter 3, here a comparison of disease location with production of key cytokines was made.

4.5.2.1 TNFα production by circulating DCs does not vary with disease location

Production of TNFα (percentage expression measured over a four-hour period) by both circulating mDC and pDC does not vary with anatomical location of the disease (comparison of L1 to L3 disease) (Fig 4.6 below).

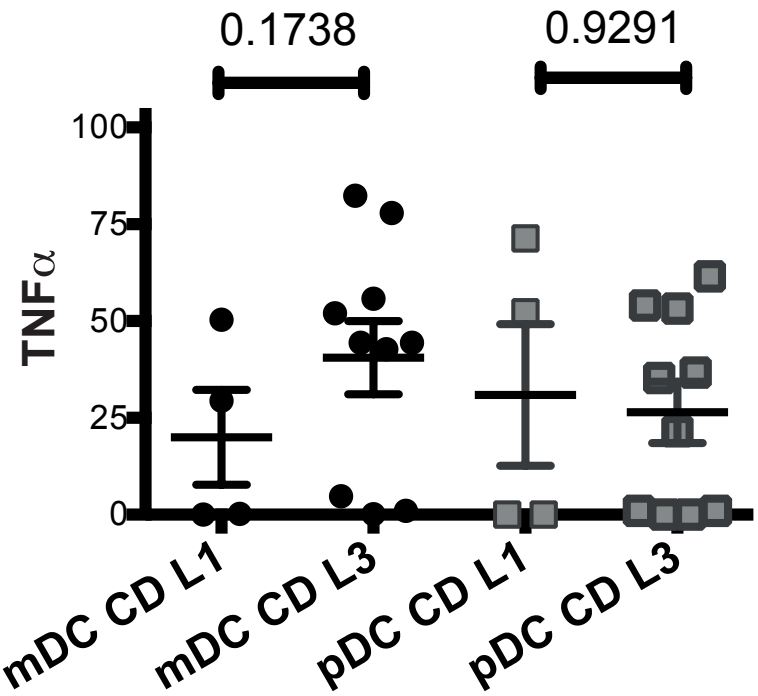


Figure 4.6: Comparison of Crohn's disease location (as per Montreal classification) demonstrating isolated ileal (Montreal L1) v ileocolonic disease (Montreal L3) with production of TNFα by mDC and pDCs. Mann-Whitney test used.

4.5.2.2 IL-6 production by circulating DCs does not vary with disease location

Production of IL-6 (percentage expression on DCs measured over a four-hour period) by both circulating mDC and pDC does not vary with anatomical location of the disease (comparison of L1 to L3 disease) (figure 4.7).

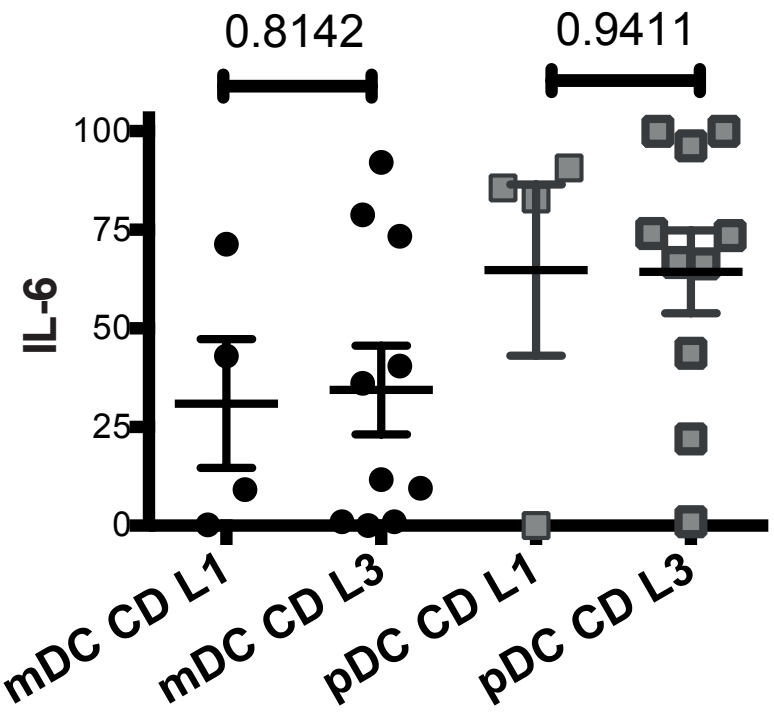


Figure 4.7: Comparison of Crohn's disease location (as per Montreal classification) demonstrating isolated ileal (Montreal L1) v ileocolonic disease (Montreal L3) with production of IL-6 by mDC and pDCs. Mann-Whitney test used.

4.5.3 Production of key cytokines does not vary with level of inflammation

As with comparison of degree of inflammation with expression of homing markers in Chapter 3, here a comparison of degree of inflammation with production of key cytokines was made.

4.5.3.1 TNFα production by circulating DCs does not vary with increased disease severity

Production of TNFα (percentage expression measured over a four-hour period) did not vary with mucosal inflammation in either the mDC or pDC subsets, with TNFα expression not discriminating between faecal calprotectin high or low values (figure 4.8).

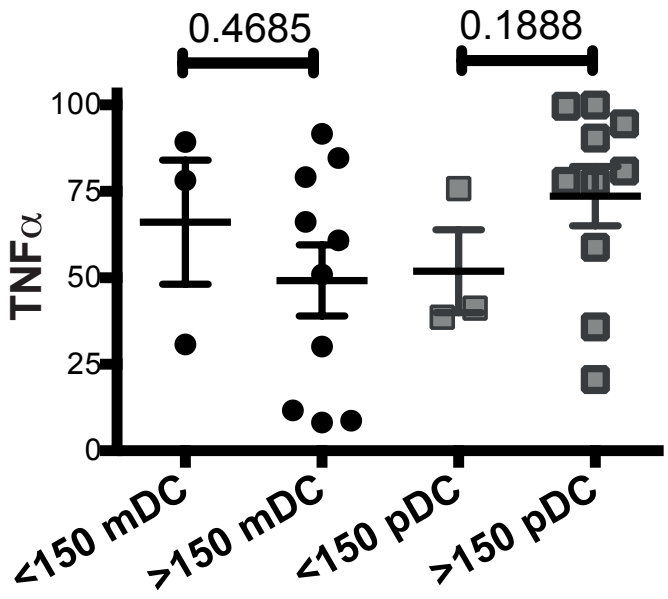


Figure 4.8: Comparison of Crohn's disease severity (assessed by faecal calprotectin value, as faecal calprotectin high (>150μg/g) or faecal calprotectin low (<150μg/g)) with expression of TNFα by mDC and pDC. Mann-Whitney test used.

4.5.3.2 IL-6 production by circulating DCs does not vary with increased disease severity

Production of IL-6 (percentage expression measured over a four-hour period) did not vary with mucosal inflammation in either the mDC or pDC subsets, with IL-6 expression not discriminating between faecal calprotectin high or low values (figure 4.8).

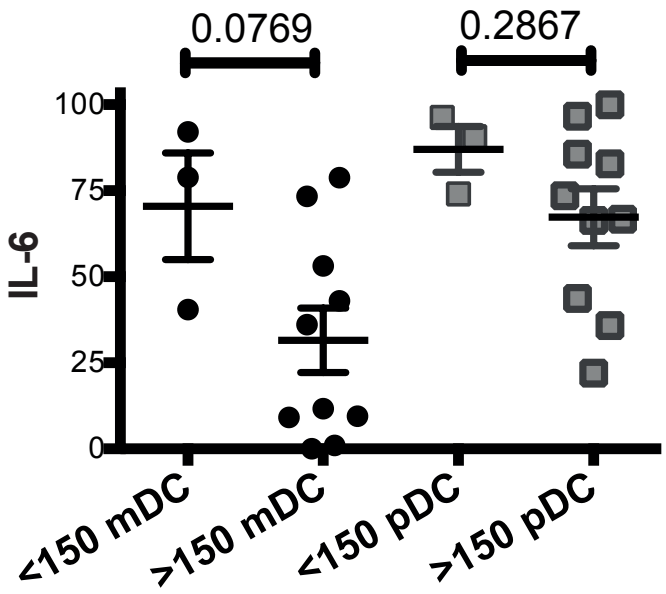


Figure 4.9: Comparison of Crohn's disease severity (assessed by faecal calprotectin value, as faecal calprotectin high (>150μg/g) or faecal calprotectin low (<150μg/g)) with expression of IL-6 by mDC and pDC. Mann-Whitney test used.

4.5.4 Expression of other examined cytokines

We examined a range of other pro- and anti- inflammatory cytokines being produced by circulating DCs in these patients (figure 4.10). There were no differences in production of IL-10, IL-12, IL-23, TGF-β, IL-1β or IFNα by either DC subgroup in Crohn's and HCs.

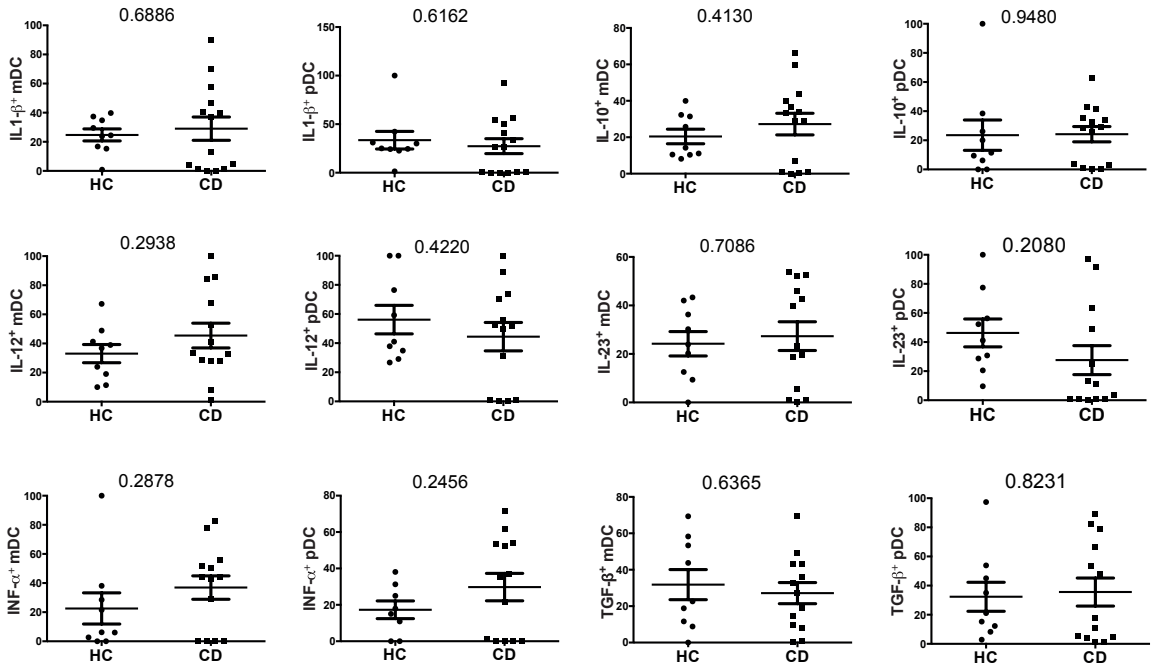


Figure 4.10: Summary population statistical data comparing expression of a range of cytokine by dendritic cells in healthy controls (HC) and subjects with Crohn's disease (CD). The examined cytokines, from left to right, were row 1: IL1-β in mDC, IL1-β in pDC, IL-10 in mDC, IL-10 in pDC; row 2: IL-12 in mDC, IL-12 in pDC, IL-23 in mDC, IL-12 in pDC; row 3: IFN-α in mDC, IFN-α in pDC, TGF-β in mDC, TGF-β in pDC. Unpaired t-test was used.

4.6 Summary of results

- Circulating DCs are functionally abnormal in Crohn's disease
    - On-going production of TNFα by circulating mDC was increased in Crohn's disease.
    - On-going production of IL-6 by circulating pDC was increased in Crohn's disease.
- There are significant differences between function of the circulating mDC and pDC populations as assessed by on-going cytokine production.
- ELISA confirms that increased DC production of TNFα and IL-6 in Crohn's disease is mirrored by increased serum concentration of these pro-inflammatory cytokines.
- On-going production of the other assessed cytokines (including IL-10, IL-12, INFα and IL-23) did not differ between healthy controls and subjects with Crohn's disease, and there was no difference in production between the two dendritic cell subtypes.

4.7 Discussion

4.7.1 Production of TNFα and IL-6 by DCs

In this chapter, circulating dendritic cells are demonstrated to have functional abnormalities, with excessive on-going production of pro-inflammatory intracellular cytokines. Functional abnormalities are also seen within the pDC subset, implicating this cell group, previously thought to have largely tolerogenic and anti-viral properties, in Crohn's aetiology.

The technique of identifying DCs by staining from within the PBMC population allowed a direct comparison of on-going intracellular cytokine production between mDC and pDC; there are no such publications in the literature.

The concept of pDCs as a minor DC population with primarily tolerogenic and anti-viral roles was challenged in chapter 3. Here in chapter 4, the study demonstrates that circulating pDC have heightened on-going intracellular production of IL-6 in Crohn's disease, suggesting that not only are circulating pDC in Crohn's disease predisposed to home to the gut (chapter 3), but that they have an inflammatory function.

The increased production of IL-6 by circulating DCs is likely pathogenic, as IL-6 is a key pro-inflammatory cytokine associated with IBD aetiopathogenesis. Indeed, IL-6 has previously been under investigation as a potential therapeutic target (anti-IL-6 was probably efficacious but the serious adverse event of intestinal perforation mandated the early termination of the trial) and there are some data to suggest that IL-6 may be the main driver of inflammation in those patients who don't respond to anti-TNFα therapy (Ito *et al.*, 2004; Bank *et al.*, 2014). Interestingly, the increased IL-6 production was pronounced in the pDC population. The pDC subtype have a

greater skin homing marker expression than mDCs, and it has been previously demonstrated that IL-6 in inflamed gut tissue can predispose to a skin homing T cell population (Bernardo, Vallejo-Díez, Elizabeth R Mann, *et al.*, 2012b). Raised IL-6 production by circulating pDCs has been demonstrated previously via stimulation studies of purified pDC, and it is useful to corroborate those findings with a different experimental technique here (D. C. Baumgart *et al.*, 2011).

Increased TNFα production by circulating DCs has previously been described in IBD and is likely to be of clinical significance here because the presence of TNFα and IL-6, amongst other inflammatory cytokines, in the local cytokine milieu at the time of antigen presentation will predispose to clonal expansion of Th1 and Th17 populations (Blanco et al. 2008).

The excessive production of TNFα and IL-6 by DCs in Crohn's disease was mirrored by higher serum concentrations of these pro-inflammatory cytokines (as expected), suggesting that, although DCs are a relatively small component of circulating PBMCs (about 1%), they might be important in shaping overall serum cytokine composition by driving effector cell responses of cell types with much greater numbers (Dauer *et al.*, 2003; Nancey *et al.*, 2008).

4.7.2 Other cytokines

Whilst there was increased basal (non-stimulated) production of TNFα and IL-6 by circulating DCs in subjects with Crohn's disease in this study, it is worth discussing the fact that several cytokines which one might expect to be abnormal were in fact not, namely IFNα, IL-12 and IL-23. Potential explanations for the lack of difference in control and disease results may be due to a need for higher numbers of subjects; due to the biology of the cell types under study or possibly because

the patients are on immunomodulators. Azathioprine and 6MP have both been shown to alter DC expression of surface receptors and production of cytokines (Aldinucci *et al.*, 2010). We attempted to mitigate against IM as a confounder by ensuring that doses were stable for 3 months prior to recruitment.

Although previously found to be raised in a study by Baumgart et al, DC production of IFNα was not altered in the Crohn's disease subjects in this study (D. C. Baumgart *et al.*, 2011). There were several experimental differences between the Baumgart study and this thesis study that might explain the discrepancy: Baumgart used purified pDC (>95% purity) following positive cell sorting and then stimulated the cells with CpG (cytosine phosphatidyl guanine). The cells in this thesis study were stained from within the PBMC population, and were unstimulated. The advantages of staining from within PBMCs are that the cytokines identified in flow cytometry can be attributed to the cell type studied; whereas with the purification method if the 5% 'impure' cells are major producers of cytokines (e.g. macrophage or T cells) then the results can be significantly skewed. A further disadvantage of using a population of cells purified by positive cell sorting is that the cells will carry the bead-bound antibodies – another variable added to the experiment.

There are no published data demonstrating whether IL-12 and IL-23 production by circulating DCs is abnormal in Crohn's disease; however basal IL-12 production by DC enriched low density cells (LDCs) in the blood was not different between Crohn's and HCs (Peake, unpublished work) and counter-intuitively increased with infliximab.

What might be causing the abnormalities in phenotype and function of circulating DCs in Crohn's disease? One explanation is that the genetic abnormalities that encode DC protein translation

lead to an abnormal circulating DC population which predisposes to development of inflammatory disease. A second explanation is that these abnormalities in the circulating DC population occur as a consequence of the luminal disease process, for example because of increased concentrations of circulating cytokines. Indeed, it is known that cytokines affect the maturation of DCs and this will critically alter the downstream activation and differentiation of T cells (Dauer *et al.*, 2003). If the second explanation were the case it seems likely that this would have the effect of propagating and maintaining the inflammatory state, as this population expresses excess gut homing marker, and produces excess of pro-inflammatory cytokine. In addition, injection with tolerogenic DCs has been shown to ameliorate colitis activity, thus there is precedent of circulating DCs modulating colitis activity (Cabezón and Benítez-Ribas, 2013b). It would be theoretically possible to further test these hypotheses: 1) by performing bone marrow (BM) biopsy to ascertain whether the BM-DC are abnormal in Crohn's disease, which would rule out the effect of circulating inflammatory markers on the population. 2) Another method might be to engineer a murine model with DC related KO genes to evaluate whether it was predisposed to spontaneous or induced colitis.

4.8 Discussion summary points – Chapter 4

- Abnormalities in function of circulating DCs such as TNFα and IL-6 production are likely to be important in the initiation and/ or the maintenance of inflammation in Crohn's disease.
- The heightened non-stimulated production of IL-6 by circulating pDCs here supports, using a different experimental technique, the results of a published study and adds weight to the hypothesis that pDC are abnormal and potentially causative in Crohn's disease.
- The difference in cytokine production between the circulating mDC and pDC subsets (alongside the difference in phenotype demonstrated in Chapter 3) demonstrates that circulating mDC and pDC are distinct phenotypically and functionally and should be studied only as separate populations.
- That increased circulating DC production of IL-6 and TNFα was mirrored by increased serum concentrations of the same cytokines is suggestive that although circulating DCs are a relatively rare cell type (about 1% of PBMCs) they are important in shaping the serum cytokine composition (by directing T cell differentiation and expansion and hence T cell cytokine production).

Chapter 5 - The Effect of Infliximab and Adjunctive Vitamin D Therapy Upon Circulating Dendritic Cell phenotype in Crohn's Disease

5.1 Abstract

Introduction

Dendritic cells (DCs) can determine whether the mucosal immune system mounts an inflammatory or regulatory response to antigen and circulating DCs have been shown here (in chapters 3 & 4) to have abnormal phenotype and function in Crohn's disease. Vitamin D is of increasing interest as a modulator of the immune system and as a therapeutic option in Crohn's disease. This study assessed the effect of high dose parenteral vitamin D treatment on circulating DC phenotype in patients with active luminal Crohn's disease receiving anti-TNFα therapy.

Methods

First, i*n vitro* studies were performed to assess the effect of vitamin D on the percentage of DCs expressing skin or gut homing markers as assessed by flow cytometry.

Next, the experiment was repeated, but in an *ex vivo* study. Peripheral blood mononuclear cells were isolated from 16 patients with active luminal Crohn's and suboptimal vitamin D levels prior to and 6 weeks after starting anti-TNFα (infliximab) therapy. Patients with low vitamin D (<50nMol/L) were also given a single high dose of parenteral vitamin D (300,000 international units 1,25(OH)2 vitamin D3) whilst those with inadequate levels (50-74nMol/L) did not receive

vitamin D supplementation. Flow cytometry was performed to identify DCs and the two DC subtypes (mDC and pDC), as in the previous chapters. Expression of phenotypic markers (including markers of homing, maturation and microbial sensing) was assessed. In addition to immunological data; disease activity, biochemical and safety data were also recorded.

Results

In the *in vitro* study, culture with vitamin D reduced the percentage of cells expressing the skin homing marker cutaneous lymphocyte antigen (CLA, $p = 0.044$).

In the *ex vivo* study, vitamin D was safe and well tolerated across the study cohort without any clinically significant sequelae. Serum vitamin D levels were significantly increased in the group which received supplementary vitamin D, but not the non-supplemented group ($p = 0.0012$). Faecal calprotectin levels fell across the whole study population following infliximab therapy, and the fall was greater in the group which also received vitamin D (294µg/g v 166µg/g).

Anti-TNFα therapy alone induced a significant upregulation from baseline in percentage of mDCs expressing skin homing marker ($p = 0.0055$). In the group which received adjunctive vitamin D therapy, this upregulation in cells expressing CLA was blocked.

Conclusion

Anti-TNF therapy causes an upregulation in CLA-expressing circulating DCs. This may be relevant to the high rate of cutaneous side-effects associated with anti-TNF therapy. Vitamin D altered

the homing marker expression on circulating DCs in both *in vitro* and *ex vivo* studies here. This may be one of the mechanisms of action of vitamin D. Further, we postulate that the down-regulation of skin homing marker CLA by vitamin D may be clinically useful in those patients suffering cutaneous sequelae of anti-TNFα therapy.

5.2 Introduction

Terminology:

There are several different forms of vitamin D which may be given as therapy, and measured as levels. In this text, unless otherwise stated, vitamin D will be taken to mean 1,25(OH)$_2$Vitamin D3 when applied to the therapy, and 25(OH)vitamin D when applied to serum levels.

5.2.1 Modulation of dendritic cells

Having demonstrated in Chapter 3 that circulating dendritic cells have an abnormal phenotype in Crohn's disease, potentially offering a "window to the gut", in Chapter 5 we sought to evaluate whether circulating DCs were amenable to modulation by different therapeutic agents to "normalise" this abnormal surface phenotype.

In vitro studies have shown that the anti-TNFα agent infliximab can exert an immunomodulatory effect on circulating DCs, and can alter homing marker expression of T cells (Peake, Bernardo, Mann, *et al.*, 2013a). Therefore, here we sought to take it one stage further and investigate the *ex vivo* effects of infliximab upon the immunophenotype of circulating DCs, paying particular attention to its effect upon homing marker expression.

Dendritic cells are already a target of therapeutic interest in other diseases. Indeed, sipuleucel-T, a dendritic cell based vaccine, is a licensed, life prolonging therapy in metastatic prostate cancer (Small, 2006). In IBD, adoptive transfer of tolerogenic (Fas-ligand modulated) DCs is a successful approach to treating inflammation in a murine model(Rivera de Jesus and A Isidro, 2016). In addition to antigen-specific DC therapies, trans-tolerance has been investigated, and DCs have been successfully modulated towards tolerogenicity *in vitro* by agents such as sirolimus,

vitamin D and corticosteroids (Naranjo-Gómez *et al.*, 2011; Cabezón and Benítez-Ribas, 2013; Ferreira *et al.*, 2015).

Vitamin D has also elicited interest for its immune function (see 1.3.2.3.7) ever since the intra-nuclear vitamin D Receptor was discovered to reside in various cells of the immune system, including DCs. Vitamin D has been shown to have immunomodulatory effects on DCs in *in vitro* studies (Lars E Bartels *et al.*, 2013b), and in a single *ex vivo* study, by Bartels et al (Lars E. Bartels *et al.*, 2014). In the *ex vivo* study, oral vitamin D was given to 10 subjects with Crohn's disease, and their monocyte-derived DCs (Mo-DCs) were studied for maturation status, and cytokine production, and compared to Mo-DCs generated from 10 placebo matched Crohn's controls. It was seen that taking the vitamin D supplement decreased Mo-DC expression of maturation markers such as CD80, and reduced production of pro-inflammatory cytokines. The Bartels study did not examine markers of homing status (indeed homing markers are lost during *in vitro* culture and so that is not possible using Mo-DC studies), and there are further significant differences between studies performed on Mo-DCs and fresh DCs stained from PBMCs (see 1.2.8). Hence, it was our intention to study vitamin D supplementation in Crohn's disease with the novel focus being upon the effect on homing marker expression, and to see the reproducibility in the fresh PBMC stained DCs, rather than Mo-DCs.

5.2.2 Why vitamin D was chosen as an adjunct rather than as monotherapy

Crohn's disease is frequently severe, indeed, up to 70% of sufferers will undergo surgery at some point in their lifetime. Thus, although there is evidence of immunomodulatory function of vitamin

D as a sole agent, it appears more likely that vitamin D, if it is to play a role in the management of Crohn's disease, will be as an adjunct to another therapy. It is hard to imagine prescribing vitamin D as monotherapy for a disease as severe and with such serious sequelae as Crohn's disease. It is also unclear as to whether the role of vitamin D in IBD, if it has one beyond bone health, would be to help induce or help maintain remission, but the latter seems more likely.

Indeed, the only true RCT of vitamin D, performed by Jørgensen et al, which narrowly missed statistical significance, was performed upon patients in remission (S. P. Jørgensen *et al.*, 2010). The Jørgensen study included 108 patients with inactive Crohn's disease who were randomised to 1,200IU daily of $1,25(OH)_2$vit D3 or placebo over 12 months. The primary endpoint was clinical relapse, and although there was a numerical difference between the two groups, the p value was 0.06. Reasons for non-significance include the study being underpowered at the start (it was powered to detect a 50% reduction in disease relapse, which would seem excessive even for biological therapy) and a loss of patients who were excluded after the start of the trial because they were later found to have active disease based on raised C-reactive protein.

In some centres vitamin D is used as an adjunct to conventional quadruple therapy in mycobacterium *tuberculosis* infection, and is seen to give extra yield. In the study by Martineau, patients with the Taq polymorphism for VDR who were randomised to adjunctive vitamin D alongside the standard antituberculous regimen, had a reduced time to clearance of the mycobacterium (whereas those without the mutation had a non-significant reduction in primary endpoint) (Martineau *et al.*, 2011).

In additional to the clinical studies, *in vitro* studies have shown that adjunctive therapy with vitamin D, alongside other agents such as corticosteroids, is more effective at inducing

immunomodulation on DCs than using either treatment as monotherapy, and these tolerogenic DCs are successful at ameliorating severe combined immunodeficiency (SCID) colitis (Pedersen *et al.*, 2009; Naranjo-Gómez *et al.*, 2011).

5.2.3 The type of Vitamin D chosen

$1,25(OH)_2D3$ was chosen over $1,25(OH)_2D2$ as it has been shown to have a more reliable effect on raising serum 25(OH)vitamin D levels in humans (Kearns, Alvarez and Tangpricha, 2013).

5.2.4 The dose of 1,25(OH)2D3 chosen

Although it has been shown that vitamin D has immunomodulatory effects, it is not known what dose of vitamin D (if any) or what serum 25(OH) vitamin D level (if any) will lead to optimal beneficial immunomodulatory effects in humans with Crohn's disease. The dose selected was chosen on the basis that a single high dose of vitamin D3 (300,000 IU) has been shown to be effective at restoring serum 25(OH) vitamin D to optimal levels from baseline deficiency, whilst remaining safe (Kearns, Alvarez and Tangpricha, 2013). In a large meta-analysis, no significant clinical adverse events were reported with supplementation at this dose. Hypercalciuria was occasionally documented, but with no clinically significant sequelae. A further study of 39 healthy females assessed the pharmacokinetic properties of vitamin D single dose 150,000 IU versus daily 5000 IU. Although both doses were safe, and without any clinical adverse events, the daily dosage was more effective for improving the vitamin D level over the course of a month, suggesting that

150,000 IU as a single dose may not be quite enough to achieve long term repletion (Meekins *et al.*, 2014). Hence, 300,000IU was examined as the treatment dose in this study.

5.2.5 The concentration of vitamin D3 chosen for the *in vitro* study

The ideal concentration of vitamin D3 to use in the *in vitro* culture study is one that exerts maximal immunomodulatory effects, whilst having a low cellular toxicity. The concentration should be within physiological human serum concentration levels of vitamin D3 – there is little benefit in finding that the ideal concentration of vitamin D for DC modulation is actually toxic to humans. Prior to commencing dosimetry, it was important to have a feasible range of concentrations to work from. In their study of *in vitro* culture of vitamin D with human immunocytes, Yamanaka et al demonstrated that 100nM vit D3 is the optimal concentration to culture with (Yamanaka *et al.*, 2008), and so this was chosen together with a dosimetry range including 10nM and 1nM.

5.2.6 The route of administration and regimen of 1,25(OH)2D3

It was deemed logical to choose intramuscular administration in a group of patients with Crohn's disease, where malabsorption from the small bowel may be an issue. A single dose was chosen for several reasons. Firstly, adherence is guaranteed. Secondly, as mentioned above, meta-analysis has shown single dose 300,000 IU to be as effective as daily dosing for up to three months. Most importantly, this dose complies with London North West Healthcare NHS Trust (LNWHT) guidelines for correction of vitamin D deficiency in a high-risk group.

5.3 Hypothesis and Aims

5.3.1 Hypothesis

Correction of low vitamin D may be beneficial as a treatment adjunct (alongside infliximab) for Crohn's disease, via its modulation of the phenotype and function of dendritic cells.

5.3.2 Aims

- Study the *in vitro* effects of vitamin D upon DC homing marker expression.
- Study the *ex vivo* effects of infliximab upon DC phenotype, especially homing marker expression.
- Assess whether there is clinical benefit in the group receiving adjunctive vitamin D (as judged by clinical activity indices, CRP and faecal calprotectin).
- To determine whether high dose IM vitamin D, as a treatment adjunct, can induce a tolerogenic dendritic cell phenotype and function in active Crohn's disease above that of the standard treatment (infliximab) alone.

5.4 Methods

Chapter 3 presented the differences between phenotype of circulating DCs from healthy subjects and from subjects with Crohn's disease. Here, Chapter 5 presents the effect of different therapies (namely vitamin D and infliximab) upon the surface marker phenotype of circulating DCs.

We look first at the effects of vitamin D upon DCs in an *in vitro* culture study and subsequently the effects of infliximab, with or without adjunctive vitamin D, in an *ex vivo* study:

1. *In vitro* study – effects of vitamin D upon circulating DCs
2. *Ex vivo* study - the effect of infliximab and vitamin D upon DCs

5.4.1 *In vitro* vitamin D study

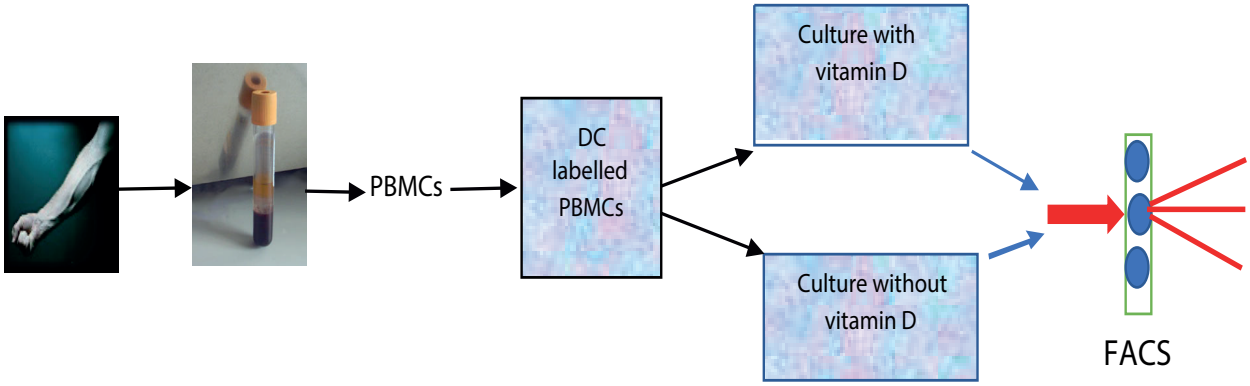


Figure 5.1: Brief diagrammatic experimental protocol. Culture of PBMCs, labelled to identify DCs, with and without vitamin D prior to flow cytometry.

5.4.1.1 Patient recruitment

Seven patients with a confirmed diagnosis of Crohn's disease were recruited to the *in vitro* arm of the study. The subjects were biologic naïve and on stable treatment for at least three months. Both active and inactive clinical disease status was accepted for the *in vitro* study (unlike the *ex vivo* study see 5.4.2.5).

5.4.1.2 Dosimetry

A range of three different vitamin D concentrations were utilized in the first four experiments to determine the optimal concentration in terms of achieving high immunological effect without causing cellular toxicity. The concentrations trialled were 100nM, 10nM, 1nM. As with the *ex vivo* study, $1,25(OH)_2$ Vitamin D3 was chosen because it has a more beneficial effect upon circulating vitamin D levels and whole-body vitamin D stores than vitamin D2 (Tripkovic *et al.*, 2012). The most suitable dose (100nM, see 5.51) was continued for the remaining *in vitro* experiments.

5.4.1.3 Laboratory methodology:

Venesection of 30mls of peripheral venous blood was performed, and isolation of PBMCs and cell counting was carried out, as per methods chapter (see 2.3).

A defined number of cells ($2x\ 10^6$) was pipetted into each of five tubes. Dosimetry experiments were performed. To create the control conditions complete medium was added to all tubes, whilst vitamin D was added in doses to achieve the three desired concentrations to all but the

negative and positive control tubes. Finally, *E coli* LPS was added to the positive control tube, as seen in table 5.1 below, to bring the volume to a standardised 2ml.

Group	**Cells (2x 10^6)**	**Media**	**Vitamin D**	**E coli**
Negative control	74.6µL	1.925ml	-	-
Vit D 100nM	74.6µL	1.825ml	100µl of 1000nM	-
Vit D 10nM	74.6µL	1.915ml	10µl of 1000nM	-
Vit D 1nM	74.6µL	1.915ml	1µl of 100nM	-
LPS - positive control	74.6µL	1.915ml	-	10µl

Table 5.1: Standardised conditions for cell culture with vitamin D.

Once the conditions were created, each of the five tubes was sub-divided into three further tubes and these fifteen tubes were cultured for pre-defined periods of time before antibody labelling. The first set of five tubes were immediately labelled with antibody (without any significant time to culture), whilst the next five tubes were cultured for 3 hours (in the incubator at 37℃) and the final five tubes were cultured for 18 hours (also at 37℃). In short, five conditions were created and each condition was examined at three time points.

Cells were labelled with the following antibodies: viability marker (LIVE-DEAD™), HLA-DR, DC lineage cocktail (CD3, CD14, CD16, CD19, CD34), DC subtype markers (CD11c, CD123), and the homing markers of interest (CLA, β7).

After the allotted period of culture, cells were stored in silver foil at 4°C until flow cytometry was performed (within 24 hours) with protocol as per the methods (see 2.3.6).

Compensation was performed using the fluorescence minus one (FMO) technique.

5.4.2 *Ex vivo* vitamin D study

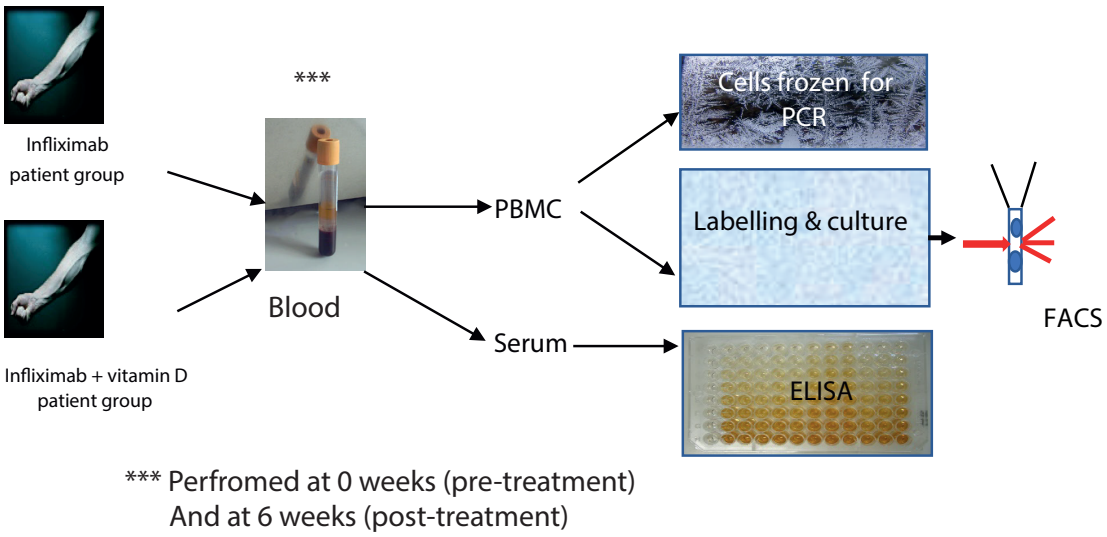


Figure 5.2: Simple diagrammatic representation of the methods used in the ex-vivo study in this chapter. Peripheral blood was taken from subjects immediately before therapy and 6 weeks after initiation of therapy. All subjects received infliximab, and half also received vitamin D. The whole blood was spun and PBMCs were labelled to identify DCs and relevant surface and intracellular markers, and serum and cells were frozen at –80 ℃ for ELISA and PCR respectively.

5.4.2.1 Patient inclusion and exclusion criteria

Inclusion criteria for subjects with Crohn's disease:

. Age 18-80

. Willing to provide their informed consent to join the study

. The subject must have a documented diagnosis of Crohn's disease, with diagnosis confirmed by histology

. Active luminal disease

. Stable immunomodulator therapy for 3 months (not being on immunomodulator for the prior 3 months also counted as stable therapy)

. The subject must be either deficient (<50nm/L) or have insufficient levels (<75 nm/L) of 25(OH) vitamin D

Exclusion criteria for subjects with Crohn's disease:

. Corticosteroid therapy within 3 months

. Vitamin D therapy within 6 months

. Biological therapy ever

. Local or systemic sepsis

. Co-existing inflammatory condition (e.g. tuberculosis, sarcoid, diverticulitis)

. Polyposis syndrome

. Current or any past history of cancer

5.4.2.2 Patient selection and recruitment for *ex-vivo* study

Recruited patients had an established diagnosis of Crohn's disease. All patients had active luminal disease (as evidenced by: raised disease activity indices, biochemical or stool inflammatory markers or typical radiological or endoscopic findings) and were awaiting escalation to infliximab therapy at the time of recruitment from the IBD outpatient clinic. Patients were currently or previously treated with an immunomodulator, stable for at least 3 months, but naïve to treatment with biological agents, and not treated with corticosteroids in the 6 months prior to the study.

The clinical and demographic data of the patients enrolled in the interventional study here is shown below in Table 5.2. It is worth noting that the patients who entered this study also entered into the healthy controls versus Crohn's disease studies in Chapters 3 and 4.

Subject ID	**Age (years)**	**Gender**	**Montreal Classification**	**HBI**	**Stoma**	**Current IM**	**Faecal Calprotectin (ug/g)**	**Vitamin D level (nmol/L)**
CD 3	31	M	A2L3B2	4	N	AZA	1100	50 (ns)
CD 4	19	F	A2L3B3p	n/a	Y	AZA	84.1	26 (s)
CD 5	28	M	A2L1B3	0	N	No	989	35 (s)
CD 6	34	F	A2L1B1p	0	N	AZA	5.3	68 (ns)

CD 7	47	F	A2L3B1p	n/a	Y	No	604	63 (ns)
CD 8	19	F	A2L1B1	0	N	AZA	439	35 (s)
CD 9	43	M	A2L3B2p	0	N	No	191	39 (s)
CD 10	24	M	A1L3B1	5	N	6MP	628	51 (ns)
CD 11	18	M	A1L3B1	1	N	6MP	2000	57 (ns)
CD 12	21	F	A3L3B3p	N/a	Y	AZA	10.9	35 (s)
CD 13	47	M	A2L3B2	1	N	AZA	187	57 (ns)
CD 14	50	M	A2L3B1p	3	N	AZA	202	55 (ns)
CD 15	49	F	A2L3B1	7	N	AZA	609	32 (s)
CD 16	19	F	A1L2B1	7	N	AZA	2000	25 (s)
CD 17	41	M	A2L3B1	3	N	AZA	-	51 (ns)

Table 5.2: Clinical characteristics of patients in therapeutic study. (s) denotes that the subject enters the vitamin D supplementation arm of the study, whilst (ns) denotes entry to the non-supplementation arm.

All of the recruited patients, in the table above, had evidence of disease activity as measured by raised faecal calprotectin (>50μg/g), with the exception of CD 6 and CD 12. CD 6 was recruited on the basis of mid-ileal Crohn's disease with luminal disease activity seen on MRI small bowel. CD12 had evidence of active disease on both ileocolonoscopy and MRI small bowel.

Detailed methodology follows, but in short, blood was taken from subjects and controls, and plasma was assessed by enzyme linked immunosorbent assay (ELISA) quantification, while fresh peripheral blood mononuclear cells (PBMC) were isolated for DC flow cytometry characterisation or DC enrichment and transmission electron microscopy (EM) analysis.

5.4.2.3 Study visits for subjects and healthy controls

Demographic data, disease characteristics (Montreal classification) and current treatment data were recorded in each case. Clinical effects and the changes in immune profile were studied, in particular dendritic cell phenotype and function before and after treatment.

5.4.2.4 Subject numbers and group characteristics

The purpose of the *ex-vivo* study was to assess whether vitamin D is an effective adjunct to standard escalation therapy (infliximab) for treatment of active Crohn's disease in promoting tolerogenic DCs. All patients were due to receive anti-TNF for the first time, for a flare of Crohn's disease. The subjects must not have received corticosteroids in the 3 months prior to recruitment, this is because steroids are known to impact upon DCs. As evidence of an active flare of disease the subject must have had one or more of the following: a raised HBI (≥6), a raised faecal calprotectin (>50), raised CRP (>5), raised ESR (>10), typical endoscopic findings or typical radiological findings.

5.4.2.5 Vitamin D supplementation – dosing by group

	Group 1 – Vitamin D Insufficient	Group 2 – Vitamin D Deficient
Vitamin D levels	**50-74**	**<50**
Intervention	**(Infliximab alone)**	**Parenteral vitamin D (+ infliximab**
Recruitment target	**8**	**8**

Table 5.3: Subject groups by vitamin D level and the therapy which they receive.

Supplementation depends upon the vitamin D status of the patient (Table 5.2). Subjects with vitamin D deficiency receive supplementation, whilst those with insufficient vitamin D levels do not, thus:

- 25(OH) vitamin D level < 50 nm/L (i.e. deficient) – 300,000 IU vitamin D3 supplement as a single IM bolus
- 25(OH) vitamin D level 50- 75 nm/L (i.e. insufficient) – no supplementation.

The HBI, CRP and calprotectin were recorded at baseline (week 0), week 12 and week 52. Serum calcium and 25(OH) vitamin D were collected at weeks 0 and 6. Additionally, urinary calcium was collected at week 6.

Study Visit		Procedures required at each visit						
	Written consent	Montreal classification	HBI	Blood (immune phenotyping)	CRP +/- Faecal calprotectin	Ca2+ and 25(OH) Vit D	Urinary calcium	Vitamin D dose
1 (week 0)	✓	✓	✓	✓	✓	✓	X	✓*
2 (week 6)	X	✓	✓	✓	✓	✓	✓	X

*Table 5.4: The procedures required for each recruited subject at each study visit. * only patients in the treatment arm received the vitamin D (patients in the control arm did not).*

5.4.2.6 Dendritic cell phenotype/function

Using the same technique as in Chapter 3, PBMCs were obtained from whole blood, and multi-colour flow cytometry was used to identify DCs. This was performed at weeks 0 and 6 (i.e. before and after infliximab treatment +/- vitamin D supplementation). Circulating DC expression of the following were again assessed: PRR status (Toll-like receptor expression TLR2 and TLR4), co-stimulatory molecule expression (CD40, CD 80, CD86), surface homing molecules expression on T cells (β7 (gut homing), CCR7 (lymph homing), CCR9 (small bowel homing) and CLA (skin homing)).

5.5 Results

5.5.1 *In vitro* experiments

5.5.1.1 Vitamin D (in vitro) and dendritic cell homing marker expression

Dosimetry – The decision to choose one dose/ concentration over another in immunology studies is not based on binary outcomes. The factors dictating which dose, from the range tested, should be continued for the rest of the *in vitro* experiments was based upon that which caused the most significant immunogenic changes, whilst demonstrating low toxicity. However, how 'immunogenic' a substance is can be open to interpretation, especially when many immune markers are under review. Generally, we found approximate levels of expression of the immune markers under study across the different vitamin D concentrations examined. As this was the case, 100nM was chosen as this had the lowest toxicity level as demonstrated by Live-Dead viability marker staining.

Culture with vitamin D (100nM $1,25(OH)_2$ vit D3) chosen as per dosimetry results, was compared to culture in the same conditions but without vitamin D, altered homing marker expression significantly across the study population.

5.5.1.2 Effect of time upon homing markers expression

As seen in the histograms below there is a natural drop off in homing receptor expression frequency with time even over a relatively short time period such as 18 hours, but enough stained cells remain to draw meaningful conclusions (figure 5.3). This loss of homing expression over time

is what makes Mo-DC (preparation of which involves >72 hours culture) unsuitable for examination of homing receptors, whilst the use of fresh PBMCs stained for DCs, as here, can bring novel data regarding homing marker expression.

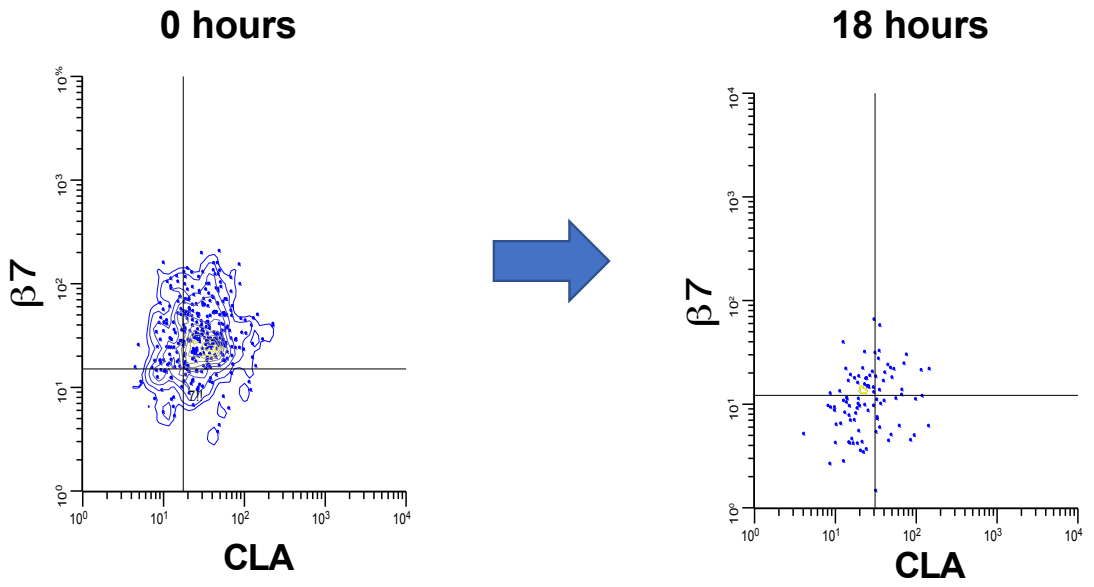


Figure 5.3: Histograms of pDC population stained for CLA (x-axis) and β7 (y-axis) at 0 hrs (left) and 18 hours (right) showing fewer cellular events at the latter time point.

5.5.1.3 In vitro vitamin D - Homing markers CLA and β7 expression on pDC

5.5.1.3.1 CLA on pDC

Frequency of expression of CLA staining on pDC was seen to be significantly down-regulated by culture with vitamin D on an individual and population basis (57.0% v 30.6%, p = 0.0104 control culture v vit D cultured, figures 5.4 and 5.5).

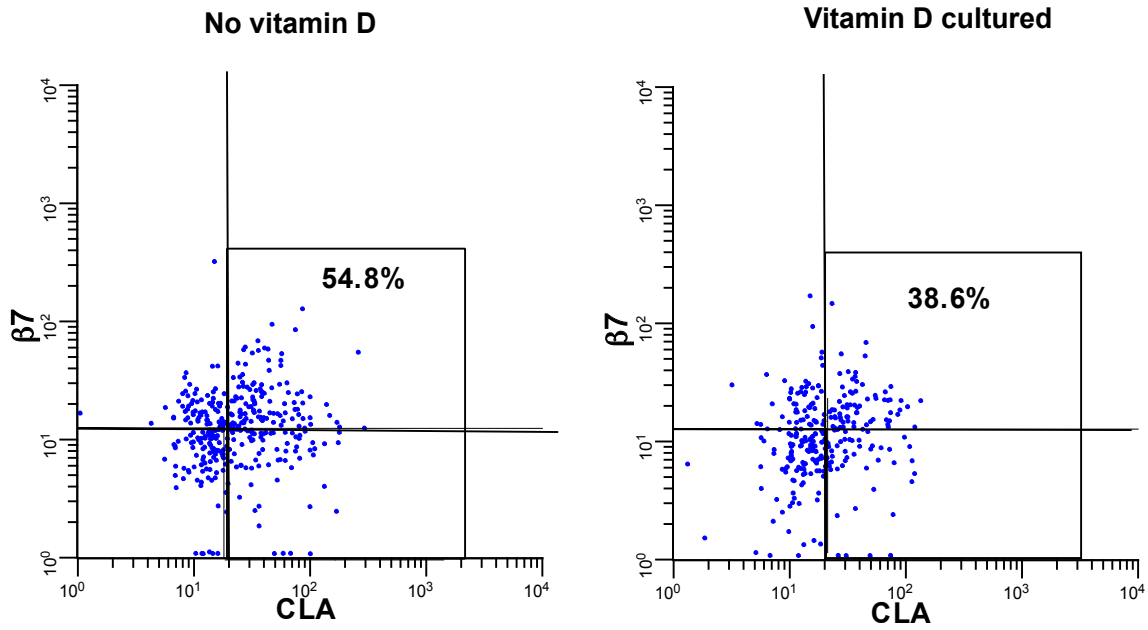


Figure 5.4: Results from a single subject - histograms of pDC stained for CLA (x-axis) and β7 (y-axis) and cultured for 18 hours in the presence (right) or absence (left) of vitamin D. Results shown in boxes are percentage of pDC expressing CLA.

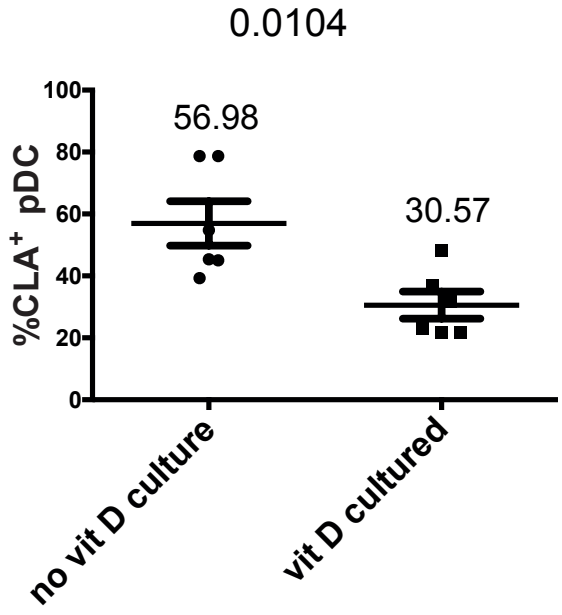


Figure 5.5: Cumulative results of in vitro studies demonstrating CLA expression on pDC after 18 hours' culture with or without 1,25(OH)$_2$ vitamin D3. N = 6 in both groups. Statistical analysis performed using unpaired t-test.

5.5.1.3.2 Gut homing marker (β7) on pDC

As with CLA, there was on-going expression of β7 despite 18 hours of culture (figure 5.6). However, culture with vitamin D did not alter the expression of CLA on pDC (32.9% v 30.0%; control cultured versus vitamin D cultured, figure 5.7).

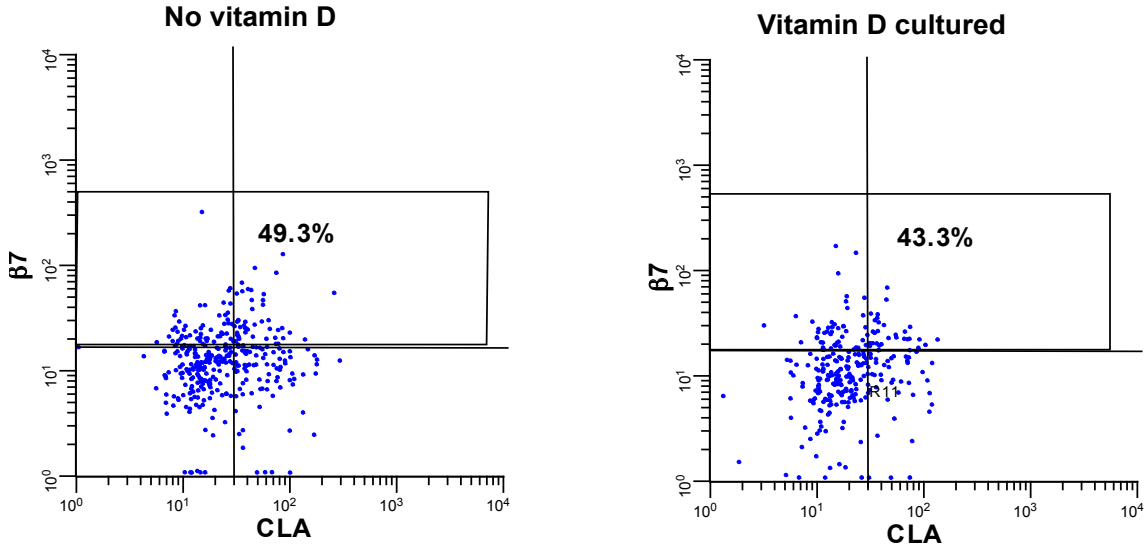


Figure 5.6: Histograms of pDC population stained for CLA (x-axis) and β7 (y-axis) and cultured for 18 hours in the presence (right) or absence (left) of vitamin D. Results shown in boxes are percentage of pDC expressing ß7.

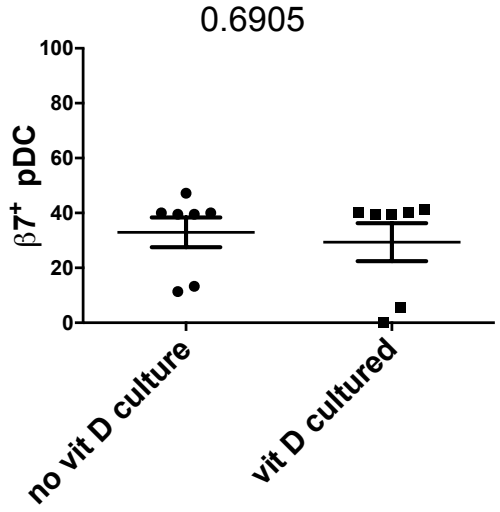


Figure 5.7: Cumulative results of in vitro studies demonstrating β7 expression on pDC after 18-hours' culture with or without 1,25(OH)₂ vitamin D3. N = 7 in both groups. Statistical analysis performed using unpaired t-test.

5.5.1.4 *In vitro* vitamin D - Homing markers CLA and β7 expression on mDC

Staining of both CLA and β7 following the 18-hour culture was too low (typically 1% and 6% respectively) to gain meaningful population data. This represents the natural loss of expression of CLA on mDC with time during culture, rather than any effect of therapy here. Example histograms are shown below (figures 5.8 and 5.9).

5.5.1.4.1 CLA on mDC

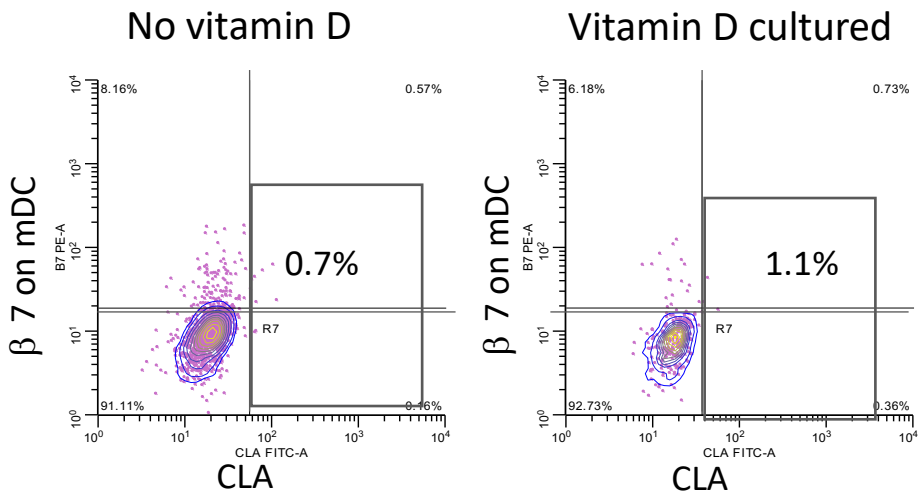


Figure 5.8: Histograms of mDC population stained for CLA (x-axis) and β7 (y-axis) and cultured for 18 hours in the presence (right) or absence (left) of vitamin D. Results shown in boxes are percentage of mDC expressing CLA.

5.5.1.4.2 β7 on mDC

In the *in vitro* studies, there was a significant fall-off in the number of mDCs expressing β7 following the period of culture, and this was not vitamin D dependent (figure 5.9).

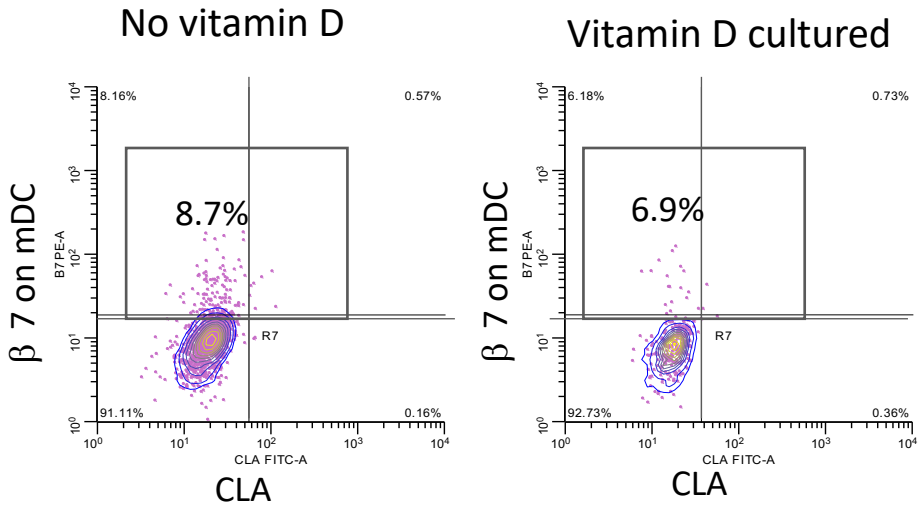


Figure 5.9: Histograms of mDC population stained for CLA (x-axis) and β7 (y-axis) and cultured for 18 hours in the presence (right) or absence (left) of vitamin D. Results shown in boxes are percentage of mDC expressing β7.

5.5.2 *Ex vivo* experiments

As per the protocol (see 5.4.2.5), subjects with active Crohn's disease who were due to start on anti-TNFα therapy were included into either the vitamin D treatment arm or the no vitamin D control arm. All patients were given anti-TNF at weeks 0,2,6 (as per standard clinical practice) and had samples and clinical indices taken at weeks 0,6 as the pre- and post- treatment study visits. Subjects in the vitamin D treatment arm received vitamin D supplementation alongside their anti-TNFα at week 0 only (Table 5.3 above). For the baseline clinical and demographic data of the patients recruited to this *ex-vivo* study see Table 5.2 above.

5.5.2.1 Clinical baseline characteristics between the two groups

There were no significant differences between the two study groups at baseline, when considering CRP, faecal calprotectin or HBI (figure 5.10).

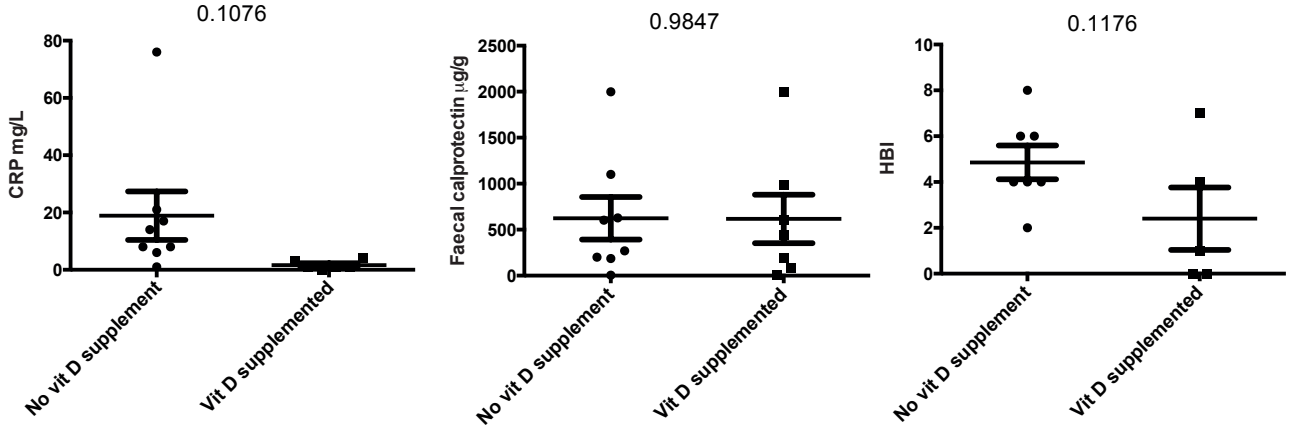


Figure 5.10: Graphs demonstrating baseline C-reactive protein (CRP), faecal calprotectin and Harvey-Bradshaw Index (HBI) for the non-supplemented and the supplemented groups. Statistical analysis performed was unpaired t-test.

There was a significant difference in baseline mean serum vitamin D levels between the groups (56.3nmol/L v 32.4nmol/L, p<0.0001, figure 5.11). This result was expected as the vitamin D levels dictated which group the subject entered.

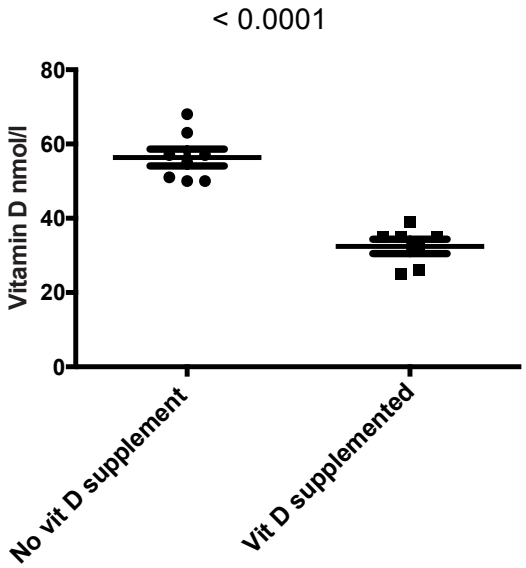


Figure 5.11: Graph demonstrating mean serum vitamin D level at baseline for the non-supplemented and the vitamin D supplemented groups. Statistical analysis performed using unpaired t-test.

5.5.2.2 Vitamin D levels post vitamin D therapy

Patients in the treatment arm were given a single IM dose of 300,000 IU vitamin D at 0 weeks, as per trust guidelines. In the vitamin D treatment group there was a statistically significant increase in serum vitamin D levels at week 6 compared to baseline levels (mean increase 30.86nmol/L, figure 5.12). There was no increase in the no-treatment arm (p=0.64). After treatment, the

vitamin D supplemented group were no longer deficient in vitamin D (mean 63.29nmol/L) and this figure was higher than that in the non-supplemented arm (54.63nmol/L, p = 0.2011, non-significant difference). As expected, the change in vitamin D levels was significantly greater in the group who received vitamin D than the control group (-2.00nmol/L v 30.86nmol/L. p = 0.0006, figure 5.13).

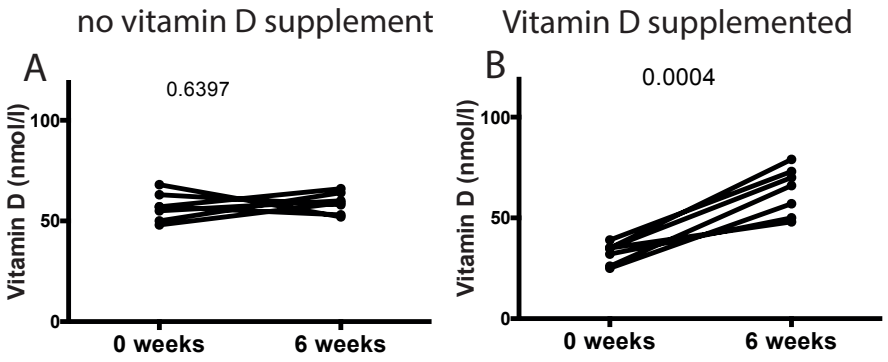


Figure 5.12: Line and dots graphs showing serum vitamin D concentrations at 0 & 6 weeks in (A) the non-supplemented control arm, and (B) the vitamin D supplemented arm. Statistical analysis performed using paired t-test.

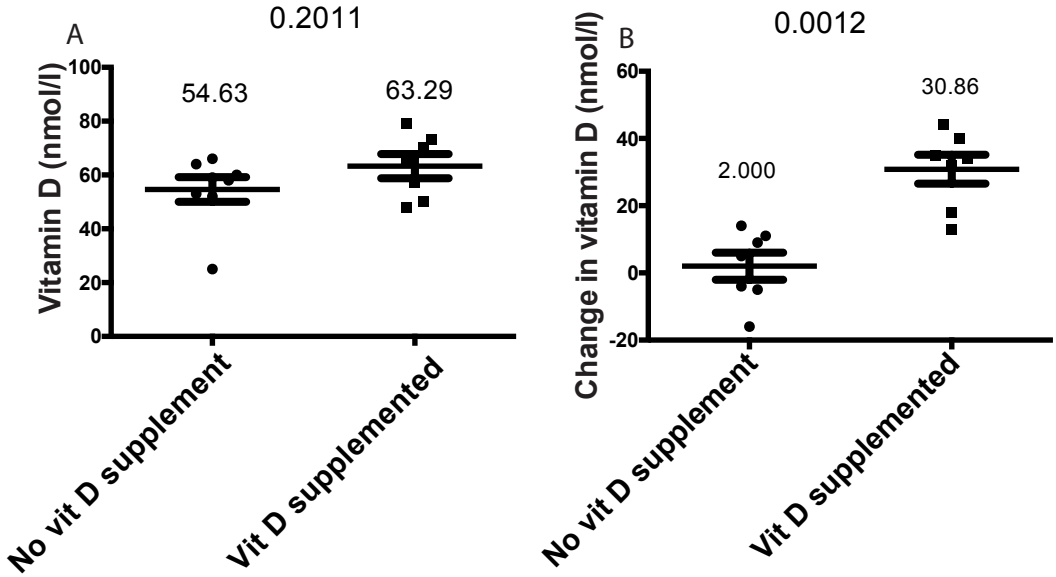


Figure 5.13: Scatter plots demonstrating (A) the week 6 serum vitamin D level in the non-supplemented v the supplemented groups, and (B) the difference in change of serum vitamin levels from 0 to 6 weeks between the control group and the vitamin D treatment group. Statistical analysis performed using unpaired t-test.

5.5.2.3 Vitamin D safety in our cohort

Patients were monitored for serum calcium concentrations and one or both of spot urine calcium concentration and calcium/creatinine ratio. Of the eight subjects who received vitamin D supplementation there were no adverse reactions and all subjects had serum calcium and urinary calcium investigations within normal range. The study was not powered for statistical analysis of safety events.

5.5.2.4 Clinical response to anti-TNFα therapy (+/- adjunctive vitamin D supplementation) in the study group

- N = 16
- 8 subjects were vitamin D supplemented
- 8 subjects were not supplemented
- 1 subject left trial before second visit (from the vitamin D supplemented arm - consent withdrawn, reason unspecified)
- 14/15 who completed trial deemed to be responders at 12 weeks (clinical decision) and continued to maintenance anti-TNFα therapy
- 1 subject discontinued anti-TNFα during induction phase (infusion reaction at 2nd dose)

5.5.2.5 Immune marker outcomes

Anti-TNFα treatment alters homing marker expression (in the whole study population – including both vitamin D supplemented and non-supplemented groups) in an *ex vivo* model

5.5.2.5.1 Gut and skin homing on mDC

Changes were seen in frequency of gut and skin homing marker expression on circulating mDC following anti-TNFα therapy. There was a 'normalisation' of the mDC phenotype at 6 weeks, following therapy. The abnormal gut homing specific phenotype seen in Crohn's subjects ($\beta7^+/CLA^-$ results described in Chapter 3, figure 3.1) reverted to that of a heathy control ($\beta7^+/CLA^+$, figure 5.14).

5.5.2.5.2 β7 (gut homing marker) on mDC is down-regulated by anti-TNF therapy

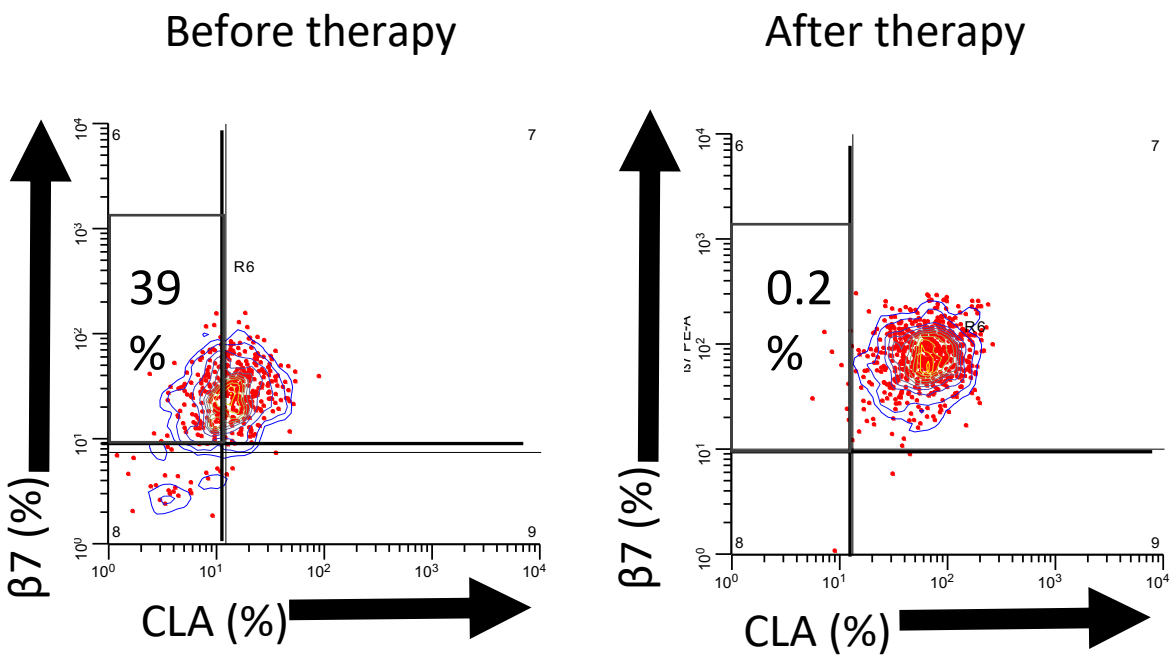


Figure 5.14: Histograms demonstrating CLA (x-axis) and β7 (y-axis) expression in mDC in a single subject before (left) and after (right) therapy. The figures in boxes are the percentage expression of gut homing phenotype ($CLA^-ß7^+$) by region gating.

There was a significant reduction in frequency of mDCs expressing gut specific homing phenotype ($CLA^{-}\beta7^{+}$) at 6 weeks in the whole study population (33.4% v 17.0%; before v after, p = 0.0081, Figure 5.15).

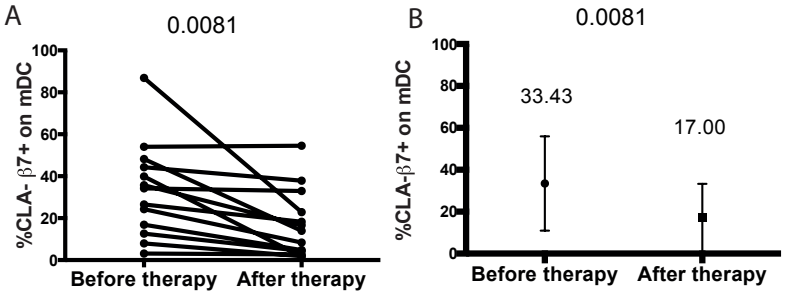


Figure 5.15: (A) Graph demonstrating before and after therapy values for gut homing phenotype $CLA^{-}\beta7^{+}$ expression on mDC. (B) Grouped data plot demonstrating the same data, and with median values shown. Statistical analysis performed using paired t-test.

5.5.2.5.3 CLA (skin homing marker) expression is increased on mDC by anti-TNF therapy

Whilst mDC expression of gut specific phenotype markers is reduced following therapy (figures 5.14 & 5.15), expression of skin homing marker (CLA) is increased (figure 5.16).

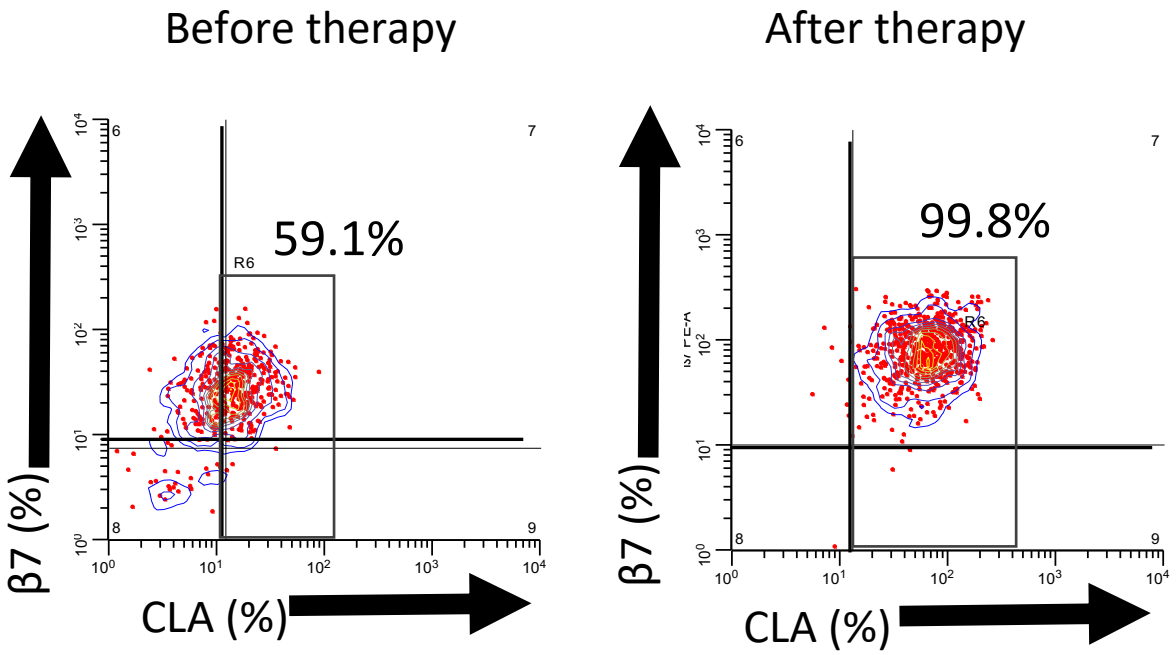


Figure 5.16: Histograms demonstrating CLA (x-axis) and ß7 (y-axis) expression on mDC in single subject before (left) and after (right) therapy. The % figures by the boxes are the percentage expression of CLA on mDC by region gating.

There was a statistically significant increase in whole study population frequency of mDC expression of CLA at 6-weeks post treatment as compared with week 0 pre-treatment (60.3% v 79.4%, 0 weeks v 6 weeks. P = 0.0037) (figure 5.17).

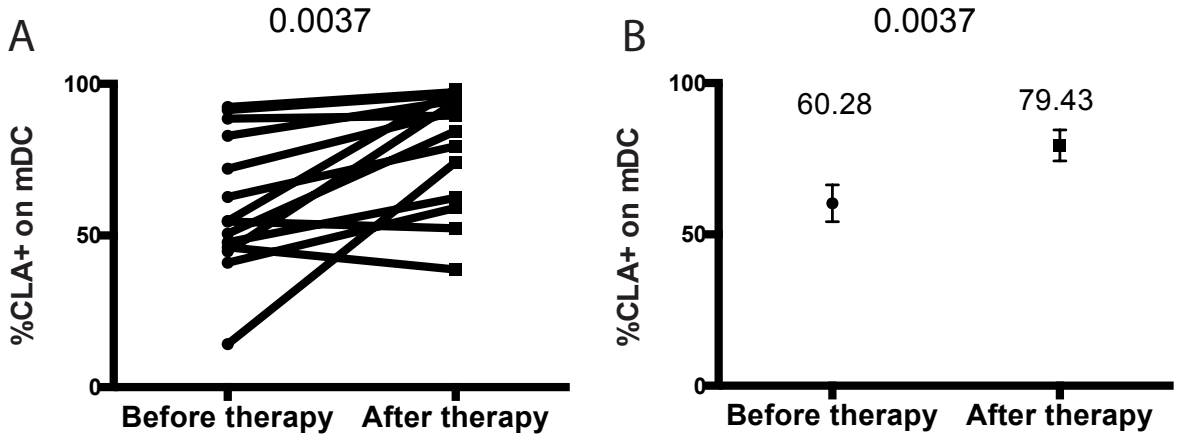


Figure 5.17: (A) Graph demonstrating before and after therapy values for frequency of skin homing phenotype (CLA) expression on mDC. (B) Grouped data column plot demonstrating the same data, and with median values shown. Statistical analysis performed using paired t-tests.

5.5.2.6 The effect of vitamin D supplementation upon immune marker outcomes

5.5.2.6.1 CLA^- $\beta7^+$ (gut homing phenotype) on mDC

The decrease in frequency of gut specific homing marker phenotype (CLA^- $ß7^+$) expression on mDC which occurs in the whole study population following anti-TNF therapy (fig 5.15) is 'blocked' by co-treatment with vitamin D, and only occurs in the anti-TNFα monotherapy arm (33.2% to 13.1%; 0 weeks v 6 weeks, p = 0.0164, fig 5.18 below). So, vitamin D blocks the skin homing phenotype that occurs with anti-TNF treatment.

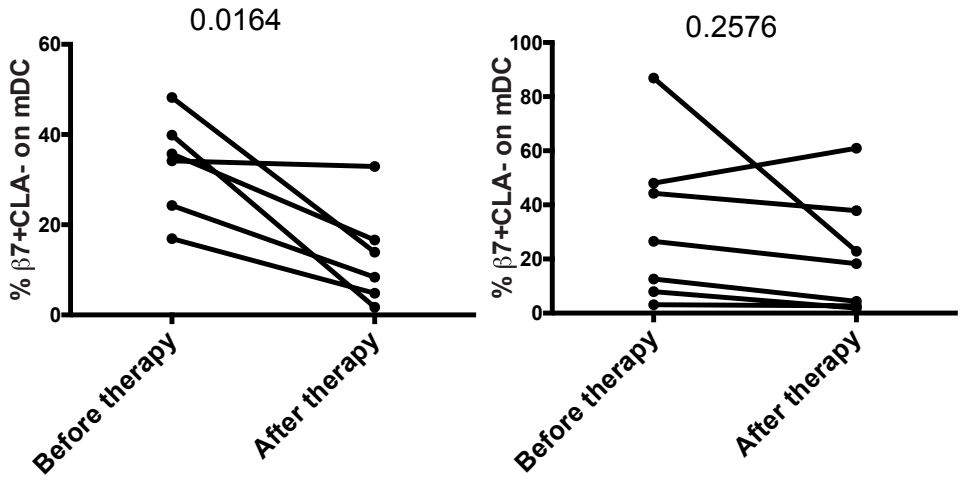


Figure 5.18: Graphs demonstrating before and after therapy values for gut specific homing phenotype ($CLA^-ß7^+$) expression on mDC in (A) the non-vitamin D treated group, and (B) the vitamin D supplemented group. Statistical analysis performed using paired t-test.

5.5.2.6.2 CLA (skin homing marker) on mDC

The increase in skin homing marker (CLA) expression on mDC which occurs in the whole study population (figure 5.22) is 'blocked' by co-treatment with vitamin D, and only occurs in the anti-TNFα monotherapy arm (61.8% to 85.0% 0 weeks v 6 weeks, p = 0.0062, figure 5.19).

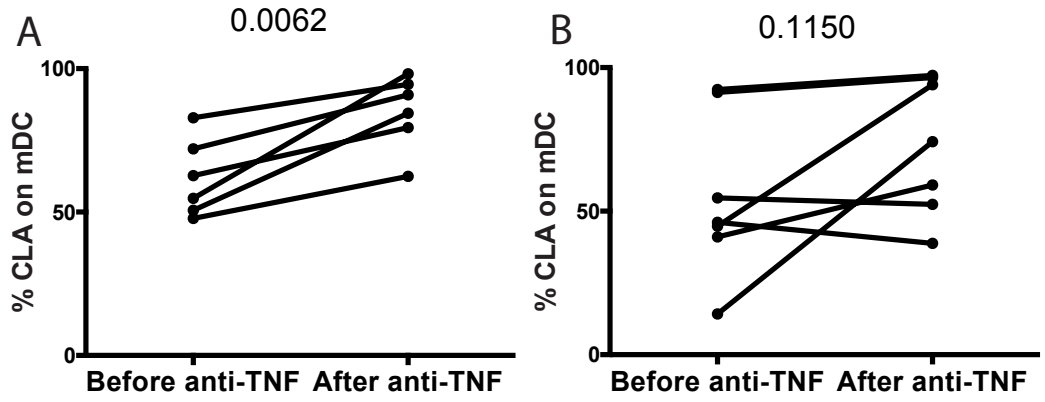


Figure 5.19: Column plots demonstrating before and after therapy values for skin homing phenotype (CLA) expression on mDC in (A) the non-vitamin D treated group, and (B) the vitamin D supplemented group. Statistical analysis performed using paired t-test.

5.5.2.6.3 $CLA^{-}\beta7^{+}$ (gut homing phenotype) on pDC

There was no change in gut specific homing marker phenotype ($CLA^{-}\beta7^{+}$) expression on pDC following anti-TNFα therapy in the whole study population (p = 0.8319, figure 5.20A). On sub-analysis of the impact of vitamin D, there was a trend toward decreased gut homing phenotype in the anti-TNF monotherapy group, but this did not reach statistical significance (11.4% v 5.3%; 0 weeks v 6 weeks. P = 0.0757, figure 5.20 B&C).

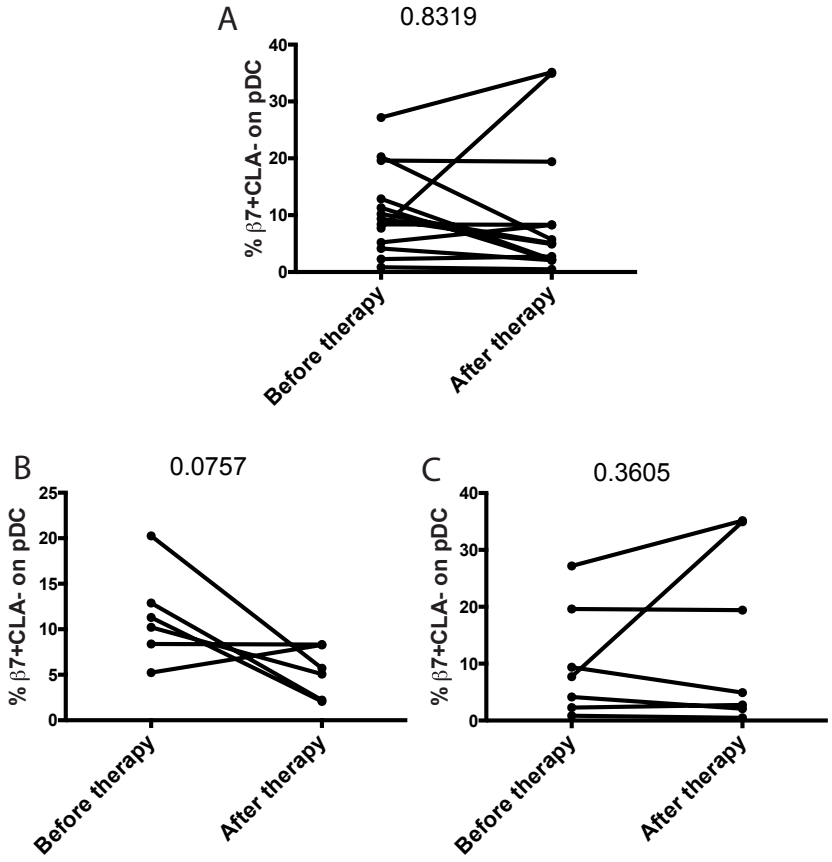


Figure 5.20: Column plots demonstrating before and after therapy values for gut specific homing phenotype ($CLA^{-}ß7^{+}$) expression on pDC in (A) the whole study population (B) the non-vitamin D supplemented control group and (C) the vitamin D treatment group. Statistical analysis performed using paired t-test.

5.5.2.6.4 CLA (skin homing marker) on pDC

There was no change in skin specific homing marker phenotype (CLA^+) expression on pDC following anti-TNFα therapy in the whole study population (p = 0.1791). On sub-analysis, supplemental vitamin D did not seem to have an impact on results (figure 5.21).

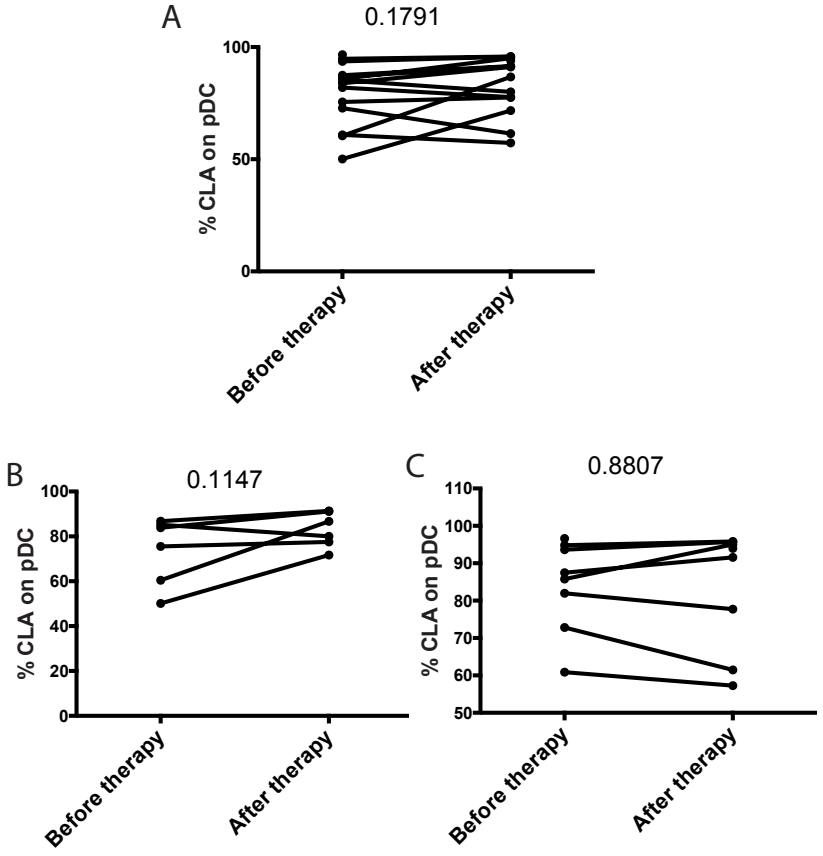


Figure 5.21: Column plots demonstrating before and after therapy values for skin homing marker (CLA) frequency of expression on pDC in (A) the whole study population (B) the non-vitamin D supplemented control group and (C) the vitamin D treatment group. Statistical analysis performed using paired t-test.

5.5.2.7 The effect of anti-TNF therapy upon DC expression of other homing markers - CCR7 and CCR9

5.5.2.7.1 CCR7 (lymphoid tissue marker) on mDC

As described in Chapter 3 (3.12), expression of CCR7 on mDC was globally low (figure 5.22), with no difference between HCs and Crohn's disease.

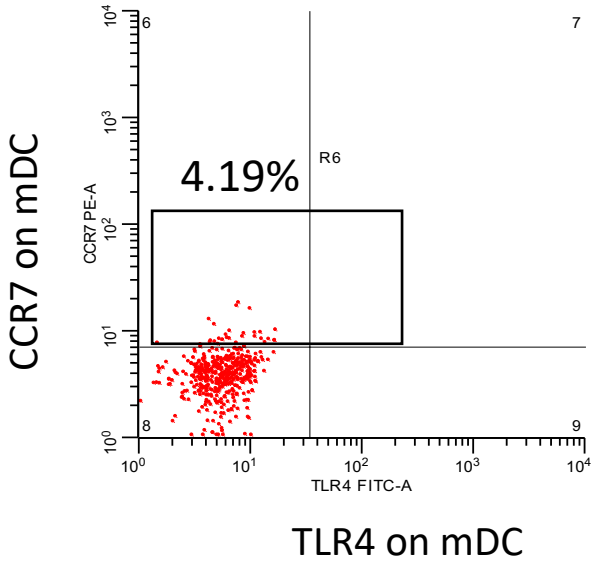


Figure 5.22: 2D histogram demonstrating mDC expression of TLR4 (x-axis) and CCR7 (y-axis) in a single subject. The % icon above the box shows the percentage expression of CCR7 on mDC by region gating.

In this *ex-vivo* study we find no significant difference in mDC CCR7 expression following anti-TNFα therapy, and no impact of vitamin D (figure 5.23).

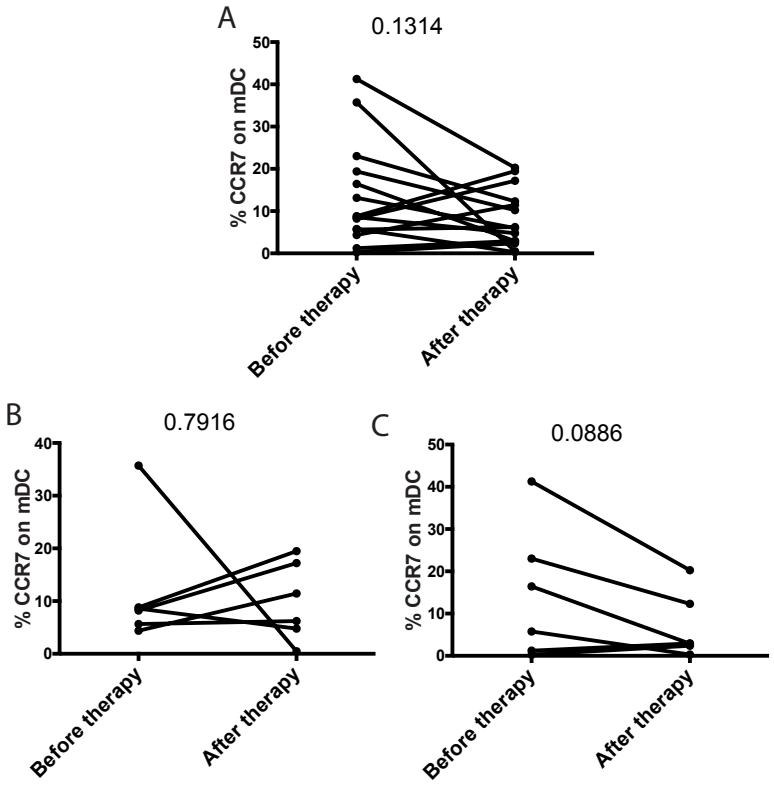


Figure 5.23: Column plots demonstrating before and after therapy values for lymphoid tissue homing marker (CCR7) expression on mDC in (A) the entire study population (B) the non-vitamin D supplemented control group and (C) the vitamin D treatment group. Statistical analysis performed using paired t-test.

5.5.2.7.2 CCR7 (lymphoid tissue marker) on pDC

There was no impact of anti-TNF on the whole group population pDC expression of CCR7, and no change with vitamin D augmentation (figure 5.24).

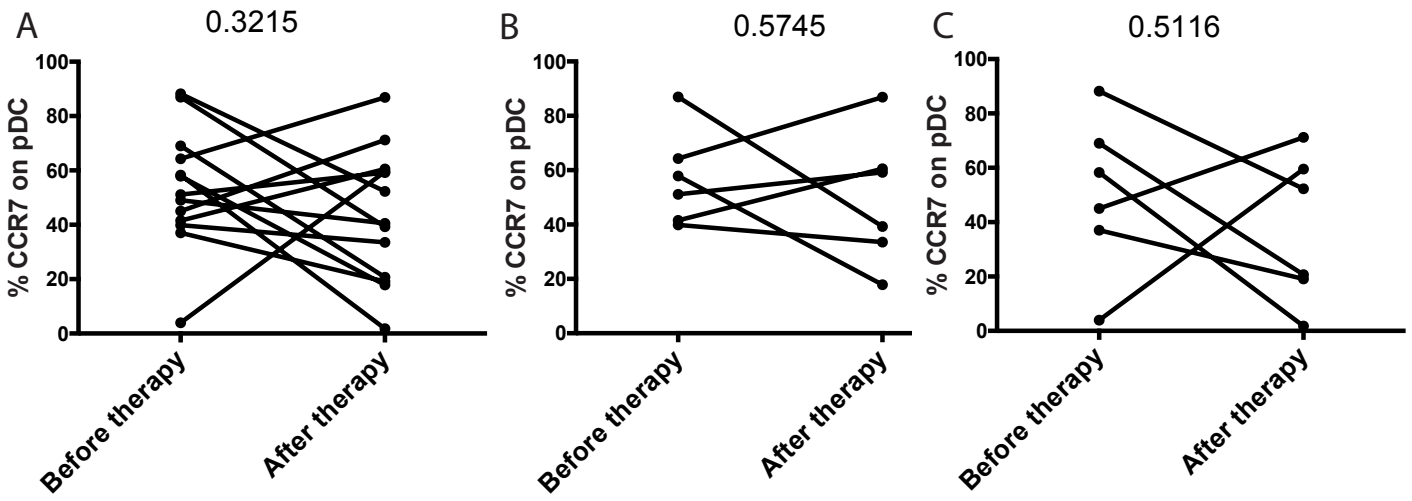


Figure 5.24: Column plots demonstrating before and after therapy values for lymphoid tissue homing marker (CCR7) expression on pDC in (A) the entire study population (B) the non-vitamin D supplemented control group and (C) the vitamin D treatment group. Statistical analysis performed using paired t-test.

5.5.2.7.3 CCR9 (gut/ small bowel homing marker) on mDC

As described in Chapter 3 (figure 3.12), expression of CCR9 on mDC was globally low across the study population (5.0% expression on mDC in Crohn's disease), with no difference between HCs and Crohn's disease (p = 0.16).

In this *ex-vivo* study we find low expression and no significant difference in mDC CCR9 expression following anti-TNFα therapy (5.5% v 4.1%, week 0 v week 6; p = 0.25), and no impact of vitamin D (figure 5.25).

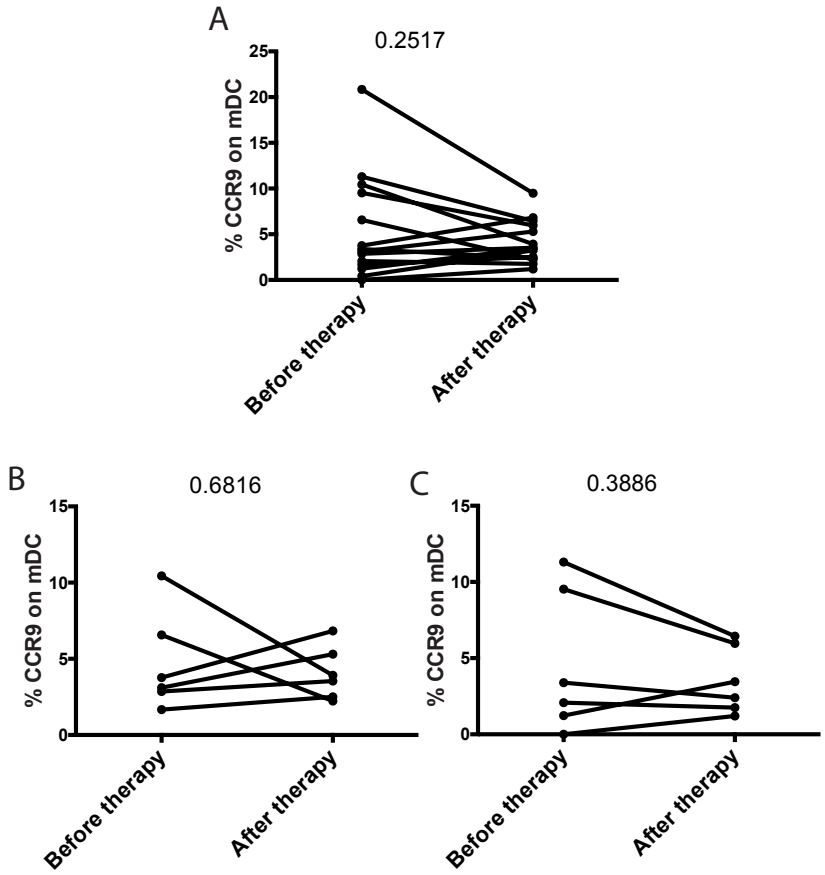


Figure 5.25: Column plots demonstrating before and after therapy values for gut/ small bowel homing marker (CCR9) expression on mDC in (A) the whole study population, (B) the non-vitamin D supplemented control group and (C) the vitamin D treatment group. Statistical analysis performed using paired t-test.

5.5.2.7.4 CCR9 (gut/ small bowel homing marker) on pDC

CCR9 frequency of expression did not alter significantly following treatment with anti-TNF therapy or vitamin D augmentation (figure 5.26).

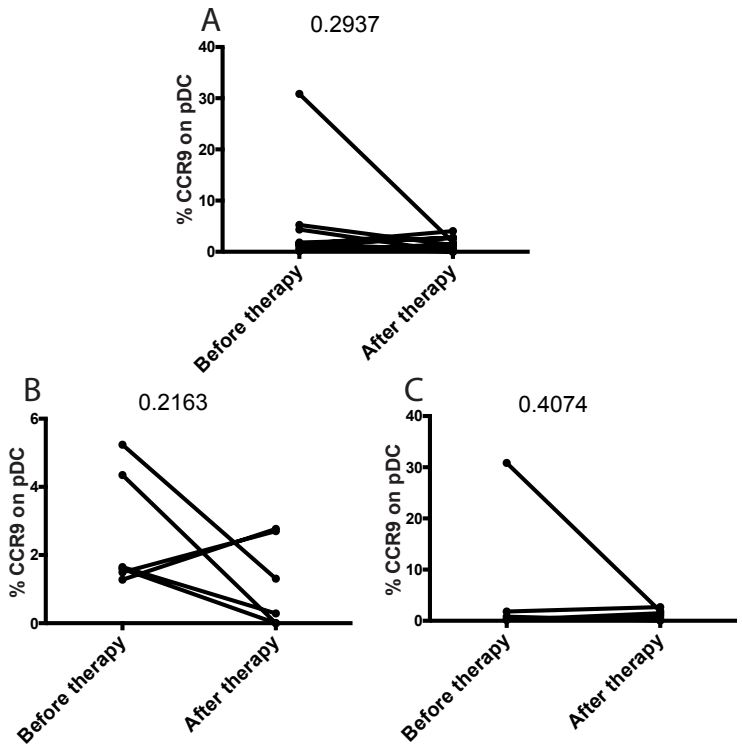


Figure 5.26: Column plots demonstrating before and after therapy values for gut/ small bowel homing marker (CCR9) expression on pDC in (A) the entire study population (B) the non-vitamin D supplemented control group and (C) the vitamin D treatment group. Statistical analysis performed using paired t-test.

5.5.2.8 Effect of therapy upon markers of function and maturity

5.5.2.8.1 TLR2 and TLR4

Neither anti-TNF therapy, nor adjunctive vitamin D, caused a change in expression of TLR2 or TLR4 on mDC (figure 5.27). The pDC graphs have not been shown because it is known that pDC do not express these particular PRRs.

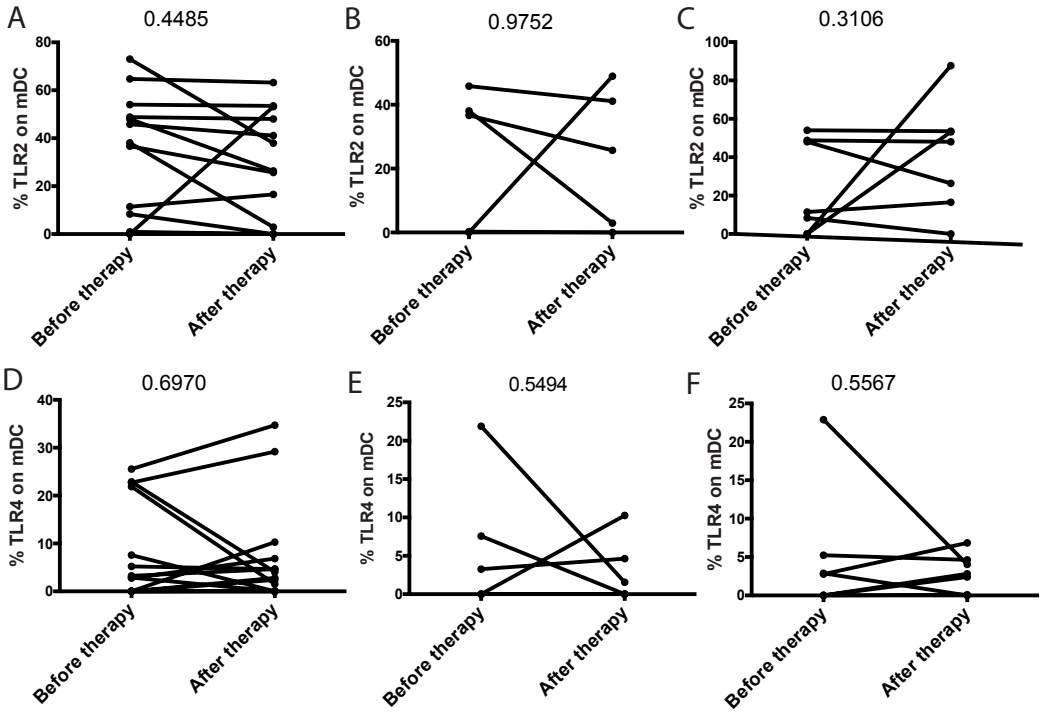


Fig 5.27: Column plots demonstrating before and after therapy values for Toll-like receptor (TLR) 2 (graphs A-C) and TLR 4 (graphs D-F) on mDC in (A&D) the entire study population (B&E) the non-vitamin D supplemented control group and (C&F) the vitamin D treatment group. Statistical analysis performed using paired t-test.

5.5.2.8.2 ILT3

ILT3 expression was globally high on both mDC and pDC and did not alter with anti-TNF therapy or vitamin D supplementation (figure 5.28).

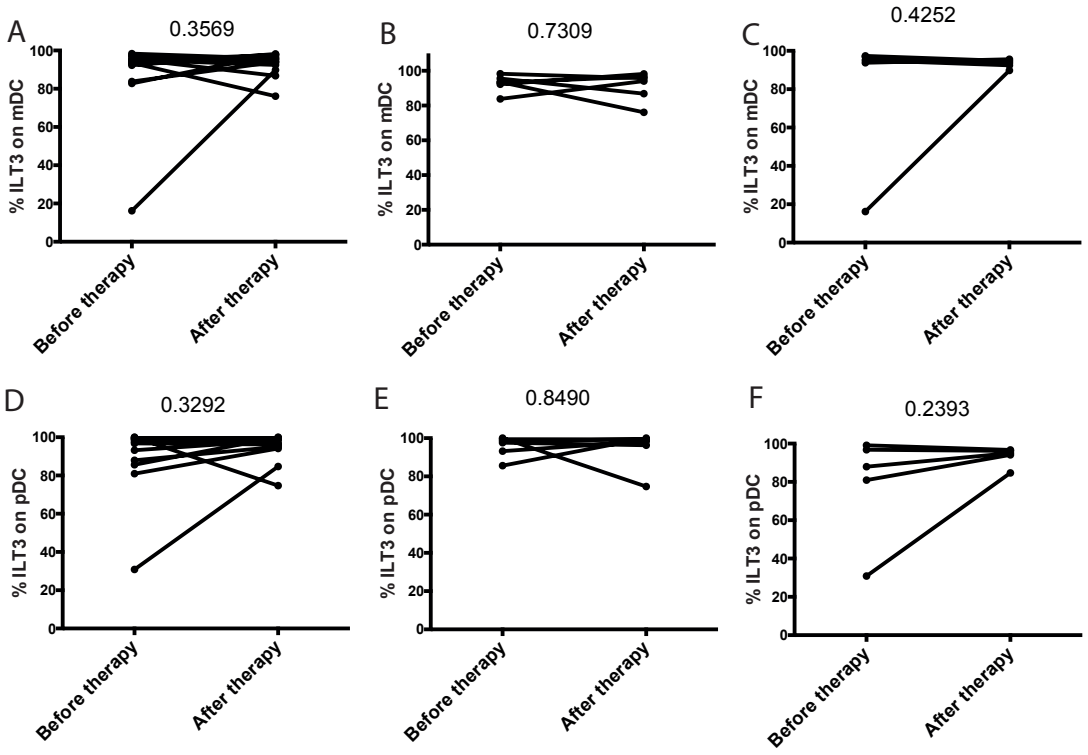


Figure 5.28: Column plots demonstrating before and after therapy expression of immunoglobulin like transcript 3 (ILT3) on mDC in (A) the whole study population, (B) the non-vitamin D supplemented control group, and (C) the vitamin D treatment group; and on pDC in (D) the whole study population, (E) the non-vitamin D supplemented control group, and (F) the vitamin D treatment group. Statistical analysis performed using paired t-test.

5.5.2.8.3 CD40, CD80 & CD86

There is negligible expression of the co-stimulatory molecules CD40, CD80 and CD86 on pDC (figure 3.21, chapter 3), and this does not alter with treatment with anti-TNF or vitamin D. Although there are recordable levels of these costimulatory molecule on mDC, there is no effect of anti-TNF therapy, or vitamin D augmentation (figure 5.29 below).

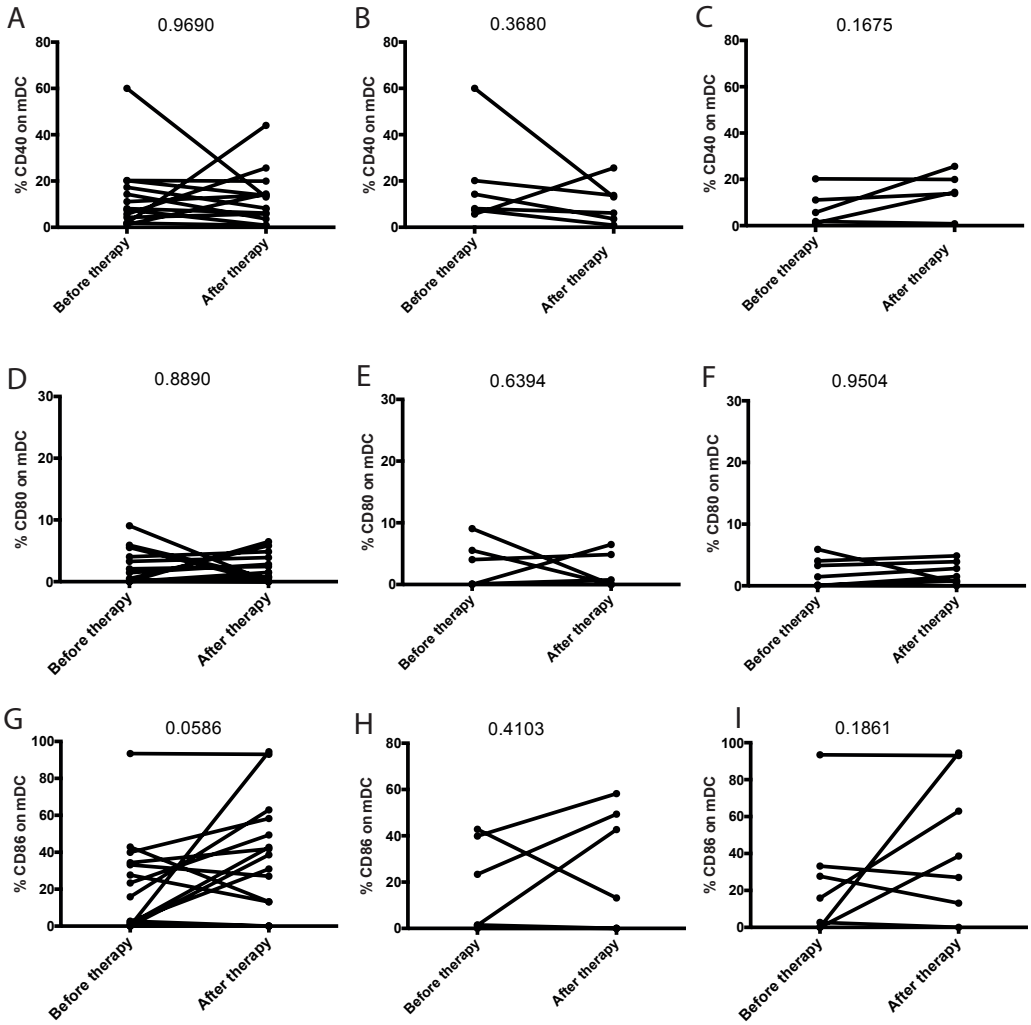


Figure 5.29: Column plots demonstrating before and after therapy mDC expression of costimulatory molecules. (A-C) demonstrate CD40 expression in whole study population, non-vitamin D supplemented control group, and the vitamin D treatment group, respectively. (D-F) demonstrate CD80 expression in whole study population, non-vitamin D supplemented control group, and the vitamin D treatment group, respectively. (G-I) demonstrate CD86 expression in whole study population, non-vitamin D supplemented control group, and the vitamin D treatment group, respectively. Statistical analysis performed using paired t-test.

5.5.3 Quantification of cellular populations

There were no significant changes in the fraction of DCs within the PBMC population following treatment to the whole cohort (i.e. Infliximab +/- vitamin D; figure 5.30).

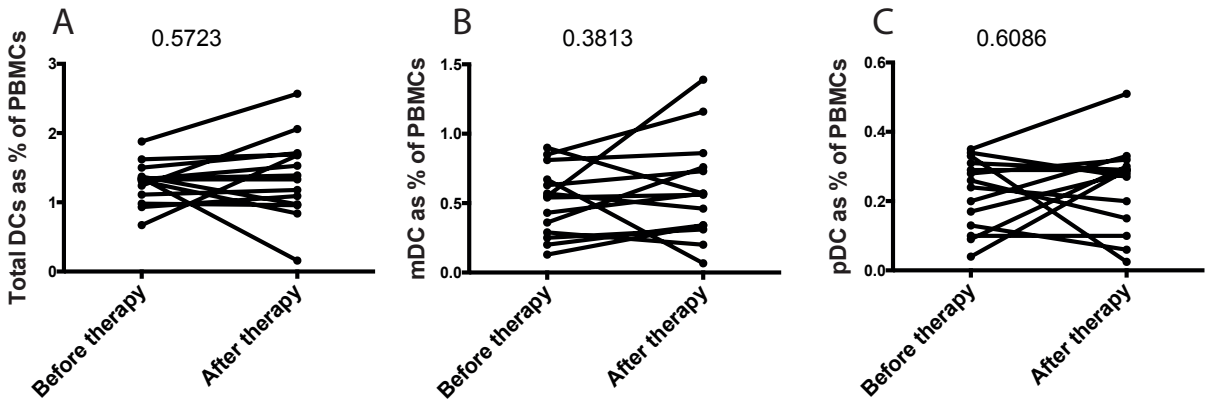


Fig 5.30: (A) total (B) myeloid and (C) plasmacytoid DCs as a percentage of the PBMC population in subjects with Crohn's disease before and after therapy.

On sub-analysis, there was no difference between the vitamin D treated group and the untreated control group within the total DC (A-C), mDC (D-F) and pDC (G-I) populations (figure 5.31).

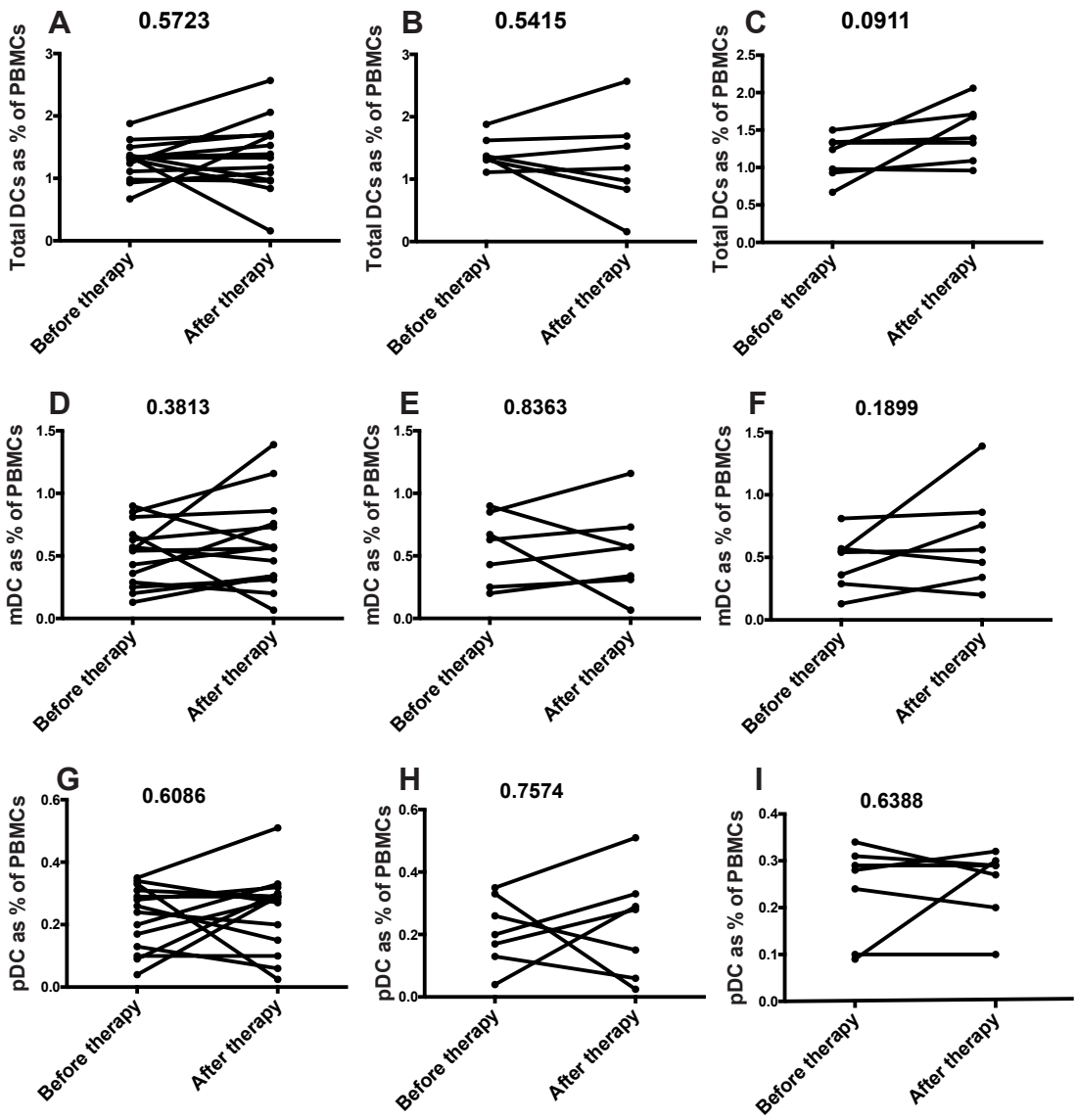


Figure 5.31: (A-C) Total DC population as a percentage of the PBMC population before and after therapy in (A) the whole cohort, (B) the non-vitamin D supplemented control group and (C) the vitamin D supplemented group. (D-F) mDC population as a percentage of the PBMC population before and after therapy in (D) the whole cohort, (E) the non-vitamin D supplemented control group and (E) the vitamin D supplemented group. (G-H) pDC population as a percentage of the PBMC population before and after therapy in (G) the whole cohort, (H) the non-vitamin D supplemented control group and (I) the vitamin D supplemented group.

5.5.4 RT-qPCR

The RT-qPCR experiments (IL-6, IL-10, TNFα) failed to yield valid, informative results. Putative reasons for this are discussed in 7.1.

Not all experiments has cells saved. This was due to insufficient cells left over after flow cytometric analysis. For all subjects where cells were saved, all samples were run as triplicates, as per MIQE guidelines (Minimum Information for Publication of Quantitative Real-time PCR Experiments) (Bustin *et al.*, 2009). Some of the data appeared skewed, with variation in values of the triplicates, so Grubb's test for outliers was used to assess for values which needed to be removed from analysis.

With outliers removed there were insufficient data points to analyse difference in the experiment group. Most samples had enough cells left after the first round of qPCR to perform repeat triplicate analysis. This was performed, with fresh, unopened media bottles used to perform the second round of experiments.

Again, there were discrepancies between replicates, and furthermore differences between values in round 1 and round 2 values for replicates from the same biological sample.

The unreliability of the triplicate data meant that no unequivocal conclusions could be drawn from differences in the biological samples.

Possible reasons for the failure of the PCR experiments are discussed in 7.1

5.6 Summary of results for Chapter 4

In the whole anti-TNFα treated study group:

- Faecal calprotectin levels fell following treatment with infliximab.
- The percentage of circulating mDC expressing skin homing maker (CLA) was upregulated following treatment.
- The percentage of circulating mDC expressing gut specific homing phenotype was reduced.

Vitamin D was shown to:

- Significantly increase the serum 25(OH)vitD3 levels from deficient range, by 30.86nMol/L. There was no change in vitamin D level in the non-supplemented group.
- Be safe when at a stat dose of 300,000IU intramuscularly (NB study not powered to demonstrate safety).
- Be associated with a reduction in faecal calprotectin of 294µg/g, compared to a reduction of 166µg/g in the non-supplemented group.
- Reduce the percentage of circulating DCs which express CLA, in the *in vitro* study.
- Impact upon homing marker status in anti-TNFα treated patients, in the *ex vivo* study:
  - By blocking the up-regulation of skin homing marker on mDC seen following anti-TNFα therapy.
  - By blocking the down-regulation of the gut specific homing mDC population following anti-TNFα therapy.

5.7 Discussion

5.7.1 DC homing marker expression with Infliximab

Skin complications of IBD are well described (occurring in up to one third of cases) (Pellicer *et al.*, 2012), and over the last couple of decades, skin complication of anti-TNFα therapy have also become increasingly recognised (Afzali *et al.*, 2014; Fréling *et al.*, 2015). In a large (n = 583), single centre, retrospective observational study of patients with IBD, Peyrin-Biroulet found that skin complications of anti-TNFα therapy occurs in one in five patients, with 10.1% of patients suffering from psoriaform reactions. Rate of development of psoriaform lesions was 1% at year 1. Risk factors for development of cutaneous sequelae of anti-TNFα therapy included young age at onset of IBD, and type of IBD (Crohn's rather than UC). Half of patients were able to remain on anti-TNFα therapy despite skin side-effects by using dermatological treatment, including topical therapies such as vitamin D derived lotions.

In this thesis it is shown in Chapter 3 that the homing marker profile on circulating DCs differs in Crohn's disease to healthy subjects. Here, in Chapter 5, we show that anti-TNFα therapy impacts significantly upon mDC expression of the skin homing marker CLA, and is associated with a significant upregulation of CLA expression. We postulate that this may be one mechanism by which anti-TNFα therapy is associated with a high burden of cutaneous complications, namely by switching trafficking of gut homing DCs towards the skin where they may propagate cutaneous lymphocyte deployment. Other theories to explain skin complications of anti-TNFα therapy include auto-immune reactions, and certainly the high rate of anti-nuclear antibody (ANA)

development (>75% after 3 infusions) would support that argument (Vermeire *et al.*, 2003). Given the complexity of the disease it is likely to be multifactorial.

Previous *in vitro* work performed by Peake et al (Peake, Bernardo, Mann, *et al.*, 2013b) has demonstrated that anti-TNFα therapy alters the homing phenotype marker expression of DCs towards a skin homing profile and away from a gut homing profile (increased CCR4, decreased $\beta7$). That the same occurs in this *ex vivo* study lends weight to this argument.

In addition to the changes in the CLA expression, there is a down-regulation in gut specific homing status of mDCs. In Chapter 3 it was shown that $CLA^{-}\beta7^{+}$ mDC are more common in Crohn's disease than healthy subjects. In this chapter, we show that the abnormal gut homing phenotype in Crohn's disease is reversed, with a decrease in the $CLA^{-}\beta7^{+}$ and an increase in the $CLA^{+}\beta7^{+}$ 'double-positive' non-tissue specific population. It would seem reasonable to postulate that circulating DCs with excessive gut homing marker expression might be one mechanism leading to the excess leucocyte trafficking to the gut seen in IBD. Taking this further, we hypothesise that a reversal of this increased gut homing capacity on mDC following anti-TNFα therapy might offer a partial explanation of the, as yet incompletely explained, mechanisms of action of anti-TNFα therapy in IBD.

It is interesting to note that vedolizumab (an $\alpha4\beta7$ anti-integrin molecule) is less effective at inducing remission in UC when the patients have previously been exposed to anti-TNFα therapy (Feagan *et al.*, 2013; William J. Sandborn *et al.*, 2013). There are several potential explanations for this, not least that this phenomenon has been seen before when other biological agents have been used sequentially, with adalimumab more efficacious in subjects not previously exposed to

infliximab therapy, and relate to a self-selected group with “difficult to treat” Crohn’s disease (Colombel *et al.*, 2007). Another plausible explanation for reduced vedolizumab treatment efficacy, considering the findings presented here, is that the therapeutic target for the anti-integrin has been reduced by anti-TNFα induced down-regulation of frequency of $\beta7$ expression.

5.7.2 Vitamin D

Vitamin D appeared to be safe and well tolerated in this study, although the experiment was not designed or powered to study adverse effects of the drug. In all patients given vitamin D, there were no symptoms related to hypercalcaemia, hypercalciuria and standard calcium monitoring investigations remained within normal range. Although vitamin D has been shown to have immunomodulatory properties previously, several questions have remained, including that of dosing. Here we show that a single 300,000IU dose of intramuscular vitamin D is effective at increasing serum 25(OH) vit D levels (by a mean of 30.1nmol/L), and at inducing immunomodulatory effects. Vitamin D is frequently prescribed in IBD, often alongside calcium as a bone protection agent for patients at risk of osteoporosis and osteomalacia, but it is often prescribed at unsuitably low doses. A typical prescription would be for one-two tablets of Calcichew D3 forte per day. This would deliver 400-800IU of 1,25$(OH)_2$vit D3 and 500mg-1000mg calcium (the lower figures being for one tablet daily, and the higher figures for two tablets daily)(BMJ Publishing Group Ltd and Royal Pharmaceutical Society, 2017). It is likely that this dose is too low to correct hypovitaminosis D, and it is unknown whether this dose will have any effect on the immune cells.

Vitamin D was seen in this study to influence homing marker expression by DCs in both the *in vitro* and *ex vivo* arms of the study. Frequency of expression of skin homing marker CLA on pDC was 52.6% in the control group versus 32.2% in the vitamin D treated tubes. In the *ex-vivo* study the increased frequency of expression of CLA on mDC that was seen with anti-TNF treatment ($p = 0.0062$) was blocked in those patients also treated with vitamin D ($p = 0.115$). This down-regulation of CLA expression on DCs may be of clinical interest. The ability to divert activated immune cells away from the skin when suffering an inflammatory skin pathology might, from a mechanistic viewpoint, reduce the cutaneous inflammatory burden. It is interesting that psoriaform lesions are the predominant cutaneous manifestation in IBD and following anti-TNFα therapy, and there are several similarities between these two autoimmune diseases. The main immune cells involved in psoriasis are the T helper cells, Th1/Th17, which produce pro-inflammatory cytokines including TNFα and IL-23. Successful treatments for both of these cytokine targets (anti-TNF and anti-IL-23 drugs of which several of each exist) are used in the fields of IBD and psoriasis. It has long been known that there is an increase in epidermal and dermal immunocytes in psoriasis, but only in the last decade or so have DCs been implicated in disease pathogenesis. In a study by Glitzner et al, activated pDCs were found in the skin of patients with psoriasis and were necessary for induction of psoriatic lesions in a murine model (psoriasis was significantly ameliorated in pDC depleted mice) (Glitzner *et al.*, 2014).

Calcipotriol is a synthetic derivative of calcitriol ($1,25(OH)_2$vitamin D3) that is widely used as a topical agent to treat psoriasis. The mechanism of action of calcipotriol is incompletely understood (Scott, Dunn and Goa, 2001). There is some evidence that it may have an effect on cutaneous DCs. Suzuki et al demonstrated that this topical vitamin D3 analogue caused decreased

pDC cytokine production in an *in vitro* murine study, and furthermore, showed reduced pDC numbers in the skin of live mice with imiquimod-induced skin inflammation(Suzuki *et al.*, 2016). There are few data on how vitamin D might down-regulate DC infiltration in the skin, but down-regulation of CLA has been studied by Yamanaka et al(Yamanaka *et al.*, 2008, 2010). In a human study, *ex-vivo* T-cells were cultured with different strengths of vitamin D3, and 100nM was seen to completely block expression of: CLA; CLA's major carrier molecule - P-selectin glycoprotein related ligand 1(PSGL-1); and e-selectin, the ligand for CLA.

5.8 Discussion summary points – Chapter 5

- Following *ex vivo* treatment with infliximab, the percentage of circulating mDC expressing gut specific homing phenotype is reduced. We postulate that this may be one mechanism of action of this class of drugs – diverting immunocytes away from the gut.
- The percentage of circulating mDC expressing skin homing maker (CLA) is upregulated following treatment with anti-TNF. This may be one explanation for the high rate of cutaneous side effects of anti-TNF therapy.
- A single intramuscular dose of 300,000IU $1,25(OH)_2$VitD3 was well tolerated and successful at raising serum levels in this cohort, and was associated with immune effects.
- Vitamin D was associated with down-regulation of CLA expression on DCs in the *in vitro* study and similarly blocked the upregulation of CLA caused by anti-TNF treatment in the *ex vivo* study. This down-regulation of DC CLA expression by vitamin D might have clinical uses at ameliorating the cutaneous inflammation associated with anti-TNF therapy.

Chapter 6 - The effect of infliximab and adjunctive vitamin D on circulating DC function (on-going intracellular cytokine production)

6.1 Abstract

Introduction

We have seen in the previous chapters that circulating dendritic cells have abnormal phenotype (Chapter 3) and function (Chapter 4) in Crohn's disease, and that this abnormal phenotype can be modulated towards normal by different treatments (Chapter 5). In this chapter, we explore whether DC function can be modulated by treatment.

Methods

Patients with active Crohn's disease were recruited as per the methods in Chapter 5 and all subjects were given anti-TNF therapy, with half also receiving co-treatment with vitamin D based upon their baseline vitamin D levels. PBMCs were isolated and on-going DC cytokine production during 4 hours' culture was assessed. ELISA was performed upon serum from the same subjects to assess serum cytokine levels before and after treatment.

Results

Production of TNFα by myeloid DCs was significantly reduced in the whole study population receiving anti-TNF therapy ($p = 0.032$); and there was a significant fall in the subgroup who

received co-treatment with vitamin D ($p = 0.016$); whilst in the subgroup without vitamin D treatment, TNFα production by myeloid DC did not decrease significantly ($p = 0.96$). There was a significant negative correlation between change in vitamin D level and change in TNFα production by myeloid DCs ($p = 0.025$, correlation coefficient = -0.65). An increase of serum 25(OH)vit D3 greater than 20 nmol/L was associated with a decrease in myeloid DC TNFα production. ELISA testing confirmed that reductions in circulating DC production of inflammatory cytokines were mirrored by similar changes in serum cytokine concentrations.

Conclusions

Infliximab down-regulation of circulating DC TNFα and IL-6 production may be one of the mechanisms of action of this drug. High doses of parenteral vitamin D given as co-treatment to patients with Crohn's disease promotes down-regulation of circulating myeloid DC production of TNFα which may influence the subsequent interaction of DCs and T cells. TNFα promotes a TH-1/ TH-17 response characteristic of Crohn's inflammation; thus, the ability of vitamin D to further block TNFα production may promote a more regulatory T cell response and improve outcomes when used as an adjunct to anti-TNFα therapy.

6.2 Introduction

Having demonstrated abnormal function of circulating DCs (increased production of pro-inflammatory cytokines) in Chapter 4, and shown that treatment (with anti-TNF +/- vitamin D) can modulate the phenotype of this abnormal population (Chapter 5), here we sought to investigate the effect of these therapies upon DC function.

Cytokines, their interaction with (and production by) DCs and their importance in IBD were discussed in some detail in the Introduction chapter (1.2.7).

There is precedent for studying the function of DCs with initiation of different treatments. *In vitro* culture with probiotics results in "tolerogenic" DCs with upregulated IL-10 and down-regulated IL-12 production (Hart *et al.*, 2004). Culture of low density cells (LDCs), containing high numbers of DCs, with infliximab has been studied previously, with levels of TNFα and IL-2 (measured in cell free culture supernatant, from LDC culture) both seen to be reduced following culture with infliximab (Peake ST, unpublished work). An *ex vivo* study examined the effects of infliximab upon circulating DCs, but this was in subjects with rheumatoid arthritis, and phenotype only (not function) was studied. It demonstrated that infliximab inhibited DC maturation and down-regulated co-stimulatory marker expression (Balanescu *et al.*, 2005).

The culture of DCs with vitamin D has also been studied previously, in a MO-DC *in vitro* experiment; again, the function of the Mo-DCs was altered towards tolerogenicity with increased IL-10 production and impaired IL-12 secretion. In addition, the vitamin D treated DCs had a reduced capacity to activate T cells (Lee *et al.*, 2016). The effect of vitamin D upon fresh DCs labelled within the PBMC population has been studied previously, and a naïve phenotype of DC

was seen, with reduced expression of co-stimulatory molecules, but in that study cytokine production was not evaluated (Milestone A, unpublished work).

The only *ex vivo* study of the effect of vitamin D supplementation on DCs, by Bartels et al, showed reduced Mo-DC production of IL-6, IL-10 and IL-1β, interesting because of the fact that there was a down-regulation of both pro- and anti- inflammatory cytokines (Lars E Bartels *et al.*, 2014b). The Bartels study was performed using oral supplementation rather than intramuscular, Mo-DC rather than fresh DCs, and assessed changes after LPS stimulation (rather than unstimulated). The novel features in our study here are the use of IM supplementation, the study in fresh PBMCs, without the confounding factors of the culture process required to produce Mo-DCs, and the study of unstimulated, immature DC function.

6.3 Hypothesis and Aims

6.3.1 Hypothesis

We hypothesised that anti-TNF treatment would reduce the production of pro-inflammatory cytokines by circulating DCs in Crohn's disease, and that the effect would be augmented by co-treatment with vitamin D.

6.3.2 Aims

- To assess the effects of anti-TNF treatment upon the function (on-going intracellular cytokine production) of circulating DCs in Crohn's disease.
- To assess the effects of adjuvant treatment with vitamin D (alongside anti-TNF treatment) upon the function (on-going intracellular cytokine production) of circulating DCs in Crohn's disease.
- To assess whether change in vitamin D concentration correlates with any decrease in pro-inflammatory cytokines (if witnessed).
- To characterise the serum cytokine profile (using ELISA), in subjects before and after treatment, and compare with the cytokine profile produced by circulating DCs.

6.4 Methods

Whilst Chapter 5 presented the effects of two different therapies (anti-TNF +/- adjunctive vitamin D) upon the phenotype of circulating DCs, this current chapter presents the effect of the same therapies upon the function of circulating DCs.

Patient recruitment and the timeline of study visits is covered in Chapter 5 (5.4), see Table 5.2 for baseline clinical and demographic data of recruited subjects. Detailed experimental methodology is covered in Chapter 2 (the Methods Chapter). A brief precis of the study methods follows:

Using the same technique as in previous chapters, PBMCs were obtained from whole blood, and multi-colour flow cytometry was used to identify DCs and DC subtypes. This was performed at weeks 0 and 6 (i.e. before and after initiation of infliximab treatment +/- vitamin D supplementation). Circulating DC expression of the following were assessed: ongoing intracellular cytokine production (IL-6, IL-10, IL-12, IL-23, IL-1β, TNF-α, IFN-α, TGF-β).

In chapter 4, ELISA was performed on thawed stored serum samples to compare serum cytokine concentrations from HCs with subjects with Crohn's disease. As expected, there were differences, with higher levels of pro-inflammatory serum cytokines in Crohn's than in HCs. In this current chapter, ELISA was also performed using the same methods described in Chapter 2, to compare pre- and post- therapy cytokine levels. Again, TNFα and IL-6 were measured.

6.4.1 Statistical analysis

Paired t-test was performed, with values <0.05 considered significant. For the correlation studies, Pearson Correlation Co-efficient was used.

6.5 Results

Abnormal and excessive on-going production of the pro-inflammatory cytokines TNFα and IL-6 by DCs (mDC and pDC respectively) was identified in subjects with Crohn's disease (see Chapter 4.5). In this current chapter, we explore whether the excessive production of cytokines can be reversed by the *ex vivo* effect of anti-TNFα therapy given intravenously to the patient as standard escalation therapy to treat their active Crohn's disease; and furthermore what adjunctive effect, if any, co-treatment with vitamin D provides. All results here are unstimulated (basal) cytokine secretion levels.

6.5.1 Effect of anti-TNF therapy on mDC TNFα production, and effect of vitamin D co-administration

TNFα expression (figure 6.1) by mDC measured over 4 hours was seen to fall across the study population at 6 weeks (post-treatment with anti-TNFα).

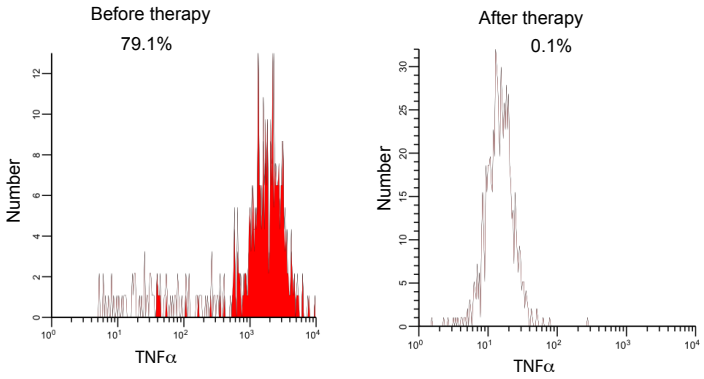


Figure 6.1: Histograms demonstrating the expression of TNFα on mDC in a subject with Crohn's disease before (left) and after (right) anti-TNFα therapy. Histograms presented are following SED_{MAX} subtraction of the isotype control (see fig 4.1 for subtraction histograms).

The reduction (56.8% v 32.0% week 0 v week 6, p = 0.033), although statistically significant, was not seen in all subjects (figure 6.2). On sub-analysis, it was seen that vitamin D co-administration had an impact on TNFα production, which significantly reduced in the vitamin D group (p = 0.016), but not the control group (p = 0.96).

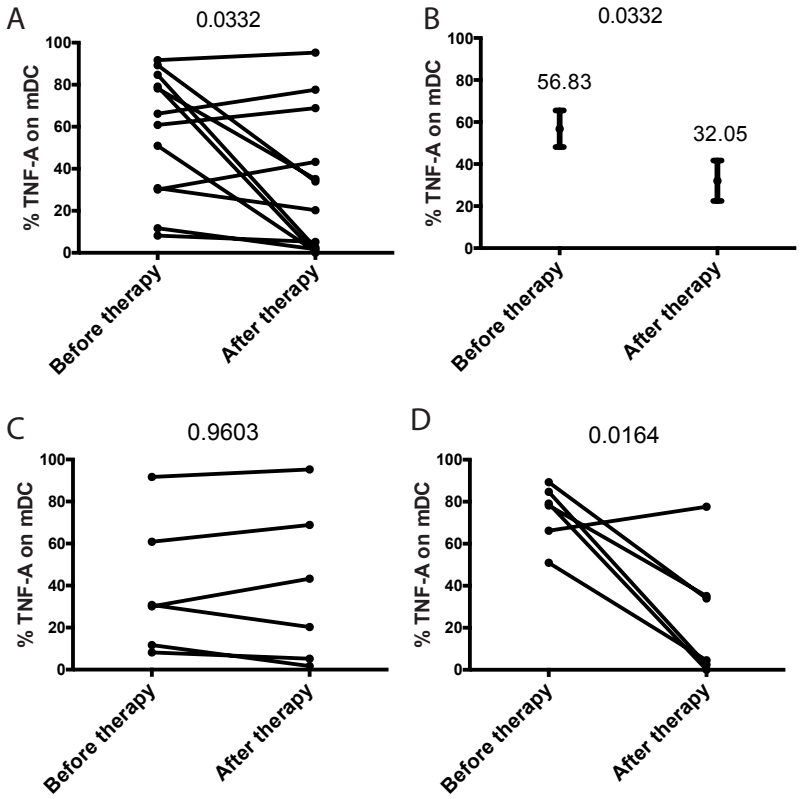


Figure 6.2: Column plots demonstrating before (week 0) and after (week 6) therapy values for TNFα expression by mDC measured over 4 hours in (A) the entire study population, (B) the entire study population shown again, but as grouped data with mean values, (C) the non-vitamin D supplemented control group and (D) the vitamin D treatment group. Statistical analysis performed using paired t-test.

6.5.2 Correlation of change in vitamin D level with reduction in on-going TNFα production by mDC

There was a statistically significant negative correlation between the change (increase) in vitamin D level between week 0 and week 6 and the change (decrease) in on-going TNFα production by mDC (Pearson's correlation coefficient -0.65, p = 0.026; figure 6.3 below).

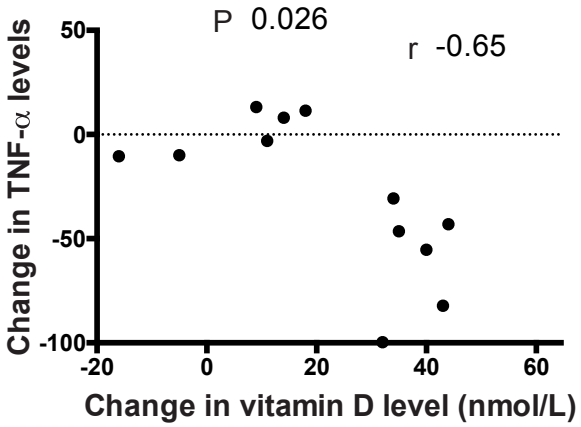


Figure 6.3: Graph plotting correlation of change in vitamin D level (nmol/L, x-axis) and change in TNFα staining on mDC between weeks 0 and 6. Statistical analysis performed using Pearson's correlation coefficient.

6.5.3 Effect of anti-TNF therapy on pDC TNFα production, and effect of vitamin D co-administration

Across the whole study group there was a trend toward reduction in TNFα production by circulating pDC following therapy (p = 0.0523; figure 6.4). On analysis of the subgroups, however, there was no significant reduction in either group. Perhaps with greater numbers in the whole study group there might have been a positive result regarding effect of anti-TNF therapy, but vitamin D did not seem to have an impact here.

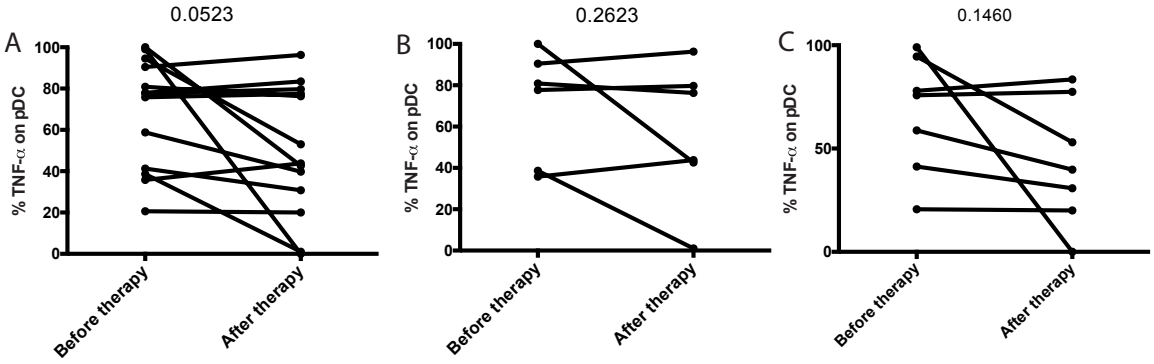


Figure 6.4: Column plots demonstrating before (week 0) and after (week 6) therapy values for TNFα production by pDC in (A) the entire study population, (B) the non-vitamin D supplemented control group and (C) the vitamin D treatment group. Statistical analysis performed using paired t-test.

6.5.4 TNFα ELISA results

Across the whole study group there was a numerical reduction in serum TNFα levels as measured by ELISA, but the results were not significant due to too few positive results (p=0.25, fig 6.5 below).

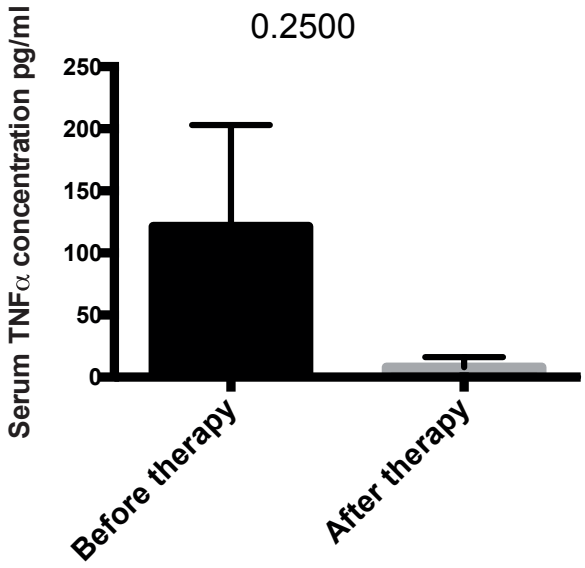


Figure 6.5: Graph representing whole population ELISA results for concentration of TNFα (units pg/ml) in serum of patients with Crohn's disease before and 6 weeks after treatment.

6.5.5 Effect of anti-TNF therapy on DC IL-6 production, and effect of vitamin D co-administration

In Chapter 4 it was shown that IL-6 production by circulating pDC was excessive within the study population with Crohn's disease (see figure 4.4 in Chapter 4). Here it is shown that there is a significant down-regulation of IL-6 production by pDC following initiation of anti-TNFα therapy (figures 6.6 & 6.7).

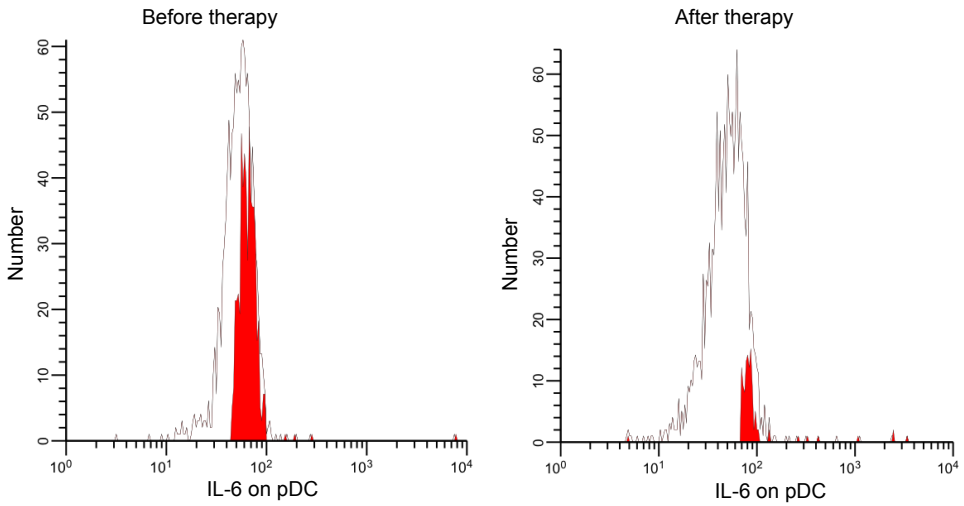


Figure 6.6: 2D Histograms demonstrating the expression of IL-6 on pDC in a subject with Crohn's disease before (left) and after (right) anti-TNFα therapy. Histograms presented are following SED_{MAX} subtraction of the isotype control (see Fig 4.1 for example of subtraction).

As with the results for TNFα production in mDC, it is seen here that changes in IL-6 production following anti-TNFα therapy are heterogeneous. Overall, there is a significant reduction in on-going production of IL-6 following anti-TNFα therapy (73.4% v 47.5%; week 0 v week 6; p = 0.04),

but as can be seen in figure 6.7 below, the changes were not seen in all subjects. Unlike with mDC, there was no significant impact of vitamin D therapy to explain this heterogeneous response, although there was a trend toward vitamin D augmenting the reduction in cytokine production (p = 0.075).

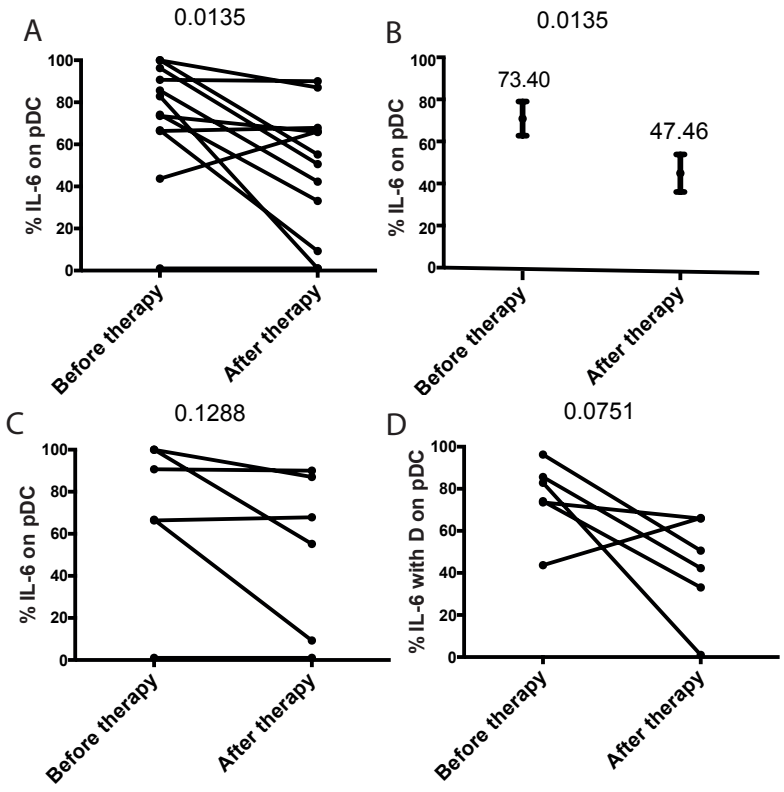


Figure 6.7: Graphs demonstrating before (week 0) and after (week 6) therapy values for IL-6 production by pDC in (A) the entire study population (B) the entire study population shown again, but as grouped data with mean values (C) the non-vitamin D supplemented control group and (D) the vitamin D treatment group. Statistical analysis performed using paired t-test.

6.5.6 IL-6 ELISA result

Across the whole study group there was a statistically significant reduction in serum IL-6 concentration following treatment, mirroring the changes in production by DCs (23.3 v 0.9 p = 0.0024, Fig 6.8).

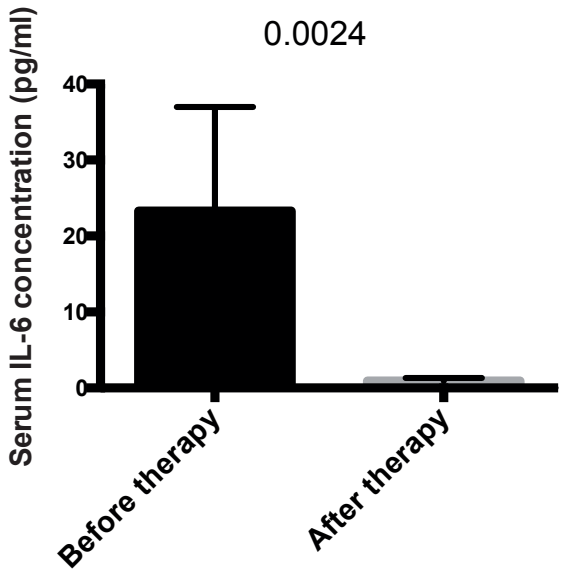


Figure 6.8: Graph representing whole population ELISA results for concentration of IL-6 (units pg/ml) in serum of patients with Crohn's disease before and 6 weeks after treatment.

6.6 Summary of results for chapter 6

- Overall in the anti-TNF treated study group: pro-inflammatory cytokine production was reduced by both mDC (TNFα) and pDC (IL-6).
- Vitamin D was shown to impact upon production of circulating TNFα but no clear impact on IL-6 was seen.
- There was a significant negative correlation between change in vitamin D level and change in TNFα production by mDCs.
- An increase of serum 25(OH)vitamin D greater than 20 nmol/L was associated with a decrease in mDC TNFα production.
- Overall in the whole treatment group, ELISA confirmed that serum levels of TNFα and IL-6 fell following treatment, mirroring the changes in production by circulating DCs.
- Production of the other cytokines under review (not shown to be abnormal at baseline in this group of patients - Chapter 4) did not alter with treatment.

6.7 Discussion

6.7.1 Infliximab

Overall in the whole study group (all of whom received anti-TNF therapy), there was a reduction in the production of some key pro-inflammatory cytokines: namely TNFα production by mDC and IL-6 production by pDC. These results are similar to the results from the study by Bartels et al, and the fact that a different experimental model (fresh DCs from PBMCs, rather than Mo-DCs) reproduces the results increases their validity (Lars E Bartels *et al.*, 2014b). As with the ELISA results from Chapter 4, (where, in the Crohn's disease group, the raised serum TNFα and IL-6 levels mirrored the increased production of the same cytokines by circulating DCs) here the decreased serum concentrations of TNFα (ns) and IL-6 following treatment also mirrored the change in production by DCs. This adds weight to the argument made in Chapter 4 that DCs, although few, have a significant role in shaping cytokine levels in whichever biomaterial they are contained (in this case blood) by driving the T-cell cytokine response. An alternative explanation would be that inflammation at a site distant to these circulating DCs (i.e. mucosal tissue in IBD) leads to an excess of circulating cytokines which may modulate the DC phenotype and function, and that the removal of this mucosal inflammation (with infliximab) will cause reduced circulating cytokines and allow down-regulation of the circulating DCs. A counter-argument against the latter explanation would be the fact that infliximab also modulates the function of circulating DCs from healthy controls, where there wouldn't be an excess of circulating pro-inflammatory cytokines, or indeed a distant inflammatory site to act as a confounder.

That circulating DCs are modulated by infliximab in terms of both phenotype (Chapter 5) and function (Chapter 6) in this *ex vivo* study (having been previously shown to cause the same modulation in *in vitro* studies) promotes the hypothesis that DC modulation may be one of the mechanisms of action of infliximab, a drug whose mechanisms are still not fully understood (Peake *et al.*, 2013).

There was a lack of effect of infliximab upon the other examined cytokines in this study, despite these cytokines being of interest in Crohn's disease. This was most likely secondary to the fact that concentration of these cytokines did not differ between Crohn's subjects and HCs at baseline, hence there was little effect to be had. The possible reasons for this lack of difference were speculated upon in 5.6.

6.7.2 Vitamin D

In addition to its effects on homing marker expression (Chapter 5), vitamin D as a co-treatment is seen here to reduce on-going mDC production of TNFα, beyond that of anti-TNFα treatment alone ($p = 0.016$). Furthermore, there was a statistically significant negative correlation ($p = 0.026$, $r = -0.65$) between the change in serum vitamin D concentration and the change in TNFα production (the higher the increase in serum vitamin D achieved, the greater the decrease in TNFα production). This would suggest that parenteral vitamin D may exert a systemic change in the function of immunomodulatory cells, with the degree of change influenced by the concentration of vitamin D. On examination of the correlation curve, immunomodulation occurs with increases of >20nmol/L. This serves to highlight the fact that there may be a minimum

dosage, serum concentration level or serum concentration increase required to achieve immunomodulatory effects (rather than simply suggesting that one must aim for an increase in serum vitamin D level of >20nmol/L in order to achieve immunomodulation – the increase needed will likely depend also upon the level of deficiency in the first place). Given that only one strength of vitamin D (300,000IU) was injected during this study it is not possible to comment on the minimum dose required to achieve immune cell down-regulation, but this would be interesting future work.

Although DCs make up a small proportion of immune cells in the blood (c1%) they are the 'master regulator' cells of immune response and heavily influence T-cell clonality, population expansion and effector functions (Mellman, 2013). It is known that the cytokine milieu will influence the direction in which DCs interact with T-cells, and the presence of TNFα will drive a Th1/Th17 response, which is characteristic of the inflammation seen in Crohn's disease (with UC historically described as a Th2 mediated disease).

6.8 Discussion summary points – Chapter 6

- The production of pro-inflammatory cytokines by circulating DCs was down-regulated by *ex vivo* treatment with anti-TNF. We postulate that this may be one of the mechanisms of actions of this class of drug.
- Vitamin D as a co-treatment caused a significant reduction in circulating mDC TNFα production, which may be of significance given the importance of DCs in shaping the T cell response, and the sensitivity of DCs to TNFα in determining this response.
- The correlation between increasing vitamin D level and decreasing mDC TNFα production suggests that immunological effects of vitamin D may require a threshold dose/ change in serum concentration.
- Neither infliximab nor vitamin D seemed to have any significant effect on the other examined cytokines, probably because they were not abnormal at baseline in this study (see Chapter 5.6).

Chapter 7 – Study Limitations and Future Work

7.1 Study limitations

The addition of a healthy control treatment group to receive vitamin D in Chapters 5&6, and of an additional Crohn's disease group to receive vitamin D without infliximab would have been positive additions to strengthen the study, but were not possible within the timeframe (or indeed the ethical scope) of the research. Using healthy subjects as controls for the subjects with Crohn's disease, and using non-vitamin D supplemented Crohn's subjects as the controls for the vitamin D supplemented group was a pragmatic approach that, whilst imperfect, allowed the study to be completed, with controls, in the allocated period. On the same theme, the decision on which subgroup to place those with Crohn's disease (i.e. to supplement with vitamin D or not) was not randomized or blinded. The decision was based on the baseline vitamin D level. Again, this was a pragmatic decision; to blindly allocate would have rendered this study a Clinical Trial of an Investigational Medicinal Product (CTIMP), which was beyond the scope of this research, and indeed the time to achieve ethical approval would have taken up an unacceptable portion of the allocated study time. Instead, vitamin D was given according to Trust guidelines, and this allowed for treatment of deficient patients (<50nmol/L) and no treatment of those considered sub-optimal (50-75nmol/L). The down-side of this approach is that the two groups had different vitamin D levels at baseline; one could argue that this is due to different biology or disease activity of each individual's Crohn's disease. In fact, the baseline HBI, CRP and faecal calprotectin were not statistically different across the two groups; but we still acknowledge the different baseline vitamin D levels between the two subgroups as a potential confounding factor.

There were some instances where higher study numbers might have clarified borderline results, and the reason that higher numbers weren't recruited was multifactorial. The main study tool used in this thesis, flow cytometry, is extremely time consuming with each experiment lasting a day and a half (once the sample is taken). There were (rare) occasions when several potential subjects presented at the same time and some were not recruited due to the difficulties in performing contiguous experiments (necessitated by the use of fresh PBMCs in our protocol). This might have been avoided, and study numbers might have been increased a little, if cryopreservation was chosen, but as mentioned in chapter 2, fresh PBMCs have an improved immunogenic assay response (Weinberg *et al.*, 2009). In addition, tight recruitment parameters were used to try to reduce the number of confounding factors that might alter DC biology. These tight parameters included: patients with active Crohn's disease; stable immunomodulator; no recent steroids; no recent vitamin D; biologic naïve; about to start anti-TNF treatment as a clinical decision for their active disease; serum vitamin D <75nmol/L. This strict inclusion policy reduced significantly the number of eligible subjects. Having said that numbers were low, however, they met recruitment targets, matched other similar studies, and were still adequate to answer most of the questions asked, especially where there was clearly a difference, or clearly no difference in results. There were a couple of occasions, however, where the result was just shy of statistical significance and greater study numbers might have clarified the outcome (e.g. Fig. 6.4).

Whilst the inclusion criteria were strict, which limited recruitment numbers somewhat, the patients recruited were still not an entirely homogeneous population. For example, whilst all of the patients recruited to the *ex-vivo* study had experienced immunomodulator therapy, and the

majority were on IM at the time of the study, there were several patients who were not currently on IM at the time of the study. Immunomodulators can alter DC biology, and so this should be regarding as a confounding factor (Aldinucci *et al.*, 2010). The three patients who were not on IM at the time of the study were distributed across both arms of the study, so it is unlikely that any changes to cell biology brought about by being on current IM impacted on statistical difference between the two groups. In addition, to further limit the confounding effect of concomitant IM, all patients had been on stable doses of IM for at least three months prior to study recruitment. A further source of heterogeneity in the study population is in Crohn's disease phenotype. Given the strict recruitment criteria and the finite time available in which to conduct the study, a pragmatic decision was made that, as long as inclusion criteria were met, any phenotype of disease would be included. This meant that patients with perianal disease, and patients with stomas, were included, as long as they had evidence of luminal inflammation in addition. All phenotypes were also included, and all degrees of inflammation (greater than no inflammation). Although this enabled improved recruitment to the study it must be mentioned as a confounding source. Location within in the colon can affect the DC population, so it is entirely feasible that DC population may be altered by presence of a stoma, or by perianal disease (Bernardo *et al.*, 2016). Ideally, large enough numbers would have been recruited to enable an adequately powered sub-analysis, but that was not possible here. For each of these confounding factors (stoma and perianal disease), there was equal distribution across the treatment and control arm, mitigating the confounding effects. Although there was a spectrum of disease severity included, CRP, faecal calprotectin and HBI did not differ between the two groups at baseline.

It is important to acknowledge that vitamin D levels are affected by multitudinous factors (discussed in Chapter 1) and it is not possible to control for all of them, e.g. season, diet, time spent outdoors et cetera. Experimental timings were chosen to limit the effect that changes in season might have on vitamin D levels: the time point for DC assessment was kept short, at 6 weeks (long enough for DC changes to take place, but short enough to limit seasonal change); and the final study point was 1 year (i.e. at exactly the same point in the season). On statistical analysis, there was no bias in the distribution of season of recruitment (using UK meteorological seasons). In future, it might be worth considering food diaries to examine vitamin D intake in the diet, but this is quite onerous and may be off-putting for subjects and make recruitment harder than it is already.

It would have been beneficial to have absolute cell count data from counting beads to accompany the flow cytometry results and give information on the effect of the treatments upon DC numbers. Unfortunately, insufficient counting bead data were gathered to draw meaningful conclusions about changes to absolute numbers. The original beads used were not reliable for cell counts (despite being in date and appropriately stored) and there was a substantial delay to obtain a good replacement with different formulation. This did not, however, compromise analysis of markers of interest. In place of counting beads a proxy technique was used: since consistent numbers of PBMCs were studied in each tube ($1x10^6$) and consistent numbers ($5x10^5$) were acquired in the flow cytometry machine a ready estimate of changes to cell populations were calculated. Whilst not as reliable as counting bead data, this is still a valid method of assessing population numbers.

RT-qPCR was performed on all samples which had frozen PBMCs stored. Unfortunately, in almost half of the RT-qPCR experiments performed there was a significant discrepancy between the technical triplicates which invalidated such samples and did not leave enough valid samples for any meaningful analysis. The reason for performing triplicates is to control for validity of the method, and variance in technical triplicates, as here, suggests inconsistencies with the methodology; but we had too few samples and insufficient DNA to explore the reasons for the variance. It is unlikely that insufficient stored DNA was the cause for experimental failure as at least $1x10^6$ cells were stored in each case. Errors in pipetting or contamination of buffers are possible causes for experimental failure. If this experiment was to be repeated it would be with new, unopened reagents only.

The monocyte work (see Appendix) is somewhat limited by its post-hoc nature, and because specific monocyte markers were not included separately in the stain panel (they were included in the lineage cocktail to exclude non-DC PBMCs). For this reason, the monocyte work was included in the appendix rather than the body of the thesis. That said, the pilot experiments with specific monocyte marker stains included ensured that the monocyte gating strategy was accurate and added validity to the data set.

7.2 Future work

It seems prudent to start by saying that there has been a lot of research into vitamin D in IBD recently, and many positive results published, but the main thing missing is an adequately powered, well-executed RCT which would answer the question as to whether vitamin D is useful

as a treatment (or as a treatment adjunct), in Crohn's disease. This author does not feel that further epidemiological studies are required. Useful mechanistic data may be obtained, however, from basic science studies, and a number of questions remain to be answered:

Given that the results show an up-regulation in frequency of expression of skin homing profile on DCs following anti-TNF therapy, it would have been interesting to study patients with cutaneous manifestations to ascertain whether any given DC phenotype predisposed to this side effect. Furthermore, given that vitamin D down-regulated this skin homing profile, it would be useful (with high study numbers) to ascertain whether vitamin D supplementation reduced the occurrence of skin reactions/rashes. However, from our study group there was only one patient who developed cutaneous manifestations during the study. It was shown in Peyrin-Biroulet's large study that incidence of psoriaform lesions, the most common cutaneous complication of TNFα, at one year is 1%, therefore a very large cohort would be required to guarantee several paradoxical complications in the one year time frame of such a study. Paradoxical inflammation should be studied further, though, using these tools and a case control study of DC phenotype and function in subjects who suffer this side effect would be useful. What would be more useful, though, would be a method of determining, before treatment is given, who is at high risk of this side effect, but as mentioned above this would take huge study numbers. Another option would be to treat subjects who develop cutaneous side effects with systemic vitamin D and study whether any clinical improvement is associated with the expected down-regulation in skin homing DC phenotype.

Vitamin D receptor status is an under-studied area, and it may be of clinical significance. If a VDR polymorphism means that vitamin D cannot exert its transcriptional functions adequately it may

well be that a higher concentration of vitamin D is required to achieve immunomodulatory effects, or indeed that, in those subjects, vitamin D is unable to exert any immune effect. This may be an unidentified confounder, therefore, and in the future studies may need to exclude those with polymorphisms, or, more usefully, include such subjects as a separate group. Ideally, a specific *ex vivo* study to determine whether VDR polymorphisms alter immune cell functions would help address this issue.

This study exclusively focuses on circulating DCs, because they are easily accessible, under-studied and they offer a "window to the gut". A lot of attention has been paid previously to gut DCs, but this experiment could be repeated in the gut DC population. In addition, we studied function of DCs through assessment of the on-going production of cytokines. This could be taken further with mixed leucocyte reaction studies (MLR) to assess the down-stream effect of the DC changes upon T cell population differentiation and function. Other methods which would augment the flow cytometry, ELISA and EM performed for this thesis include transcriptional analysis of DC phenotype and function using qPCR or microarray and RNA-Seq technologies.

Further functional studies would also be beneficial. Transwell migration assays assessing whether there is altered migration of DCs to CLA/β7 ligands in the presence of active vitamin D were attempted in this study, but were unsuccessful and time pressures ended that line of experimentation. Mouse models could also be used to give *in vivo* data to augment the functional aspects of this study.

It remains unclear whether changes in any given population of DCs are due to upregulation of markers within the cells of that population; due to a particular cell type from that population

moving elsewhere (and leaving an altered population in its wake); or because new cells with different marker expression are joining the population from elsewhere (e.g. de novo cells from the bone marrow). Whilst bone marrow (BM) DC studies cannot realistically be performed in humans for this indication, it would be of interest to assess DCs from BM and blood in IBD mice models following treatment with vitamin D, to clarify whether the altered BM DC population are driving the changes in circulation, or whether the changes occur due to local factors in the blood.

Chapter 8 – Appendix

8.1 End of study (12 month) clinical data

The following data were collected for each subject at 12 months from study entry date: continuation or discontinuation of infliximab (as a proxy for therapy success); HBI; CRP; faecal calprotectin; development of infusion reaction or extra-intestinal disease manifestation (EIM), with particular attention to be paid to dermatological sequelae.

8.1.1 Continuation of infliximab after the one year assessment

Discontinuation of anti-TNF is deemed a proxy marker for failure of therapy (Bouhnik *et al.*, 2018). In this study, one patient from the non-supplemented control arm, and two patients from the vitamin D supplemented arm discontinued during the study, giving no statistical difference between the two groups ($p = 0.5692$, figure 8.1).

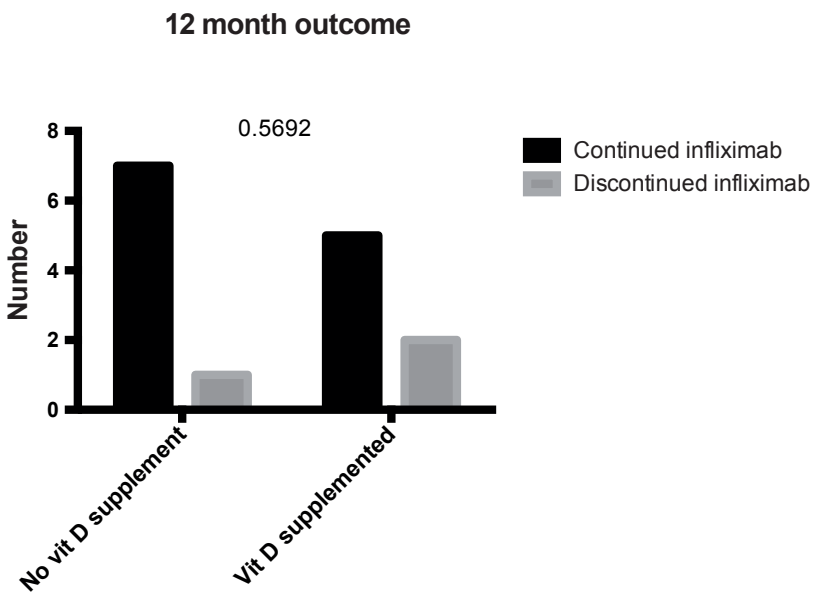


Figure 8.1: Graph showing binomial data for continuation or discontinuation of infliximab beyond 1 year for both the non-supplemented and the vitamin D supplemented groups. Fisher's exact test with two-tailed p-value performed.

8.1.2 Twelve month CRP data

Although there was a trend towards reduction of CRP following treatment, in the whole study group, it did not reach statistical significance (p=0.0586) possibly because CRP was not excessively elevated at baseline (<20mg/L in both groups). There was no statistical difference in CRP levels at 12 months in the vitamin D supplemented and non-vitamin D supplemented groups (figure 8.2).

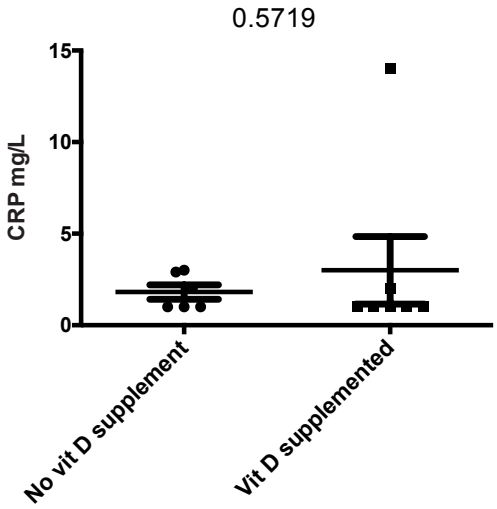


Figure 8.2: Dot plot graph demonstrating C-reactive protein (CRP) levels in the non-supplemented and the vitamin D supplemented groups at 12 months after initiation of therapy. Statistical evaluation performed using unpaired t-test.

8.1.3 Twelve-month faecal calprotectin data

In the whole study population, there was a significant reduction in faecal calprotectin (FC) level between the 0 and 12-month study visits (mean 649µg/g v 235µg/g; before v after treatment, p=0.0437; figure 8.3). The mean decrease in FC in the vitamin D treated group was 437µg/g, compared with 386µg/g in the non-supplemented group and this difference was not statistically significant. Although there was a numerical reduction in the 12-month FC level in the vitamin D supplemented group (294µg/g v 166µg/g) this difference was not statistically significant (figure 8.4). Similarly, there was no difference in representation between the two study groups when separating by FC high and FC low status and no difference in change in FC levels between the groups (figure 8.4).

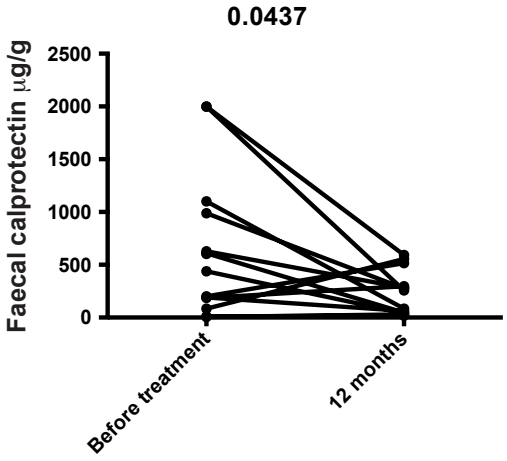


Fig 8.3: Line and dots graph showing a fall in faecal calprotectin 12 months after treatment start, in the whole study group.

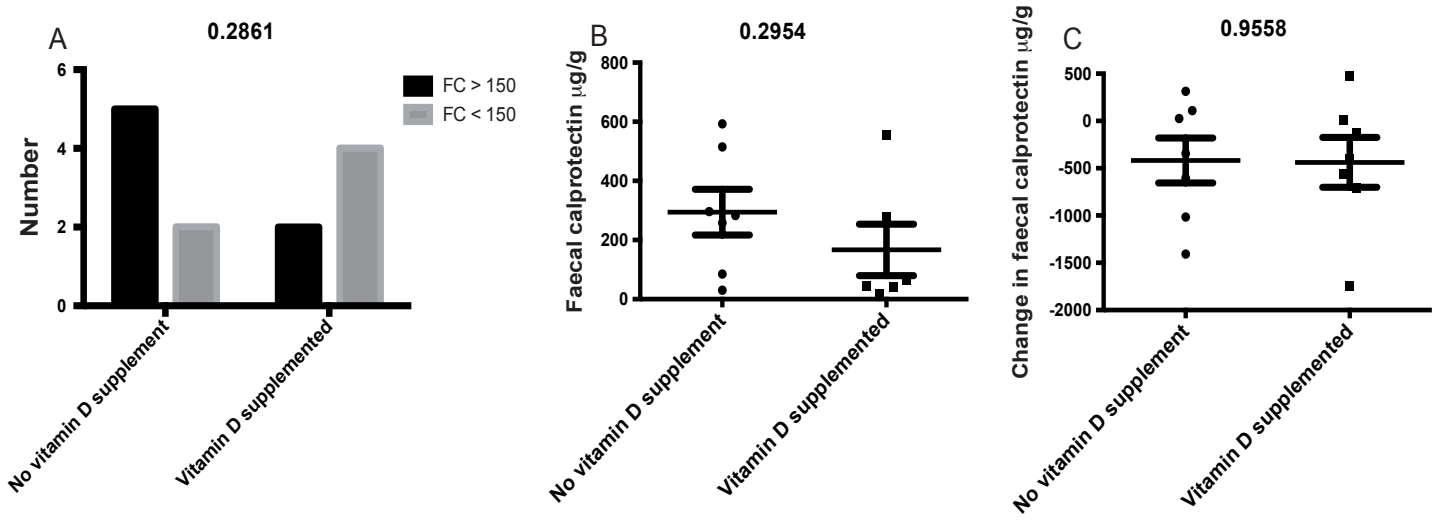


Figure 8.4: (A) Column graph demonstrating faecal calprotectin (FC) high v low in the two treatment arms (non-supplemented and vitamin D supplemented); (B)& (C) Scatter plots demonstrating (A) FC level at 12 months in the non-supplemented and the supplemented groups, and (B) demonstrating change in FC level between 0 & 12 months in the non-supplemented and the supplemented groups.

8.1.4 Twelve-month HBI data

There was no difference in HBI between the two groups at 12 months (figure 8.5A, p=0.3676), with the patients generally having well controlled disease. There was a significant fall in HBI in the whole anti-TNF treated study population (mean fall in HBI 3.18, p=0.0117, figure 8.5B), but the numbers were too small to identify any difference between the two subgroups (vitamin D supplemented v not supplemented).

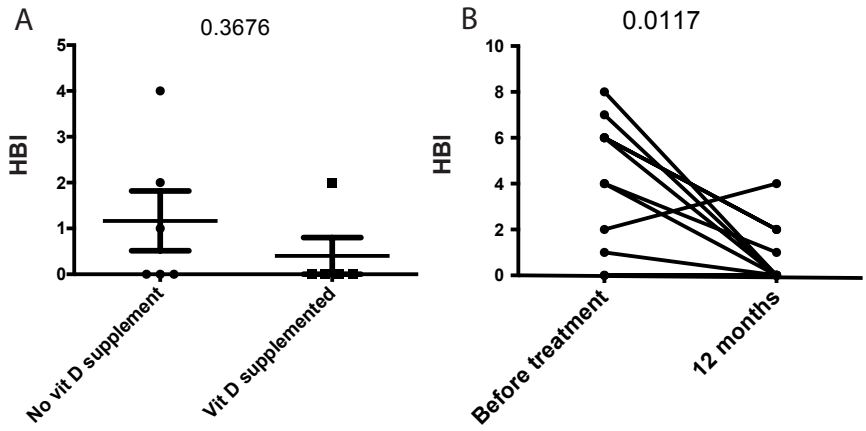


Figure 8.5: Graphs demonstrating (A) Harvey-Bradshaw index (HBI) at month 12 after initiation of treatment in the non-supplemented and the vitamin D supplemented group and (B) the fall in HBI with treatment in the whole study group between month 0 & 12. Statistical analysis was performed using unpaired t-test and paired t-test respectively.

8.1.5 Extra-intestinal manifestations (EIMs) of Crohn's disease

The only EIM which developed during the 12-months follow-up was a psoriatic skin reaction, described below.

Skin reaction

During the 12-month trial follow up, one patient from the control arm (non-vitamin D supplemented) and zero patients from the vitamin D arm developed a dermatological condition. The subject with a skin complication developed guttate psoriasis which was treated with topical vitamin D and emollients. This subject had a good clinical response to anti-TNF therapy with HBI reducing from 4 to 0, faecal calprotectin reducing from 100 to 80, and the infliximab being continued beyond the 1 year study end-point. Interestingly the same patient later developed Henoch Schonlein Purpura which necessitated discontinuation of infliximab, during the second year of treatment.

The DC migration marker phenotype of the subject who developed psoriasis did not reveal any major differences compared to other subjects who did not develop skin disease.

With the skin sequelae N=1 it is not possible to comment upon the clinical efficacy of vitamin D optimisation at reducing dermatological complaints in IBD (although it is worth mentioning that topical vitamin D [as prescribed by the dermatologist], alongside drug cessation, was successful at treating the skin rash).

8.2 Supplementary Data - Monocytes

The reasons for including the results from the monocyte studies in the appendix rather than the body of the thesis are that the initial remit of the thesis did not include monocyte study, and the analysis was performed *post-hoc* rather than following an *a-priori* hypothesis.

8.2.1 Introduction to monocytes and intestinal macrophages

Monocytes and intestinal macrophages have a pivotal defensive role at the interface between innate and adaptive immune systems and both are implicated in the aetiology of inflammatory bowel disease.

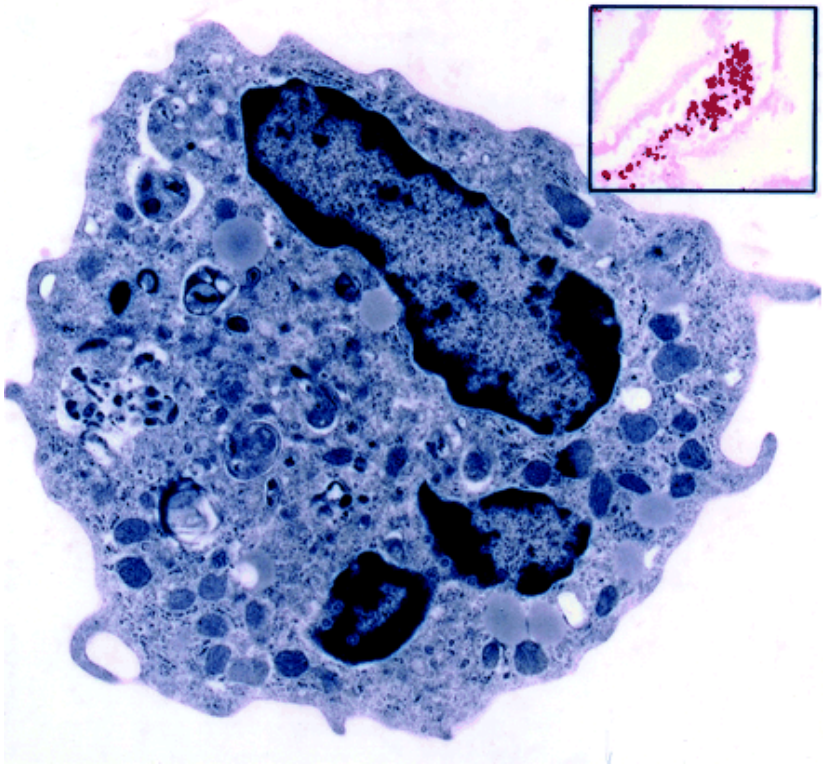


Figure 8.6: Electron microscope image of an intestinal macrophage. Note the large phagosomes and the smaller dendritic-like projections(Smith *et al.*, 2001).

8.2.2 Monocyte and macrophage lineage, development and life cycle

Both the monocyte (literal meaning: "single cell") and its progeny, the macrophage (literal meaning: "*big eater",* figure 8.6) are cells of myeloid lineage which play crucial roles in innate

immunity. Both perform three main function: phagocytosis, antigen presentation and production of cytokines.

Monocytes are produced in the bone marrow from precursors called monoblasts which are themselves differentiated from haematopoietic stem cells (figure 8.7). They tend to remain in the blood circulation for 1- 3 days before migrating to the tissues where they differentiate into macrophages and DCs.

There are three main monocyte populations in human blood, based upon relative expression of the LPS receptor CD14 and the FCγIII receptor CD16 (see Table 8.1 below): Classical monocytes are phagocytic but with no inflammatory characteristics; non-classical monocytes have inflammatory and antigen-presentation abilities and the intermediate subtype have both phagocytic and inflammatory properties (Mukherjee *et al.*, 2015). All subtypes of monocyte are capable of differentiation to macrophages, but only classical monocytes may differentiate into Mo-DCs (Boyette *et al.*, 2017).

Monocyte subtype	**Identifying surface receptor expression**
Classical monocyte	$CD14^{++}CD16^{-}$
Non-classical monocyte	$CD14^{+}CD16^{++}$
Intermediate monocyte	$CD14^{++}CD16^{+}$

Table 8.1: Identification of monocyte subtypes (Ziegler-Heitbrock *et al.*, 2010).

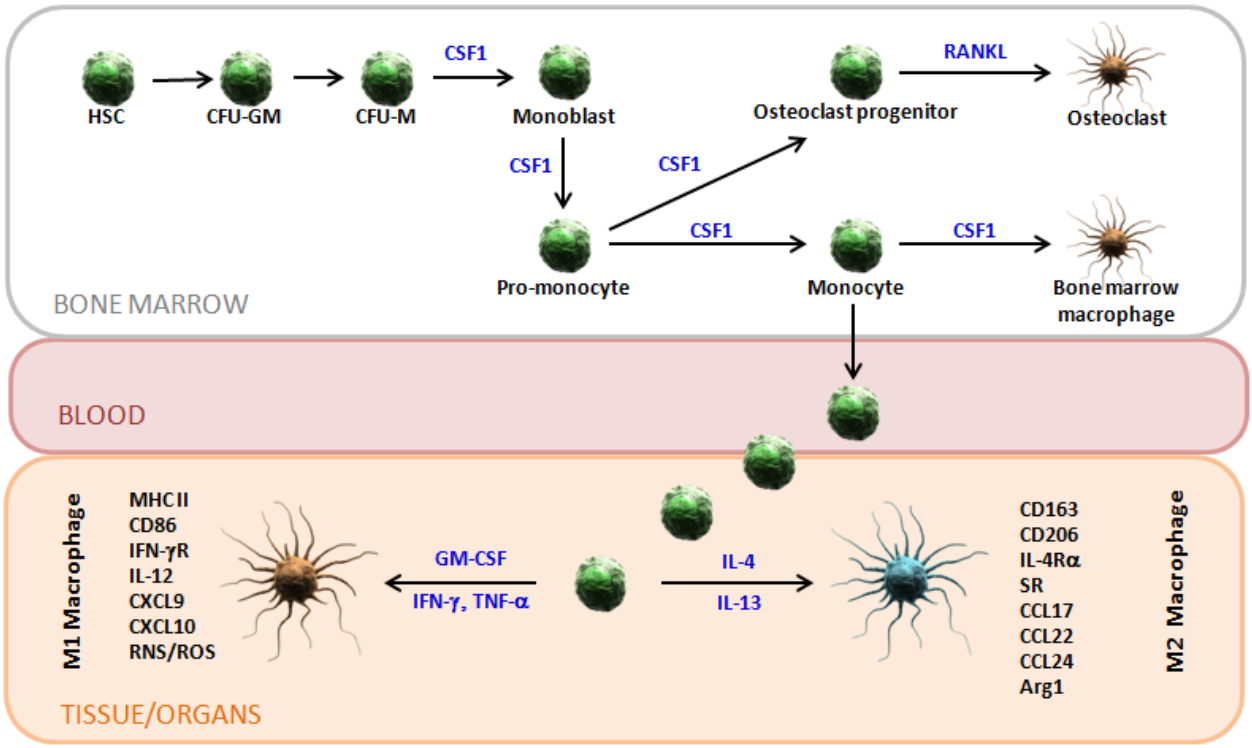


Figure 8.7: Monocyte and macrophage development pathways (Image courtesy of Biolegend, see permissions in appendix).

Most tissue resident macrophages originate from circulating monocytes which undergo major transformational change once leucocyte extravasation has occurred, mainly in response to inflammation (the yolk sac and fetal liver are additional sources for small numbers of macrophages) (Isidro and Appleyard, 2016). Whilst primarily known for their phagocytic activities, macrophages are now known to have a wide-range of 'house-keeping' roles and are essential for homeostasis in health as well as for defence against pathogens (Okabe and Medzhitov, 2016). These house-keeping roles include: removal of apoptotic cells; initiation of tissue repair; development of some tissues; and as a source of growth factors for other cells in tissue.

The phenotype of macrophages differs depending upon the tissue in which they reside; there are significant differences between tissue-resident macrophages and gut-resident macrophages, because of the requirement for tolerogenicity in the gut. In general, though, macrophages carry the following markers which can be used for identification: CD11b, CD14, CD16, CD40, CD64, CD68. A brief precis of some of the protein markers used to identify monocytes and exclude other cells (for identification and gating strategy see Methods 2.3.2.9):

CD11b (also known as Integrin Alpha M, ITGAM) is a protein which is expressed on the surface of many leukocytes of the innate immune system, including monocytes, macrophages, NK cells and granulocytes. It has a role in leukocyte adhesion.

CD11c, a transmembrane protein (and an integrin), is highly expressed on dendritic cells, and found at lower concentrations on various other immune cells, including monocytes. Monocytes may upregulate CD11c, but it tends to be at lower levels than mDC; and monocytes may significantly upregulate expression of CD11c when they migrate into tissues and differentiate into tissue mDCs and tissue macrophages.

CD14 is a protein of the innate immune system which acts as a co-factor, with TLR4, for the detection of bacterial LPS. It is expressed predominantly on monocytes and macrophages, but also (in much smaller quantities) on neutrophils and DCs.

CD16 is a FcγRIII receptor, a member of the immunoglobulin superfamily and is involved in immune cell signal transduction. It is expressed on non-classical and intermediate, but not classical monocytes

CD40, a member of the TNF superfamily, is ubiquitous on APCs and is responsible for activation of APC upon its binding to CD40L on Th cells(Grewal and Flavell, 1998).

Whilst CD64 may be expressed on DCs in tissue, it is at a much lower level, and in general CD64 is associated with inflammatory monocytes and activated M1 macrophages (it is expressed at lower levels on M2 macrophages). Indeed, CD64 is commonly used to distinguish between intestinal macrophages and DCs.

Macrophages have been sub-characterised into M1 (high IL-12, low IL-10) and M2 (high IL-10 low IL-12) subtypes, to fit with the Th1/ Th2 sub-categories of T-cells (although this paradigm is being challenged as understanding of macrophage biology advances) (Mills *et al.*, 2000; Martinez and Gordon, 2014). M1 macrophages, activated by TNF-α, IFN-γ and LPS, are proinflammatory by way of their ability to metabolise arginine to nitric oxide. M2 macrophages, stimulated by IL-4, IL-13, IL-10, and glucocorticoids, are considered anti-inflammatory (arginine is metabolised to ornithine) (Akinrinmade *et al.*, 2017). Macrophages can engulf and destroy many potential pathogens and can attract other APC's by secreting cytokines. Cytokine production includes TNF, IL-1, IL-6, IL-8, IL-12 and IL-23 (all of which are implicated in Crohn's disease). Macrophages also acquire specialist adaptations including class A scavenger receptors and transmembrane integrins. The former is involved in the removal of foreign substances whilst the latter allows for responses to a variety of cell surface events. Macrophages are relatively long lived, staying active for 6 to 16 days (Levin, Grinstein and Canton, 2016).

8.2.3 Monocytes and macrophages in Crohn's disease

Despite a dearth of study data, some abnormalities in the monocyte population have been identified in Crohn's disease. The proportion of the classical monocytes is reduced whilst the proportion of non-classical monocytes is increased, as compared with healthy controls (Thiesen Gren, 2015). The authors postulated that classical, but not non-classical, monocytes migrate to the gut in Crohn's disease. There were no major differences between monocyte function in health and in Crohn's disease in terms of phagocytosis, migration and cytokine production, although further data are needed (Koch *et al.*, 2010; Schwarzmaier *et al.*, 2013).

Intestinal macrophages, expressing CD64, act as first defence against luminal micro-organisms and potentially allergenic or toxic food particles presenting to the mucosa (Cerovic *et al.*, 2014; Steinbach and Plevy, 2014). Like intestinal DCs, they reside in the lamina propria, just one cell layer away from the luminal contents. These macrophages must react speedily and concertedly to contain and remove large immunological loads. Thus, they must show plasticity and powers of recruitment. It is the recruitment of pro-inflammatory CD14hiHLA-DR macrophages, which resemble and which are likely to be derived from classical blood monocytes, that have been shown to dominate the inflamed intestinal mucosa of Crohn's disease (Thiesen *et al.*, 2014). Although the pathogenesis underlying this macrophage influx is yet to be wholly defined, it is likely that these cells are driving inflammation by up-regulating IL-1β, IL-6, TNFα and MMP whilst down-regulating IL-10 production (Thiesen Gren, 2015). The net result is a pro-inflammatory milieu resulting in intestinal mucosal disease.

Conversely type M2 (tissue repair) populations of macrophage fail to develop characteristic activated dendritic cell morphology. Intra-cellular E *coli* survival is increased and co-stimulatory CD40 expression (required for APC stimulation) is reduced (Karaiskos *et al.*, 2011).

Thus, in Crohn's disease there is evidence for macrophage populations exhibiting restricted M2 activity and concomitantly enhanced M1 activity.

8.2.4 Monocytes, macrophages and Vitamin D

In vitamin D deficiency, there is a shift of monocyte populations to a more activated phenotype, with lower frequency of $CCR2^+$ monocytes and higher frequency of non-classical and $CX3CR1^+$ monocytes (Manion *et al.*, 2017). Pre-treatment with $1,25(OH)_2D3$ inhibited LPS induced production of TNFα and IL-6 by human monocytes and induced mitogen-activated protein kinase phosphatase-1 (MKP-1) expression. MKP-1 is known to inhibit LPS p38 activation, so the upregulation of MKP-1 may be casual in the reduction of inflammatory cytokines (Zhang *et al.*, 2012).

When the bowel mucosa is not inflamed, there is a steady state whereby intestinal macrophages are hyporesponsive to inflammatory signals and produce fewer inflammatory mediator molecules in comparison with their counterparts in less antigen rich tissues. In addition, Tregs are promoted and show benign immunoregulatory activity (Baeke *et al.*, 2010).

As discussed in 1.3.2.2.3, there is growing evidence that vitamin D enhances intestinal barrier function (Bischoff *et al.*, 2014). It achieves this by several mechanisms including the enhancement of innate immune responses in several cell types including macrophages. Macrophages gain

increased chemotactic and phagocytic activity under the influence of vitamin D. Genes encoding for IL-2 and IL-10 are primary target genes for vitamin D. IL-10 is a regulatory cytokine which inhibits antigen presentation and subsequent pro-inflammatory cytokine release, and is associated with M2, rather than M1, macrophage populations (De Calisto, Villablanca and Mora, 2012; Paul, Khare and Gasche, 2012). Pattern recognition receptors are promoted and peptide proinflammatory factor production are inhibited by vitamin D. Macrophage production of antibacterial proteins such as cathelicidin inhibit surface expression of MHC II complex antigen and costimulatory molecules thus reducing pro-inflammatory IL-1, IL-6, IL-8 and TNFα (Baeke *et al.*, 2008; Sun, 2010).

8.2.5 Methods

The flow cytometry gating strategy for identifying the monocyte population from amongst the PBMCs is described in Chapter 2 the Methods Chapter (2.3.2.6-2.3.2.9). Data analysis was performed as for the DC experiments.

8.2.6 Results

8.2.6.1 Monocyte studies – comparison of healthy controls to subjects with Crohn's disease

8.2.6.1.1 Homing markers

CLA (skin homing) expression on monocytes was reduced in Crohn's disease (25.1% in HC versus 9.2% in CD; p = 0.048) whilst β7 (gut homing) was increased (15.3% in HC versus 25.2% in CD; p = 0.0228) (figure 8.8).

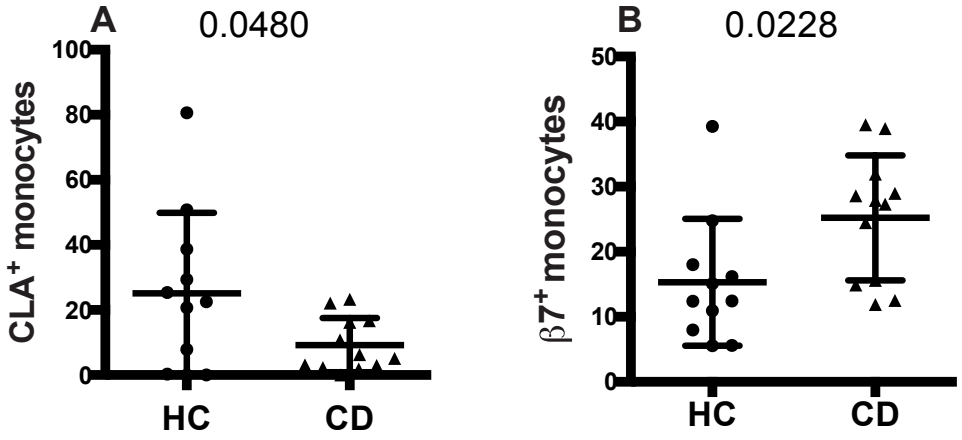


Figure 8.8: Pooled population data demonstrating monocyte expression of (A) CLA and (B) β7 in healthy controls (HC) and subjects with Crohn's disease (CD).

8.2.6.1.2 Markers of maturity

Monocyte expression of TLR2 did not differ between healthy controls and subjects with Crohn's disease (p = 0.37), but monocyte expression of TLR4 was greater in the Crohn's population (2.36% in HC versus 24.78% in CD; p = 0.018) (Figure 8.9).

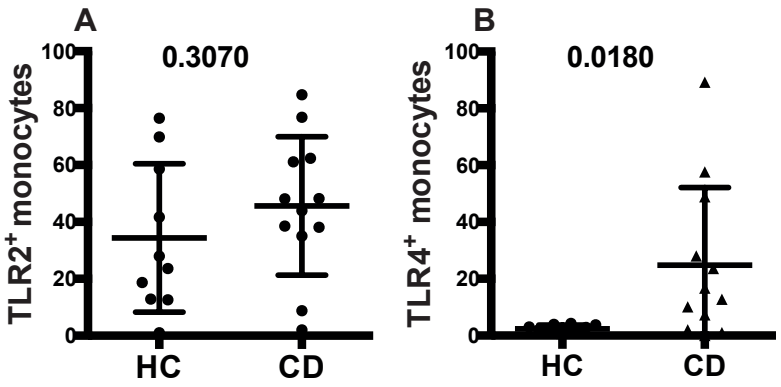


Figure 8.9: Pooled population data demonstrating monocyte expression of (A) TLR2 and (B)TLR4 in healthy controls (HC) and subjects with Crohn's disease (CD).

Monocyte expression of costimulatory markers CD80 and CD86 was increased in Crohn's disease (p = 0.0165 and 0.0406 respectively), but there was no difference with expression of CD40 (figure 8.10).

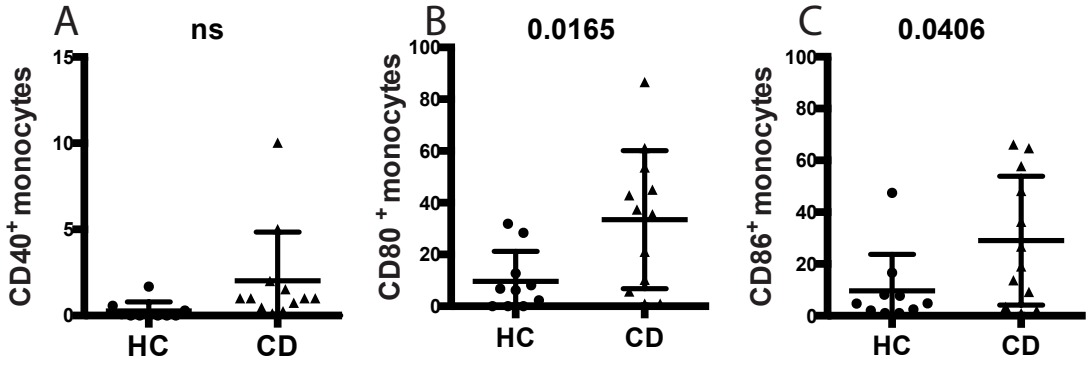


Figure 8.10: Pooled population data demonstrating monocyte expression of (A) CD40, (B)TCD80 and (C) CD86 in healthy controls (HC) and subjects with Crohn's disease (CD).

8.2.6.1.3 Cytokines

Monocyte production of cytokines in Crohn's disease (measured as expression over four hours of ICS) was increased for TGFβ, TNFα and IL-6 (p = 0.0367, 0.0185 and 0.0458 respectively, figure 8.11).

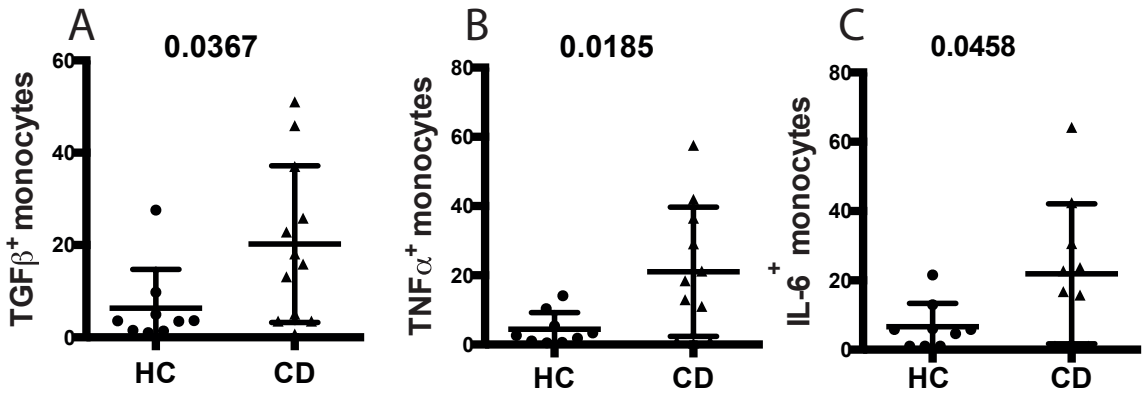


Figure 8.11: Pooled population data demonstrating differences in monocyte production of cytokines (expression over four hours intracellular staining) between healthy controls (HC) and subjects with Crohn's disease (CD) for (A) TGFβ, (B) TNFα and (C) IL-6.

8.2.6.2 The effects of therapy upon monocyte phenotype and function

8.2.6.2.1 Homing markers

There was an increase in monocyte expression of both CLA (from 9.21% to 32.9%; p = 0.052) and β7 (from 25.2% to 52.0%; p = 0.0069) in the whole population group following therapy (figure 8.12). Vitamin D mitigated this effect.

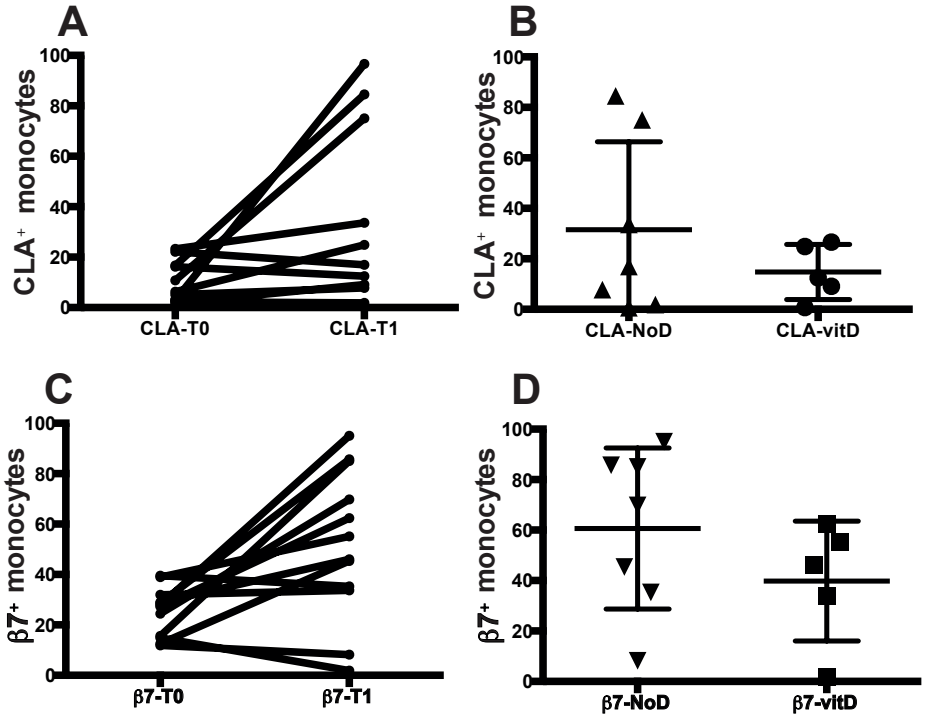


Figure 8.12: (A)& (C) Population data graphs demonstrating before and after therapy values for monocyte expression of (A) CLA and (C) β7 in the whole study population. (B) & (D) population data graphs comparing post-therapy values for monocyte expression of (B) CLA and (D) β7 in the vitamin D non-supplemented control group and the vitamin D treated group.

8.2.6.2.2 Cytokines

There was a non-significant numerical reduction of monocyte expression of both TNFα and IL-6 following therapy in the whole study population (infliximab +/- vitamin D) (figure 8.13). Vitamin D enhances this effect for TNFα, but unable to see additive effect with IL-6.

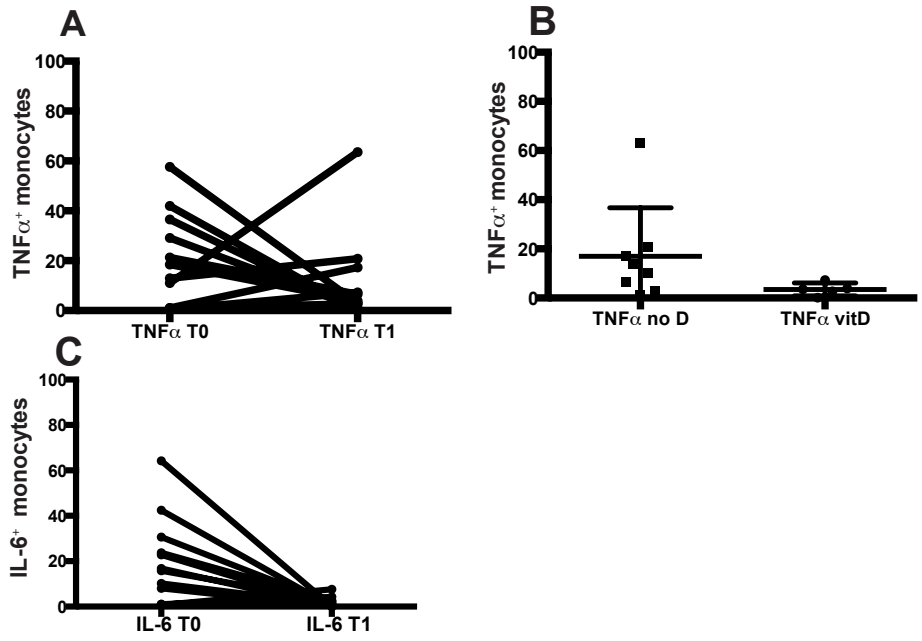


Figure 8.13: (A)& (C) Population data graphs demonstrating before and after therapy values for monocyte expression of (A) TNFα and (C)IL-6 in the whole study population. (B) population data graph comparing post-therapy values for monocyte expression of TNFα in the vitamin D non-supplemented control group and the vitamin D treated group.

8.2.7 Discussion

There were differences in the monocyte population in Crohn's disease in terms of both expression of phenotypic markers, and on-going production of inflammatory cytokines. Whilst β7 is not expressed at any great level on monocytes in health, we see here that expression is

higher in Crohn's disease. This would fit with the monocytes maturing and heading to gut tissue to become tissue macrophages (Tiisala, Paavonen and Renkonen, 1995). Monocytes are known to express CLA, and this is the first comparison of expression in CD and HC. The lower expression of CLA in CD may be due to down-regulation to enable homing to the gut (increased gut tissue specific macrophages are seen in Crohn's disease) (Kieffer *et al.*, 2001).

Both TLR2 and TLR4 are expressed on monocytes, unlike CCR2 which has low expression. The increased TLR4 expression seen in this study would be commensurate with an upregulated inflammatory monocyte population. Previous studies regarding inflammatory cytokine production have been inconclusive, but here upregulated production of TNFα, TGFβ and IL-6 are seen (Gren and Grip, 2016).

As with the DC studies performed in this thesis, anti-TNF treatment had an effect on monocyte cytokine production, and it is possible that vitamin D also had an additional effect, but other studies would be needed to confirm this, given the *post-hoc* nature of this monocyte study. This would be worth looking at further because, as discussed in 1.4, pre-treatment with 1,25$(OH)_2$D3 is known to inhibit LPS induced production of TNFα and IL-6 by human monocytes and to induce mitogen-activated protein kinase phosphatase-1 (MKP-1) expression. MKP-1 is known to inhibit LPS p38 activation, so vitamin D has a plausible mechanism for causing a reduction of inflammatory cytokines (Zhang *et al.*, 2012).

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