Modulation of Intestinal Dendritic Cells by Manipulation of Enteric Bacteria in Intestinal Inflammation

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Abstract

Inflammatory bowel disease (IBD) involves dysregulated immune responses to intestinal microbiota. Intestinal dendritic cells (DC) play a pivotal role in bacterial recognition, tolerance induction, T cell homing and differentiation.

We hypothesized that alterations in human colonic DC are central to the inflammatory process, lymphocyte homing, and therapeutic responses in patients with IBD. Colonic $CD11c^+$ cells have been shown to be activated in IBD but $CD11c^-$ cells have not been characterised. We identified, in ulcerative colitis (UC), a $CD11c^-$ population that had morphological features of DC, expressed MHC class II and Natural Killer cell marker CD56, expressed less activation markers and produced less cytokine, and were weakly stimulatory. Few were plasmacytoid DC. Their number increased in UC and Crohn's disease (CD) but decreased after inflammation resolved.

We explored function and homing properties of colonic $CD11c^+$ DC, and their relationship with intestinal microbiota in IBD. In acute UC, $IL\text{-}10^+$ and $IL\text{-}12p40^+$ $CD11c^+$ DC increased, and fewer $CD11c^+$ DC expressed the homing molecule α-E (CD103). In active CD, $IL\text{-}12p40^+$ DC increased and the ratio of pro:anti-inflammatory bacteria, namely bacteroides:bifidobacteria, correlated positively with $IL\text{-}12p40^+$ DC; $IL\text{-}6^+$ DC also increased and correlated with increased C-reactive protein, but negatively with anti-inflammatory *Faecalibacterium praustnitzii.*

In IBD probiotics and corticosteroids may work, in part, by modulating DC function. In UC patients treated with the probiotic mixture VSL#3, $TLR\text{-}2^+$ DC and $IL\text{-}12p40^+$ DC

decreased while IL-10^{+} DC increased. In patients on corticosteroids similar changes were seen. Such effects were however not seen in patients on placebo.

In conclusion, intestinal inflammation in IBD is associated with novel human colonic cells that share features of DC and NK cells. Intestinal DC function is influenced by composition of the commensal microbiota. Probiotics and corticosteroids are associated with altered "favourable" DC function; these effects may contribute to therapeutic benefit in patients with IBD.

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ABBREVIATIONS

APC	Antigen presenting cells
ASA	Aminosalicylic acid
ATG16L1	Autophagy-related 16-like-1
AZA	Azathioprine
B.	Bifidobacteria
β7	Beta 7 integrin
CD	Crohn's disease
CCR	CC Chemokine Receptor
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLA	Cutaneous leucocyte antigen
CRP	C- reactive protein
CX_3CR1	CX_3C-chemokine receptor 1
CyC	Cytochrome c
DAPI	4' 6-diamidino-2-phenylindole
DC	Dendritic cell(s)
DC-SIGN	Dendritic cell specific ICAM-3 grabbing non-integrin
DNA	Deoxyribonucleic acid

DSS	Dextran sulfate sodium
DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoabsorbent assay
ENS	Enhanced normalised subtraction
EM	Electron microscopy
EPEC	Enteropathic *E. coli*
ER	Endoplasmic reticulum
EREC	*Clostridium coccoides-Eubacterium rectal*
Fab	Fragment of antibody
FACS	Fluorescence assisted cell sorter
F. prausnitzii	*Faecalibacterium prausnitzii*
FCS	Foetal calf serum
FISH	Fluorescence in situ hybridization
FITC	Fluorescent activated cell scanner
Flt3L	Flt3-ligand
FOS	Fructo-oligosaccharide
Foxp3	Forkhead box p3 transcription factor
FSc	Forward scatter
GALT	Gut associated lymphoid tissue
GM-CSF	Granulocyte macrophage colony stimulating factor
GOS	Galacto-oligosaccharide
H	Hour(s)
HBSS	Hank's balance salt solution

HEV	High endothelial venules
HLA	Human leucocyte antigen
ICAM	Intracellular adhesion molecule
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IKDC	IFN-producing killer cells
IL	Interleukin
IR	Intensity ratio
IRGM	Immunity-related GTPase family M
L.	Lactobacillus
LDC	Low density cells
Lin	Lineage cocktail of monoclonal antibody (CD3, CD14, CD16, CD19, CD34)
LPMC	Lamina propria mononuclear cells
LPS	Lipopolysaccharide
MACS	Magnetic cell sorting
MadCAM-1	Mucosal addressin cell adhesion molecule-1
MALT	Mucosa-associated lymphoid tissue
MAP	Mycobacterium avium subspecies paratuberculosis
M cell	Microfold cell
MDP	muramyl dipeptide
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MIF	Macrophage inhibitory factor
Min	Minutes
MLN	Mesenteric lymph node(S)
ml	Mililitre(s)
MLR	Mixed leucocyte reaction
MNC	Mononuclear cells
MoDC	Monocyte-derived dendritic cells
MP	Mercaptopurine
MyD88	Myeloid differentiation primary-response gene 88
NBD	Nucleotide binding domain
NFκB	Nuclear factor kappa-light chain enhancer of activator B cells
NK	Natural killer
NOD	Nucleotide binding oligomerisation domain
ODN	Oligodeoxynucleotides
PAMP	Pathogen associated molecular pattern(s)
PBMC	Peripheral mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1 receptor
PE	Phycoerythrin
PC-Cy5	Phycoerythrin-cyanin 5.1 conjugate
PGN	Peptidoglycan
PIR	Positive intensity ratio
PPAR	Peroxisome proliferator activated receptor
PRR	Pattern recognition receptor

PSC	Primary sclerosing cholangitis
RA	Retinoic acid
RANK	Receptor activator of NFκB
RALDHs	Retinal dehydrogenase
RCT	Randomised controlled trial
RNA	Ribodeoxynucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room temperature (ambient)
SCID	Severe combined immunodeficiency
SED	Super-enhanced D_{max}
SSc	Side scatter
S.	Streptococcus
TCR	T-cell receptor
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNBS	trinitrobenzene sulfuric acid
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
u	Units
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
vs	Versus (compared with)
ZO	Zonula occludens

Chapter 1

General Introduction

1.1 Dendritic Cells

Dendritic cells (DC) are a heterogenous population of uniquely equipped cells that specialized in antigen presentation, and they are widely distributed in small numbers throughout the body. DC act as sentinels, acquiring antigens in peripheral tissues before migrating to secondary lymphoid organs where they interact with lymphocytes (Randolph *et al*., 2005). Their migratory capacity distinguishes them from macrophages (Randolph *et al*., 2008). DC are also sensors, responding to a spectrum of environmental cues by extensive differentiation or maturation. They play a critical role in the initiation and regulation of immune responses (Banchereau *et al*., 2000). Unlike other antigen presenting cells such as B cells and macrophages, DC have the unique ability to stimulate the proliferation and differentiation of both naive and memory T cells. They can determine whether non-responsiveness (tolerance) or an active immune response occurs to a particular antigen, as well as influence whether a T helper (Th) 1, Th 2, Th17 or a regulatory response predominates (Kelsall, 2008; Steinman, 2003). New data have suggested that basophils can also function as professional antigen presenting cells, at least, for the generation of Th2 responses (Wynn, 2009). DC also control tissue specific homing of effector T cells (Mora, 2008).

DC present in all lymphoid organs can be divided into subsets according to their phenotype, anatomical location and functions (Dudziak *et al*., 2007). These subsets may display distinct predetermined functions and plasticity depending on their local environment. Several intricate and innate properties account for their sentinel and sensor roles in the immune system. Firstly they have special mechanisms for antigen capture and processing; secondly they are able to migrate to defined sites in lymphoid organs to initiate immunity, and lastly, they can rapidly differentiate or mature in response to a

spectrum of stimuli ranging from Toll-like receptor (TLR) ligands to many other non-microbial factors such as cytokines, innate lymphocytes, and immune complexes (Steinman and Banchereau, 2007).

DC were first described in 1868 by a medical student, Paul Langerhans. The "Langerhans cell" was initially observed in gold-stained human skin and had DC like projections in their cytoplasm (Langerhans, 1868); these cells have now been identified as a unique subset of DC that contained Birbeck's granules in their cytoplasms (Birbeck *et al*., 1961). In 1973, Steinman and Cohn discovered and characterised an unsual population of cells that were widely distributed in the lymphoid organs. In the murine spleen these large stellate cells had properties distinct from those of phagocytes, lymphocytes and granulocytes. They had large nuclei but lacked features of active endocytosis and their cytoplasms bore pseudopods of various forms, sizes and numbers; they were therefore named "dendritic cell" (Steinman and Cohn, 1973). Steinman and Cohn showed that DC expressed major histocompatibility complex (MHC) class II molecules required for antigen presentation and stimulated mixed lymphocyte response (Steinman *et al*., 1983). They later demonstrated that DC exist in human as well as in mice (Banchereau and Steinman, 1998; Van Voorhis *et al*., 1982). These enriched splenic cells, now known as conventional DC, are present in all lymphoid organs (Dudziak *et al*., 2007). Until the 1990s, progress in the DC field has been slow due to the small numbers of DC *in vivo,* the lack of markers that distinguish them from macrophages or monocytes, and the technical difficulties in purifying DC. However in the last decade, methods have been developed to isolate and generate DC from the blood and bone marrow, and studies have begun to establish the role of DC in the maintenance of immune homeostasis and their importance in human diseases (Caux *et al*., 1992; Inaba *et al*., 1992; Sallusto and Lanzavecchia, 1994;

Steinman, 2003; Steinman and Banchereau, 2007). Multiple DC subsets have been identified in mice and humans; these subsets differ in cellular origin, half life in peripheral tissues, and possibly, the mechanism by which they are renewed (Ardavin, 2003; Geissmann, 2007; Shortman and Liu, 2002).

1.1.1 Dendritic Cell Origin, Development and Subsets

Human DC originate from haematopoietic stems cells in the bone marrow and occasional peripheral blood stem cells (Geissmann, 2007; Inaba *et al*., 1993; Katz *et al*., 1979; Reid, 1997). DC develop *in vivo* from immature precursors into two major populations: "conventional" or myeloid DC, and plasmacytoid DC. DC development from haematopoetic progenitors is associated with Flt3 expression and their ability to respond to Flt3-ligand (Flt3L) (D'Amico and Wu, 2003; Karsunky *et al*., 2003). Flt3L enhances the generation of both myeloid DC and plasmacytoid DC *in vitro* (Blom *et al*., 2000) and *in vivo* (Maraskovsky *et al*., 2000; Pulendran *et al*., 2000). In healthy human volunteers administration of Flt3L increased the frequencies of blood myeloid and plasmacytoid DC (Pulendran *et al*., 2000). In contrast, Flt3L-deficient mice showed a decrease in numbers of DC in both peripheral and lymphoid tissues (McKenna *et al*., 2000). These data indicate that Flt3L is a critical factor for DC development in both humans and mice. **Figure 1.1** illustrates the origin and development of DC in steady state conditions.

Myeloid DC - *In vivo,* human myeloid DC can be divided into *peripheral tissue resident DC, secondary lymphoid organ resident DC*, and *circulating blood myeloid DC* (Merad and Manz, 2009). In the skin epidermis myeloid precursors differentiate into $CD11c^{+}$

$CD1a^+$ Langerhan cells whereas in other tissues they differentiate into $CD11c^+$ $CD1a^-$ interstitial/dermal cells (Caux *et al*., 1997; Ito *et al*., 1999; Strunk *et al*., 1997). Skin DC subsets express different sets of molecules. Epidermal Langerhan cells express Langerin, CD1a and E-Cadherin, while dermal DC express DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), CD11b, factor XIIIa, and CD14 (Merad *et al*., 2008). DC in secondary lymphoid tissues are the most studied DC population in mice, but little information is available on the human counterparts. In the steady state, peripheral and lymphoid tissue DC are maintained by circulating blood precursors from the bone-marrow, with the exception of Langerhans cells which can repopulate locally through self-renewal (Merad and Manz, 2009). Myeloid DC maintain self tolerance and induce immune response against invading pathogens (Banchereau and Steinman, 1998; Steinman, 2003).

Most studies with human myeloid DC subsets have been performed with *in vitro*-generated DC. Human myeloid DC can be derived from peripheral blood monocytes *in vitro* by stimulation with granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-4 (Sallusto and Lanzavecchia, 1994), or following transmigration through endothelial cells and phagocytosis (Randolph *et al*., 1998). These cells express CD11c and other myeloid markers such as CD11b, CD33, CD13, and they lack expression of lymphoid markers. They are $CD45RA^-$ and $CD45RO^+$ and express low levels of IL-3 receptor (CD123), and are immature based on their low stimulatory capacity and low expression of co-stimulatory molecules. When these cells are cultured, they upregulate co-stimulatory molecules and become highly stimulatory (Sallusto and Lanzavecchia, 1994).

Plasmacytoid DC - Human plasmacytoid DC express very low or no levels of CD11c. Instead, they express CD4 and CD45RA, the c-type lectin receptor, BDCA-2, and the neuronal receptor, BDCA-4, as well as high levels of CD123 (Banchereau and Steinman, 1998; Shortman and Liu, 2002). Plasmacytoid DC express little GM-CSF receptor and require activation before displaying characteristic stimulatory activity *in vitro*, and they can express TLR-9 that responds to bacterial deoxyribonucleic acid (DNA) and demethylated CpG deoxyoligonucleotides (Bauer *et al*., 2001; Krug *et al*., 2001a; Krug *et al*., 2001b). In the steady state, they circulate in the blood and can be found in the bone marrow, spleen, thymus, liver and lymph nodes. Human and mouse plasmacytoid DC enter the lymph nodes through high endothelial venules (HEV) (Yoneyama *et al*., 2004) to accumulate in the paracortical T cell-rich regions (Colonna *et al*., 2004). In the spleen and lymph nodes, plasmacytoid DC have a short lifespan and continuous replacement from the blood is necessary (Merad and Manz, 2009). *In vitro,* they act as antigen presenting cells (APC) but their role in T cell priming *in vivo* has not been established. They are known for their role in secreting large amount of type 1 interferon (IFN) upon viral exposure (Cella *et al*., 2000; Grouard *et al*., 1997; Liu, 2005). Recently, IFN-producing killer cells (IKDC) have been identified as a novel subset of mouse plasmacytoid DC that is capable of killing target cells and secreting large quantities of type 1 and 2 IFN (Chan *et al*., 2006; Taieb *et al*., 2006).

Blood DC have been mostly studied in humans and contains both myeloid and plasmacytoid DC. These cells have been characterized as human leucocyte antigen (HLA)-DR^+ Lineage $(lin)^-$ [Lineage = $CD3^-$, $CD19^-$, $CD14^-$, $CD16^-$, $CD34^-$, $CD56^-$) cells with the expression of CD11c in myeloid DC, and the expression of IL-3Ra chain and CD123 in plasmacytoid DC (Olweus *et al*., 1997; Siegal *et al*., 1999). During

inflammation, plasmacytoid DC appear to migrate directly into the inflamed secondary lymphoid tissues through HEV (Yoneyama *et al*., 2004), while myeloid DC migrate into the secondary lymphoid tissues via afferent lymph at inflammatory sites (Randolph *et al*., 2005; Randolph *et al*., 2008)

The factors regulating DC development and homeostasis, and the nature of DC precursor that migrates from bone marrow to peripheral lymphoid organ are incompletely understood. These questions are difficult for several reasons; firstly, monocytes can develop phenotypic characteristics of DC *in vivo* during inflammation or *in vitro* in the presence of cytokines (Geissmann, 2007; Randolph *et al*., 1998). Secondly, monocytes, plasmacytoid DC and myeloid DC in lymphoid tissue share a common progenitor known as the macrophage and DC precursor (Fogg *et al*., 2006; Varol *et al*., 2007), and lastly DC subsets are functionally and phenotypically diverse (Shortman and Liu, 2002). Recent murine data suggest that in the steady state, monocytes do not develop into myeloid DC; instead they only make a minor contribution to the lymphoid-organ DC network (Geissmann, 2007).

Traditionally, many DC subsets have been thought of as non-dividing terminally differentiated cells, generated from the bone marrow, that circulate in the blood as committed precursors, where they migrate to peripheral tissues to maintain the DC pool (Geissmann, 2007). Two studies have now challenged this view (Bogunovic *et al*., 2006; Kabashima *et al*., 2005); Kabashima *et al.* showed that in parabiotic mice, only about five percent of splenic DC were undergoing cell division at any time, and only a small proportion was replenished by circulating precursors over a six week period (Kabashima *et al*., 2005), whereas Bogunovic *et al.* showed that dermal DC proliferated *in situ* in mice

and normal human dermis (Bogunovic *et al*., 2006). These results support a new paradigm that tissue-resident DC have local proliferation properties in the steady state. In contrast, Liu *et al.* recently showed that spleen and lymph node DC from parabiotic mice were indeed continually replaced by blood borne precursors but that these precursors had a short half-life and were rapidly cleared from the circulation. They also revealed that regulatory T cells control DC development in the peripheral lymphoid organs in a Flt3-dependent manner (Liu *et al*., 2009). Taken together these studies suggest that tissue DC subsets probably have different half lives and could be maintained or be renewed through replenishment from bone marrow precursors, or through the proliferation of resident or local progenitors.

There is no single specific marker in humans that identifies all DC, but they can be recognised by excluding other cell types. In human, these cells are characterised by their high expression of HLA-DR and they lack the expression of lineage markers; T cells (CD3), B cells (CD19), monocytes (CD14), macrophages (CD16), progenitor cells (CD34) and natural killer (NK) cells (CD56) (Bell *et al*., 2001). Throughout this thesis, human DC have been defined as HLA-DR^+ Lin^- cells.

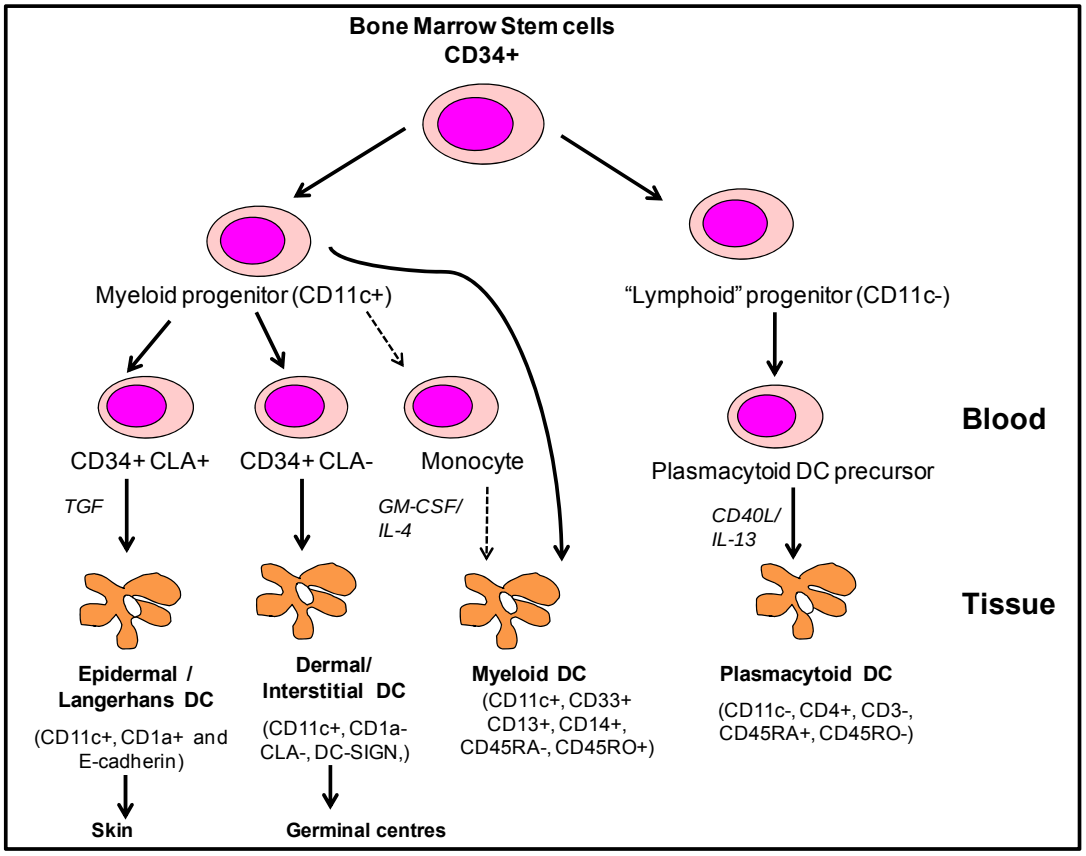

Figure 1.1: Dendritic cell (DC) origin and development

Haematopoietic stem cell produces myeloid and lymphoid DC progenitors in the bone marrow, following mobilisation by Fit3L. In the skin, two myeloid DC subsets, Langerhans cells and interstitial cells, are present. Blood contains two major DC subsets, myeloid and plasmacytoid DC. New data suggest that most conventional or myeloid DC in lymphoid tissue originate, without a monocytic intermediate, from bone marrow-derived myeloid precursor (Merad and Manz, 2009). Plasmacytoid DC originate from lymphoid progenitors and enter lymphoid tissues via high endothelial venules.

1.1.2 Dendritic Cell Maturation

A major feature of the life history of DC is maturation. Maturation is used to describe the intricate differentiation process whereby DC respond to environmental stimuli, acquire potent immunostimulatory functions and subsequently elicit an adaptive immune response.

This process involves the loss of their endocytic and phagocytic receptors (Garrett *et al*., 2000), the extension of dendrites to expand surface area for T cell interaction, the up-regulation of surface MHC class II and co-stimulatory molecules (eg. CD40, CD80, CD86), and a change in chemokine receptor expression from chemokine receptor CCR6 to CCR7, to promote DC migration to lymphoid tissues (Sallusto *et al*., 1999; Sallusto, 1999). In concert with these processes, DC acquire the ability to activate naive T cells. For example, during inflammation or infection, DC can be activated directly by interacting with pattern recognition receptors (PRR) of pathogens, or indirectly (Iwasaki and Medzhitov, 2004), by exposure to inflammatory cytokines such as α, type 1-IFN, or IL-1 (Honda *et al*., 2003). Exposure to pathogen components results in fully activated DC that promote Th differentiation. In contrast, indirect activation by inflammatory mediators generates DC to support T cell clonal expansion (Sporri and Reis e Sousa, 2005).

When DC encounter microbial stimuli, maturation may also occur following their interaction with CD40-CD40 ligands, resulting in cytokine release and prolongation of DC survival (Banchereau *et al*., 2000; Kapsenberg, 2003; Reis e Sousa *et al*., 2003; Rescigno, 2002). Signalling through receptor activator of nuclear factor kappa-light chain enhancers of activated B cells (NFkB) and RANK, by the RANK ligand (RANKL) on T cell surface promotes DC survival whereas TNF-related apoptosis-inducing ligand (TRAIL), a TNF family member produced by activated T cells induces DC apoptosis via the TRAIL receptor. In addition chemokines directly provide maturation signals to DC. For instance CCL19, a chemokine receptor CCR7 ligand, induces maturation of activated murine DC, but not those in the steady state, as well as rapid endocytosis and dendrite formation (Yanagawa and Onoe, 2002; Yanagawa and Onoe, 2003).

Innate lymphocytes, NK cells, NKT cells and γδ T cells can also trigger DC maturation (Reschner *et al*., 2008). This cross talk between DC and innate lymphocytes occurs largely in secondary lymphoid organs, which leads to innate lymphocyte activation and DC maturation. This process is multi-directional, involving not only cell-to-cell contact but also the presence of soluble factors (Reschner *et al*., 2008). Initially innate cells induce DC maturation via cell contact mechanisms, DC then enhance and expand the numbers of innate lymphocytes. Lastly, maturing DC process antigens from cells lysed by innate lymphocytes to elicit T cell adaptive immunity. The interaction of DC with innate lymphocytes appears to represent a major control mechanism for immunity that is independent of TLR ligands (Munz *et al*., 2005).

1.1.3 Dendritic Cell Migration and Recruitment

DC migration is dependent upon the coordinate expression of distinct chemokine receptors that direct immature precursor cells into the peripheral tissues and translocation of mature DC in regional lymphoid follicles (Randolph *et al*., 2008). In the absence of inflammation or infection, immature or "steady state" DC within tissues are continuously on patrol and migrate to organised lymphoid tissues via the lymphatic channels as non-activated or partially activated cells; these immature cells present self-antigens for the induction and maintenance of self-tolerance (Steinman *et al*., 2000; Steinman *et al*., 2003). These cells process self antigens which silences potentially autoreactive T cells that may have escaped thymic deletion (Lutz and Schuler, 2002; Steinman and Nussenzweig, 2002). Non-responsiveness is not only confined to self antigens but can also be induced to foreign antigens (Hawiger *et al*., 2001). Immature tissue DC are endocytically active but express

low levels of MHC class II and co-stimulatory molecules, and are weak stimulators of T cell proliferation (Coombes and Maloy, 2007).

In contrast, when exposed to inflammatory signals or microbial products, DC mature and migrate at higher rates to secondary organised lymphoid tissue where stimulation of naive T cells takes place. This pathway is mediated by the interaction between PRR, such as TLR on DC, and pathogen-associated molecular patterns (PAMPs) present on the surface of microrganisms (Kaisho and Akira, 2003). When migrating from peripheral tissues to lymph nodes DC mature by which they downregulate their antigen acquisition machinery and enhance ability to stimulate T cells (Banchereau and Steinman, 1998). Inflammatory cues also tigger maturation of DC and concomittant upregulation of CCR7, the chemokine receptor centrally require for DC migration to lymph nodes (Forster *et al*., 1999; Sallusto *et al*., 1999).

Historically, all lymph node DC were thought to migrate from upstream tisues via afferent lymphatic channels, based on several observations: DC were abundant in cannulated afferent lymph and were involved in transporting antigens, whereas commited DC precursors were sparse in the circulation of humans (Steinman, 1991). When afferent lymphatics were severed, lymph nodes were voided of DC and antigen presentation was inhibited (Mebius *et al*., 1991). Some DC populations have now been shown to enter lymph nodes directly from the circulation, via specialised high endothelial venules (HEV), notably plasmacytoid DC (Cella *et al*., 1999; Villadangos and Schnorrer, 2007) and mouse $CD8\alpha^+$ DC (Kamath *et al*., 2002; Randolph *et al*., 2008). However, their true trafficking patterns remain to be determined. In the steady state, DC in lymph nodes do not orginate

from upstream tissue DC, but it appears that during inflammation tissue-derived DC comprise a major fraction of lymph node DC (Jakubzick *et al*., 2008).

Different chemokines receptors are important for the process of DC migration; CCR2 is involved in splenic DC translocation into T cell rich areas of lymphoid tissues during infection with *Leishmania Major* (Sato *et al*., 2000). In the intestine, CCR2 may be responsible for recruitment of circulating blood DC to the epithelium and lamina propria (Vanbervliet *et al*., 2002). CCR5 is important in DC recruitment to inflammatory sites (Aliberti *et al*., 2000; Sallusto *et al*., 1998). CCR6 is essential for DC positioning at epithelial surfaces (Cook *et al*., 2000; Vanbervliet *et al*., 2002) and CCR7 drives the migration of lamina propria DC to mesenteric lymph nodes (MLN) (Dieu *et al*., 1998; Forster *et al*., 1999; Jang *et al*., 2006; Sallusto *et al*., 1999). In the mouse, mucosal DC drive intestinal immune compartmentalisation by imprinting α4 Beta7 (β7) integrin and CCR9 on T cells that they activate resulting in homing to the small intestine (Mora *et al*., 2003; Stagg *et al*., 2002).

In summary, the trafficking patterns of DC are highly regulated and differ among different types of DC and these processes are influenced by distinct signalling cascade. Many DC from peripheral tissue enter lymph nodes through the afferent lymph, whilst mouse $CD8\alpha^+$ DC and plasmacytoid DC enter lymph nodes through HEV. Only a few DC enter blood either through reentry into venules present within peripheral tissues or via escape into efferent lymph (Randolph *et al*., 2008).

1.1.4 Stimulation of Adaptive Immunity

DC can contribute to the expansion and differentiation of most classes of lymphocytes (Ueno *et al*., 2007). Apart from T cells, they play a role in the differentiation of B cells, innate NK, (Gerosa *et al*., 2002) and NKT cells (Vincent *et al*., 2002) although little is known about the mechanism for stimulating these other lymphocytes. Both the type of DC and its maturational status influence the subsequent T cell responses and their effects on adaptive immunity. In addition to driving the clonal expansion of proliferative T cells, DC can shape the functional differentiation of dividing T cells.

Over 20 years ago, Mossman and Coffman showed that effector $CD4^+$ T cells differentiated into two subsets, T helper (Th) 1 and Th2 cells based on distinct patterns of cytokine production (Mosmann *et al*., 1986). Most recently the identification of an additional subset, known as Th17 cells, has further illustrated the complexity and diversity of effector $CD4^+$ T cells (Maloy and Kullberg, 2008).

Th1 cells secrete high levels of IL-2, GM-CSF, TNF-α and IFN-γ which are instrumental cytokines in cell-mediated immunity against intracellular virus, bacteria or protozoa and/or promotion of cytoxocity in NK cells and $CD8^+$ T cells (Mosmann *et al*., 1986). Signal transducer and activator transcription (STAT) proteins are pivotal in the signalling of Th subset differentiation. STAT-1 and STAT-4 maintain and amplify the Th1 responses (Kaplan *et al*., 1996; Meraz *et al*., 1996). The production of IL-12 by DC has been shown to drive a Th1 response (Macatonia *et al*., 1995). IL-12, a heterodimeric cytokine, composed of two subunits known as IL-12p35 and IL-12p40, induces and maintains Th1 cells (Oppmann *et al*., 2000). IL-12 is closely related to IL-23, a relatively recent

discovery, which is composed of IL-23p40 and the unique IL-23p19 subunit (Kastelein *et al*., 2007; Oppmann *et al*., 2000). Studies in murine models of autoimmune diseases and inflammatory bowel disease (IBD) demonstrated that IL-23 played a key role in driving autoimmune tissue pathology (Cua *et al*., 2003; Murphy *et al*., 2003), and intestinal inflammation (Hue *et al*., 2006). The autoimmune and inflammatory processes were associated with the production of a novel subset of $CD4^+$ T cell secreting IL-17A, subsequently termed Th17 cells (Bettelli *et al*., 2008; Langrish *et al*., 2004; Weaver *et al*., 2007).

IL-23 is secreted by DC in response to microbial stimulation but the factors that determine whether an activated DC produce IL-12 or IL-23 remain unclear. These responses may be dependent on distinct expression of PRR and associated signal transduction pathways (Maloy and Kullberg, 2008). For instance, commensal gram-negative bacteria or peptidoglycan activation of TLR2 or nucleotide binding oligomerisation domain (NOD) pathways resulted in IL-23 production (Smits *et al*., 2004; van Beelen *et al*., 2007). Another IL-12 related cytokine member includes IL-27 which consists of p28 and EBI3 subunits. IL-27 is produced by DC and it induces the proliferation of naive T cells (Pflanz *et al*., 2002; Smits *et al*., 2004).

Th2 cells predominantly secrete IL-4, IL-5 and IL-13 and drive humoral immunity directed towards clearing extracellular pathogens (Mosmann and Coffman, 1989). They stimulate B cells to produce immunoglobulin (IgG)-E and enhance the maturation of eosinophils and the degranulation of mast cells and basophils. STAT-6 activation is necessary for Th2 development (Kaplan *et al*., 1996). The production of IL-4 and IL-10 by DC contributes to a Th2 response; these cytokines are important against multicellular

parasites, such as helminths. However, three recent papers showed that DC were not required for the generation of $CD4^+$ Th2 responses to protease allergens (Sokol *et al*., 2009a), helminths (Perrigoue *et al*., 2009), or antigen IgG-E complexes *in vivo* (Yoshimoto *et al*., 2009). Instead, basophils that expressed MHC-II and produced IL-4 were required and sufficient for the generation of Th2 immunity by functioning as APC in draining lymph nodes (Wynn, 2009).

Th17 cells represent a new heterogenous population, with effector functions distinct from those of Th1 and Th2 cells, which is capable of secreting a diverse range of cytokines including IL-6, IL-17A, IL-17F, IL-21, IL-22, IFN-γ and TNF-α (Bettelli *et al*., 2008; McGeachy and Cua, 2008; Weaver *et al*., 2007). It is primarily characterised by the secretion of the cytokine IL-17. Human Th17 cells may produce IL-26 and CCL20 (Wilson *et al*., 2007). IL-23 is no longer necessary to induce differentiation of Th17 cells. TGF-β together with IL-6 or IL-21 initiates the differentiation of Th17 cells, while IL-23 may act as an expansion or maintenance factor for Th17 cells (Awasthi and Kuchroo, 2009; Langrish *et al*., 2005; McGeachy and Cua, 2008). TGF-β is essential for the production of both Th17 and regulatory T cells (Treg) from naive T cells (Maloy and Kullberg, 2008). *In vitro* Treg cells can promote the development of Th17 cells in a TGF-β dependent manner (Awasthi *et al*., 2008; Xu *et al*., 2007). The generation of Th17 cells also involves STAT-3 and retinoic-acid receptor related orphan receptor γt (RORγt). In the intestine, the precise relationship between Treg and Th17 cell lineages, and the functions of Th-17 associated cytokines remains unclear. The local cytokine microenvironment is likely to determine the tissue protective and pro-inflammatory roles of Th17 cells (Maloy, 2008; Maloy and Kullberg, 2008).

DC can also promote negative selection in the thymus, (Brocker *et al*., 1997) and influence peripheral tolerance and the generation of ***regulatory T cells (Treg)*** (Dhodapkar and Steinman, 2002). Treg is characterised by the expression of forkhead box transcription factor (Fox) p3 the and high expression of CD25. Treg can downregulate several types of immune responses (Curotto de Lafaille and Lafaille, 2009). Naturally occurring $CD4^+$ $CD25^+$ Treg cells that express Foxp3 differentiate in the thymus and migrate to peripheral tissues with their regulatory function already intact (Hori *et al*., 2003), whereas adaptive or induced $Foxp3^+$ Treg cells differentiate in secondary lymphoid organs and tissues from naïve $CD4^+$ T cells following the oral administration of antigens (Coombes and Powrie, 2008; Curotto de Lafaille and Lafaille, 2009). Cytokines that induce the differentiation of Th1, Th2 or Th17 cells antagonise $Foxp3^+$ Treg cell differentiation (Zhou *et al*., 2009). In the mouse, lamina propria and MLN $CD103^+$ DC produce both TGF-β and retinoic acid (RA) and mediate the differentiation of naïve T cells into $Foxp3^+$ Treg cells in the absence of any exogenous factors (Coombes *et al*., 2007; Sun *et al*., 2007). However this feature was not common to $CD103^-$ DC in the MLN.

Distinction between various Th cell subsets, however, is not absolute and T cell subsets that produce intermediate patterns of cytokine exist (Gor *et al*., 2003; Lyakh *et al*., 2008). In summary, DC play an integral role in shaping the nature of immune response in the gut. DC conditioning in the intestine are influenced by local environmental factors. In the steady state, commensal bacteria signaling via PRR activates NFκB expression on intestinal epithelial cells, which act on DC to downregulate IL-12/23p40 production in response to microbial stimuli. IL-10 and TGF-β can limit the responsiveness of intestinal DC to bacteria signals. Defective conditioning of DC in the intestine may result in intestinal inflammation.

1.2 Intestinal Dendritic Cells and Immune Regulation

The gastrointestinal (GI) tract is in constant contact with a dynamic and diverse luminal environment that contains an enormous number of commensal bacteria and potentially a variety of pathogens (Guarner and Malagelada, 2003). In this highly antigenic environment, the ability to maintain immune tolerance to commensal bacteria or self antigens as well as to mount effector responses to invading pathogens is a key feature of the gut immune system (Macpherson *et al*., 2005). Mucosal DC located at the intersection between the innate and adaptive immune systems are likely to be central to this process. They survey the microbial environment, coordinate immune responses to danger signals (Niess and Reinecker, 2005), and prime naive T cells to control overwhelming infections or tissue inflammation (Nagler-Anderson, 2001; Steinman *et al*., 2003). The decision between the induction of tolerance and active immunity depends on the subpopulation of DC and the surface receptors involved, and the tissue environment during DC activation and T cell priming. During the steady state, DC traffic through intestinal tissues with the turnover time of a few days (Pugh *et al*., 1983).

Within the GI tract DC are found in organised lymphoid tissue; this includes the Peyer's patches and MLN where the induction of T and B cell response occur, and within generalised tissues interspersed with effector cells which include the lamina propria and intestinal epithelium (Iwasaki and Kelsall, 2001; Maric *et al*., 1996). DC in intestinal tissues include conventional $CD11c^{+}$ DC and plasmacytoid DC (Iwasaki, 2007). A previously unrecognised network of DC that respond to microbial stimuli and upregulate CD80 and CD86 *in vivo* has been identified within the muscular layer of the mouse intestine (Flores-Langarica *et al*., 2005).

As in other tissues, intestinal DC comprise a heterogenous population. Although diverse DC lineages with distinct morphology have been recognised in the lamina propria and organised lymphoid tissues, their specific role in antigen sampling and presentation remains largely unknown.

In the murine intestine, three subsets of $CD11c^+$ DC have been described in the MLN and Peyer's patches: $CD11b^+$ $CD8\alpha^-$ DC in the subepithelial dome, $CD11b^-$ $CD8\alpha^+$ in the interfollicular regions and $CD11b^-$ $CD8\alpha^-$ (double negative) subsets in both areas (Iwasaki and Kelsall, 2000; Iwasaki and Kelsall, 2001). DC from Peyer's patches can also be described in terms of their expression of the chemokine receptors CX3C-chemokine receptor 1 (CX3CR1) and CCR-6 (Niess *et al*., 2005; Salazar-Gonzalez *et al*., 2006). Myeloid-derived mucosal DC in mice expressed the chemokine receptor CX3CR1 to form transepithelial dendrites for direct sampling of luminal antigens (Niess *et al*., 2005). CX3CR1 controlled the clearance of entero-invasive pathogens by DC, suggesting that specialised mucosal DC control host bacteria interaction via a CX3CR1-dependent process (Niess *et al*., 2005). The murine small intestinal lamina propria also contains similar DC subsets to Peyer's patches DC apart from $CD8\alpha^+$ DC (Niess *et al*., 2005). Lamina propria DC that express the integrin CD103 or αE have been identified in the mouse intestine (Annacker *et al*., 2005; Johansson-Lindbom *et al*., 2005) and their functions will be discussed in the next section 1.2.4.

In the human colon, lamina propria DC are characterised by their expression of CD83 and DC-SIGN and they have the phenotype of $CD11c^+$ Lin^- with an immature state and low TLR-2 and -4 expression compared with their blood counterparts (Bell *et al*., 2001). In the

human intestinal lamina propria, DC are largely HLA-DR^{+} CD11c^{+} lin^{-} myeloid cells with few identifiable plasmacytoid DC (CD11c^{-} CD123^{+}) (Bell *et al*., 2001; Hart *et al*., 2005).

During inflammation, DC are recruited to the intestine but whether these cells represent a separate lineage to those already present in the steady state, and whether the ability of these "inflammatory DC" to drive pro-inflammatory responses relate to their exposure to microbial signals and pro-inflammatory cytokines on arrival in the intestine remains unclear (Coombes and Powrie, 2008). The tissue microenvironment, among other factors, influences DC responses to stimulation. In addition, the subsets, phenotype and functions of DC are likely to be influenced by tissue specific factors produced by intraepithelial lymphocytes and epithelial cells in response to signals from commensal or pathogenic microorganism (Rescigno *et al*., 2008; Rescigno, 2008; Rimoldi *et al*., 2005). DC display regional specialisation that enables them to function appropriately in diverse settings.

Macrophages are present in high numbers in the intestinal tract of mice and humans. Both DC and macrophages are members of the mononuclear phagocyte systems. Intestinal DC can be distinguished from macrophages based on their dendritic morphology, their ability to capture, process and present antigens to naïve T cells (Kelsall, 2008). Macrophages, on the other hand, capture and kill microbes, scavenge apoptotic cells and secrete regulatory cytokines. However, the phenotype and functions of DC and macrophages can vary in the presence of inflammation or exposure to microbial stimuli (Kelsall, 2008).

In summary, intestinal DC are flexible. They have unique functions compared with DC from other sites. These properties include the shaping of intestinal immune responses

mediated by environmental cues, the imprinting of mucosal homing receptors on T and B cells, and the induction of regulatory cells to soluble antigens.

1.2.1 Sampling of Luminal Antigen by Intestinal Dendritic Cells

DC are present as immature cells with high phagocytic activity within peripheral tissues and discrete regions of organised secondary lymphoid organs. Immature DC act as sentinels of the immune system where they constantly sample foreign and self antigens from the intestinal lumen (Liu and MacPherson, 1993).

Within the GI tract, several pathways have been described by which DC can sample and process specific antigens or pathogens. Traditionally the main route for microorganisms or macromolecules to gain access to the mucosal immune system is via specialised epithelial cells, known as M (microfold) cells (Kraehenbuhl and Neutra, 2000). These cells are scattered among conventional epithelial cells overlying the dome of the Peyer's patches follicle and they shuttle luminal antigens to DC in the subepithelial dome regions. In this regard, DC have been shown to phagocytose orally administered *Salmonella typhimurium* into the tissue via the M cells (Hopkins *et al*., 2000; Hopkins and Kraehenbuhl, 1997). DC are early cellular targets for *Listeria monocytogenes* infections in rats, and are responsible for bacterial spread to the host (Pron *et al*., 2001). A population of $CD11c^{+}$ $CD11b^{-}$ $CD8^{-}$ DC residing in the subepithelial dome take up fluorescent polystyrene microparticles given orally and subsequently migrate to B cell follicles or T cell parafollicular zones following the ingestion of *cholera toxin* or live salmonella bacteria (Shreedhar *et al*., 2003). Macrophages in close proximity were however not involved in this process. These data

suggest that DC can migrate in response to enterotoxin adjuvants and live bacteria that enter the mucosa via M cells (Shreedhar *et al*., 2003). Antigen uptake by M cells can be enhanced by IgA (Neutra and Kraehenbuhl, 1993; Weltzin *et al*., 1989). The observation that DC express IgA receptors suggests that this interaction may help to increase antigen uptake (Geissmann *et al*., 2001; Heystek *et al*., 2002). M cells covering the Peyer's patches express chemokines such as CCL20 and CXCL16 which may facilitate DC migration (Hase *et al*., 2006; Iwasaki and Kelsall, 2000).

Secondly DC may acquire antigen indirectly via the internalisation of apoptotic epithelial cells (Huang *et al*., 2000). Alternatively they may take up antigen exosomes shed from epithelial cells (Karlsson *et al*., 2001; Van *et al*., 2001). In addition DC in the subepithelial follicles can sample antigens directly from apoptotic epithelial cells. For instance, in mice infected with type 1 Reovirus, $CD11c^{+}$ $CD11b^{-}$ $CD8^{-}$ $CD4^{-}$ DC in the Peyer's patches capture viral antigens from infected apoptotic epithelial cells (Fleeton *et al*., 2004).

DC within the lamina propria can sample luminal contents directly by opening tight junctions between enterocytes and extending dendrites into the gut lumen, an observation that was first made in the 1970s through electron-microscopic (EM) studies of rat ileum (Collan, 1972; Rescigno *et al*., 2001a). This process of sampling by DC has now been demonstrated in the terminal ileum of mice both in the steady state (Niess *et al*., 2005), as well as in studies of bacterial uptake *in vivo* by *Salmonella typhimurium* and non pathogenic *Escherichia coli (E. Coli)* (Rescigno *et al*., 2001a). The formation of transepithelial dendrites is thought to require myeloid differentiation primary response gene 88 (MyD88)-dependent signalling through TLR and the expression of CX3CR1 (Chieppa *et al*., 2006; Niess *et al*., 2005). These lamina propria DC may include subsets

that express IL-12p40 and IL-23p19, suggesting that they may be producers of IL-23 (Becker *et al*., 2003). It is likely that antigen sampling is induced by signals from epithelial cells that have been in contact with luminal bacteria. **Figure 1.2** shows an electron microscopy picture of a human lamina propria colonic DC projecting dendrites across epithelium into the intestinal lumen.

Finally, the identification of a population of DC within the epithelial layers supports the notion that bacteria may gain access to lamina propria DC directly following the breakdown of epithelial barrier by infection and/or inflammation, as seen IBD (Maric *et al*., 1996). Here DC penetrate the epithelium and are in direct competition with epithelial cells in the processing and presentation of antigens to intraepithelial lymphocytes (Rescigno *et al*., 2001b). Intestinal epithelial cells may also be involved directly with antigen uptake pathways by delivering antigens or exosomes to lamina propria DC (Morelli, 2006). Intestinal epithelial cells express lectins, NOD 1 and 2, which helps with the recognition of bacterial products (Cario *et al*., 2000; Hisamatsu *et al*., 2003; Inohara *et al*., 2001).

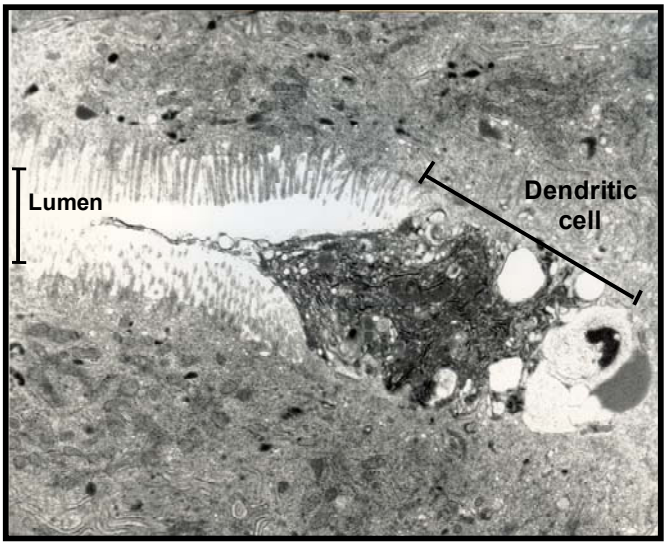

Figure 1.2: Electron Microscopy (EM) of a human lamina propria DC
This EM picture shows a human colonic lamina propria DC from a control subject, projecting dendrites into the intestinal lumen.

1.2.2 Induction of Tolerance and Active Immunity by Intestinal Dendritic Cells

Oral tolerance induction is a key feature of intestinal DC in generating systemic non-responsiveness to particular antigens. During the "steady state" such as in the absence of inflammation, infection or vaccination with mucosal adjuvants, intestinal DC migrate constitutively from the lamina propria and /or Peyer's patches to the MLN. When DC are activated, this process of migration occurs at a faster rate. However the nature of DC migrating in the "steady state" versus the "stimulated" state remains unclear. It is likely that "steady state" mucosal DC are involved in tolerance induction via the induction of regulatory T cells that produce TGF-β or IL-10, or via functional T cell anergy/deletion. In contrast, upon exposure to pathogens or cytokines such as IL-1 or TNF-α, activated DC that migrate to secondary lymphoid tissues become inducers of effector cells (Cerovic *et al*., 2009). One recent study, however, has shown that DC which migrate from the intestine in the steady state, are paradoxically able to induce strong inflammatory

responses from naive T cells, despite their role in the maintenance of oral tolerance (Milling *et al*., 2009). Intestinal lymphatic DC from the thoracic ducts of rats stimulated strong proliferative responses, induced secretion of IFN-γ and proliferation of FoxP3-positive lymphocytes (Milling *et al*., 2009).

Enhanced tolerance to intestinal antigens is observed after expansion of DC following stimulation with Fit3-ligand (Viney *et al*., 1998). Various pathways have been described by which DC achieved tolerance which involves the interaction between commensal bacteria and distinct DC populations, both in the periphery and local mucosal tissue. The induction of tolerance by DC can be achieved by the downregulation of CD80 and CD86 (Steinman *et al*., 2003), the interaction between DC and novel co-stimulatory molecules such as CD200 and CD200R (Gorczynski *et al*., 2005), and the signalling through novel receptor ligand interactions (Hoyne *et al*., 2000). In addition induction of tolerance can be achieved by the control of T cell proliferation through the release of immunosuppressive cytokines, IL-10, TGF-β and IFN-γ (Levings *et al*., 2001; Levings *et al*., 2002; Yamagiwa *et al*., 2001), and the production of metabolites such as indoleamine 2,3 deoxygenase (IDO) (Grohmann *et al*., 2000).

Worbs *et al.* showed that in mice after the administration of oral soluble antigens, antigens were transported via afferent lymphatics by DC into the draining MLN, this process was obligatory for the induction of oral tolerance (Worbs *et al*., 2006). The majority of DC entering the MLN originated from the lamina propria suggesting that these were the cells likely to contribute to antigen presentation for oral tolerance induction (Bimczok *et al*., 2005; Turnbull *et al*., 2005).

The same DC population derived from different tissues can exhibit different responses to the same stimuli. For example, in the mouse, mucosally-derived plasmacytoid DC induces differentiation of "T reg" like cells that release IL-10 and IL-4 after DC maturation with CpG (Bilsborough *et al*., 2003), whereas bone marrow and spleen-derived plasmacytoid DC induce a Th1 T cell phenotype when stimulated by CpG (Boonstra *et al*., 2003). Moreover within the same mucosal tissue, different DC populations display distinct functional phenotypes under the same stimulation conditions. In the Peyer's patches $CD11c^+$ $CD11b^+$ DC produced IL-10 whereas $CD11c^+$ $CD8\alpha^+$ DC and $CD11c^+$ $CD11b^-$ $CD8\alpha^-$ DC produced IL-12p70 following stimulation with CD40L (Iwasaki and Kelsall, 2001; Kellermann and McEvoy, 2001). All together, these data suggest that the induction of tolerance versus active immunity may be complex and is dependent on the tissue microenvironment, the types of DC the signalling events and response to microbial stimuli.

Figure 1.3 illustrates a model where intestinal DC migrate to secondary lymphoid tissue following the sampling of luminal antigen, and subsequently determine the type of T cell responses: tolerance (non responsiveness), Th1, Th2, Th17 or a regulatory response.

It remains unclear whether the immune environment in the human gut is the same as that in animal models. Ways in which DC interact with microbes may be different in humans and mice. Furthermore, it remains to be determined whether functional distinct properties of intestinal DC have been predetermined by local microenvironment or whether they are functionally committed precursors that have been directed to migrate to the intestine.

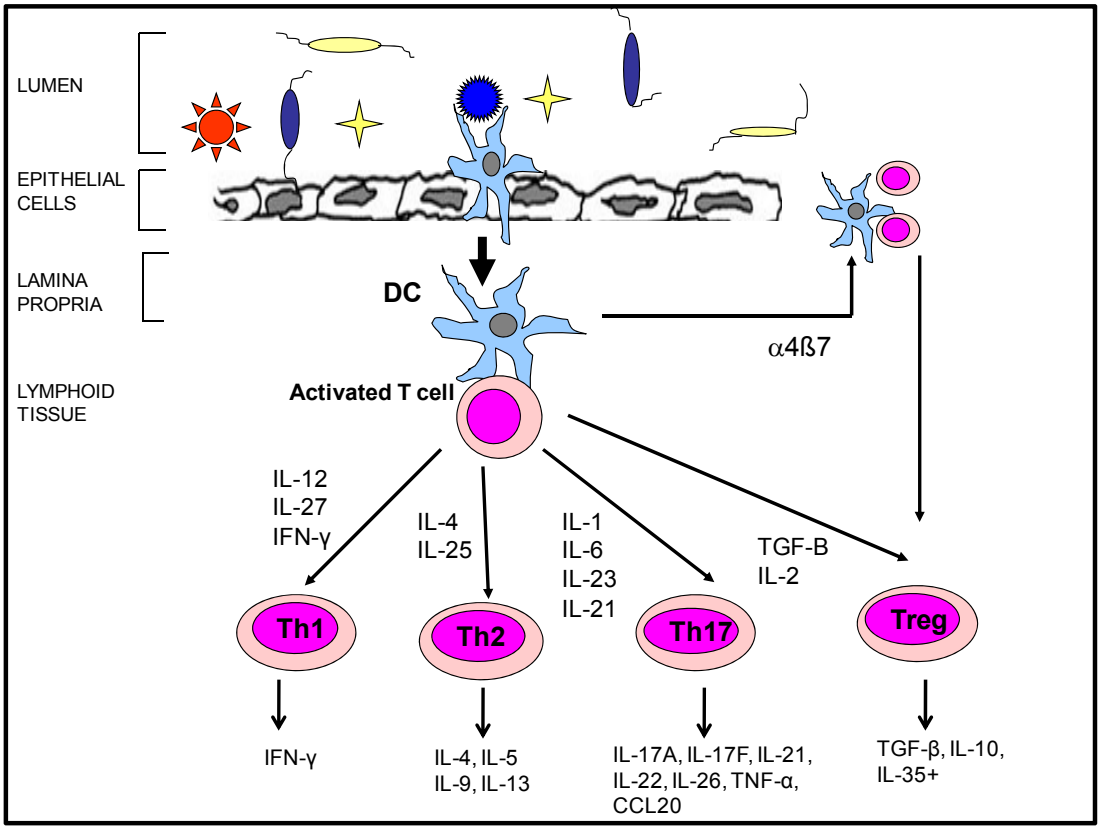

Figure 1.3: Proposed mechanisms of control of T cell responses by intestinal dendritic cells

This figure illustrates the development of Th1, Th2, Th17 and Treg cells from naïve $CD4^+$ T cells following activation by intestinal DC. The location and precise phenotype that distinguish these different subsets remain to be understood. Excessive Th1 response in the absence of adequate regulatory response lead to inflammatory pathology. It remains unclear whether resident DC lose their non-inflammatory properties, or whether fresh non-conditioned DC are recruited from blood as differentiated cells or monocytic precursor during inflammation. DC promote the development of $Foxp3^+$ Treg cells in the GALT in the presence of TGF-β. They can also dictate the homing potential of recently activated T cells by imprinting α4β7 on these cells.

1.2.3 Intestinal Dendritic Cells and Microbial Interaction

DC display significant plasticity in their ability to respond to microbial stimuli. The outcome of stimulation is dependent on the local interaction between DC populations and lymphocytes (Pulendran, 2004). The ability of DC to sample luminal bacteria suggest that they play a critical role in the surveillance of pathogens in the gut environment.

Microbial products including lipopolysaccharide (LPS) stimulate DC maturation. In mice, LPS from *E coli* stimulated IL-12 DC production whereas LPS from *Porphyromonas gingivalis* did not, suggesting that LPS from different bacteria activate DC subsets to produce different cytokines, and induce distinct types of adaptive immunity *in vivo* (Pulendran *et al*., 2001). Apart from distinguishing between microorganisms, DC can also differentiate between closely related microbial structures of the same organism. Murine DC lines produced IL-12 to prime a Th1 response when *Candida albicans* was ingested, whereas IL-4 was produced and IL-12 inhibited when the hyphae form of the yeast was ingested (d'Ostiani *et al*., 2000). Enteric pathogens can also alter intestinal DC function (Kapsenberg, 2003). For example, *Heligmosomoides polygyrus* infection induced DC activation and IL-10 expression, which impaired host protection against *Citrobacter rodentium* infection, resulting in an enhanced bacterial infection and more severe colonic inflammation (Chen *et al*., 2006). It is likely that exposure to microbial products alone is insufficient to activate intestinal DC and there may be a need for inflammatory signals from cytokines to indicate local tissue damage.

Studies using oligonucleotide microarrays to compare gene expression of DC exposed to candida, influenza virus or *E. coli* have demonstrated that DC are able to elicit tailored immune response to certain organism (Huang *et al*., 2001). For instance when human monocyte-derived DC were exposed to different pathogens, organism-specific responses occurred in addition to a temporal cascade of common core response to all tested antigens (Huang *et al*., 2001).

In healthy individuals, $CD4^+$ T cells from the lamina propria exhibit reactivity to commensal bacteria including *Enterobacter*, *E. coli* and *Enterococcus* species, as well as to the pathogen, *Salmonella typhimurium*. Depletion studies showed that bacteria-specific $CD4^+$ T cell activation and proliferation *in vitro* were dependent on intestinal DC, which themselves exhibited a pro-inflammatory cytokine profile upon bacterial stimulation (Howe *et al*., 2009).

DC sense their microbial environment by expressing a series of surface PRR that recognise a limited set of conserved molecular patterns, referred to as PAMPs, on the surface of bacteria (Akira *et al*., 2006; Akira, 2006). PAMPs are recognised through at least three PRR families of molecules: TLR, cell surface c-type lectin receptors and intracytoplasmic NOD-like receptors. It is unclear how PRR monitoring and response distinguish between the abundant normal microbiota and the rare pathogen. Microbes can directly activate DC through PRR, or indirectly, by the capture of apoptotic or necrotic cells dying in response to microbial stimuli (Neish, 2008).

Toll-like Receptors (TLR)- The best characterised of all PRR are the TLR. Ten TLR have been identified in humans and 13 TLR have been detected in mouse (Akira *et al*., 2006;

Akira, 2009). TLR can be divided based on their cellular localisation and differential expression by distinct DC subsets. TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 are expressed on the cell surface, whereas TLR-3, TLR-7, TLR-8 and TLR- 9 are expressed in intracellular compartments (Akira *et al*., 2001). TLR-2 expressed by $CD11c^+$ DC and its ligands are required for recognition of gram-positive cell wall components, including lipoproteins and peptidoglycans (Kadowaki *et al*., 2001). TLR-4 recognises LPS from *E. coli* (Takeuchi *et al*., 1999); TLR-5 recognises flagellin from gram-negative bacteria, and TLR-9 recognises GpG motifs bacterial DNA and is expressed by plasmacytoid DC and B cells. Different DC recognise different PAMPs to induce a distinct immune response. *E. coli* LPS stimulates DC via TLR-4 to induce IL-12 secretion and a Th1 response, whereas *Porphyromonas gingivalis* LPS activates DC via TLR-2 to secrete IL-10, and the development on Th2 response (Pulendran *et al*., 2001).

C-type lectin receptors – C-type lectins act as anchors for bacteria, viruses, parasites and fungi, and allow internalisation of these microorganisms. They also act as adhesion molecules between DC and other cell types. Distinct DC subsets express different c-type lectins: BDCA-2 is specific to plasmacytoid DC (Dzionek *et al*., 2001), langerin/CD207 is specific to langerhans cells (Valladeau *et al*., 2000), and DC-SIGN is specific to interstitial DC (Geijtenbeek *et al*., 2000). Bacteria *(Lactobacillus reuteri* and *Lactobacillus casei)* and its cell surface compounds can bind to DC-SIGN, and lead to the induction of regulatory T cells (den *et al*., 2009). DC-SIGN is implicated to play a role in the induction of various responses mediated by DC. Konstantinov *et al.* have shown that the major S layer protein, SlpA, of *L. acidophilus* NCFM binds to DC-SIGN and induced a concentration-dependent production of IL-10 and low IL-12p70. A knockout mutant of *L. acidophilus* NCFM which lacked SlpA was significantly reduced in binding to DC-

SIGN (Konstantinov *et al*., 2008). These data suggest that the interaction of probiotic bacteria with DC-SIGN ligand is functionally involved in the modulation of DC and T cell functions.

In ileal tissues from patients with CD, enhanced infiltrates of DC-SIGN$^+$ and CD83$^+$ DC have been found in the subepithelial dome overlying the Peyer's patches (Salim *et al*., 2009). CD83$^+$ cells in Crohn's tissues showed reduced expression of the lymph node migratory receptor, CCR7, possibly contributing to their retention within the epithelium. These cells expressed TLR-4 and produced TNF-α. When exposed to *E coli*, CD83$^+$ DC co-localized with translocated bacteria (Salim *et al*., 2009). Thus non-migrating DC in the subepithelial dome can internalize non-pathogenic bacteria which may be important for the onset and perpetuation of intestinal inflammation in CD (Salim *et al*., 2009).

NOD-like receptors – NOD-like receptors recognise intracellular microbial components and signal pathways that produce pro-inflammatory cytokines (Delbridge and O'Riordan, 2007; Mariathasan and Monack, 2007). DC express NOD 1, which recognises muramyl-tripeptides from gram-negative bacteria, and NOD 2, a key cytoplasmic sensor of intracellular bacterial peptidoglycan and a mediator of innate immunity. Mutation in NOD2 has been associated with ileal Crohn's disease (CD) (Hugot *et al*., 2001; Ogura *et al*., 2001) suggesting that the dysregulated recognition of intestinal microbiota bacteria results in inflammatory disease in genetically predisposed subjects.

1.2.4 Regulation of Lymphocyte Homing by Intestinal Dendritic Cells

Lymphocytes continuously migrate around the body to meet antigens. T cells trafficking to lymphoid and extralymphoid tissues involves a multistep process and multiple signals that is regulated by the coordinated interaction between cell surface molecules on T cells with their respective ligands on the surface of vascular endothelial cells (Butcher, 1992; Butcher *et al*., 1999). In the intestine, lymphocytes primed in MLN draining intestinal sites have the propensity to home back to the intestine (Stagg *et al*., 2002). Two homing molecules promote specific homing to the gut: α4β7 which is attracted to its ligand, mucosal addressin cell adhesion molecule-1, MadCAM-1, expressed on HEV, Peyer's patches and MLN in the intestine (Butcher *et al*., 1999), and the chemokine receptor CCR9, attracted to its ligand CCL-25 (TECK). The latter plays an important role in the recruitment of effector T cells to the small intestine (Zabel *et al*., 1999). Specific homing pathways also exist for other organs. For instance, cutaneous leukocyte antigen (CLA, a carbohydrate selectin ligand), CCR4, CCR8 and CCR10 have been implicated in homing to the skin (Campbell *et al*., 1999; Campbell *et al*., 2007; Ohmori *et al*., 2006; Schaerli *et al*., 2004). RA is required for the induction of α4β7 and CCR9 by mucosal lymphocytes. RA binds to intracellular retinoic receptors, which activates the transcription of genes encoding α4β7 and CCR9 (Iwata *et al*., 2003; Iwata *et al*., 2004). Gut DC play an important role in the homing process (Dudda *et al*., 2005; Sigmundsdottir and Butcher, 2008). **Figure 1.4** illustrates proposed mechanisms of control of lymphocyte homing by DC.

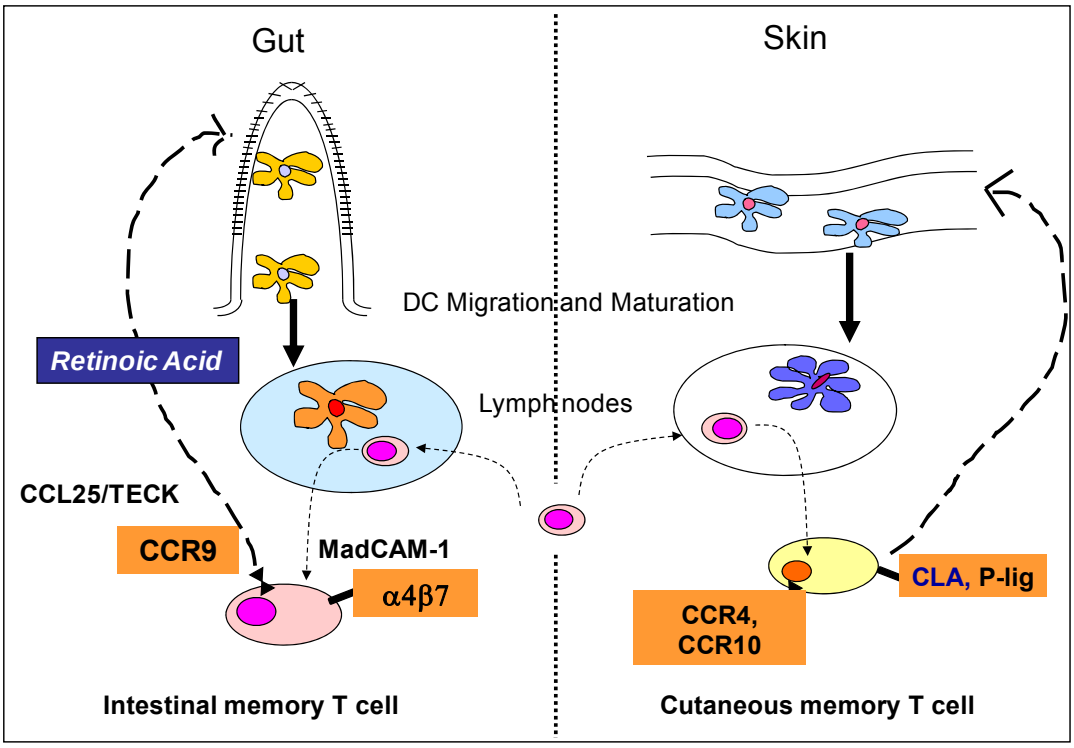

Figure 1.4: Control of lymphocyte homing by dendritic cells

DC in gut-related lymphoid organs imprint T cells with gut-homing specificity by providing RA during antigen presentation. MLN-DC and Peyer's patches DC produce RA from retinol (vitamin A), and imprint gut-homing specificity on T cells upon activation. The imprinted T cells express both α4β7 and CCR9, which bind to MAdCAM-1 and CCL25, respectively, and migrate into the small intestinal tissues (left panel). Conversely, DC in skin-draining LN imprint CLA, CCR4 or CCR10 on T cells, which subsequently migrate to the skin (right panel).

DC not only activate and imprint antigen specificity by presenting antigen to lymphocytes, but they also direct lymphocytes to the site where the antigen is most likely to be encountered by imprinting tissue specificity (Johansson-Lindbom *et al*., 2003; Mora *et al*., 2003; Stagg *et al*., 2002). Work from our laboratory has demonstrated that in mice DC from MLN, but not from peripheral lymph nodes, induce α4β7 on T cells, thus targeting T cells back to intestinal tissue *in vivo* (Stagg *et al*., 2002). Others have shown that murine intestinal DC from Peyer's patches, MLN and the lamina propria induce α4β7 and CCR9

expression on T cells (Johansson-Lindbom *et al*., 2003; Johansson-Lindbom *et al*., 2005). In contrast, T cells activated by DC from skin-draining nodes express ligands for skin homing molecules, P- and E-selectins and lack expression of gut homing markers (Mora *et al*., 2003). In the mouse, the ability to generate $CCR9^+$ $\alpha4B7^+$ gut homing T cells appears to be confined to a functionally distinct subset of MLN DC that express the integrin α chain CD103, which make up about 40 percent of DC in the MLN; these specialised cells most likely originate from the small intestinal lamina propria (Johansson-Lindbom *et al*., 2005). Most small intestinal lamina propria DC express CD103, and DC derived from these sites are more potent at generating gut trophic effector T cells than MLN DC, implying that DC are imprinted with the ability to generate gut trophic T cells before entering MLN (Jaensson *et al*., 2008). In contrast, $CD103^-$ MLN DC did not generate gut trophic T cells. The heterodimer αE(CD103) β7 is expressed on the majority of of human and mouse intestinal lymphocytes and CD103 mediates adhesion of lymphocytes to intestinal epithelial cells via interactions with E-cadherin (Cepek *et al*., 1994).

The mechanisms and specific signals involved in DC imprinting appear to be dependent on vitamin A (Iwata, 2009). The vitamin A metabolite, RA, is converted from retinal by retinal dehydrogenase (RALDHs). RA induces gut homing markers on mouse T cells together with concomitant suppression of the skin-homing molecules E- and P-selectin (Iwata *et al*., 2004).

T cells with regulatory properties (Treg) play a central role in maintenance of immunological homeostasis and tolerance in the gut. Most studied of these cells include the naturally occurring population of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells that develops in the thymus. Similar to conventional T cells, Treg have homing receptors allowing migration

into specific tissues and DC are thought to play an important role in the generation of Treg cell responses (Coombes *et al*., 2007; Siddiqui and Powrie, 2008).

Foxp3^{+} Treg cells can also be induced from naive FoxP3^{-} CD4^{+} T cells; this process appears to be dependent on RA (Kang *et al*., 2007). In the mouse, the induction of retinoid-induced FoxP3^{+} Treg cells involved T cell activation driven by mucosal DC and costimulation through CD28 (Kang *et al*., 2007). RA can promote TGF-β1-dependent generation of FoxP3+ Treg cells but decrease the TGF-β1- and IL-6-dependent generation of inflammatory Th17 cells. RA-induced FoxP3^{+} Treg cells express gut-homing receptors important for migration to the small intestine. Altogether, these results identify RA as positive regulatory factors for generation of gut-homing FoxP3^{+} T cells (Kang *et al*., 2007).

In particular, α4β7 is induced on Treg when exposed to DC from MLN but not from peripheral lymph nodes, and RA appears to be central to this induction of α4β7 and homing to the intestine (Annacker *et al*., 2005). As with conventional T cells, the DC subset, CD103^{+} DC, is equipped for converting antigen-specific T cells into Treg cells (Coombes *et al*., 2007; Sun *et al*., 2007). The CD103^{+} DC population displays an enhanced ability to generate Treg compared with their CD103^{-} counterparts *in vitro,* and this ability is inhibited by RA receptor (RAR) antagonist (Benson *et al*., 2007).

In summary, intestinal DC play a pivotal role in lymphocyte homing; this process is dependent on RA and TGF-β. The role of lymphocyte trafficking in the pathogenesis of IBD has been discussed separately in section 1.4.3.

1.3 Intestinal Microbiota

The term "microbiota" refers to the community of living micro-organisms present in the ecological niche of a host individual. The human GI tract is home to an inconceivable number of micro-organisms (Backhed *et al*., 2005; Neish, 2008; Sonnenburg *et al*., 2004). It represents the largest surface area of the body which is colonized by a diverse and dynamic microbial community, including the resident microbiota as well as foreign bacteria. Bacterial diversity in the gut consists of eight phyla; members of the gram negative Bacteroidetes and gram-positive Firmicutes consist between 60 to 80 percent of the total faecal microbial community (Eckburg *et al*., 2005). The composition of bacteria within different regions of the GI tract varies (Eckburg *et al*., 2005).

Although we are all born germ free, as adults our microbiota census exceeds the total number of our own human cells by 10-fold and the microbes contribute to many physiological and metabolic activities (Gill *et al*., 2006). They endow us with functional features that we have not had to evolve ourselves (Backhed *et al*., 2005). There are approximately 10^{14} microbes that reside in each of our bodies. The aggregate genomes of these gut species may contain >100 fold more genes than our own genome. Our relationship with components of this microbiota is often described as commensal (one partner benefits and the other is apparently unaffected) as opposed to mutualistic (both partners experience increased fitness) (Gill *et al*., 2006).

Apart from environmental factors, there is growing evidence that genetic factors may contribute to the composition of the gut microbiota. Molecular studies on the faecal microbiota have shown that three bacterial divisions dominate: bacteroidetes, firmicutes,

and actinobacteria. However, microbial diversity between individuals is remarkably different and each individual has a unique suite of bacteria (Guarner, 2005).

The benefits of our microflora are well known which include important metabolic, trophic and protective functions. Our microbiota assist the host in the fermentation of non-digestible residue and the production of short chain fatty acids, which serves as a major energy source for colonic enterocytes. They also control epithelial cell proliferation and differentiation; as well as provide protection against pathogens (Guarner and Malagelada, 2003).

The vast majority of microbial communities in the human gut are represented by organisms that are unculturable using standard methods in the laboratory (Rehman and Jasmer, 1998). Culture-independent methods include metagenomics and molecular inventories of 16S rRNA genes. Recently, metagenomics have allowed us to study the genes contained in this vast uncultured majority via the isolation and sequencing of DNA directly from the community in order to assess diversity and profile (Marchesi and Shanahan, 2007). This method helps to indicate the presence or absence of specific lineages and allows for community comparisons. Gloux *et al.* screened a metagenomic library of DNA extracted from the distal gut and identified genes that modulate the growth of epithelial cells (Gloux *et al*., 2007). Other culture independent technique includes fluorescent in-situ hybridisation (FISH). This technique includes group specific fluorescent probes, used to detect and localize the presence or absence of specific DNA sequences on chromosomes. The probes bind to only those parts of the chromosome with which they show a high degree of sequence similarity (Tannock, 2007). Metagenomic

profiling is important to identify specific functional categories of genes (Kurokawa *et al*., 2007; Tringe *et al*., 2005).

The above discussion emphasizes the importance of the host dialogue with our gut microbiota and their interaction with mucosal homeostasis (Marchesi and Shanahan, 2007). Methods are now available to identify the stability and variation in the microbial community in health. A breakdown in the interaction between immune homoestasis and intestinal microbiota results in chronic inflammatory bowel disorders such as IBD. In the next section, the role of intestinal microbiota in IBD will be discussed.

1.4 Inflammatory Bowel Disease

Ulcerative colitis (UC) and Crohn's disease (CD), collectively termed IBD, results from a dysregulated response of the mucosal immune system to components of the luminal microbiota in individuals who are genetically predisposed to the disease (Bamias and Cominelli, 2007; Baumgart and Carding, 2007; Sartor, 2006).

1.4.1 Role of Intestinal Microbiota in Inflammatory Bowel Disease

In human IBD, several studies have shown that the composition of faecal microbiota differs between patients with IBD and healthy controls (Seksik *et al*., 2003). It remains unclear whether changes of the microbial community in IBD are linked to disease aetiology, or a result of changes in gut environment due to disease (Macfarlane *et al*., 2009). In CD, analysis of faecal bacteria showed an increase in enterobacteria but more than 30 percent of the dominant flora belonged to a yet undefined phylogenetic groups. Using a metagenomic approach, Manichanch *et al.* have shown that there was a marked reduction in the Firmicutes in CD patients in remission, but no difference in numbers of bacteroides and *C. coccoides*, when compared with controls (Manichanh *et al*., 2006).

In patients with UC, there is reduced diversity of faecal microbiota (Andoh *et al*., 2007). In UC, higher serum antibody titres to the outer membrane protein of *Bacteroides vulgates* have been reported (Matsuda *et al*., 2000), and higher numbers of *E. coli* have been found in the mucus layer but not adherent to the surface layer of epithelium (Burke and Axon, 1988; Schultsz *et al*., 1997) whilst other *E. coli* subtypes have shown no difference in

adherence properties (Macfarlane *et al*., 2004). Swidsinski *et al.* recently demonstrated that lower faecal levels of *Faecalibacterium (F.) prausnitzii* along with faecal leucocyte counts differentiated active CD from UC. There was a depletion of *F. prausnitzii* with a normal leukocyte count in CD and a high *F. prausnitzii* in patients with UC (Swidsinski *et al*., 2008).

Apart from intestinal bacteria in the gut lumen, mucosal microbial communities may play a role in IBD. Distinct microbial communities have been established in biofilms on mucosal surfaces of the large bowel (Campieri and Gionchetti, 2001; Matsuda *et al*., 2000). Human studies have shown that mucosal bacterial populations in UC may be altered towards a more pro-inflammatory phenotype (Sartor, 2001; Sartor, 2008). In patients with active UC, baseline reduction, temporal instability, and decrease of bacterial richness were observed in the mucosal flora towards disease relapse whereas the colonic mucosal-associated flora for controls appeared stable over time (Ott *et al*., 2008). In tissues from IBD patients, species richness increased from control to non-inflamed tissue, but declined in fully inflamed tissue (Sepehri *et al*., 2007). An increased prevalence of mucosa *E. coli* and *Bacteroides* has been observed in CD patients (Barnich and rfeuille-Michaud, 2007; Kotlowski *et al*., 2007; Swidsinski *et al*., 2005). Invasive *E. coli* was associated with ileal CD, and the number of *E. coli* in situ correlated with severity of disease (Baumgart *et al*., 2007).

CD has been thought to relate to defective bacterial killing (Packey and Sartor, 2009b). Mutation of NOD2 in a subset of patients with CD results in decreased α-defensin production and impaired clearance of intracellular bacteria. CD is also associated with genetic polymorphisms of at least two components of the autophagy pathway, autophagy-

related 16-like-1 (ATG16L1) and immunity-related GTPase family M (IRGM) (Prescott *et al*., 2007; Rioux *et al*., 2007), which lead to decreased antimicrobial peptide secretion, inefficient killing of phagocytosed bacteria, persistent intracellular bacteria antigens and increased mucosa permeability (Packey and Sartor, 2009a). Although several studies have suggested a link between *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and IBD (Clancy *et al*., 2007; Juste *et al*., 2009; Ren *et al*., 2008), there is no evidence that MAP is a causative agent in patients with CD (Packey and Sartor, 2009c).

The search for a causative disease pathogen in IBD has been complicated by the marked individuality of the gut microbiota with minimal overlap between individuals (Dicksved *et al*., 2007; Eckburg *et al*., 2005). Host genotype may partly determine the microbial composition in the gut supported by a study showing high similarity in the fecal microbial communities in monozygotic twins (Stewart *et al*., 2005). However, in discordant monozygotic twins, analysis of the mucosa-associated microbiota in twin wih predominantly ileal CD revealed lower abundance of *F. prausnitzii* and increased levels of *E. Coli* compared to healthy co-twins. Overall these twin data suggest that dysbiosis is likely to be associated with disease phenotype instead of genotype, and that reduced *F. prausnitzii* and increased *E .coli* are indicative of ileal CD phenotype (Willing *et al*., 2009).

To summarise, IBD is characterised by altered microbial composition, defective clearance of bacteria in a subgroup of patients, and enhanced mucosa bacteria uptake; the dysbiosis is associated with host genetic alterations in immunoregulation and innate bacterial killing (Sartor, 2008).

1.4.2 Role of Dendritic Cells in Inflammatory Bowel Disease

Tolerance is developed towards resident intestinal flora but this tolerance is broken in intestinal inflammation (Duchmann *et al*., 1995; Duchmann *et al*., 1997). In IBD in which there is a dysregulated immune response to microbial flora, DC are likely to be of fundamental importance. ***Animal models of colitis*** have provided strong evidence that the interaction between the intestinal microbiota and mucosal immune system plays an important role in the pathogenesis of IBD (Karlis *et al*., 2004). In murine models of colitis, DC accumulate throughout the entire lamina propria and MLN (Leach *et al*., 1996; Strober *et al*., 2002).

One of the best characterised animal models involves the transfer of CD45RB ^{high} $CD4^+$ T cells into severe combined immunodeficiency (SCID) mice which results in chronic intestinal lesions similar to those seen in human IBD (Malmstrom *et al*., 2001). In such a model, colitis was associated with increased $CD11c^+$ DC in the MLN and up to one third of the DC expressed high levels of activation marker OX40L (CD134L). Co-transfer of $CD45RB^{low}$ $CD4^+$ T cells inhibited the accumulation of $CD134L^+$ DC suggesting that regulatory T cells may function, in part, to prevent DC activation. Moreover, blocking CD134-CD134L interactions ameliorated the colitis, reduced T cell proliferation and the numbers of $\alpha4\beta7^+$ T cells in the MLN (Malmstrom *et al*., 2001).

DC are likely to be crucial for the activation and expansion of $CD25^+$ $CD4^+$ T cells in both draining lymph nodes and at sites of inflammation to suppress functions of effector T cells. Using the $CD45RB^{high}$ $CD4^+$ T cell transfer model of colitis, Mottet *et al.* showed that the transfer of $CD25^+$ $CD4^+$ T cells into mice with colitis resulted in resolution of the

lamina propria infiltrate and $CD25^+$ $CD4^+$ T cells proliferated between clusters of $CD11c^+$ DC in the MLN and inflamed colon (Mottet *et al*., 2003).

DC are also involved in the early events of intestinal inflammation demonstrated in a slightly different model in which total $CD4^+$ T cells were transferred to RAG1-/- mice resulting in colitis. Transplanted T cells formed aggregates with $CD11c^+$ DC in the lamina propria, and subsequently underwent proliferation, approximately 8 to 11 days post transfer before the manifestation of colitis. The degree of expansion within the DC clusters was proportional to the severity of intestinal inflammation supporting the role of DC in the initiation, and possibly the maintenance of inflammation (Leithauser *et al*., 2001).

Other investigators have shown the up-regulation of co-stimulatory molecules CD40, CD80 and CD86 together with the expansion of colonic lamina propria DC in murine models of colitis. In addition lamina propria DC from inflamed tissue produced higher levels of IL-12p40, IL-23p19 and IL-10 (Becker *et al*., 2003).

IL-23 is a heterodimer which shares the p40 subunit with IL-12. IL-23 consists of IL-23p19 and IL23p40 whereas IL-12 comprises IL-12p35 and IL-12p40. Both cytokines, produced by activated macrophages and DC, are potent regulators of the adaptive immune responses (Lyakh *et al*., 2008). IL-12 is required for antimicrobial responses to intracellular pathogens, whereas IL-23 is likely to be important for the recruitment and activation of a range of inflammatory cells required for the induction of chronic inflammation and granuloma formation (Iwakura and Ishigame, 2006). In studies of transgenic mice expressing firefly luciferase under the influence of the IL-12p40 promoter, CD8α and CD11b double-negative $CD11c^+$ lamina propria DC represented the

major source of IL-12p40 production in the small intestine whereas additional IL-23 was produced in response to bacterial flora (Langrish *et al*., 2004). One other possibility is that $CD11b^+$ DC in the inflamed mouse colon produced IL-23 and not IL-12, which helped to expand pathogenic T cells in situ (Oshitani *et al*., 1997; Oshitani *et al*., 1998; Seldenrijk *et al*., 1989; Waraich *et al*., 1997). B7-H1, a ligand for programmed death-1 receptor (PD-1) has also been described to be involved in the development of colitis. Inhibition of B7-H1 suppressed chronic intestinal inflammation in mice (Kanai *et al*., 2003).

Furthermore, when DC were selectively depleted in mice before the initiation of DSS-induced colitis, the disease was more severe, compared with that of DC-intact mice (Qualls *et al*., 2009). Increased IL-6 expression in colon tissues correlated positively with increased colitis severity in DC-depleted mice. Thus resident DC can suppress the severity of acute DSS colitis and regulation of IL-6 production may contribute to DC-mediated control of intestinal inflammation (Qualls *et al*., 2009).

Similar to findings in murine models of colitis, ***studies of DC in human IBD*** have shown a role of activated DC at sites of intestinal inflammation (Bell *et al*., 2001; Kaser *et al*., 2004), suggesting that they are likely to contribute to the generation of IBD in humans. Early studies have focussed on the comparison of cells in inflamed and normal colonic lamina propria.

Several investigators have shown an increased in the number and maturation of DC within inflamed IBD tissue (Kaser *et al*., 2004), whilst others have suggested enhanced recruitment of immature DC into inflamed tissue (te Velde *et al*., 2003). In CD, there was an increase in $CD83^+$ and DC-$SIGN^+$ lamina propria DC, which may produce IL-12 and

IL-18 during intestinal inflammation (de *et al*., 2003). Infiltration of $CD83^+$ $CCR7^-$ DC have been reported in the subepithelial dome; these cells internalized translocated *E. coli* HB101 in the Peyer's patches of ileal CD, and may be important in the onset and perpetuation of mucosal inflammation (Salim *et al*., 2009). In addition, $MDC8^+$ monocytes, which were possible precursors of DC, have been identified as a potential source of TNF-α. There were increased TNF-α producing cells in the ileal and colonic lamina propria of patients with CD than controls. Furthermore, abundant numbers of $Langerin^+$ immature DC have been found in the subepithelial space of IBD tissue and were associated with enhanced expression of CCL20 in the intestinal epithelium. Thus CCL20 might regulate the attraction of T lymphocytes and DC in IBD (Kaser *et al*., 2004).

Vuckovic *et al*. have reported increased number of $CD40^+$ $CD86^+$ lin^- DC in the peripheral blood and lamina propria of patients with UC and CD implying the presence of activated DC in the blood and tissues (Vuckovic *et al*., 2001), whereas Baumgart *et al.* have demonstrated that patients with active IBD lacked immature blood plasmacytoid and myeloid DC, suggesting recruitment of these cells to the tissue (Baumgart *et al*., 2005).

The expression of TLR-2 and -4, and the activation of CD40, was enhanced on lamina propria DC of patients with CD and UC (Hart *et al*., 2005). In CD, more colonic DC produce pro-inflammatory cytokines, IL-6 and IL-12p40, than controls. In addition, treatment of patients with CD patients with anti-TNF therapy resulted in a reduced expression of CD40 by lamina propria DC (Hart *et al*., 2005).

One study in UC has identified an increased number of $CD83^+$ and $CD86^+$ lamina propria cells, most likely DC, which produced macrophage inhibitory factor (MIF). MIF can then

induce the production of IL-1 and IL-8 by DC and monocytes, which may enhance neutrophil recruitment and activation (Murakami *et al*., 2002). In addition colonic lamina propria in UC contained numerous basal aggregates consisting of lymphocytes and $CD80^+$ dendritiform cells that most likely represented activated DC (Yeung *et al*., 2000). DC generated *in vitro* from peripheral blood monocytes show increased immuno-stimulatory capacity, and produced more nitric oxide in patients with UC (Ikeda *et al*., 2001).

Lastly, DC can be the source of cytokine production. IL-12-related cytokine, IL-27, is increased in lamina propria of UC patients (Christ *et al*., 1998). UC was also associated with an atypical Th2 response mediated by non-classical NKT cells producing IL-13 suggesting that DC may regulate NKT cell activity through IL-27 in UC (Fuss *et al*., 2004).

Unlike mouse models, studies of human DC in IBD have previously been hampered by the lack of adequate tissue, inconsistent phenotype or function of specific DC populations with the human intestine. Nonetheless emerging evidence now supports the role of DC as important players of the regulation of intestinal immunity in humans.

1.4.3 Dysregulated Lymphocyte Homing in Inflammatory Bowel Disease

In IBD, both tissue specific homing of lymphocyte populations and an imbalance of regulatory and effector responses are likely to contribute to the dysregulated immune response (Eksteen *et al*., 2008). In animal models of IBD, immune cell trafficking pathways play a role in these diseases. Gut-specific adhesion molecules are increased in

CD which makes them potential therapeutic targets (Apostolaki *et al*., 2008), whereas in UC, leukocyte recruitment is less well defined. Thus far no adhesion molecule combination is unique to the colon in the way that CCR9/CCL25 is in the small bowel.

In experimental models of colitis such as DSS-induced intestinal inflammation, upregulation of MAdCAM-1 expression in the intestinal lamina propria has been demonstrated (Farkas *et al*., 2006; Goto *et al*., 2006; Kato *et al*., 2000; Matsuzaki *et al*., 2005; Picarella *et al*., 1997), and anti-MAdCAM-1 treatment resulted in reduction of lymphocyte recruitment to the intestine together with an improvement in intestinal inflammation (Hesterberg *et al*., 1996; Podolsky *et al*., 1993; Rivera-Nieves *et al*., 2006).

In the cotton top tamarine, a primate which develops spontaneous and chronic intestinal inflammation resembling that of UC in humans, treatment with anti-α4 and anti-α4β7 monoclonal antibodies ameliorated intestinal inflammation and was associated with reduced density of mucosal $\alpha4\beta7^+$ lymphocytes (Rivera-Nieves *et al*., 2005).

In other models, the inhibition of several integrins is neccessary for effective treatment and resolution of inflammation. For example in models of CD-like ileitis (SAMP1/YitFc and $CD4^+$ T cell transfer models) pathogenic T cells recirculated to the chronically inflamed small intestine via the α4β7/MAdCAM-1 pathway. In addition T cells also engaged other recruitment pathways such as α4β1 and L-selectin (Park *et al*., 2007). In a T cell transfer colitis model the ability of T cells to induce colitis was decreased when lymphocyte migration into the gut was paralysed due to a mutation in β7 integrin (Schon *et al*., 1999).

In human IBD, organ-specific homing pathways have been described (Arihiro *et al*., 2002; Souza *et al*., 1999). MAdCAM-1 was constitutively expressed on intestinal lamina propria high endothelial venules of healthy mucosa but expression was enhanced in IBD tissue. The proportion of venular endothelium within lamina propria that expressed MAdCAM-1 was also enhanced in inflamed tissue of patients with UC or CD compared with non-inflamed tissue (Arihiro *et al*., 2002; Souza *et al*., 1999).

In patients with CD affecting the small intestine but not the colon, there were increased $CCR9^+$ lymphocytes in the peripheral blood. In the inflamed small intestine of CD, CCL25 expression was altered but these findings were not detected in normal or inflamed colon of CD patients. Additionally in CD, $CCR9^+$ T cells from small intestine lamina propria demonstrated a predominant Th1 and Th17 cytokine profile (Jaensson *et al*., 2008).

Alterations in the number and function of $\alpha4\beta7^{hi}$ and $CCR9^+$ gut-homing cells occur in both active CD and UC (Arihiro *et al*., 2002; Briskin *et al*., 1997; Souza *et al*., 1999). Relapse of disease in UC patients was associated with an increase in both Th1 (TNFα) and Th2 (IL-4) cytokines by intestinal homing $\beta7^+$ memory T cells (Hart *et al*., 2004a).

There is emerging evidence that extra-intestinal manifestations of IBD including skin, eye, liver and biliary complications are related to aberrant homing of immune cells. It has been suggested that long-lived populations of memory lymphocytes that arise as a consequence of bowel inflammation migrate not only to the gut but also to the liver; MAdCAM-1 and CCL25, previously thought to be restricted to the gut, were up-regulated in the liver in primary sclerosing cholangitis (PSC) (Eksteen *et al*., 2004; Eksteen *et al*., 2009), providing a mechanism for infiltration of immune cells into the liver. Human gut-derived

DC, like their murine counterparts, induced RA-dependent expression of α4β7 and CCR9 on CD8 T cells whereas DC from the liver induced only modest levels of these homing molecules. Thus the inability of liver DC to imprint gut-tropism implies that $\alpha4\beta7^{+}$ $CCR9^{+}$ T cells that infiltrate the liver in PSC are primed in the gut (Eksteen *et al*., 2009).

The therapeutic efficacy of interupting trafficking of effector lymphocyte populations has been demonstrated in controlled clinical trials. Natalizumab, a recombinant humanised monoclonal antibody against α4 integrin, was effective in inducing and maintaining remission in active CD (Ghosh *et al*., 2003; Sandborn *et al*., 2005; Targan *et al*., 2007). Natalizumab inhibits the shared α4 integrin moiety of both α4β7 and α4β1, which imparts a broader tissue effect on immune function. Caution is required when inhibition of lymphocyte trafficking is not tissue-specific, but has overlap with other organs. It may be that inhibition of lymphocyte trafficking and impaired immune surveillance lead to reactivation of infections such as progressive multifocal leukoencephalopathy (Van *et al*., 2005; Yousry *et al*., 2006). A second humanised monoclonal antibody, MLN-02 (LDP-02), developed selectively against α4β7, was effective in patients with UC and associated risks of more widespread immunosuppression have not been observed (Feagan *et al*., 2005). More recently, the highly specific CCR-9 antagonist (CCX282-B; Traficet-EN) has also shown efficacy in CD (Schreiber *et al*., 2008).

1.5 Therapeutic Management for Inflammatory Bowel Disease

1.5.1 General Principles of Medical Therapy

The treatment of patients with IBD is dependent on several important factors, including the anatomical extent of disease (proctitis, left sided colitis, extensive or pancolitis), the severity of disease (mild, moderate, severe, fulminant), disease chronicity (acute as opposed to chronic active) and disease complications (Kornbluth and Sachar, 2004; Stange *et al*., 2008; Stange and Travis, 2008). Therapy is sequential, first to treat acute disease, then to maintain remission.

1.5.2 Ulcerative Colitis

UC affects only the large intestine and is characterised by a chronic relapsing disease course. For the treatment of mild to moderately active UC, oral and / or rectal 5-aminosalicylic acid (ASA) is the first line therapy (Marshall and Irvine, 1997; Marshall and Irvine, 2000; Regueiro *et al*., 2006a). Combination therapy with oral and rectal 5-ASA may achieve higher remission rates than either rectal 5-ASA or oral 5-ASA alone in distal UC. In patients with active extensive disease, combined rectal and oral 5-ASA is the most effective (Marteau *et al*., 2005; Regueiro *et al*., 2006b). Up to 90 percent of patients can be maintained in remission using oral once daily 5-ASA therapy (Dignass and Veerman, 2008; Kamm *et al*., 2008a; Kamm *et al*., 2008b; Kruis *et al*., 2008).

In patients with persistently active, steroid-dependent or steroid–refractory UC, immunomodulators, azathioprine (AZA) or 6-mercaptopurine (6-MP) should be started. Half of the patients who are intolerant of AZA can tolerate 6-MP. Second line therapy

includes infliximab and alternative third line options include tacrolimus, methotrexate, 6-thioguanine or leucocyte apheresis (Ng and Kamm, 2008b). Surgical options should be considered and discussed. Continuing pharmacological therapy that does not achieve steroid-free remission is not recommended. Probiotics or curcumin may be useful adjunctive therapies in patients with active UC (Makharia *et al*., 2008; Ng and Kamm, 2008a; Ng and Kamm, 2008b).

1.5.3 Crohn's Disease

Crohn's disease (CD) encompasses a multisystem disorder with distinct clinical and pathogical features. It is characterised by transmural and occasional granulomatous inflammation of the GI tract, as well as a relapsing and remitting disease course(Baumgart and Carding, 2007). CD is not medically or surgically "curable" and therapeutic approaches are aimed mainly at the induction and maintenance of disease remission, improvement of quality of life, prevention of disease complications, and recently mucosal healing has emerged as a therapeutic goal. Guidelines and recommendations for the medical management of CD have been extensively reviewed (Lichtenstein *et al*., 2009; Travis *et al*., 2006).

In patients with mild to moderate active ileal, ileocolonic or colonic CD, 5-ASA have been used as first line therapy although it is only minimally effective compared with placebo (Hanauer and Stromberg, 2004). In such patients, controlled-release budesonide has demonstrated short term efficacy in controlled clinical trials (Kane *et al*., 2002; Otley and Steinhart, 2005). The hypothesis that CD may be caused or exacerbated by bacteria has led

to the use of antibiotics (metronidazole or ciprofloxacin) to treat active luminal or perianal disease (Ishikawa *et al*., 2003b; Rubin and Kornblunth, 2005). In patients with moderate to severe CD, induction of remission can be achieved with 40 to 60mg of prednisolone daily. More than 50 percent of patients treated with steroids will develop steroid-dependent or steroid-refractory disease (Faubion, Jr. *et al*., 2001) and AZA, 6-MP, or parenteral methotrexate are effective in maintaining steroid-free remission in such patients (Lichtenstein *et al*., 2006; Travis *et al*., 2006). The anti-TNF antibodies, infliximab, adaliumumab and certolizumab pegol have been shown to be effective in patients who are intolerant to, or fail to respond to immunosuppressants (Colombel *et al*., 2007; Hanauer *et al*., 2002; Schreiber *et al*., 2007). Infliximab monotherapy and infliximab combined with AZA is more effective than AZA in patients with moderate to severe disease who have failed to respond to first line therapy with mesalazine or corticosteroids, and who are naive to immunosuppressive or biological agents (Colombel *et al*., 2008; Lemann *et al*., 2006).

1.6 Probiotics and Prebiotics

1.6.1 The Concept of Probiotics and Prebiotics

Since Metchnikoff's time more than 100 years ago, it was well recognised that certain organisms appear to have therapeutic value to human health. In 1907 Metchnikoff suggested that fermented milk containing *lactobacilli* were the reason for the longevity of the Balkan people (Morelli, 2000).

Experiments in murine models and clinical observations in man suggest that luminal contents provide the constant antigenic stimulus for intestinal inflammation. The rationale for using probiotics and prebiotics in IBD stems from studies of dysbiosis in the intestinal microbiota in UC, CD and pouchitis, detected using conventional anaerobic culture or molecular probes (Manichanh *et al*., 2006; Swidsinski *et al*., 2002). The dysbiosis theory, reviewed by Tamboli *et al*., suggests a breakdown in the balance between putative species of "protective" versus "harmful" intestinal bacteria, which leads to chronic intestinal inflammation (Tamboli *et al*., 2004). The number of different commensal bacteria is altered in IBD patients with increased bacteroides, adherent or invasive *E. coli*, and enterococci, and reduced bifidobacterium, lactobacillus species and *F. prausnitzii* (Neut *et al*., 2002; Sokol *et al*., 2008; Sokol *et al*., 2009b; Swidsinski *et al*., 2002) (Section 1.4.1).

Probiotics have been defined as living non-pathogenic microorganisms which, when ingested, in sufficient quantities exert a positive influence on host health or physiology (Schrezenmeir and de, 2001). Probiotics may be used either as a single strain, a combination of various strains or as genetically modified probiotics. They can also be used in food or as food supplements. Beneficial effects are demonstrated with dead organisms or bacterial DNA, and the current definition should be broadened to include these other microbial factors (Hord, 2008).

Another approach to therapeutically modify the microbiota involves the manipulation of its energy sources. This can be achieved by altering the collective metabolic output of the microbiota, using dietary supplementation with bacterial fermentative substances such as complex carbohydrates, in the form of prebiotics (Lim *et al*., 2005). ***Prebiotics*** have been defined as non-digestible food ingredients that beneficially affect the host by selectively

stimulating the growth and/or number of bacteria in the colon, such as *bifidobacteria* or *lactobacilli*, thus improving host health (Macfarlane *et al*., 2008). The most widely studied of these are the non-digestible oligosaccharides, which include galacto-oligosaccharides (GOS), lactulose, inulins and their fructo-oligosaccharides (FOS) derivatives (Macfarlane *et al*., 2006). Unlike probiotics, prebiotics can only affect the growth of organisms that are already present in the gut. The use of ***Synbiotics*** which consist of a combination of probiotics and prebiotics is a relatively a new concept in the treatment of IBD. **Table 1** shows the examples of commonly used prebiotics, prebiotics and synbiotics.

Examples of common probiotics, prebiotics and synbiotics

Probiotics	**Prebiotics**
Lactobacilli	FOS (eg. Oligofructose and neosugar)
L. acidophilus	Inulin
L. casei	GOS
L. delbrueckii subsp. bulgaricus	Lactulose
L. reuteri	Lactitol
L. brevis	
L. cellobiosus	**Synbiotics**
L. curvatus	
L. fermentum	Bifidobacteria + FOS
L. plantarum	Lactobacilli + Lactitol
	Bifidobacteria + GOS
Gram positive cocci	
Lactococcus lactis subsp. cremoris	
Streptococcus salivarius subsp. thermophilus	
Enterococcus faecium	
S. diaacetylactis	
S. intermedius	
Bifidobacteria	
B. bifidum	
B. adolescentis	
B. animalis	
B. infantis	
B. longum	
B. thermophilum	

FOS, fructooligosaccharides; GOS, galactooligosaccharides

Table 1: Examples of common probiotics, prebiotics and synbiotics

1.6.2 Probiotics in Inflammatory Bowel Disease

1.6.2.1 Animal Studies of Probiotics

Illuminating work from Gordon's laboratory provided evidence that manipulating the microbiota with probiotics can influence the host (Sonnenburg *et al*., 2006). Germ free mice were colonized with *Bifidobacterium (B) thetaiotaomicron*, a prominent component of the adult human gut microbiota and *B. longum*, a commonly used probiotic. *B. longum* repressed *B. thetaiotaomicron* expression of antibacterial proteins which may promote its own survival in the gut, as well as influenced the composition, structure, and function of its microbial community (Sonnenburg *et al*., 2006). Altogether these data suggested that exogenously administered bacteria can influence the composition of host bacteria. A single microbial molecule from *Bacterial fragillis* protected its host from inflammatory disease caused by *Helicobacter hepaticus* in an animal model of experimental colitis, suggesting that natural anti-inflammatory molecules from the bacteria microbiota can actively promote human health, and may potentially be therapies for human inflammatory disorders (Mazmanian *et al*., 2008). Metabonomic studies showed that probiotics can modulate the gut microbiome and the metabolism of short-chain fatty acids (SCFA), amino acids, bile acids and plasma lipoproteins; these findings emphasized the diversity of synbiotic co-metabolic connections between the gut microbial content and the host (Martin *et al*., 2008).

1.6.2.2 Clinical Studies of Probiotics

Evidence that probiotic bacteria provide therapeutic benefits in human IBD is emerging. Several recent articles have comprehensively reviewed clinical trials using probiotic bacteria to treat patients with IBD (Ewaschuk and Dieleman, 2006; Hedin *et al*., 2007; Isaacs and Herfarth, 2008; Jonkers and Stockbrugger, 2007; Mallon *et al*., 2007). The main studies of probiotics used to treat adult patients with IBD have been shown in detail in **Tables 2 - 4.**

Author	Study	Therapeutic goal	Patient no.	Probiotic strain	Daily dose (no of organisms)	Response	Duration
Gionchetti *et al*.	RCT	Remission maintenance (post antibiotic - induced remission)	40	VSL#3	6g	15% VSL#3 vs. 100% placebo relapsed ($p<0.001$)	9 months
Mimura *et al*.	RCT	Remission maintenance (post antibiotic - induced remission)	36	VSL#3	6g	15% VSL#3 vs. 94% placebo relapsed ($p<0.0001$)	12 months
Gionchetti *et al*.	RCT	Prophylaxis of pouchitis post pouch formation	40	VSL#3	3g	10% VSL#3 vs. 40% placebo developed pouchitis ($p<0.01$)	12 months

Table 2: Controlled clinical trials of probiotics in pouchitis

Author	Study	Therapeutic goal	Patient no.	Probiotic strain	Daily dose (no of organisms)	Response	Duration
Kruis *et al*	RCT	Remission maintenance	120	*E. Coli Nissle*	5 x 10^{10}	16% *E coli* vs. 11% mesalazine relapsed (ns)	3 months
Kruis *et al*	RCT	Remission maintenance	327	*E. Coli Nissle*	5 x 10^{10}	36% *E coli* vs. 34% mesalazine relapsed (p=0.003)	12 months
Rembacken *et al* .	RCT	Remission maintenance	116	*E. Coli Nissle*	5 x 10^{10}	67% *E coli* vs. 73% mesalazine relapsed (ns)	12 months
Ishikawa *et al.*	RCT	Remission maintenance	21	Bifido-fermented milk	-	27% probiotic vs. 90% relapsed (p=0.01)	12 months
Venturi *et al*	Open-label	Remission maintenance	20	VSL#3	1 x 10^{12}	75% remission	12 months
Bibiloni *et al*	Open-label	Treatment of active mild-moderate UC	34	VSL#3	3,600 billion	53% remission 24% response	6 weeks
Makharia *et al* (abstract)	RCT	Treatment of active mild-moderate UC	147	VSL#3	3,500 billion	42% VSL#3 vs. 18% placebo responded (p<0.05)	3 months
Guslandi *et al*	Open-label	Treatment of active mild-moderate UC	25	*S. Bourlardii*	750mg	71% remission	1 month
Kato *et al*	RCT	Treatment of moderate active UC	20	Bifido-fermented milk	100ml	Decrease endoscopic and histolgic score	3 months

Table 3: Clinical studies of probiotics in ulcerative colitis

Author	Study	Therapeutic goal	Patient no.	Probiotic strain	Daily dose (no of organisms)	Response	Duration
Plein *et al*	RCT	Moderately active CD	17	*S. bourardii*	750mg 3x 10^{11}	Significant decrease CDAI in *S. bourlardii*	7 weeks
Guslandi *et al*	RCT	Remission maintenance	32	*S. bourlardii*	1000mg 4 x 10^{11}	6% *S bourladii* vs 38% mesalazine relapsed (p=0.04)	6 months
Prantera *et al*	RCT	Prophylaxis of post-operative CD	32	*L. GG*	12 billion	60% *L. GG* vs. 35% placebo endoscopic recurrence (p=0.29)	12 months
Marteau *et al*	RCT	Prophylaxis of post-operative CD	98	*L. johnsonii LA1*	4 x 10^{9}	49% *L. jonhsonii* vs. 64% placebo endoscopic recurrence (p=0.15)	6 months
Van Gossum *et al*	RCT	Prophylaxis of post-operative CD	70	*L. johnsonii LA1*	1X10^{10}	Similar endoscopic and clinical recurrence rates	3 months

Table 4: Controlled clinical trials of probiotics in Crohn's disease

In summary, the most convincing evidence of the use of probiotics is in the treatment of pouchitis. Pouchitis is a complication after ileo-anal pouch anastomosis in patients with UC. Positive controlled clinical trials have demonstrated that the probiotic mixture, VSL#3, which consists of four strains of Lactobacillus (*L. acidophilus, L. casei, L. plantarum, L. delbrueckii),* three strains of *Bifidobacterium (B. infantis, B. longum, B. breve),* and one strain of *Streptococcus salivarius* subsp. *thermophilus*, was beneficial in maintaining remission in pouchitis (Gionchetti *et al*., 2000; Mimura *et al*., 2004), and preventing the development of pouchitis in patients following ileo-anal pouch formation (Gionchetti *et al*., 2003) .

In UC, *E. coli Nissle 1917* was effective, and equivalent to mesalazine, in maintaining remission in UC (Kruis *et al*., 1997; Kruis *et al*., 2004; Rembacken *et al*., 1999). Bifidobacteria-fermented milk was superior to placebo in maintaining remission in UC (Ishikawa *et al*., 2003a), and in a small pilot study *Saccharomyces bourladii* was effective in the treatment of active UC (Guslandi *et al*., 2003).VSL#3 has also recently been shown to be effective in the treatment of acute mild to moderately active UC (Bibiloni *et al*., 2005; Makharia *et al*., 2008; Venturi *et al*., 1999).

In CD, small studies showed that *Saccharomyces bourladii* may be useful in the treatment and maintanence of disease remission (Guslandi *et al*., 2000; Plein and Hotz, 1993), but *Lactobacillus GG* (Prantera *et al*., 2002) and *Lactobacillus johnsonii LA1* (Marteau *et al*., 2006; Van Gossum *et al*., 2007) were not beneficial in preventing post-operative recurrence in CD.

1.6.3 Prebiotics in Inflammatory Bowel Disease

1.6.3.1 Animal Studies of Prebiotics

Prebiotics ameliorate inflammation in animal models of colitis. In rats with DSS-induced colitis, oral prebiotics (400mg inulin/day) resulted in less mucosal inflammation, reduced inflammatory prostaglandin E2, thromboxane B2, leukotriene B4 and tissue myeloperoxidase activity, when compared with control rats (Videla *et al*., 2001). In a separate study, rats fed either whey-derived or lactose-derived GOS for ten days before the induction of TNBS showed increased levels of faecal bifidobacteria, but no reduction in severity of inflammation (Holma *et al*., 2002). The therapeutic effects of probiotics and prebiotics have been compared with controls in 82 mice with TNBS-induced colitis. Both FOS and a lactic acid bacteria probiotic (*Lactobacillus acidophilus, L. casei subsp rhamnosus and Bifidobacterium animalis*) demonstrated similar beneficial effects with a significant reduction in inflammatory score, myeloperoxidase activities, gut pH and an increased in faecal lactate and butyrate concentrations (Cherbut *et al*., 2003).

Inulin and oligofructose alleviate acute inflammation in animal models of colitis. In transgenic HLA-B27 rats, a prebiotic preparation, Synergy I (combination of inulin and oligofructose), resulted in reduced pro-inflammatory IL-1B in the caecum, diminished production of bacterially stimulated IFN- γ by mesenteric lymphocytes, and increased mucosal levels of TGF-β. Prebiotics also stimulated the growth of lactobacilli and bifidobacterium in the caecum, and reduced IFN-γ in MLN (Hoentjen *et al*., 2005). These immunoregulatory activities of prebiotic were associated with improved tissue inflammation scores and the prevention of colitis. Osman *et al.* showed that oligofructose and inulin together with bifidobacterium induced lower IL-1B and myeloperoxidase

production in the colon as well as significantly less bacterial translocation to MLN in Spague-Dawley rats with DSS-induced acute colitis (Osman *et al*., 2006).

1.6.3.2 Clinical Studies of Prebiotics

Three controlled trials and have assessed the use of prebiotics in human IBD, two in UC (Casellas *et al*., 2007; Furrie *et al*., 2005) and one in pouchitis (Welters *et al*., 2002). Although underpowered, all of them showed promising results **(Table 5).**

Author	Study	Therapeutic goal	Patient number	Prebiotic preparation	Daily dose (no of organisms)	Response	Duration
Welters *et al*	RCT	Chronic pouchitis	24	Inulin	24g	Inulin-treated patients: Reduced endoscopic and histological pouch disease activity index Reduced faecal pH and faecal *bacteroides fragilis* Increased faecal butyrate	3 weeks
Furrie *et al*	RCT	Treatment of mild UC	18	*B. longum*+ FOS/inulin	200 billion *B. longum* + 12g FOS/ inulin	Prebiotics-treated patients: Lower endoscopic score and reduced inflammatory mediators	1 month
Casellas *et al*	RCT	Treatment of mild to moderately active UC	40	Oligofructose-enriched inulin	12g	Inulin-treated patients: Reduction in calprotectin and disease activity score	2 weeks
Lindsay *et al*	Pilot	Treatment of moderately active ileocolonic CD	10	FOS	15g	Decreased Harvey Bradshaw index Increased faecal bifidobacteria concentration Increased TLR-2 and TLR-4 production by DC and IL-10+DC	4 weeks

Table 5: Clinical studies of prebiotics in inflammatory bowel disease

One possible mechanism of prebiotics in IBD may relate, in part, to the modulation of DC. In one uncontrolled study of patients with CD, three weeks of FOS resulted in a significantly less disease activity, the proportion of IL-10^{+} mucosal DC and the proportion of DC expressing TLR-2 and -4 increased significantly (Lindsay *et al*., 2006). A multi-centre placebo-controlled trial of prebiotics for the treatment of moderately active CD is currently in progress.

1.6.4 Mechanisms of Action of Probiotics

As described in section 1.6.2.2, controlled clinical trials of probiotic bacteria have demonstrated benefit in IBD. The precise mechanisms influencing the cross-talk between the microbe and the host remain unclear but there is growing evidence to suggest that the functioning of the immune system at both a systemic and a mucosal level can be modulated by bacteria in the gut. Recent compelling evidence has demonstrated that manipulating the microbiota can influence the host. In summary, several new mechanisms by which probiotics exert their beneficial effects have been identified and it is now clear that significant differences exist between different probiotic bacterial species and strains; organisms need to be selected in a more rational manner to treat disease. Mechanisms contributing to altered immune function *in vivo* induced by probiotic bacteria may include modulation of the microbiota itself, improved barrier function with consequent reduction in immune exposure to microbiota and direct effects of bacteria on different epithelial and immune cell types (Ng *et al*., 2009) .

Probiotic bacteria have multiple and diverse influences on the host as proposed in **Table 6.** Different organisms can influence the intestinal luminal environment, epithelial and mucosal barrier function, and the mucosal immune system. They exert their effects on numerous cell types involved in the innate and adaptive immune responses, such as epithelial cells, DC, monocytes or macrophages, B cells, T cells including T cells with regulatory properties, and NK cells (Bohm and Kruis, 2006; Cummings and Kong, 2004). **Figure 1.5** provides a simplified illustration of the proposed antimicrobial activities of probiotic bacteria.

Mechanisms of Action of Probiotics
Antimicrobial Activity
Decrease luminal pH
Secrete antimicrobial peptides
Inhibit bacterial invasion
Block bacterial adhesion to epithelial cells
Enhancement of Barrier Function
Increase mucus production
Enhance barrier integrity
Immunomodulation
Effects on epithelial cells
Effects on dendritic cells
Effects on monocytes / macrophage
Effects on lymphocytes
- B lymphocytes
- NK cells
- T cells
- T cell redistribution

Table 6: Mechanisms of action of probiotics

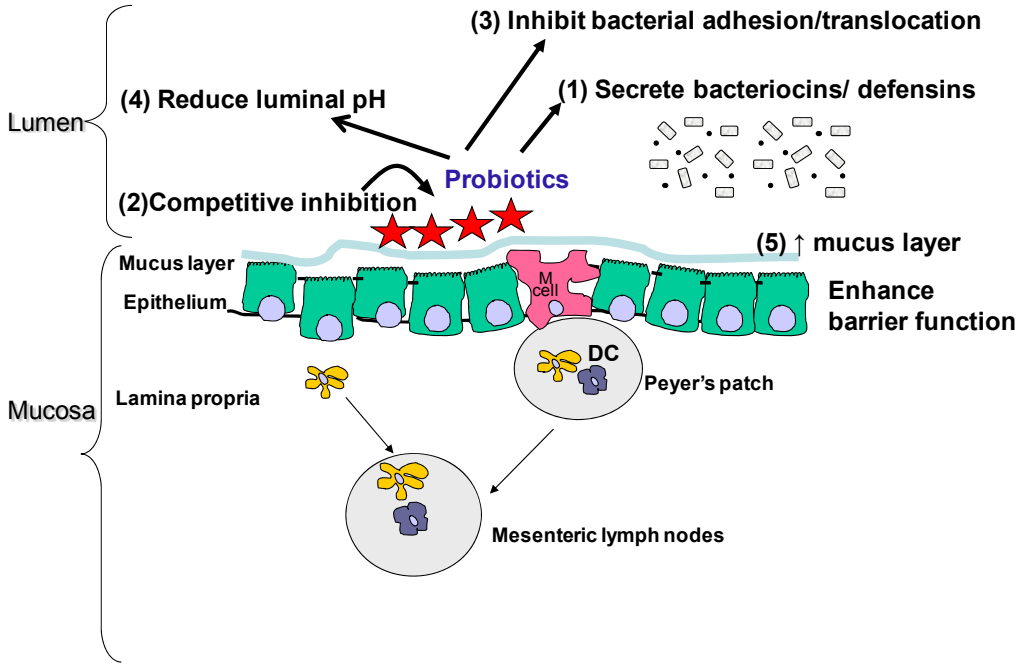

Figure 1.5: Inhibition of enteric bacteria and enhancement of barrier function by probiotic bacteria. Schematic representation of the cross-talk between probiotic bacteria and the intestinal mucosa. Antimicrobial activities of probiotics include the (1) production of bacteriocins/defensins; (2) competitive inhibition with pathogenic bacteria; (3) inhibition of bacterial adherence or translocation and; (4) reduction of luminal pH. Probiotic bacteria can also enhance intestinal barrier function by (5) increasing mucus production.

1.6.4.1 *In Vitro* and Animal Studies

In animal models of IBD the requirement for bacterial colonization to induce an inflammatory phenotype is virtually universal (Mizoguchi *et al*., 2003). Support for a "favourable" action of probiotics on intestinal inflammation comes mainly from animal models, including DSS- and hapten-induced colitis, HLA-B27 transgenic rats, and IL-10 and IL-2-deficient mice (Dieleman *et al*., 2003; Madsen *et al*., 1999; Rachmilewitz *et al*., 2002; Waidmann *et al*., 2003).

Studies in various animal models of colitis are useful in providing mechanistic and therapeutic proof of concept. For instance, in the IL-10 knockout mouse model of colitis, lactobacillus and bifidobacterium species were effective in reducing established intestinal inflammation (Madsen *et al*., 1999; McCarthy *et al*., 2003) and in HLA-B27 transgenic mice, *Lactobacillus GG* effectively prevented the relapse of colitis after antibiotic treatment (Dieleman *et al*., 2003).

1.6.4.2 Modification of the Intestinal Microbiota

Probiotic bacteria can antagonise pathogenic bacteria by reducing luminal pH, inhibiting bacterial adherence and translocation, or producing antibacterial substances and defensins. One of the mechanisms by which the gut flora resists colonisation by pathogenic bacteria is by the production of a physiologically restrictive environment, with respect to pH, redox potential and hydrogen sulphide production. Probiotic bacteria decrease the luminal pH, as has been demonstrated in patients with UC following ingestion of the probiotic preparation VSL#3 (Venturi *et al*., 1999).

In a fatal mouse Shiga toxin-producing *E coli* O157:H7 infection model, the probiotic *B. breve* produced a high concentration of acetic acid, consequently lowering the luminal pH. This pH reduction was associated with increased animal survival (Asahara *et al*., 2004).

Production of antimicrobial compounds, termed bacteriocins, by probiotic bacteria is also likely to contribute to their beneficial activity. Several bacteriocins produced by different species from the genus lactobacillus have been described (Klaenhammer, 1988). The

inhibitory activity of these bacteriocins varies; some inhibit other lactobacilli or taxonomically-related Gram positive bacteria, and some are active against a much wider range of Gram-positive and Gram-negative bacteria as well as yeasts and moulds (Nemcova, 1997). For example, the probiotic *L. salivarius* subsp. *salivarius* UCC118 produced a peptide which inhibited a broad range of pathogens such as Bacillus, Staphylococcus, Enterococcus, Listeria and Salmonella species (Flynn *et al*., 2002).

Lacticin 3147, a broad-spectrum bacteriocin produced by *Lactococcus lactis* subsp., inhibited a range of genetically distinct *Clostridium difficile* isolates from healthy subjects and patients with IBD (Rea *et al*., 2007). A further example is the antimicrobial effect of *Lactobacillus* species on *Helicobacter pylori* infection of gastric mucosa, achieved by the release of bacteriocins and the ability to decrease adherence of this pathogen to epithelial cells (Gotteland *et al*., 2006).

Probiotics can reduce the epithelial injury following exposure to *E. coli O157:H7* and *E. coli O127:H6.* The pre-treatment of intestinal (T84) cells with lactic acid-producing bacteria reduced the ability of pathogenic *E. coli* to inject virulence factors into the cells or to breach the intracellular tight junctions (Sherman *et al*., 2005).

Adhesion and invasion of an intestinal epithelial cell line (Intestine 407) by adherent invasive *E. coli* isolated from patients with CD was substantially diminished by co- or pre-incubation with the probiotic strain *E coli Nissle 1917*. These findings demonstrated that probiotics prevent epithelial injury induced by attaching-effacing bacteria (Malchow, 1997; Rembacken *et al*., 1999).

Defensins are anti-microbial peptides involved in innate defence mechanisms. The probiotic *E. coli Nissle* strain induced expression of human beta-defensin 2 (hBD-2) in Caco-2 intestinal epithelial cells (Wehkamp *et al*., 2004); this type of effect may contribute to an improved mucosal barrier and provide a means of limiting access of enteric pathogens. Induction of human beta-defensin 2 by *E. coli Nissle 1917* was dependent on the NF-kB and AP-1-pathways, mediated through bacterial flagellin (Schlee *et al*., 2007).

1.6.4.3 Enhancement of Barrier Function

In addition to the inhibition of growth of "conventional" organisms or potential pathogens, probiotics can influence mucosal cell-cell interactions and cellular "stability" by the enhancement of intestinal barrier functions through modulation of cytoskeletal and tight junctional protein phosphorylation.

Intestinal barrier function is maintained by several interrelated systems including mucus secretion, chloride and water secretion and binding together of epithelial cells at their apical junctions by tight junction proteins. Disruption of epithelial barrier function is seen in several conditions including IBD, both active and inactive, in the healthy relatives of patients with IBD (Hilsden *et al*., 1996; Hollander *et al*., 1986; May *et al*., 1993; Peeters *et al*., 1997; Schmitz *et al*., 1999; Wyatt *et al*., 1993), enteric infections (Sakaguchi *et al*., 2002), coeliac disease and some autoimmune diseases such as Type 1 diabetes (Watts *et al*., 2005). Enhancement of mucosal barrier function may be an important mechanism by which probiotic bacteria benefit the host in such diseases (Meddings, 2008).

Enhancement of barrier function by probiotic bacteria has been observed both in *in vitro* models and *in vivo* in the whole animal. The probiotic mixture VSL#3 normalised barrier integrity as assessed by short circuit currents, transepithelial potential differences and mannitol fluxes in excised tissue from mice (Madsen *et al*., 2001). Furthermore, in an *in vitro* culture using T84 epithelial cells, VSL#3, but not the other probiotic bacteria, *L. reuteri, S. bovis* and a non-pathogenic *E. coli*, decreased monolayer permeability and conductance, indicating that the increase in resistance was specific to one or more of the bacteria in VSL#3. Increased barrier integrity in response to probiotic bacteria has been observed in healthy animals and in animal models of colitis. For example, in healthy rats, *L. brevis* enhanced barrier function as assessed by permeability to mannitol in excluded colonic loops (Garcia-Lafuente *et al*., 2001). In IL-10 deficient mice with chronic colitis lactobacillus improved barrier function *in vivo* (Madsen *et al*., 1999). In a methotrexate-induced model of colitis, *L. plantarum* and *L. reuteri* enhanced barrier function (Mao *et al*., 1996). However, enhancement of barrier function was not observed in all models of colitis studied. *L. plantarum* did not enhance the barrier function in the context of TNBS colitis (Kennedy *et al*., 2000).

The mechanisms by which probiotics bacteria enhance gut mucosal barrier function are unclear, but may relate to alterations in mucus or chloride secretion or changes in tight junction protein expression by epithelial cells**.** Some probiotic bacteria modify MUC gene expression and mucus secretion. For example, *L. plantarum* 299v increased MUC2 and MUC3 mRNA expression when incubated with the epithelial cell line HT-29 (Mack *et al*., 1999). VSL#3 and *E. coli* Nissle strain increased MUC2, MUC3 and MUC5AC gene and protein expression (Otte and Podolsky, 2004). Some probiotic bacteria can limit chloride and water secretion. For example, *S. Thermophilus* and *L. acidophilus* reversed the

increase in enteroinvasive *E. coli*-induced chloride secretion by an epithelial cell line (Resta-Lenert and Barrett, 2003).

Tight junction proteins are dynamic structures subject to structural changes that dictate their functional status. In epithelial cells, the tight junction protein, zonula occludens-1 (ZO-1) redistributed when exposed to pathogenic bacteria such as *S. Dublin* (Otte and Podolsky, 2004). However, co-culture of epithelial cells with VSL#3 probiotic bacteria in addition to *S. Dublin* prevented the redistribution of ZO-1 and stabilised the barrier function, suggesting that probiotic bacteria may be important in the preservation of the cytoskeleton architecture. Other probiotic bacteria altered other cytoskeleton structures; for example *L. acidophilus* protected against F-actin rearrangement, which was induced in an epithelial cell line on exposure to a pathogenic *E. Coli* (Lievin-Le Moal *et al*., 2002). *S. thermophilus* and *L. acidophilus* maintained (actin, ZO-1) or enhanced (actinin, occludin) cytoskeletal and tight junctional protein structures in epithelial cell lines (Resta-Lenert and Barrett, 2003).

E. coli Nissle 1917 counteracted the disruptive effects of *Enteropathic E. coli* (EPEC) on T-84 epithelial cells monolayers. This effect was achieved by altering protein kinase C signaling and increasing the redistribution and expression of zonulaoccludens-2 (ZO-2), a crucial factor in maintaining epithelial tight junction function (Zyrek *et al*., 2007).

1.6.4.4 Immunomodulation

Effects of Probiotic Bacteria on Epithelial Cells

There may be intrinsic differences in how epithelial cells sense commensal or probiotic bacteria versus pathogenic bacteria at the level of signal transduction pathways and cytokine production. This concept has been demonstrated by Lammers *et al*. and Otte *et al.* who showed that probiotic bacteria in the VSL#3 combination did not induce IL-8 secretion by epithelial cells compared with intestinal pathogens such as enteropathogenic *E. coli*, *Salmonella dublin*, *Shigella dysenteriae* and *Listeria monocytogenes,* all of which induced secretion of IL-8 (Lammers *et al*., 2002; Otte and Podolsky, 2004). Furthermore, co-culture of the pathogenic bacteria *S. Dublin* with VSL#3 probiotic bacteria decreased IL-8 secretion seen with the pathogenic bacteria alone, indicating that probiotic bacteria can override the effects of pathogenic bacteria. However, the probiotic bacteria *E. coli Nissle 1917* induced IL-8 secretion by intestinal epithelial cell lines in a dose dependent manner suggesting that the ability to prevent secretion of IL-8 from epithelial cells was not a feature of all probiotic bacteria (Lammers *et al*., 2002; Otte and Podolsky, 2004).

One other effect of probiotic bacteria on epithelial cells was the ability of commensal organisms to act through TLR, such as TLR-2 and TLR-4, possibly on epithelial cells. Such interactions induced the production of protective cytokines that enhanced epithelial cell regeneration and inhibited epithelial cell apoptosis (Rakoff-Nahoum *et al*., 2004). *L. casei* prevented the development of acute DSS-induced colitis in TLR-4 mutant (lps-/lps-) mice by inhibiting myeloperoxidase activitiy and IL-12p40, and increasing TGF- β and IL-10 mRNA (Chung *et al*., 2008).

The signalling pathways which allow epithelial cells to distinguish commensal or probiotic organisms from pathogenic organisms appear to be different. Pathogenic bacteria induce pro-inflammatory responses in intestinal epithelial cells by activating the transcription factor NFκB. In contrast, non-pathogenic species can attenuated pro-inflammatory responses by blocking the degradation of the counter-regulatory factor IκB. This method of blocking proinflammatory responses was shown by non-pathogenic *Salmonella pullorum* which attenuated IL-8 secretion elicited by pathogenic *Salmonella typhimurium* (Neish *et al*., 2000). Another method of avoiding pro-inflammatory responses to commensal bacteria has been demonstrated for *Bacteroides thetaiotaomicron* which induced an anti-inflammatory response in epithelial cells by shuttling transcription factor, NFκB out of the nucleus by a pathway involving the nuclear hormone receptor, peroxisome proliferator activated receptor (PPAR)-γ, resulting in attenuation of NFκB-mediated inflammatory gene expression independent of the IκB pathway (Kelly *et al*., 2004). VSL#3 probiotic bacteria produced soluble factors which inhibited proteosome in intestinal epithelial cells thereby inhibiting the NFκB pathway (Petrof *et al*., 2004). Furthermore, DNA derived from the probiotic mixture VSL#3 delayed NFκB activation, stabilised levels of IκB, and inhibited proteasome function (Jijon *et al*., 2004).

Probiotic bacteria may also enable epithelial recovery or prevent apoptosis, as suggested by a study in which cytokine-induced apoptosis was prevented in intestinal epithelial cells in the presence of *L. rhamnosus* GG (Yan and Polk, 2002). Culture of probiotic bacteria with either mouse or human colon cells activated anti-apoptotic Akt/protein kinase B and inhibited activation of the pro-apoptotic p38/mitogen-activated protein kinase by TNF-α, IL-1β, or IFN-γ. Inhibition of apoptosis may enhance survival of intestinal cells and promote proliferation during recovery from epithelial injury. In addition, a non-pathogenic

commensal bacterium *S. pullorum*, influenced epithelial cell proliferation by "injecting" factors into the gut epithelium that blocked β-catenin degradation, a substance which has been implicated in epithelial growth control (Neish *et al*., 2000).

Effects of Probiotic Bacteria on Dendritic Cells

The pivotal position of DC at the intersection of innate and adaptive immunity with their ability to recognise and respond to bacterial components, to initiate primary immune responses and to direct developing T and B cell responses underlines the importance of understanding the functional effects of different bacteria on DC. Various ways of antigen sampling by DC have been discussed in Section 1.2.1. The effects of different probiotic bacteria on DC have been studied in different experimental systems (whole blood DC, freshly isolated lamina propria DC, monocyte-derived DC and bone-marrow derived DC) and in different species (human and mouse).

Figure 1.6 illustrates mechanisms of the modulation of DC function by probiotic bacteria. In humans the probiotic combination VSL#3 was a potent inducer of IL-10 by both blood and lamina propria DC *in vitro* (Hart *et al*., 2004c). DC were defined as a population of cells which were HLA-DR^+ and negative for a set of lineage markers thereby excluding T cells, B cells, macrophages, NK cells and myeloid progenitor cells (Bell *et al*., 2001).

In patients with pouchitis treated with the probiotic combination VSL#3 there were enhanced levels of IL-10 in their mucosa and decreased levels of TNF-α, IL-1, inducible nitric oxide synthase and matrix metalloproteinase (Ulisse *et al*., 2001). In agreement with the observation that VSL#3 is a potent inducer of IL-10 by DC, Drakes *et al.* have shown

that murine bone marrow-derived DC incubated with VSL#3 increased IL-10 detected by ELISA (Drakes *et al*., 2004).

Individual strains within VSL#3 displayed distinct immunomodulatory effects on DC; the most marked anti-inflammatory effects were produced by bifidobacteria strains (*B. longum, B. infantis* and *B. breve*) which upregulated IL-10 and downregulated IL-12p40 production by DC. Anti-inflammatory effects of bifidobacteria strains have been described in other studies. Using DC derived from human cord blood monocytes, Young *et al*. demonstrated that *B. longum, B. bifidum* and *B. pseudocatenulatum*, but not *B. infantis*, induced high levels of IL-10 (Young *et al*., 2004).

Rigby *et al.* demonstrated that murine freshly isolated lamina propria DC incubated with *B. longum* secreted IL-10 and IL-12, but a greater proportion of DC produced IL-10 than IL-12 (Rigby *et al*., 2002). In a different experiment, purified human monocytes and monocyte-derived DC stimulated with ultra violet-inactivated gram-positive (*L. plantarum* and *B. adolescentis*) and gram-negative (*E. coli and Veillonella parvula*) bacterial strains, *B. adolescentis* induced low amounts of IL-12, TNF-α, IL-6 and IL-8; *L. reuteri* and *L. casei*, but not *L. plantarum*, primed monocyte-derived DC to result in a regulatory response (Braat *et al*., 2004b; Karlsson *et al*., 2004).. These Treg cells produced increased levels of IL-10 and were able to inhibit the proliferation of bystander T cells in an IL-10-dependent fashion. Strikingly, both *L. reuteri* and *L. casei*, but not *L. plantarum*, bound the C-type lectin DC-SIGN. Blocking antibodies to DC-SIGN inhibited the induction of the Treg cells by these probiotic bacteria, implying that ligation of DC-SIGN can actively prime DC to induce Treg cells. Thus the targeting of DC-SIGN by certain probiotic

bacteria might explain their beneficial effect in the treatment of a number of inflammatory diseases, including atopic dermatitis and CD (Smits *et al*., 2005).

In contrast to the anti-inflammatory activity of some strains of bifidobacteria and lactobacilli, other lactobacilli strains have pro-inflammatory activity. When cultured with human monocyte-derived DC, *L. reuteri, L. gasseri* and *L. johnsonii,* induced activation and maturation of DC, enhanced IL-12 production and induced allogeneic T cell priming (Mohamadzadeh *et al*., 2005).

However, Christensen *et al*. demonstrated that *L. reuteri* induced little or no IL-12 production by DC in an experimental system using murine bone-marrow derived DC and inhibited proinflammatory cytokine production (IL-12, IL-6 and TNF-α) by *L. casei* which was a potent inducer of IL-12. These varying effects of probiotic bacteria highlight differences which arise when different experimental systems in different animals were used (Christensen *et al*., 2002). However, there appeared to be different responses of different bacterial strains even within a genus. In human monocyte-derived DC, *L. rhamnosus* and *L. plantarum* induced no or low levels of IL-12 (Braat *et al*., 2004a; Braat *et al*., 2004b; Karlsson *et al*., 2004; Veckman *et al*., 2004). in contrast to the high levels of IL-12 noted with *L. gasseri* and *L. johnsonii* (Mohamadzadeh *et al*., 2005).

Braat *et al.* demonstrated that *L. rhamnosus* may 'educate" DC to stimulate proliferation of peripheral $CD4^+$ T cells and reduced CD3/CD28 stimulated cytokine production *in vitro*. *L. rhamnosus* resulted in a reduction of IL-4 from peripheral $CD4^+$ T cells of normal individuals, and a decreased in IFN-γ and IL-2 production by $CD4^+$ T cells from patients

with CD. This observation suggested that a comparable mechanism may exist in humans (Braat *et al*., 2004b).

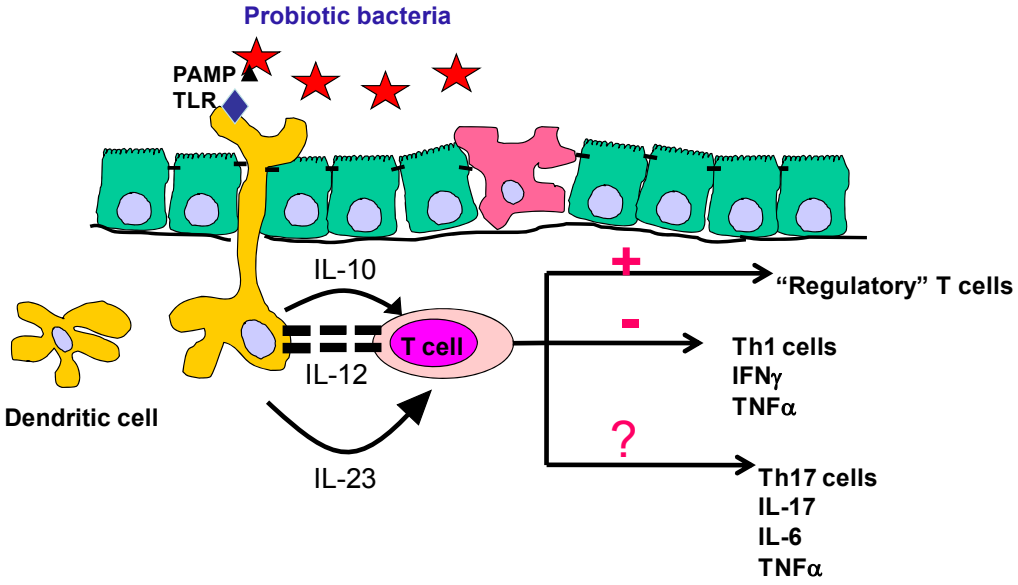

Figure 1.6: Modulation of dendritic cell function by probiotic bacteria. Pathogen associated molecular patterns (PAMPs) derived from probiotic bacteria are recognized by pattern recognition receptors, such as toll-like receptors (TLRs) on DC in the epithelium or lamina propria. Probiotic bacteria can shape the mucosal immune system towards a non-inflammatory, tolerogenic pattern through the induction of T cells with regulatory properties. Probiotics can also downregulate Th1 response and inhibit the production of proinflammatory cytokines, IL-12, TNF-α and IFN-γ by DC. The predominant cytokine profile depends on the nature of the stimulus and the types of probiotic bacteria. The IL-23/IL-17-mediated inflammatory axis has recently been implicated in the pathogenesis of inflammatory bowel disease but there remain gaps in our knowledge on how probiotics influence the differentiation of Th17 cells. This diagram is a simplified synthesis of data derived *in vitro* and in *vivo*.

Effects of Probiotic Bacteria on Monocytes and Macrophages

Blood monocytes and tissue macrophages are effective secondary presenters of antigens to memory T cells. *L. plantarum* increased IL-10 synthesis and secretion in macrophages derived from the inflamed colon (Pathmakanthan *et al*., 2004). In contrast, *L. rhamnosus* GG promoted the production of IFN-γ, IL-12 and IL-18 (Miettinen *et al*., 1998), and induced NFκB and STAT DNA-binding in primary human macrophages. He *et al.* have shown that *B. bifidum, B. breve* and *B. infantis* stimulated more IL-10 and less IL-12 and TNF-α from a murine macrophage-like cell line than *B. Adolescentis* (He *et al*., 2002), again suggesting strain differences within a genus. DNA derived from the probiotic mixture VSL#3 activated NFκB and induced low levels of IL-6 and IL-12 by bone-marrow derived macrophages compared with immunostimulatory oligonucleotides (Rachmilewitz *et al*., 2004).

Effects of Probiotic Bacteria on Lymphocytes

Probiotic bacteria may affect lymphocytes directly or secondarily via changes in stimulation induced by alterations in antigen presenting DC or macrophages. These effects have been demonstrated for different types of lymphocytes.

***B lymphocytes* -** Probiotic bacteria may exert beneficial effects and modulate the immune response to potentially harmful antigens via B lymphocytes and antibody production. For example, *L. rhamnosus* GG administered to children with acute gastroenteritis resulted in an increase in IgG, IgA and IgM secretion from circulating lymphocytes (Kaila *et al*., 1992). *B. bifidum* enhanced the antibody response to ovalbumin, and yoghurts containing

L. acidophilus, *L. bulgaricus, S. thermophilus, B. bifidum* and *B. infantis* stimulated the IgA response to cholera toxin in mice (Tejada-Simon *et al*., 1999).

The effects of probiotic bacteria on B lymphocytes and antibody production have also been evaluated in vaccination trials. The immunogenicity of rotavirus vaccination was enhanced in children who received *L. rhamnosus* GG compared with those who received placebo (Isolauri *et al*., 1995). Similarly increased *Salmonella*-specific IgA levels were found in subjects who received a combination of *L. rhamnosus* GG and *Salmonella* vaccination (Fang *et al*., 2000).

Natural Killer (NK) Cells **-** The synbiotic *L. casei ssp. casei* with dextran (prebiotic) significantly elevated the NK cell activities in spleen mononuclear cells from BALB/c mice, and oral administration of this symbiotic to healthy volunteers induced NK cell activities, and increased production of IL-12 in human peripheral blood mononuclear cells (PBMC) (Ogawa *et al*., 2006). Takeda *et al.* showed that *L. casei* Shirota can enhance NK cell activity *in vivo* and *in vitro* in humans; this effect may be dependent on IL-12 (Takeda *et al*., 2006).

T Cells **-** The type of T cell response, whether it be a Th1, Th2, Th3 / Tr1 or Th17 response is controlled predominantly by interactions between DC and T cells (Maloy and Kullberg, 2008). In humans, VSL#3 potently induced IL-10 by DC and co-culture of naïve T cells with probiotic-treated DC led to a decrease in Th1 polarised cells (Hart *et al*., 2004c). In a different experimental system in which monocyte-derived DC were cultured with the probiotic *L. rhamnosus* and the subsequent effect on T cells assessed, decreased T cell proliferation and T cell cytokine production, particularly IL-2, IL-4 and IL-10, has

been demonstrated (Braat *et al*., 2004b). This *in vitro* effect of *L. rhamnosus* on DC and subsequent T cell hyporesponsiveness was reflected in *in vivo* studies in which healthy controls and patients with CD were fed *L. rhamnosus* for two weeks. Ingestion of *L. rhamnosus* reduced IFN-γ and IL-2 production by peripheral T cells in CD patients and also reduced IL-4 production in healthy controls (Braat *et al*., 2004b).

Probiotic bacteria influenced the generation of regulatory T cells in a murine model of contact dermatitis. Daily oral administration of fermented milk containing the probiotic *L. casei DN-114 001* reduced antigen-specific skin inflammation by controlling the antigen-specific T cell response in hapten 2,4-dinitrofluorobenzene, a model of allergic contact dermatitis mediated by $CD8^+$ CTL and controlled by $CD4^+$ regulatory T cells. The alleviation of contact hypersensitivity by prior feeding with *L. casei* was due to downregulation of the hapten-specific $CD8^+$ T cell response as indicated by a decrease in expansion of hapten-specific IFNγ-producing $CD8^+$ effectors. Furthermore, experiments in mice deficient in $CD4^+$ cells indicated that $CD4^+$ cells were mandatory for the effect of *L. casei* on contact hypersensitivity. It has been proposed that *L. casei* reduced contact hypersensitivity by direct or indirect activation of regulatory $CD4^+$ T cells (Chapat *et al*., 2004). Von der Weid *et al.* have also reported that *in vitro* a different probiotic organism, *L. paracasei* NCC2461, induced the development of a population of $CD4^+$ T cells with regulatory properties in that they had low proliferative capacity and produced TGF-β and IL-10 (von der *et al*., 2001).

Probiotic bacteria also induced regulatory T cells in the context of intestinal inflammation. In TNBS colitis, the probiotic combination VSL#3 ameliorated intestinal inflammation; the beneficial effect of VSL#3 was attributable to IL-10-dependent-regulatory $CD4^+$ T

cells bearing surface TGF-β (Di Giacinto *et al*., 2005). These cells appeared to be similar to $CD25^+$ regulatory T cells that inhibited cell-transfer colitis by a TGFβ - dependent mechanism (Oida *et al*., 2003).

Effects of Probiotic Bacteria on T Cell Redistribution

Dalmasso *et al.* have reported a novel biological property of probiotics, the capacity to affect immune cell redistribution by improving the competence of lymphatic endothelial cells to trap T lymphocytes. In *Saccharomyces boulardii*-fed mice, IFN-γ production by $CD4^+$ T cells was reduced in the colon but increased in the MLN. *S. boulardii* has a unique action on inflammation by a specific alteration of the migratory behavior of T cells causing accumulation of these cells in MLN (Dalmasso *et al*., 2006; Nakamura *et al*., 2004).

1.6.4.5 Mechanisms of Probiotics in Clinical Diseases

A number of studies in IBD have shown that probiotics can induce regulatory cytokines, including IL-10 and TGF-ß, and suppress pro-inflammatory cytokines, such as TNF, in the mucosa of patients with CD and pouchitis (Borruel *et al*., 2002; Pathmakanthan *et al*., 2004; Ulisse *et al*., 2001).

In a study aimed at assessing the anti-inflammatory effects of probiotics, healthy controls and IBD patients consumed *L. rhamnosus GR-1* and *L. reuteri RC-14* supplemented yogurt for 30 days. The proportion of putative regulatory $CD4^+$ $CD25^{high}$ T cells in

peripheral blood increased significantly in IBD patients, after treatment, but not in controls (Lorea *et al*., 2007). The basal proportion of TNF-α^+ IL-12^+ monocytes and myeloid DC decreased in patients with IBD and healthy controls. In addition serum IL-12 concentrations and the proportion of IL-2^+ and $CD69^+$ T cells from stimulated cells decreased in IBD patients. Thus probiotic yogurt intake was associated with significant anti-inflammatory effects that parallel the expansion of the peripheral pool of putative Treg cells in IBD patients with few effects in controls (Lorea *et al*., 2007).

Adhesive *E. Coli* has been implicated in the pathogenesis of UC. Studies using a 16S rRNA technique have shown reductions in bifidobacteria (Macfarlane *et al*., 2005; Mylonaki *et al*., 2005), and lactobacilli in patients with UC (Bullock *et al*., 2004). Using a short term synbiotic treatment, there was an increased bifidobacterial colonization of the rectal mucosa. In a separate experimental system, colonic biopsies from UC have been co-cultured for 24 hours with *B. longum.* The concentrations of TNF and IL-8 in supernatants of inflamed UC tissue co-cultured with probiotics were lower than those cultured alone. The number of LPMC with NFκB p65 positive in co-cultured tissues was also reduced (Bai *et al*., 2006). Release of TNF-α by inflamed CD mucosa was significantly reduced by co-culture *L. casei* or *L. bulgaris,* but not *L. crispatus* and *E coli.* No change in TNF-α production was seen in experiments with non-inflamed Crohn's mucosa and control mucosa. Certain probiotic bacteria may therefore interact with immunocompetent cells at the mucosal interface and thus modulate local production of pro-inflammatory cytokines by inflamed tissue (Borruel *et al*., 2002).

When the mucosal explants of CD were incubated with *L. casei* or its genomic DNA, or *E coli,* live *L. casei* counteracted the proinflammatory effects of *E. coli* on CD inflamed mucosa by specific downregulation of key proinflammatory mediators including TNF-α,

IFN-γ, IL-2, IL-6, IL-8 and CXCL1; these effects have not been reproduced by *L. casei* DNA (Llopis *et al*., 2008).

1.6.4.6 Systemic Anti-inflammatory Activities of Probiotics

The anti-inflammatory effects of probiotic bacteria could be systemic, at least in part, rather than localized. Beneficial effects have been observed after parenteral administration of inactivated and fractionated bacteria (Rachmilewitz *et al*., 2004; Sheil *et al*., 2004). In one study, co culture of *L. casei* or *L. bulgaris* with mucosal explants from affected intestinal mucosa of CD reduced the inflammatory response induced by co-culture of bacteria. This was associated with a significant reduction in pro-inflammatory cytokines including TNF-α, a reduction in the number of CD4 cells as well as TNF-α expression among intraepithelial lymphocytes, suggesting that the anti-inflammatory effect might be systemic (Borruel *et al*., 2002).

Investigators from Cork, Ireland, administered *L. salivarius* subcutaneously to IL-10 knockout (KO) mice, which ameriolated the severity of colitis and suppressed collagen induced arthritis, suggesting that the oral route may not be essential for probiotic anti-inflammatory effects and that responses were not disease specific (Sheil *et al*., 2004). Probiotics may not need to encounter the mucosal immune system directly to exert an effect. The faecal microflora remained unchanged following subcutaneous administration, but TNF and IL-12 levels from splenocytes stimulated by *Salmonella typhimurium* decreased and TGF-β levels were maintained, suggesting a mechanism of action distinct from colonic flora modulation (Sheil *et al*., 2004).

1.6.4.7 Probiotic Bacterial DNA

One of the tenets of bacteriotherapy is that viable bacteria are required to have a beneficial effect. Recent studies suggest that bacterial DNA sequences may provide the same effects as live bacteria (Jijon *et al*., 2004; Rachmilewitz *et al*., 2002). Bacterial DNA contains non-methylated CpG motifs which bind to TLR-9. TLR-9 signaling is dependent on the adaptor protein MyD88. In the presence of both TLR-9 and MyD88, non viable bacteria may have the ability to signal and elicit beneficial effects. In an experiment that used methylated and non-methylated genomic DNA isolated from the probiotic preparation VSL#3, DNAse-treated probiotics, and *E. coli* genomic DNA, the authors demonstrated that genomic DNA (but not methylated DNA, calf thymus DNA or DNAse-treated probiotics) ameliorated the severity of colitis in DSS-induced, TNBS-induced and spontaneous colitis in IL-10 knock-out mice. In the same study, intragastric and subcutaneous administration of gamma-irradiated nonviable bacteria and live bacteria produced similar beneficial effects (Rachmilewitz *et al*., 2004). Lammers *et al*. demonstrated that Bifidobacterium genomic DNA induced the secretion of IL-10 by PBMC from healthy donors, demonstrating the immunomodulatory effects of bacteria DNA (Lammers *et al*., 2003). By using a VSL#3 conditioned medium, Petrof *et al.* showed that early proteasome inhibition may account for NFkB inhibition and heat shock protein induction in cell line from the intestinal epithelium of mice. The use of VSL#3 conditioned medium further challenged the concept that to exert beneficial effects probiotics must be live bacteria (Petrof *et al*., 2004).

1.6.5 Mechanisms of Action of Prebiotics

The definition and clinical efficacy of prebiotics in IBD have been described in Section 1.6.3. Prebiotics can influence host immunity and defence (Saulnier *et al*., 2009). Several potential mechanisms of prebiotic-induced immunomodulation have also been reported (Gibson and Roberfroid, 1995; Macfarlane and Cummings, 1999; Macfarlane *et al*., 2006). Firstly, prebiotics can selectively ***increase specific bacteria*** that modulate local cytokine and antibody (IgA) production. Prebiotics influence host defence by selectively stimulating the growth and/or activity of beneficial intestinal bacteria which include *bifidobacteria* and *lactobacillus* (Lomax and Calder, 2009). The increase in beneficial bacteria leads to increased competition with pathogenic bacteria for binding sites on the intestinal epithelium and nutrients. Furthermore, bifidobacteria (Berg, 1996), or microbial substances such as cell wall components and cytoplasmic antigens (De *et al*., 1987), may also cross the intestinal barrier directly into Peyer's patches to activate immune cells. Antibacterial substance produced by bifidobacterium and lactobacillus species can also suppress the growth and survival of pathogens (Gibson and Wang, 1994). Prebiotics in the form of FOS stimulated the growth of both faecal and mucosal bifidobacteria in healthy subjects (Ichikawa *et al*., 2002; Kripke *et al*., 1989).

Secondly, prebiotics can potentially ***increase intestinal short-chain fatty acids*** (***SCFA)*** production and enhance binding of SCFA to G-coupled protein receptors on immune cells within the gut-associated lymphoid tissues (Wong *et al*., 2006). SCFA are produced by microbial fermentation of inulin or oligofructose in the colon and terminal ileum. High concentrations of SCFA affect immune cell functions in the Peyer's patches. In addition, colonic infusion of SCFA or butyrate enhanced epithelial proliferation in distant intestinal

segments, suggesting that SCFA produced in the colon induced beneficial effects throughout entire GI tract (Bourriaud *et al*., 2005; Probert *et al*., 2004).

Fermentation of prebiotics by bifidobacteria species *in vitro* resulted in the production of SCFA, including lactate and butyrate. The generation of SCFA provided an acidic colonic environment which was detrimental to pathogenic bacteria such as bacteroides, clostridia and coliforms (Gibson and Wang, 1994; Millard *et al*., 2002). In addition, acidification of the colon enhanced mucin production which strengthened barrier function (Barcelo *et al*., 2000).

In vitro butyrate suppressed lymphocyte proliferation, inhibited Th1 cytokine production by lymphocytes, induced T cell apoptosis and up-regulated DC IL-10 production (Cavaglieri *et al*., 2003; Kurita-Ochiai *et al*., 2003; Saemann *et al*., 2000).

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In one study, butyrate affected DC maturation and cytokine production *in vitro,* altering the balance of IL-12 and IL-10 secretion towards a regulatory profile (Millard *et al*., 2002). Butyrate is also a putative ligand for PPAR-γ (Kinoshita *et al*., 2002), a nuclear receptor expressed by DC amongst other cells (Desreumaux *et al*., 2001; Dubuquoy *et al*., 2003). Therefore, prebiotics may have effects on the intestinal immune system that are independent from direct manipulation of the intestinal microbiota. The generation of bifidobacteria or SCFA-producing bacteria may also alter the presence of PAMPs in the intestinal lumen including endotoxin or LPS, and unmethylated CpG motifs of DNA (Welters *et al*., 2002) (Akira *et al*., 2001).

Enhanced SCFA production in the gut after prebiotic supplementation increased SCFA supply to immune cells located along the GALT; these cells can be activated via SCFA receptors (Bach Knudsen *et al*., 2003). Another mechanism of prebiotics includes the interaction of prebiotic carbohydrates with carbohydrate receptors on intestinal epithelial cells and immune cells.

In animal models of colitis increasing dietary FOS decreased disease activity, enhanced luminal bifidobacteria and inhibited intracellular transcription factors such as NFkB (Gibson *et al*., 1995; Van *et al*., 1995). Healthy rats fed with a mixture of FOS and a probiotic bifidobacteria demonstrated enhanced IL-10 release from Peyer's patches and increased caecal secretory IgA levels (Roller *et al*., 2004). A small randomised double blind controlled trial of patients with UC showed that supplementation with *B. Longum,* inulin and oligofructose improved inflammation and significantly reduced intestinal mRNA levels of the proinflammatory cytokines IL-1B and TNF-α (Furrie *et al*., 2005).

Altogether prebiotics can enhance the proliferation of beneficial microbes or probiotics, to maximize sustainable changes in the human microbiome. Immune tolerance, intestinal barrier function and the bioavailability of nutrients, may be modified by changing the composition and functions of the microbial communities by such strategies.

We have cited evidence throughout the first chapter that intestinal DC are central immunoregulators of the response to commensal bacteria. In the intestine, DC can be divided into $CD11c^+$ myeloid cells and $CD11c^-$ cells. Most studies on intestinal DC, in particularly in human IBD, have focused almost exclusively on myeloid DC. The nature of the $CD11c^-$ cells in the human intestine has not been formally characterised. We therefore examine the phenotype and functional properties of these cells in IBD **(Chapter 3).** Secondly, in view of the importance of DC in controlling T cell responses, we explore cytokine production by DC in IBD, and correlate these cytokines with disease acitivity and the intestinal microbiota **(Chapter 4).** *In vitro,* probiotic bacteria modulate DC to increase regulatory cytokines and to downregulate proinflammatory cytokines but whether similar effects can be seen in the *in vivo* setting remains unknown. We determine the role that DC may play in the modification of gut inflammation by therapy for IBD which include probiotic bacteria, and a conventional immunosuppressant, corticosteroids. We assess the effects of exogenously administered probiotic bacteria and corticosteroids on DC functions *in vivo* in patients with active UC **(Chapter 5).** Lastly, DC control lymphocyte homing to sites of intestinal inflammation, and we examine the expression of homing molecules on colonic DC in UC **(Chapter 6).**

1.7 Hypothesis

Colonic dendritic cells are central to gut bacterial recognition, orchestrating the inflammatory process, T cell homing to the gut, and the response to medical therapy in patients with inflammatory bowel disease.

1.8 Aims

To test the above hypotheses, the specific aims of the project were:

(1) To identify and characterise colonic $CD11c^-$ $HLA\text{-}DR^+$ $lin^{-/dim}$ cells in UC and controls, and to compare their phenotype and function with that of $CD11c^+$ myeloid DC population

(2) To assess the functions of $CD11c^+$ DC in IBD, and to correlate cytokine production with disease activity and faecal micobiota

(3) To determine the effects of (i) probiotic preparation VSL#3, (ii) corticosteroids and (iii) placebo on intestinal DC function *in vivo,* as part of a controlled clinical trial assessing the effects of VSL#3 in active UC

(2) To assess CD103 expression on human intestinal DC in healthy controls and UC, and to compare the repertoire of homing markers on T cells activated by lamina propria mononuclear cells from UC

Chapter 2

General Materials and Methods

2.1 Materials

2.1.1 Human Intestinal Tissue

Human intestinal biopsies were obtained at colonoscopy or flexible sigmoidoscopy from patients with UC (active and inactive), CD (active) and healthy controls. Between six to ten mucosal biopsies (30 to 60 mg of tissue) were taken per patient. Tissues were stored in medium and ice and transported immediately for processing. Patients who had a colonoscopy had received bowel preparation with Citramag and Senna; patients with active UC who underwent flexible sigmoidoscopy did not receive any bowel preparation. Patients' demographics have been described in Section 2.2.1. Ethical approval and written informed consents were obtained from all patients.

2.1.2 Whole Blood Samples

Five millilitres (ml) human venous blood samples were obtained from healthy volunteers in lithium heparin tubes.

2.1.3 Buffers and Media

***Complete medium*:** RPMI-1640 Dutch Modification (Sigma Aldrick Co. Ltd, Irvine, UK) containing 10% FCS, 20mM L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin and 25μg/ml gentamicin (for processing and culture of intestinal biopsy).

***Culture medium*:** RPMI-1640 Dutch Modification (Sigma Aldrick Co. Ltd, Irvine, UK) containing 10% FCS, 20mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin (For processing and culture of whole blood cells or peripheral mononuclear cells).

FACS Buffer: PBS supplemented with 2% FCS, 0.02% sodium azide and 1mM EDTA.

Phosphate Buffered Saline *(PBS) (Dulbecco's):* Calcium- and magnesium-free balanced salt solution - maintains the integrity of mammalian cells *in vitro* (Sigma, Poole).

2.1.4 Reagents

Antibody beads: Used in multiplex ELISA. Anti-cytokine monoclonal antibody-conjugated microbeads – each bead type is filled with a distinct combination of fluorescent dyes that confer a unique spectral signature (Bender Medsystems, Austria).

Collagenase digestion medium: contains 1mg/mL collagenase D (Roche Diagnostics Ltd, Lewes, England), 20µg/mL deoxyribonuclease I (Roche Diagnostics Ltd) and 2% fetal calf serum in RPMI 1640 Dutch modification used for enzymatic digestion of the tissue.

4',6-diamidino-2-phenylindole (***DAPI)***: a fluorescent stain that binds strongly to DNA, used in fluorescent microscopy

Dithiothreitol (DTT): Supplemented to calcium- and magnesium-free HBSS to remove mucus and faeces. Working concentration 1mM (Sigma-Aldrich).

EDTA: Chelates ions to prevent cell clustering (Sigma, Dorset). Supplemented to calcium- and magnesium-free HBSS to remove epithelial cells.

Foetal calf serum (FCS): supplement for cell culture media. FCS blocks non-specific binding during monoclonal antibody labelling. Stored in aliquots at -80ºC (Tissue culture systems).

Ficoll-Paque: Density gradient used to separate PBMC from whole blood (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Flow-count^{TM} Fluorespheres: Flow cytometry beads (Beckman Coulter, Bucks) used as a reference population for the calculation of absolute cell numbers.

Hanks' balanced salt solution (HBSS): calcium- and magnesium-free HBSS (Gibco BRL, Paisley, Scotland) used to wash tissue cell suspension.

Leucoperm A: Cell fixative reagent used in intracellular cytokine staining of lamina propria mononuclear cells (LPMC) (Serotec, UK).

Leucoperm B: Cell permealisation agent used in intracellular cytokine staining of LPMC (Serotec, UK).

Monensin: Inhibits intracellular protein transport and causes newly synthesized cytokine to be trapped with the Golgi apparatus of cells. (Sigma, MO, USA).

Nycoprep: Density gradient – used to separate low density cell from the non-adherent fraction of PBMC following overnight culture (Axis-Shield, Oslo).

Optilyse C: Erythrocyte lysing solution (Beckman Coulter, UK) used to disrupt red blood cells during intracellular labeling of whole blood cells.

Paraformaldehyde (PFA): Fixative (BDH chemicals, Poole). 1% PFA dissolved in saline by slow heating to 60ºC to pH 7.0-7.4. Stock solution stored at 4ºC.

Sodium azide: Prevents capping and shedding of monoclonal antibody bound to target antigens on viable cells (Sigma, MO, USA).

Tryptan blue: 0.4 % working concentration. Dye excluded by live cells that is used to calculate then proportion of viable cells in suspension (Sigma, Poole).

2.1.5 Antibodies

Antibodies (Abs) with the following specificities and fluorochrome labels were used and isotype- matched controls were obtained from the same manufacturers **(Table 7).**

Ab Specificity	Clone	Conjugate	Isotype	Manufacturer
HLA-DR	G46-6	APC	mIgG2a	BD Pharmingen
CD11c	B-ly6	PE		BD Pharmingen
CD11c	KB90	FITC	mIgG1	Dako Cytomation
DC Exclusion Cocktail	S4.1/TUK4/3G8/SJ25-C1/581	PC5		Serotec
CD40	LOB7/6	FITC	mIgG1	Serotec
CD86	BU63	FITC	mIgG1	Serotec
BDCA-2	AC144	PE	mIgG1	Miltenyi Biotec
BDCA-4	AD5-17F6	PE	mIgG1	Miltenyi Biotec
CD123	9F5	PE	mIgG1	BD Bioscience
TLR-2	TLR2.1	FITC	mIgG2a	Serotec
TLR-4	HTA125	FITC	mIgG2a	Serotec
CD138	B-A38	FITC		Serotec
CD56	N901	PE	mIgG1	Beckman Coulter
NKG2D	1D11	FITC	mIgG1	Abcam
NKp44	PNIM3710	PE	mIgG1	Beckman Coulter
NKp30	FAB1849P	FITC	mIgG1	R&D systems
CD103		FITC	mIgG1	BD Bioscience
B7 Integrin	FIB504	PE	rIgG2a	BD Pharmingen

CD3	UCHT1	PE/PE-Cy5	mIgG1	BD Pharmingen
CD8	RPA-T8	PE	mIgG1	BD Pharmingen
CD8	SK1	FITC/APC	mIgG1	BD Bioscience
CD8	B9.11	PE-Cy5	mIgG1	Beckman Coulter
CD14	RMO25	PE-Cy5	mIgG2a	Beckman Coulter
CD16	3G8	PE-Cy5	mIgG1	Beckman Coulter
CD19	J4.119	PE-Cy5	mIgG1	Beckman Coulter
CD34	581	PE-Cy5	mIgG1	Beckman Coulter
CD45RA	HI100	PE-Cy5	mIgG2b	BD Pharmingen

IL-10	JES3-9D7	PE	rIgG1	Serotec
IL-12p40	C11.5	PE	mIgG1	BD Pharmingen
IL-6	#1936	PE	mIgG2b	R&D systems
IL-13	32007.111	PE	mIgG1	R&D systems

mIgG1	X40	FITC/PE	N/a	BD Bioscience
mIgG1	679.1Mc7	PE-Cy5	N/a	Beckman Coulter
mIgG2a	G155-178	PE/PE-Cy5	N/a	BD Pharmingen
mIgG2a	X39	APC	N/a	BD Bioscience
rIgG2a	LODNP-16	PE/PE-Cy5	N/a	Beckman coulter
mIgG2b	133303	PE	N/a	R&D systems
mIgG2b	27-35	PE-Cy5/APC	N/a	BD Pharmingen
rIgG2b	A95-1	APC	N/a	BD Pharmingen

Table 7: Monoclonal antibody list

2.1.6 Study Drug

VSL#3 (VSL Pharmaceuticals, Inc., Fort Lauderdale, USA) sachets contained 900 billion viable lyophilized bacteria consisting of four strains of lactobacillus (*L. casei, L. Plantarum, L. acidophilus, and L delbrueckii* subsp. *bulgaricus*), three strains of Bifidobacterium (*B. longum, B. breve*, and *B. infantis*), and one strain of *Streptococcus salivarius* subsp. *thermophilus*. The placebo was provided in the form of identical sachets containing maize starch. For patients with UC, each patient received two sachets twice per day of oral VSL#3 (3,600 billion bacteria), or placebo, daily for eight weeks. Patients treated with oral corticosteroids received prednisolone 40mg each day. The steroid dose was reduced by 5mg each week over eight weeks.

2.2 METHODS

2.2.1 Patients and Controls

Diagnosis for patients with IBD was made using clinical parameters, radiographic studies, endoscopic and histologic criteria. Disease activity for UC was assessed using the UC disease activity index: UCDAI, range of scores 0 (no active disease) to 12 (maximum disease) (Singleton, 1987). Disease activity for patients with CD was assessed using the Crohn's disease activity index (CDAI). The CDAI incorporates 8 variables: numbers of stools per day, severity of abdominal pain, general well-being, extraintestinal manifestation, abdominal mass, use of anti-diarrhoeal drugs, haematocrit and body weight. These items yield a composite score ranging from 0 to 600. A score of <150 is generally regarded as reflecting clinical remission, whereas a score above 400 indicates severe disease (Best *et al*., 1976a; Best and Becktel, 1981). The control group consisted of patients with macroscopically and histologically normal intestine who had been referred with symptoms of rectal bleeding or a change in bowel habits.

Demographic details of each patient and full medical history were obtained. Baseline characteristics of patients, including concurrent medical therapy, are described in Table 8. Table 8 represents patients analysed in Chapters 3 and 6.

A subgroup of patients with active UC were recruited as part of a multi-centre, double blind, placebo-controlled therapeutic trial evaluating the effectiveness of the probiotic preparation VSL#3 for the treatment of mild to moderately acute UC (Harrow Research Ethics Committee: 05/Q40405/71). Twenty-eight patients with acute UC were included in

this sub-study and their baseline characteristics have been described separately (Table 12 - Chapter 5). Written, informed consent was obtained from all patients and the study was approved by the Local Research Ethics Committee.

	Active UC (N=44)	Inactive UC (N=10)	Active Crohn's Disease (N=28)	Controls (N=22)
Age(mean)	43	37	44	45
Sex (F/M)	20/24	5/5	16/12	9/13
Disease location				
Distal	24	0	-	-
Extensive	7	2	-	-
Total	13	8	-	-
UCDAI score				
<3	-	10	-	-
3 to 8	39	-	-	-
>8	5	-	-	-
CDAI score (mean)	**-**	**-**	280	-
Medication				
5-ASA	29	8	13	-
AZA/6-MP	6	-	21	-
Steroids	2	-	4	-
No medication	10	2	6	-

5-ASA: 5-aminosalicylic acid
AZA: azathioprine
6-MP: 6-mercaptopurine

Table 8: Patients' baseline characteristics

2.2.2 Intestinal Tissue Sampling and Processing

Isolation of Lamina Propria Mononuclear Cells (LPMC)

The method used was described in detail in previous work from the Antigen Presentation Research group (Bell *et al*., 2001). Approximately ten rectal biopsies were taken from each UC patient and control. Biopsy specimens were collected in ice-cold RPMI 1640 Dutch

modification (Sigma-Aldrich, Dorset, England) supplemented with 10% fetal calf serum, 25μg/mL gentamicin, and 100U/mL penicillin/streptomycin (complete medium). Tissues were incubated with occasional agitation in calcium- and magnesium-free Hank's balanced salt solution (HBSS) (Gibco BRL, Paisley, Scotland) containing 1 mmol/L dithiothreitol (Sigma-Aldrich) for 20 minutes in a T25 tissue culture flask to remove mucus and faeces. Biopsies were blotted and weighed. The epithelium was removed using two 30-minute treatments with 1 mmol/L EDTA in HBSS at 37°C with gentle agitation. The biopsy samples were washed in HBSS between each treatment until the supernatant was macroscopically free of released epithelial cells.

Tissue digestion was performed using 25ml of collagenase digestion medium in a T25 flask on a shaker for 90 to 150 minutes at 37°C in a tightly capped flask. LPMC released from the tissue samples were passed through a cell strainer and washed in complete medium. The total number of mononuclear cells in suspension was calculated by Trypan blue viability count. Approximately $2X10^6$ LPMC were extracted from ten colonic biopsies.

2.2.3 Cell Surface Labelling

LPMC were labelled in phosphate-buffered saline (PBS) containing 1 mmol/L EDTA and 0.02% sodium azide (fluorescence-activated cell sorter FACS buffer). A minimum of 50,000 LPMCs were used per antibody labeling. Antibodies were added at predetermined optimal concentrations and labelling was performed on ice for 20 minutes. The cells were then washed twice by centrifugation in FACS buffer (300g, 10 minutes, 4°C), and fixed in

paraformaldehyde (500µL of 1%). The samples were stored protected from the light at 4°C until acquisition on the flow cytometer within 24 hours.

2.2.4 Cytokine Labelling

For intracellular cytokine labelling, paired cultures of LPMC, one incubated with monensin to maintain cytokine within the golgi apparatus of cells and the other incubated without monensin, were cultured for 4 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were labelled for surface markers for 20 minutes on ice, fixed with 50µL leucoperm A and permeabilized with100µL leucoperm B. Five µL of anti-cytokine antibody (IL-10-PE, IL-12-PE, IL-6-PE, IL-13-PE) were added for 20 minutes on ice. The cells were then washed twice in FACs buffer and fixed in 1% paraformaldehyde (500 µL of 0.5%). Samples were stored at 4°C until acquisition within 24 hours.

2.2.5 Dendritic Cell Sorting

LPMCs were extracted as previously described, washed for 5 minutes and resuspended in Mini Macs buffer (PBS supplemented with 2mmol/L EDTA and 0.5% bovine serum albumin). Cells were labelled with an antibody mixture containing $CD11c^-$ PE, lineage cocktail, and HLA-DR-APC for 20 minutes on ice before sorting on a Becton Dickinson FACS Calibur machine (Oxford, England) as a $CD11c^-$ $HLA\text{-}DR^+$ $lin^{-/dim}$ population.

2.2.6 Peripheral Blood Mononuclear Cells (PBMC)

Human whole blood was obtained by venipuncture into sodium-heparin Vacutainers (Beckton-Dickinson) and diluted 2:1 with RPMI-1640 Dutch modification. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of whole blood over Ficoll-paque density gradient 650g for 20 minutes at room temperature. PBMC were harvested from the interface and washed twice by centrifugation at 650g for 10 minutes, and re-suspended in 1ml complete medium. The total number of mononuclear cells in suspension was calculated by Trypan blue viability count.

2.2.7 Enrichment of Blood Dendritic Cells (Low Density Cells)

For use in functional assays, DC-enriched low density cells (LDC) were prepared from blood of healthy donors. LDC were prepared by culturing PBMC overnight in complete medium at $4X10^6$/ml in T25 flasks (Falcon) at 37°C in a humidified atmosphere of 5% CO2 and then separating non-adherent cells on hypertonic nycoprep (14.5g added to 100ml of complete medium). Cells recovered from the interface were washed twice by centrifugation (650g for 10 minutes), re-suspended in complete medium, counted and used as a source of blood DC in stimulation assays.

2.2.8 Mixed Leucocyte Reaction (MLR)

The stimulatory capacity of LPMC, blood LDC and sorted $CD11c^-$ cells was assessed in an allogeneic MLR. Carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled

naïve ($CD45RO^-$) $CD4^+$ T cells or CFSE-labelled $CD14^-$ cells were used as responders, and LPMC, sorted $CD11c^-$ cells and LDC, enriched for DC, as stimulators. To obtain responder cells, PBMC were obtained from healthy controls as described above and resuspended in 0.5ml MiniMACS buffer (PBS containing 0.5% BSA and 2mM EDTA) and labelled with a mixture of immunomagnetic microbeads coupled with anti-CD19 (40 µl), anti-CD14 (40 µl) and anti-HLA-DR (50 µl) (All from Miltenyi biotech, Bisley, UK) and with allophycocyanin (APC) conjugated antibodies to CD45RO (20 µl) and CD8 (20 µl) (BD Biosciences, Oxford, UK) for 20 minutes at 4°C. The cells were washed twice in cold mini-Macs buffer (480g, 5 min) and labelled with anti-APC microbeads (Miltenyi) on ice for 30 minutes. After washing the cells twice in Mini-Macs buffer, the resultant cells were separated on LD magnetic Mini-MACS columns (Miltenyi Biotec), washed twice (500g, 4°C, 5 min) and resuspended in 0.5ml PBS. Cells not retained on the column were collected as a source of naïve $CD4^+$ T cells. An equal volume of CFSE in PBS solution was added to the cell suspension to give a 5µM final concentration. After incubation for 3 minutes at room temperature, 1ml FCS was added to the reaction mixture to prevent further labelling. The CFSE-labelled cells were then washed twice in complete medium and enumerated.

The CFSE-labelled $CD4^+$ naïve T cells were plated out in 96-well round bottom plate at a concentration of $4X10^5$ cells per well. The stimulator cell populations were added at a concentration of ~8000 cells per well in 200µl total volume Complete medium (medium alone was added as a negative control). After 5 days of culture, cells were harvested and analysed by flow cytometry to assess for lymphocyte proliferation. Dividing cells were identified by the reduction in CFSE staining intensity. The numbers of dividing cells were determined by reference to FlowCount beads.

2.2.9 Multiple Enzyme Linked Immunoabsorbent Assay (ELISA)

Supernatants were harvested from tissues cultured overnight in the incubator. The cytokine contents of the supernatants were measured using multiplex ELISA kits (human Th1/Th2 10plex Kit II BMS7 16FF, Bender Medsystems, Austria). The amount of ten cytokines in the supernatant was quantified. The ten cytokines assayed and respective limit of detection of each analyte are shown in Table 9. According to manufacturer's instructions, the antibody beads, biotinylated detector antibodies and streptavidine-PE were diluted to a 1 X working concentration. 25µl aliquots of supernatant (or corresponding standards of known analyte concentration) were pipetted into a 96-well filter bottom microplate and incubated with 25µl mixed antibody beads and 50µl biotinylated conjugate mixture per well in the dark for 2 hours at room temperature on a plate shaker (500rpm). After washing, the beads were stained with streptavidine-PE (50) for 1 hour in the dark on a plate shaker. After further two washes with 100µl assay buffer using vacuum manifold to aspirate, 200µl of assay buffer was added to each well, the contents were mixed and transferred into a FACS tube. The mixture was topped up with 300µl assay buffer to a final volume of 0.5ml per tube. The samples were stored at 4ºC and resuspended thoroughly before acquisition on flow cytometer within 24 hours. The quantities of cytokine in the supernatant were determined against a standard curve for known concentrations of that cytokine.

Analyte	Sensitivity
IL-1β	4.5 pg/ml
IL-2	8.9 pg/ml
IL-4	6.4 pg/ml
IL-5	5.3 pg/ml
IL-6	4.7 pg/ml
IL-8	6.4 pg/ml
IL-10	6.9 pg/ml
TNF-α	7.9 pg/ml
IL-12p70	9.7 pg/ml
IFN-γ	7.0 pg/ml

Table 9: Analytes and sensitivity for Multiplex ELISA

2.2.10 Cytospins and Immunofluorescence Staining

Assisted by Hafid Omar-Al-Hassi of the Antigen Presentation Research Group

Indirect immunofluorescence labelling was performed on sorted $CD11c^{-}$ $HLA\text{-}DR^{+}$ $lin^{-/dim}$ cells using cytospins. Slides were washed in PBS for 5 minutes and fixed in 1:1 acetone / methanol for 4 minutes at room temperature. Cytospins were incubated with anti-HLA-DR, followed by anti-CD123 and anti-BDCA-2. Slides were then rinsed in PBS for 5 minutes and mounted under coverslips.

2.2.11 Electron Microscopy

Performed by Nicholas English of the Antigen Presentation Research Group

Approximately $12X10^3$ sorted $CD11c^-$ cells were mixed with a 10-fold excess human red blood cells in order to provide bulk for subsequent processing, and fixed in 3% glutaraldehyde in 0.1M sodium phosphate buffer (pH7.4) for 12 hours at 4°C. Cells were embedded as a pellet in low gelling temperature agarose (Sigma). Cells were washed twice with the buffer and fixed in 1% osmium tetroxide in 0.1% (pH 7.4) sodium phosphate buffer for 1 hour. After washing with water to remove the sodium phosphate and keeping in water overnight, cells were block stained in 2% uranyl acetate for 2 to 4 hours. The cells were washed with distilled water, dehydrated using an acetone gradient, and gradually infiltrated with araldite resin. After at least 2 changes of araldite over 4 to 8 hours, the cells were embedded in the araldite resin and cured for 18 hours at 65°C. The blocks were cut into 100nm sections using a Reichert-Jung Ultracut E microtome and collected on 200 mesh copper grids. The ultrathin sections were stained with Reynold's lead citrate and viewed on a Jeol JEM-1200 EX electron microscope.

2.2.12 Flow Cytometry

Multi-colour flow cytometry characterizes and measures the physical properties of individual cells in suspension as they pass through one or more focused laser beams.

During acquisition, 10µl of flow-count fluorospheres at a known concentration was added to each tube. Blood samples were acquired for approximately 5 minutes at high flow rate,

and tissue samples were acquired for approximately 8 minutes. All data were then saved as list-mode data files and transferred to a computer for analysis.

(i) FACs Analysis

Data were acquired using a FACs Calibur flow cytometer (Becton Dickinson, Oxford, England) using CellQuest software for partial online colour compensation. Completion of compensation and analysis of listmode data was carried out offline using WinList software (Verity Software House, Maine). For compensation, a region was made around the lymphocyte area of the side scatter (SSS) versus forward scatter (FSC) plot and the CD8 marker was evaluated in each channel using the compensation toolbox on the Winlist software programme. Live cells (viability gauged by size and granularity) were analysed on a plot of HLA-DR versus lineage cocktail staining. Lineage cocktail comprised specific monoclonal antibody labels for T cells (CD3), monocytes (CD14, CD16), B cells (CD19), and stem cells (CD34). DC were identified as HLA-DR^+ $lin^{-/dim}$ cells. Using multi-colour analysis, $CD11c^+$ and $CD11c^-$ populations within a HLA-DR^+ $lin^{-/dim}$ gate were assessed for expression of surface markers and cytokine production. Two methods were used to analyse the flow cytometry data: *(a) Region Gating* and, (b) Enhanced Normalized Subtraction (ENS).

(ii) Region Gating

The percentage of cells expressing a given surface marker was measured by determining the proportion of antibody stained cells falling beyond the distribution of staining with an isotype matched control antibody. For example, a region is drawn that excludes irrelevant, isotype matched control mAb or non specific staining. All events that fall within this

region exceed istopye control staining intensity and is regarded as positively labeled cells **(Figure 2.1).** WinList^{TM} calculates the proportion of positive cells amongst the total DC population. This method was used because it (1) discounts all events that fall within the distribution of the control histogram; (2) more accurately delineates subpopulations based on surface antigen expression; (3) reduces impact of differences in fluorochrome (antibody binding and free fluorochrome variation between batches of antibody). The region method was used to generate percentage positive cells in all phenotypic analysis.

(iii) Enhanced Normalised Subtraction

Enhance Normalised Subtraction (WinList software) was used to measure (1) the *level* of staining for cell surface marker and; (2) the percentage of cytokine-positive cells.

The ***level of staining for surface markers***, expressed as an intensity ratio (IR) representing the ratio of median value of positive events in the test histogram to median value of staining with an isotype-matched control antibody was measured using Enhanced Normalised Subtraction (ENS) on WinList software (Verity Software House, Maine) (Panoskaltsis *et al*., 2002) **(Figure 2.2).**

The ***percentage of cytokine-positive cells*** was determined by superenhanced D_{max} (SED) normalized subtraction. Normalized cumulative histograms of staining of cells cultured without monensin (control sample - used as a reference sample allowing for gain or loss of cytokine to be measured) were subtracted from histograms of the staining in the presence of monensin (test histogram), allowing the build-up of trapped synthesized protein to be detected in a 4 hour window (Holden *et al*., 2008); To minimise the effects of non-specific

binding, control cells were treated in the absence of monensin but fixed, permeabilised and labelled with identical antibody as the test sample staining, hence we felt that the inclusion of an isotype control was not necessary. Monensin is an ionophore originating from *Streptomyces cinnamonensis* that disrupts protein transportation from the Golgi apparatus leading to the trapping of newly synthesised protein within the cytoplasm of cells (Mollenhauer *et al*., 1990).

To discriminate positive from negative events in the test histograms, we used Winlist™ software and Enhanced Normalised Subtraction technique which can sensitively detect intracellular cytokine in non-stimulated DC. When there is an obvious separation between the test and control histograms, flow cytometry analysis software can generate cut off markers to accurately define the positive events. In some cases, secreted protein binds back to the surface of cell in the no monensin sample together with the loss of pre-existing cytokine from cells in the monensin sample, which contribute to the overall loss of cytokine. In such situation the subtraction process can be reversed to generate negative results. In this system, the control sample does not represent zero but represents a reference point on which to quantify the build up of cytokine in our test sample. The no monensin sample may contain cytokine positive cells. It is therefore important to quantify the reduction in cytokine production compared with background control as labelling these negative results as zero would be inaccurate. The optimisation of this technique to detect ongoing cytokine production in un-stimulated DC has been described, and this modified technique may complement both secretion and gene expression techniques (Holden *et al*., 2008).

The subtraction technique allows a more accurate calculation of the proportion positively labeled cells especially when events are low or when the test and controls histograms overlap. An example is shown in **Figure 2.3.**

The ***level of staining*** (IR) for cytokine positivity was determined using ENS, as a ratio of median value of positive events in test histograms to median value of control histograms (Gupta *et al*., 2007).

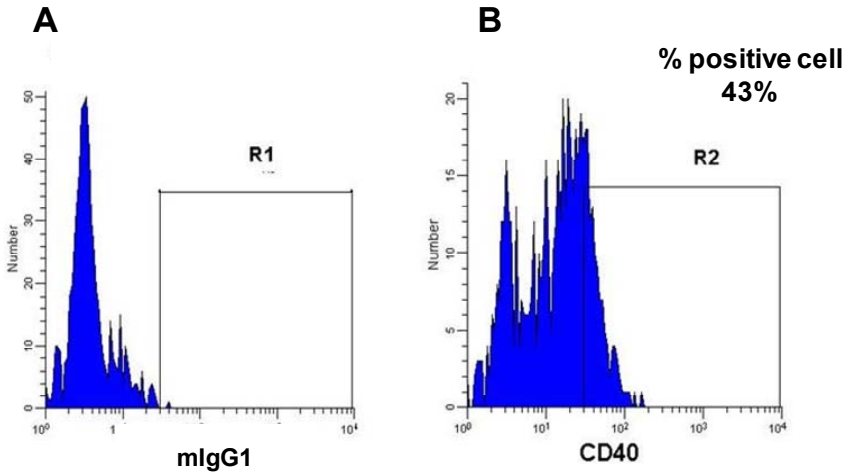


Figure 2.1: Cell surface labelling by region gating. A region is in drawn that excludes non-specific staining or isotype matched control monoclonal antibody staining (A-R1). The same region (R2) is applied to the phenotype staining histogram (B). All events that fall within this region and exceed isotype control staining (with a cut-off of <0.5% cells within R1) was calculated by WinList and regarded as positively labelled cells. This method was used in the generation of all percentage positive cells data for phenotypic analysis as it more accurately delineates subpopulations based upon surface antigen expression.

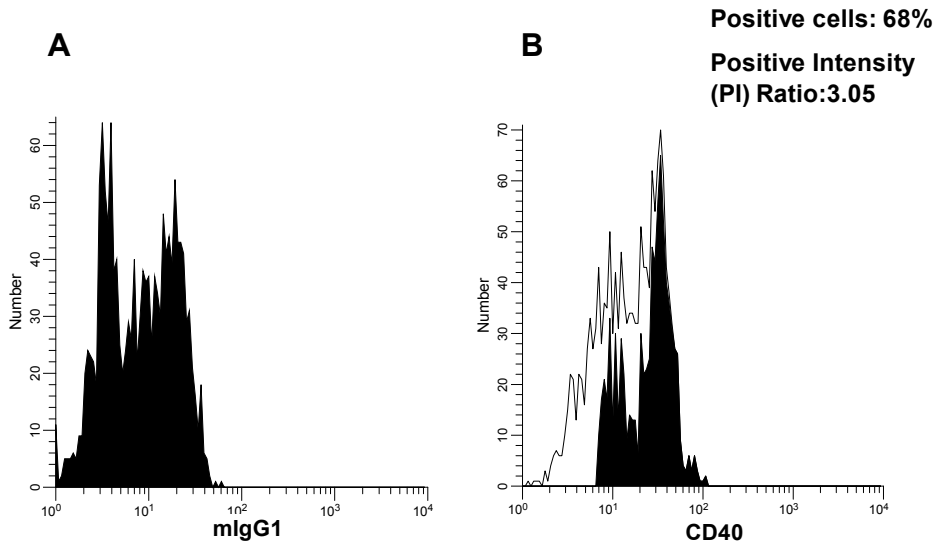


Figure 2.2: Positive Intensity Ratio (PIR). (A) is the isotype control which represents the level of non specific staining with an irrelevant isotype matched control monoclonal antibody whereas (B) is the staining of the surface marker CD40; this represents specific labelling for CD40 combined with an element of non specific staining. Using Enhanced Normalised Subtraction (ENS), WinList software generates positive IR after subtracting the non specific binding component from the phenotype labelling histogram. The median channel of fluorescence and distribution of each histogram is compared and the proportion of positive events calculated (B-shaded area). This is the ratio of linearised fluorescence median of only the positive events after subtraction to the linearised control median. The IR compare the relative intensities of positive events in a test sample (B) compared with all events in control distributions (A).

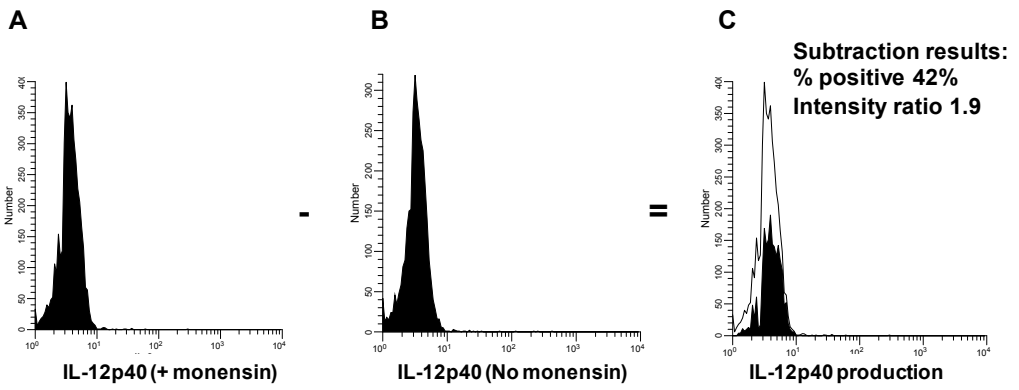


Figure 2.3: Intracellular cytokine production by colonic DC measured with Enhanced Normalised Subtraction (ENS). Detection of ongoing intracellular cytokine production in unstimulated DC was measured by ENS. This figure shows one-parameter histograms for the intracellular staining of IL-12p40 in $CD11c^+$ DC in the absence (B - No Monensin), and presence (A + Monensin) of monensin. Using Super-enhanced Normalised Subtraction from Winlist software, cytokine production was determined by the subtraction of staining in the sample with monensin from staining in sample without monensin. The shaded area (C) on the right panel represents the proportion of cells staining positive for IL-12p40 after subtraction. When more "positive events" in the control histogram were detected compared with test histogram, subtraction was reversed with the generation of negative events in the test histogram. Staining cells from control and test histogram in the same way ensured minimal difference in non-specific binding between test and control samples. The result is representative of ongoing cytokine production in the absence of exogenous stimulation. Specificity of antibody labelling has been previously confirmed in competition experiments with unlabelled relevant and irrelevant antibodies. Using unlabelled antibodies, specific blocking antibodies blocked cytokine staining but irrelevant blocking antibodies were not able to inhibit staining production.

(iv) Absolute Cell count

Absolute cells counts were obtained by simultaneous acquisition of Flow-Count fluorospheres (Coulter Immunotech). Fluorescent counting beads were identified and enumerated on a FL1 versus SCC plot. Based on the number of events in the cell region of interest and on the number of events in the bead region, the number of cells per µl can be calculated.

Number of cells per µl

$$= \frac{\text{Number of events in gated region}}{\text{Number of beads in gated region} \times \frac{\text{volume of sample}}{\text{volume of beads}}} \times \text{bead conc/µl}$$

2.2.13 Fluoresecent in-situ Hybridisation (FISH)

Performed by Jane Benjamin/Kevin Whelan (collaborators at Kings College Hospital)

Constituents of the fecal microbiota were quantified using FISH (Franks *et al*., 1998b; Harmsen *et al*., 2002). The fecal microbiota were fixed and hybridized with indocarbocyanin (Cy3) labelled oligonucleotide DNA probes targeting total bacteria cells 4' 6-diamidino-2-phenylindole (DAPI), bifidobacteria, *Bacteroides-prevotella, Clostridium coccoides-Eubacterium rectal (EREC),* and *Faecalibacterium (F.) praustnitzii* (Microsynth, Switzerland). These bacterial groups were chosen because they have been shown to be altered in patients with IBD (Seksik *et al*., 2003; Sokol *et al*., 2008; Sokol *et al*., 2009b; Swidsinski *et al*., 2002; Tamboli *et al*., 2004).

Faecal bacteria from fresh faecal samples were immediately harvested in PBS (0.1mol/L; pH 7.0) with the use of glass beads and centrifugation, and fixed overnight in 4% (w/v) paraformaldehyde. Bacteria were then washed with PBS (0.1mol/L; pH 7.0) three times and suspended in a 1:1 solution of PBS and 96% ethanol. This solution was then stored at -20ºC. Fixed bacteria were spotted on 3-aminopropyltriethoxysilane treated eight-well slides, air dried and serially dehydrated in 60%, 80%, and 96% ethanol (Maddox and Jenkins, 1987). Total cells counts were quantified using a nucleic acid stain, 4, 6-

diamidino-2-phenlindole (Kapuscinski, 1995). Individual species were quantified using hybridisation with indocarbocyanin (Cy3)-labelled oligonucleotide probes targeting specific regions of 16SrRNA **(Table 10).** The fixed bacteria was then hybridised with the oligonucleotide probes according to a previously published protocol (Amann *et al*., 1990). Briefly, probes were diluted to a concentration of 4.5 ng/µl in sterile hybridisation buffer (0.9 M NaCl; 0.02M Tris/HCl; 0.01% sodium dodecycl sulphate) and 10 µl added to each well which was then incubated at 46°C overnight in a light-free saturated humidity chamber. Slides were washed to remove unbound probe prior to quantification within 24 hours (Mylonaki *et al*., 2005).

Hybridized fecal microbiota were manually quantified by viewing the slides under an Axioplan 2 imaging microscope (Zeiss, Germany) equipped with an HBO-100 fluorescent lamp (Osram, Germany) and filterset number 15 (Zeiss, Germany). Fifteen random fields were selected in duplicate for each probe. Faecal bacterial concentrations were expressed as cells per gram of dry faeces in order to standardise the comparison between patient samples with varying consistency. Faecal bacterial concentrations were expressed as cells per gram of dry faeces in order to standardise comparison between samples.

Target bacterial Group	**Probe**	**Sequence (5'-3')**
Bifidobacteria	Bif 164 (Langendijk *et al*., 1995)	-CAT CCG GCA TTA CCA CCC-
Bacteroides	Bac 303 (Manz *et al*., 1996)	-CCA ATG TGG GGG ACC TT-
Clostridium coccoides-Eubacterium rectal	EREC 482 (Franks *et al*., 1998a)	-GCT TCT TAG TCA RCT ACC G
Faecalibacterium prausnitzii	Fprau-0645 (Suau *et al*., 2001)	-CCT CTG CAC TAC TCA AGA AAA AC-

Table 10: Target bacteria group, indocarbocyanin (Cy3)-labelled oligonucleotide probes and targeted regions of 16SrRNA

2.2.14 Statistical Analysis

Assisted by Paul Bassett

Statistical analyses were carried out using Sigma stat software (SPSS Inc Chicago). Pooled data were expressed as median values ± mean standard error. Two-tailed t tests were employed to compare normally distributed data and Mann-Whitney Rank-Sum tests were used to analyse non-normally distributed data.

Stata (version 9.2) by StataCorp (4905 Lakeway Drive, College Station, Texas 77845 USA) software was used for statistical analyses of pre and post treatment values using the Wilcoxon matched-pair test. Association between variables was examined using Pearson correlation. Associations between variables were assessed using Pearson's (for normally distributed data) or Spearman's (for skewed distribution) rank correlation. These methods measure the strength of the association between two variables on a scale between -1 and +1. A correlation coefficient of near to -1 implies a strong negative relationship (as one measure increases, the other decreases), a correlation coefficient near to 0 implies little evidence of a relationship between the two measures, whilst a correlation coefficient near to +1 implies a strong positive relationship (as one measure increases, the other also increases). For most data, values $p<0.05$ were considered significant, whereas for multiplex and correlation analyses, due to the large number of different analyses there is more possibility of a significant result due to chance alone and the Bonferonni correction is important, and so a more stringent level of significance is used. Only analyses with a p-value of less than 0.01 were considered to be statistically significant in this analysis.

2.2.15 Definition of Clinical Outcome

The Ulcerative Colitis Disease Activity Index (UCDAI) is the sum of scores from four criteria (stool frequency, rectal bleeding, sigmoidoscopic mucosal appearance, physician's global assessment), each ranked zero to three giving a maximum of 12 points (Singleton, 1987). Clinical remission was defined as a UCDAI of ≤ 2 points. Clinical response was defined as a decrease in UCDAI ≥3 points or a final score ≤3.

2.2.16 Grading of Histologic Inflammation

The severity of histologic inflammation in each rectal biopsy specimen was reported by an expert pathologist. The degree of inflammation for each rectal biopsy was graded as: 0, inactive/quiescent/normal (No epithelial infiltration by neutrophils); 1, mild (neutrophil infiltration of <50% of sampled crypts or cross sections, no ulcers or erosions); 2, moderate (neutrophil infiltration of ≥50% of sampled crypts or cross sections, no ulcers or erosions, or 3, severe (erosions or ulceration, irrespective of other features) (Gupta *et al*., 2007).

Chapter 3

Characterisation of Human Colonic CD11c^{-} Cells in Inflammatory Bowel Disease

3.1 Abstract

Introduction: Intestinal DC are heterogeneous, comprising phenotypically and functionally distinct subpopulations. They sample luminal bacteria and are key players in the regulatory events that normally limit inflammatory responses to commensal bacteria. In human IBD, colonic myeloid DC, identified as $CD11c^+$ cells within a $HLA\text{-}DR^+$ $lin^{-/dim}$ population, are activated. The nature of $CD11c^-$ cells, also present within this population, remains unclear. We hypothesized that this hitherto poorly characterized population in the gut may be involved in intestinal inflammation.

Methods: $HLA\text{-}DR^+$ $lin^{-/dim}$ cells were identified in freshly isolated lamina propria mononuclear cells by multicolour flow cytometry. Proportion and number of $CD11c^+$ and $CD11c^-$ cells, surface expression of activation markers CD40, CD86, TLR-2, TLR-4, and CD56 (natural killer - NK marker), were determined on these cells extracted from tissue of patients with UC (54), CD (28), and controls (22).

Results: $HLA\text{-}DR^+$ $lineage^{-/dim}$ cells represented approximately 3% and 0.7% of all lamina propria mononuclear cells in UC and controls, respectively. Lamina propria colonic $CD11c^-$ $HLA\text{-}DR^+$ $lin^{-/dim}$ cells were significantly increased in tissues from UC (inflamed and "non-inflamed") and CD compared with controls. In contrast, numbers of $CD11c^+$ $HLA\text{-}DR^+$ $lin^{-/dim}$ cells were unchanged. $CD11c^-$ cells decreased after inflammation resolved in UC. Fewer $CD11c^-$ cells expressed activation markers, TLR or produced IL-10, IL -12p40, IL-6 and IL-13, compared with their $CD11c^+$ counterparts. $CD11c^-$ cells were weakly stimulatory in mixed leucocyte reactions. Few colonic $CD11c^-$ cells expressed plasmacytoid DC markers (BDCA-2, BDCA-4 and CD123) but a major subset expressed high levels of CD56.

Conclusions: Intestinal inflammation in IBD is associated with the presence of cells that share phenotypic features of both DC and NK cells. This novel population of human colonic $CD56^+$ $HLA\text{-}DR^+$ cells may function in immune regulation or tissue repair. Their increase in inactive UC may be a marker of sub-clinical inflammation.

3.2 Introduction

The pathogenesis of UC remains unclear but emerging evidence suggests that it involves a dysregulated immune response to the enteric intestinal microflora in genetically predisposed individuals (Podolsky, 2002).

Discrimination between the beneficial commensal organisms and potentially harmful pathogens is a central component of the essential role that gut immune cells play in maintaining the balance between oral tolerance and immune activation. In the gut, DC are likely to play a pivotal role in the initiation and perpetuation of immune responses (Steinman, 1991). Within intestinal mucosa tissues, aggregates of DC, ultimately derived from bone marrow precursors, are present in small numbers in organized lymphoid tissues, MLN, and the lamina propria of the small and large intestine (Banchereau *et al*., 2000; Iwasaki and Kelsall, 1999; Kelsall *et al*., 2002; Maric *et al*., 1996). Intestinal DC have tissue-specific properties distinct from those of their non-mucosal counterparts, probably as a reflection of adaptations to their microbe rich external environment (Stagg *et al*., 2003).

Functionally distinct subsets of DC have been defined in mice and humans based on their cell-surface phenotype and functional properties. In humans, these subsets include conventional myeloid ($CD11c^{+}$ $BDCA2^{-}$, $BDCA4^{-}$ $CD123^{lo}$) DC and plasmacytoid ($CD11c^{-}$, $BDAC2^{+}$, $BDCA4^{+}$, $CD123^{hi}$) DC. DC present in inflammatory sites are functionally different from those found in the tissues under steady state conditions (Le *et al*., 2006; Salazar-Gonzalez *et al*., 2006) and studies in animal models of colitis and in human IBD strongly suggest a role for DC in driving intestinal inflammation (Krajina *et*

al., 2003; Leithauser *et al*., 2001). In patients with active IBD, DC are reduced in number in the peripheral blood (Baumgart *et al*., 2005), but increased in the intestinal tissue (Middel *et al*., 2006; te Velde *et al*., 2003) suggesting DC recruitment to and / or retention within the GI tract during inflammation.

Previous work from this laboratory has characterised HLA-DR$^+$ lineage (lin)$^{-/dim}$ (lin = anti-CD3, 14, 16, 19, 34) cells isolated from the human colonic lamina propria (Hart *et al*., 2005). This population comprises a CD11c$^+$ subset together with a variable number of CD11c$^-$ cells. The CD11c$^+$ cells have phenoptypic, morphologic and functional properties of myeloid DC, and disease-associated changes in these cells occur in IBD (Bell *et al*., 2001). For instance, in CD, more colonic CD11c$^+$ DC expressed TLR-2, TLR-4 and produced more IL-12/23p40 (Hart *et al*., 2005). In contrast to the detailed analysis of the CD11c$^+$ HLA-DR$^+$lin$^{-/dim}$ colonic subset, the CD11c$^-$ population has not been characterised. In peripheral blood, cells with a CD11c$^-$ HLA-DR$^+$lin$^{-/dim}$ phenotype comprise plasmacytoid DC but the nature of the cells with this phenotype in colonic tissue and the potential contribution of such cells to the development or regulation of intestinal inflammation, remain to be investigated.

3.3 Hypothesis

Lamina propria CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ cells are involved in the inflammatory process that underlies active inflammatory bowel disease.

3.4 Aims:

- To identify human intestinal lamina propria DC in inflamed and normal tissue.
- To characterise lamina propria $CD11c^-$ population in patients with UC and controls, and to compare these cells with their $CD11c^+$ counterparts.

3.5 Identification of Human Intestinal Lamina Propria Dendritic Cells

LPMC were obtained from colonic biopsies by immediate collagenase digestion. DC were identified in LPMC as $HLA\text{-}DR^+$ $lineage^{-/dim}$ populations on four-colour flow cytometry **(Figure 3.1).** In the human intestine, DC have been categorized into two subsets: $CD11c^+$ myeloid DC and $CD11c^-$ cells. $HLA\text{-}DR^+$ $lineage^{-/dim}$ cells represented approximately 3% and 0.7% of all LPMC in UC and controls, respectively. In controls, lamina propria DC were predominantly myeloid cells.

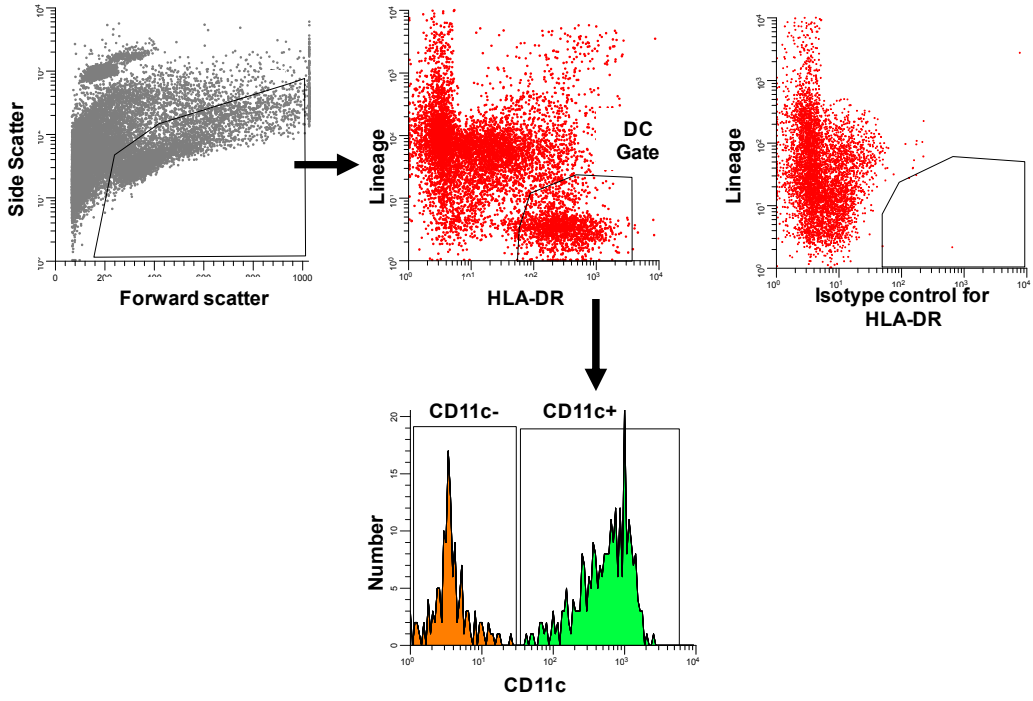


Figure 3.1: Identification of intestinal lamina propria dendritic cells. The LPMC are shown on a forward scatter (FSC) versus side scatter (SCC) plot. DC were identified in LPMC as HLA-DR^{+} lineage$^{-/dim}$ (lin = CD3^{-}, CD14^{-}, CD16^{-}, CD19^{-}, CD34^{-}) populations, labelled DC gate. HLA-DR^{+} cells were gated with reference to the isotype control antibody labelling. Within the DC gate, CD11c^{+} and CD11c^{-} cells were present.

3.6 Lamina Propria CD11c^{-} HLA-DR^{+} lineage$^{-/dim}$ Cells are Increased in Ulcerative Colitis

The number of HLA-DR^{+} lineage$^{-/dim}$ cells and DC subsets in both UC and control tissues were determined with reference to simultaneous acquisition of FlowCount beads. Based on the number of events in the cell region of interest and on the number of events in the bead region and a known bead concentration, the number of cells per milligram tissue was determined as described in Section 2.2.12(iv). There was a significant increase in the number of HLA-DR^{+} lin$^{-/dim}$ cells from inflamed tissue in UC (median± SEM, 503±101 / mg tissue) compared with control tissue (164±22 cells / mg tissue; n=44; $p<0.05$)

(Figure 3.2). In Figure 3.2, even when the two high "outliers" (> 2000 HLA-DR$^+$ lin$^{-/dim}$ cells/mg) were excluded, the number of cells remained significantly different between UC and controls. In addition, the proportion of HLA-DR$^+$ lin$^{-/dim}$ cells that was CD11c$^-$ was significantly greater in LPMC from UC patients compared with the proportion in healthy controls ($p<0.001$) **(Figure 3.3).**

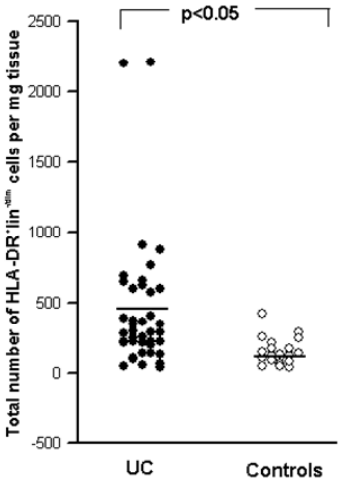


Figure 3.2: Number of HLA-DR$^+$ lineage$^{-/dim}$ cells in UC and controls. This figure shows the total number HLA-DR$^+$ lineage$^{-/dim}$ cells per milligram tissue in active UC patients and controls. Each dot represents one subject. Horizontal line represents median value (UC=44; controls=22).

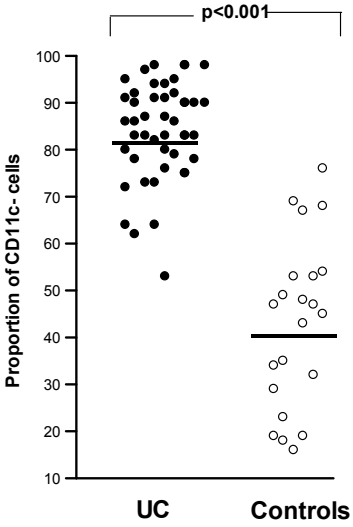


Figure 3.3: Proportion of CD11c$^-$ cells in active UC and controls. This figure shows the proportion of CD11c$^-$ cells within the HLA-DR$^+$ lineage$^{-/dim}$ region (putative DC gate). Horizontal line represents median value. Each dot represents one subject (UC=44; controls=22).

The increase in proportion of $CD11c^-$ cells was due to increased numbers of this population rather than a loss of $CD11c^+$ cells in disease **(Figure 3.4A).** The number of colonic $CD11c^-$ $HLA\text{-}DR^+$ $lin^{-/dim}$ cells was significantly greater for UC patients than controls (UC 447±94 vs. control 102±17 / mg tissue, $p<0.001$). In contrast, the number of $CD11c^+$ DC did not differ significantly between UC patients and controls (p=ns) **(Figure 3.4B).** Overall, these data indicate that there were more $HLA\text{-}DR^+$ $lin^{-/dim}$ cells in the colons of UC patients and that this difference was attributable to a selective increase in $CD11c^-$ cells.

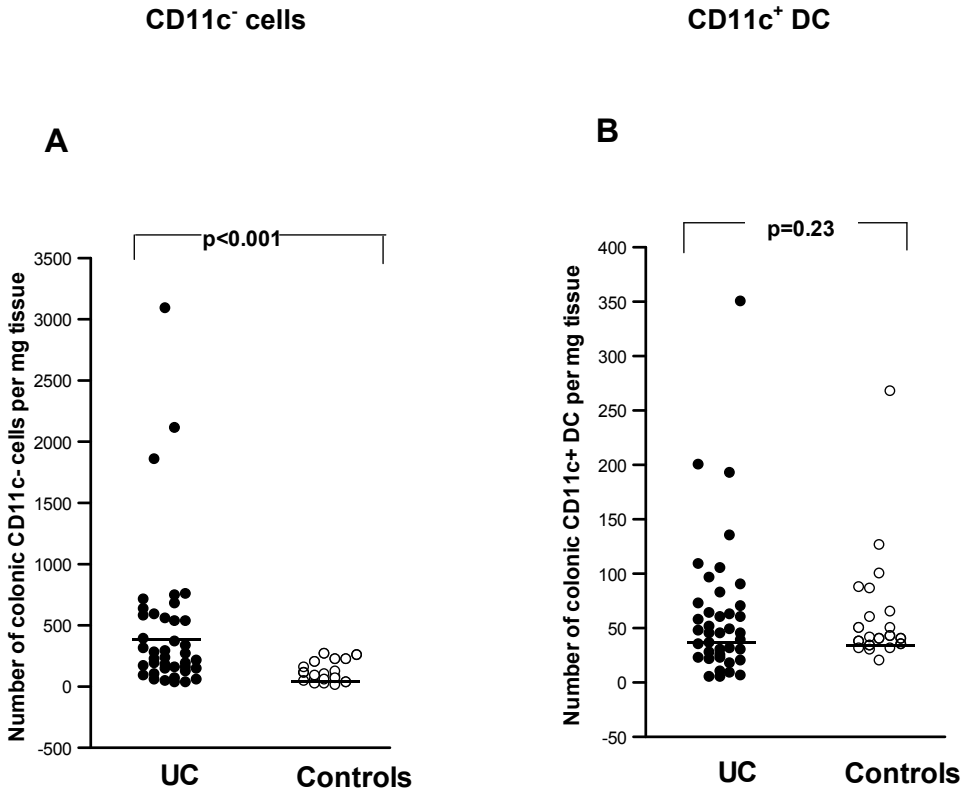


Figure 3.4: Absolute numbers of $CD11c^-$ cells and $CD11^+$ DC in active UC and controls

(A) Number of $CD11c^-$ cells and; **(B)** Number $CD11c^+$ DC per mg tissue in UC (n=44) and controls (n=22). All samples were taken from colonic tissue. Horizontal line represents mean value.

To determine whether the increase in CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ cells was also present in "inactive" or quiescent disease, two approaches were taken. Firstly, in 10 active UC patients, repeat tissue sampling was performed following symptom resolution. Resolution in macroscopic inflammation (sigmoidoscopy score ≤ 2) was associated with decreased numbers of CD11c$^-$ cells (399±154 to 92±23 / mg tissue; $p<0.05$). Conversely, there was a non-significant trend towards increased number of CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ cells in patients who had persistent or worsening macroscopic inflammation at repeat sampling **(Figure 3.5).** There was, however, no significant change in the number of CD11c$^+$ DC, from either tissue with resolved inflammation (69±29 to 40±7 / mg tissue; p=ns) or from those with persistent inflammation (56±17 to 77±12 / mg tissue; p=ns). Thus CD11c$^-$ cells, but not CD11c$^+$ DC, are likely to be markers of intestinal inflammation.

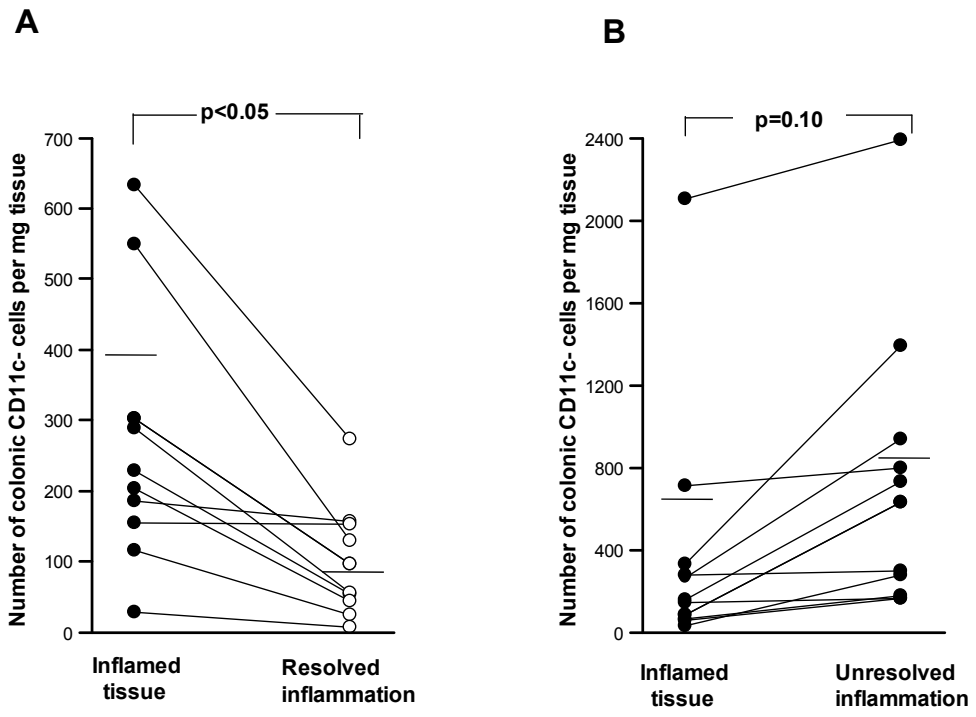


Figure 3.5: Changes in number of CD11c$^-$ cells with and without reduction of macroscopic inflammation. In ten active UC patients, repeat tissue sampling was performed to determine the changes in the number of CD11c$^-$ cells in tissue **(A)** after resolution of inflammation and **(B)** with persistent inflammation.

Secondly, numbers of CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ cells in 'non-inflamed' tissue from UC patients were directly compared with numbers from inflamed UC tissue and control tissue. Non-inflamed UC tissue was from patients with inactive UC undergoing surveillance (n=6) or from macroscopically uninvolved tissue from patients with subtotal colitis in which there was a clear demarcation between inflamed and 'non-inflamed' tissue (n=4). There was significantly more CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ cells in 'non-inflamed' tissue from UC patients compared with tissue from healthy controls ($p<0.05$) **(Figure 3.6).** In the patients where paired biopsy specimens were taken from both non-inflamed and inflamed sections, the number of CD11c$^-$ cells was significantly higher in inflamed than in 'paired' "non-inflamed" UC tissue ($p<0.05$).

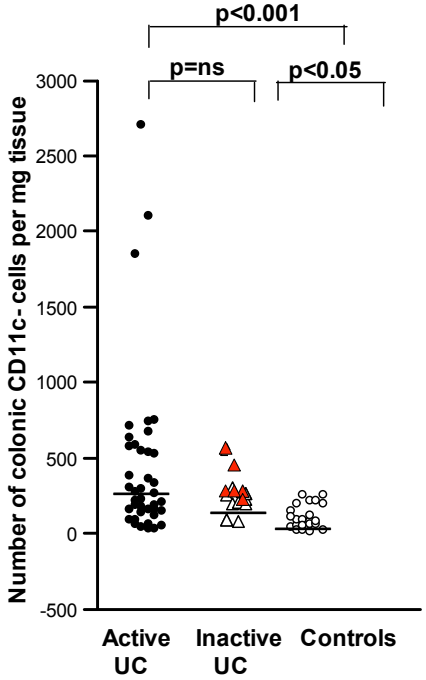


Figure 3.6: Number of CD11c$^-$ cells in active UC, inactive UC and controls. This figure shows the absolute numbers of CD11c$^-$ cells from *inflamed* tissue from patients with active UC, *non-inflamed tissue* from patients with inactive UC (red triangles represent biopsies from previously involved colonic tissue but currently macroscopically normal; unfilled triangles represent uninvolved colonic tissue), and *normal* tissue from controls.

3.7 Lamina Propria CD11c$^-$ Cells are Increased in Crohn's Disease

To assess whether this population is specific for UC, we used patients with CD as our inflammatory controls. There was a significant increase in the absolute number of HLA-DR$^+$ lineage$^{-/dim}$ cells from inflamed CD tissue (1701±635 / mg tissue) compared with both non-inflamed CD tissue (187±28 / mg tissue; $p<0.001$) and control tissue (152±18 / mg tissue; $p<0.001$). Numbers of putative DC was comparable between non- inflamed CD tissue and control tissue (152±18 / mg tissue; $p=0.348$) **(Figure 3.7A).**

When we analysed CD11c$^-$ cells from inflamed CD tissue the number of these cells were markedly higher (1602±621 / mg tissue) compared with non inflamed CD tissue (91±17 / mg tissue $p<0.001$) and control tissue (89±13 / mg tissue; $p<0.001$) **(Figure 3.7B).**
Consistently, the increase in proportion of DC was due to an increase in the number of CD11c$^-$ cells as there were no differences between numbers of CD11c$^+$ DC between inflamed CD, non inflamed CD and control tissues **(Figure 3.7C).** We assessed whether there was an association between disease activity in patients with UC and Crohn's disease and CD11c$^-$ cells. There were no significant correlation between the UCDAI and CDAI and the number of CD11c$^-$ cells.

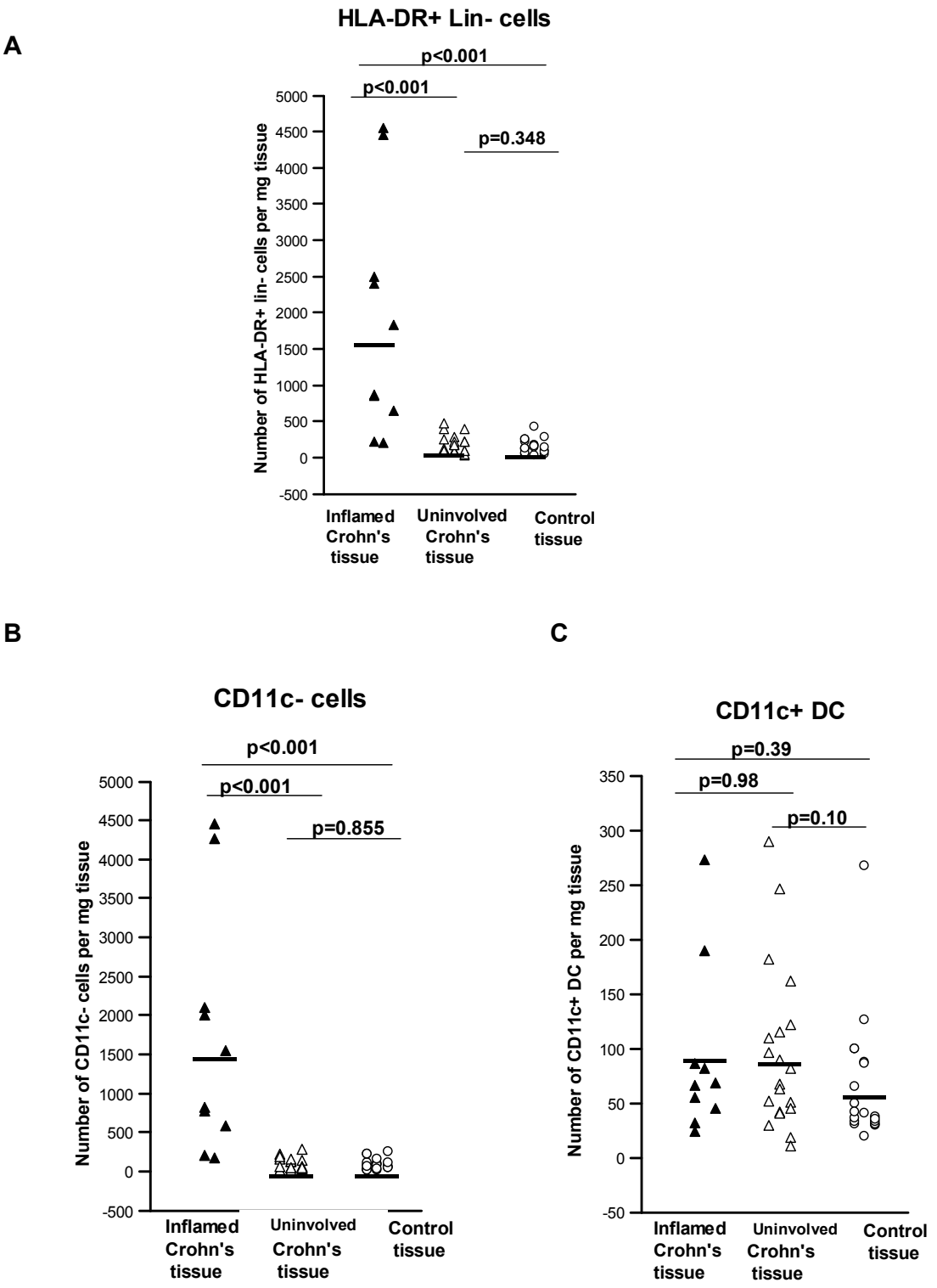


Figure 3.7: Absolute number of HLA-DR$^+$lin$^-$ cells, CD11c$^-$ cells and CD11c$^+$ DC in CD and controls. (A) Numbers of HLA-DR$^+$ lin$^-$ cells; **(B)** Numbers of CD11c$^-$ cells; **(C)** Numbers of CD11c$^+$ DC ; inflamed Crohn's tissue (n=10); non inflamed Crohn's tissue (n=18); control tissue (n=22).

3.8 Few Lamina Propria CD11c$^-$ Cells in Ulcerative Colitis are Plasmacytoid Dendritic Cells

Having established increased numbers of CD11c$^-$ cells in UC, we further determined the phenotype and functions of this population in the human gut. In peripheral blood cells with a phenotype CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ are BDCA2$^+$, BDCA4$^+$ CD123^{hi} plasmacytoid DC (Dzionek *et al*., 2000). However, very few colonic CD11c$^-$ cells from UC patients expressed CD123 (4.3±2.7%; n=5), BDCA-2 (1.8±1.1%; n=5), and only a small proportion expressed BDCA-4 (12±10.5%; n=5) suggesting that the majority were unlikely to be equivalent to the CD11c$^-$ DC population identifiable in peripheral blood (Patterson *et al*., 1991) **(Figure 3.8)**. No detectable staining of CD11c$^-$ cells was obtained with antibodies to IgG kappa light chain or to plasma cells marker CD138. Together with the lack of staining with anti-CD19 in the lineage cocktail (CD3$^-$, CD14$^-$, CD16$^-$, CD19$^-$, CD34$^-$) these findings argued against the presence of B cells in the CD11c$^-$ population.

To confirm that a small subset of colonic CD11c$^-$ cells expressed plasmacytoid DC markers, CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ cells were purified by cell sorting and cytospins prepared. Immunofluorescent labeling with antibody to HLA-DR, CD123 and BDCA-2 showed expression of these surface markers on some of the CD11c$^-$ cells **(Figure 3.9).** As the immunofluorescence studies suggested that sorted CD11c$^-$ cells express BDCA-2 in intracellular compartments, we repeated the flow cytometric analysis using permeabilised cells. However, there was no increased detection of BDCA-2 expression on CD11c$^-$ cells following permeabilisation.

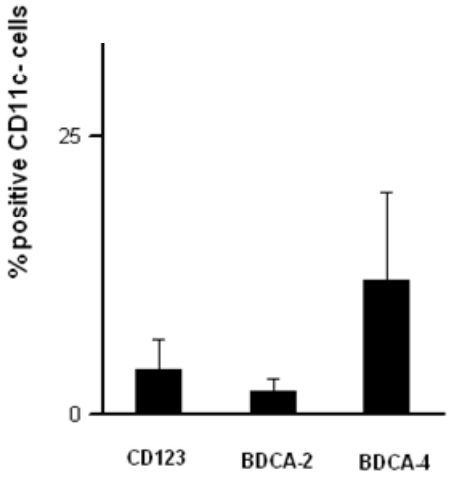


Figure 3.8: Surface expression of CD123, BDCA-2 and BDCA-4 on colonic CD11c$^-$ cells in UC patients. The percentage of expression of CD123, BDCA-2 and BDCA-4 on CD11c$^-$ cells was assessed on multi-colour flow cytometry. The figure shows pooled data from 5 experiments.

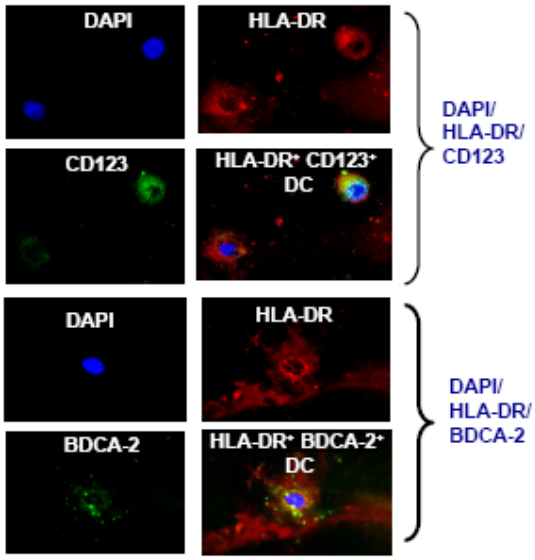


Figure 3.9: Immunohistochemistry of BDCA-2 and CD123 expression on sorted colonic CD11c$^-$ cells. Immunofluorescence was carried out on cytospins of sorted CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ cells derived from inflamed tissue of a patient with UC**.** In the upper 4 panel, sorted CD11c$^-$ cells from UC tissue were stained for HLA-DR and CD123. The lower 4 panels showed expression of HLA-DR and BDCA-2 by equivalent cells from UC tissue. DAPI stained theDNA of cells.

3.9 Tissue Processing Does Not Affect Plasmacytoid Marker Staining

To rule out the possibility that exposure to dithiothreitol, EDTA, collagenase or DNase removes these markers from the surface of colonic cells during their extraction, expression of CD123, BDCA-2 and BDCA-4 was examined on PBMC that had been exposed to the enzymatic mixture used for colonic tissue. Expression of CD123, BDCA-2, BDCA-4, CD138 and IgG kappa light chain was not affected by exposure to the colonic tissue processing **(Figure 3.10).** There was also no enhancement of expression of plasmacytoid markers on colonic cells following culture for four hours at 37°C to allow for potential re-expression of these markers following isolation. These data strongly suggested that the majority of the CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ cells extracted from colonic DC did not belong to the plasmacytoid DC lineage. Analysis by electron microscopy, flow cytometry and immunofluorescence staining of sorted cells did indicate the presence of a few plasmacytoid DC (~5%) but there were insufficient of these cells for further detailed analysis. It is conceivable that they represented blood-derived contaminants.

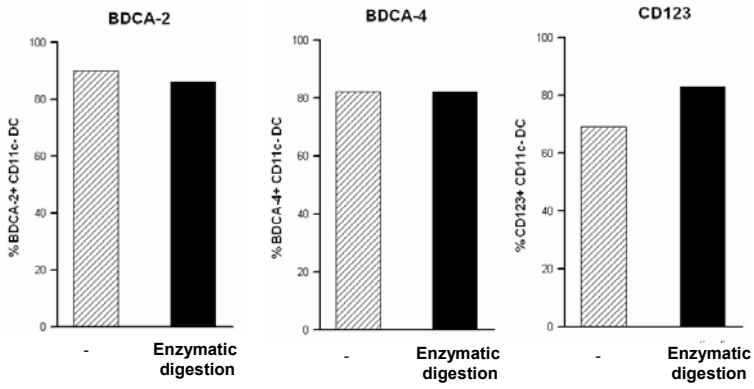


Figure 3.10: Effect of enzymatic digestion on CD123, BDCA-2 and BDCA-4 expression on blood DC. PBMC were exposed to the tissue digestion process which include DTT, EDTA and collagenase used for intestinal biopsy digestion. The proportion of CD123$^+$, BDCA-2$^+$ and BDCA-4$^+$ CD11c$^-$ cells within PBMC in the presence of enzymatic digestion processing is shown.

3.10 CD11c$^-$ Cells have Morphology of Immature Dendritic Cells

We next attempt to define more precisely the nature of the UC-associated CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ colonic population. Following cell sorting the morphology of this population was assessed by electron microscopy. Although only a small number of cells were present, CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ cells appeared homogenous, and had features of small myeloid cells, possibly immature DC with a heterochromatic nucleus or partly euchromatic nucleus (small type 1 and small type 1 to 2 DC) (Patterson *et al*., 1991). Type I DC have numerous short pseudopodia, irregularly shaped nuclei, abundant mitochondria, irregular surfaces with numerous projections and cytoplasmic vacuoles but exhibit few lysosomes or endocytic vacuole. In contrast, type 2 DC have a paler nucleus with a thin rim of dense heterochromatin, cytoplasm devoid of organelles, less vacuoles and a smooth cell boundary with few processes (Van Voorhis *et al*., 1982; Van Voorhis *et al*., 1983). No rough endoplasmic reticulum was present in our cells, and these cells contained some vacuoles of different sizes which covered a large proportion of their cytoplasm **(Figure 3.11).**

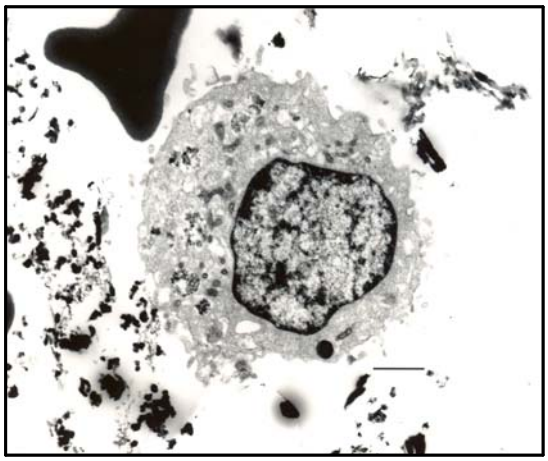


Figure 3.11: Electron Microscopy of sorted CD11c$^-$ cells. Sorted CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ cell from inflamed tissue of a patient with active UC patient (magnification X11500). Scale bar=1μm.

3.11 In Ulcerative Colitis Lamina Propria $CD11c^-$ Cells Express Less CD40 and CD86 than Their $CD11c^+$ Counterparts

Expression of HLA-DR by the $CD11c^-$ LPMC population suggests they may function as antigen presenting cells. To explore this potential function further, the expression of co-stimulatory molecules and pattern recognition receptors was assessed. An example of a histogram of surface marker expression (CD40) on $CD11c^-$ and $CD11c^+$ cells determined by the gating method is shown in Section 2.2.12(ii). $CD40^+$ and, to a lesser degree, $CD86^+$ cells were detectable within the $CD11c^-$ $HLA\text{-}DR^+$ $lin^{-/dim}$ population from UC patients. In **Figure 3.12**, a smaller proportion of these $CD11c^-$ cells expressed CD40 (26±4%, n=30) and CD86 (7±2%, n=30; $p<0.05$) compared with their $CD11c^+$ counterparts (CD40: 72±5%; CD86: 58±5%). The level of expression of CD40 (Positive-intensity ratio [IR]: 6±0.6) and CD86 (3±0.3) by $CD11c^-$ cells was also lower than the $CD11c^+$ cells (CD40: 19±2; CD86: 16±2; $p<0.05$). However, the proportion of $CD11c^-$ cells that expressed CD40 (UC 26±4% vs. control 30±9%) and CD86 (UC 7±2% vs. Control 10±4%) did not differ significantly between UC patients and controls (data not shown).

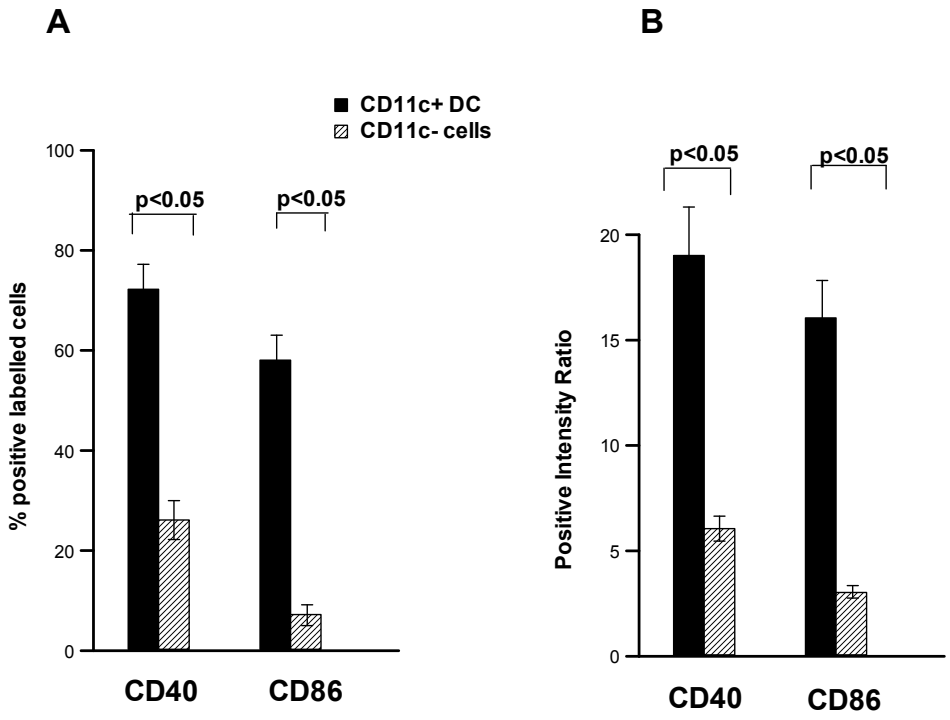


Figure 3.12: Percentage and level of expression of CD40 and CD86 on CD11c$^-$ cells and CD11c$^+$ DC in UC. This figure shows (A) percentage of expression and (B) level of staining expressed as positive intensity ratio (PIR) of CD40 and CD86 on colonic CD11c$^-$ cells and CD11c$^+$ DC in patients with UC (n=30).

3.12 Few Lamina Propria CD11c$^-$ cells from Ulcerative Colitis Express TLR-2 and TLR-4

In addition, very few CD11c$^-$ cells expressed pattern recognition molecules, TLR-2 (CD11c$^-$ 3±1% versus CD11c$^+$ 14±3%; $p<0.05$) and TLR-4 (CD11c$^-$ 3±1% versus CD11c$^+$ 12±2%; $p<0.05$) compared with their CD11c$^+$ counterparts in UC patients **(Figure 3.13).** Although there was no statistical significant difference between the level of expression (PIR) of TLR-2 and TLR-4 between CD11c$^-$ and CD11c$^+$ cells, there was a trend towards lower intensity of TLR expression on CD11c$^-$ cells compared with CD11c$^+$ DC. When the level of staining is low as seen with the TLR, the subtraction method used to generate the

PIR may underestimate the true value. There was no significant difference between the proportion of CD11c$^-$ TLR-2$^+$ and CD11c$^-$ TLR-4$^+$ cells between UC patients and controls (data not shown). Overall, the percentage of expression and level of staining of CD40, CD86, TLR-2 and TLR-4 on CD11c$^-$ cells were lower compared with their CD11c$^+$ counterparts but these data were more convincing for CD40 and CD86 compared to TLR-2 and TLR-4.

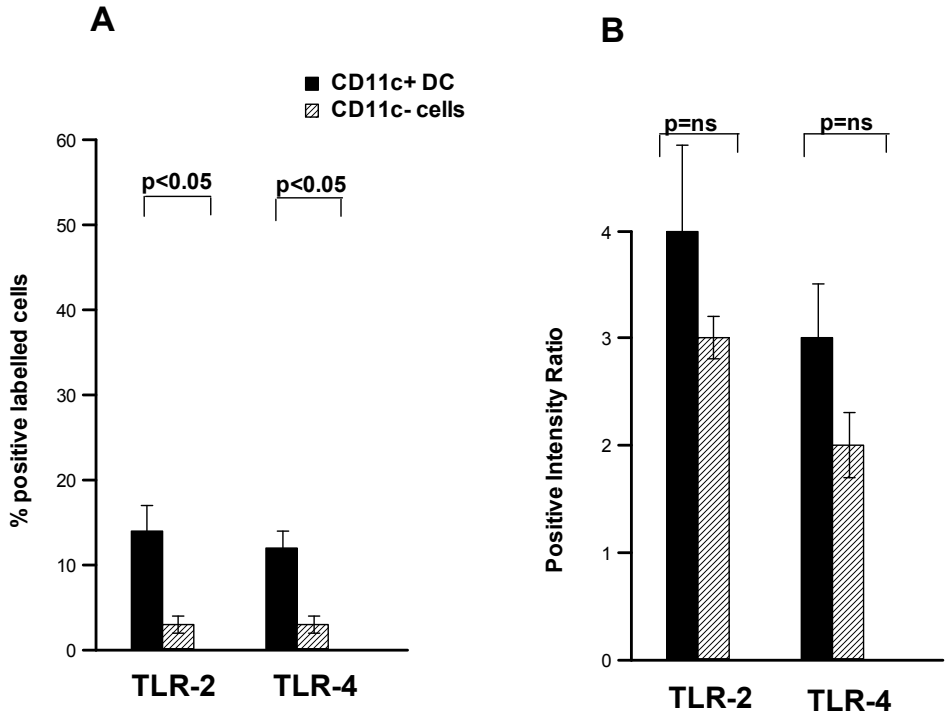
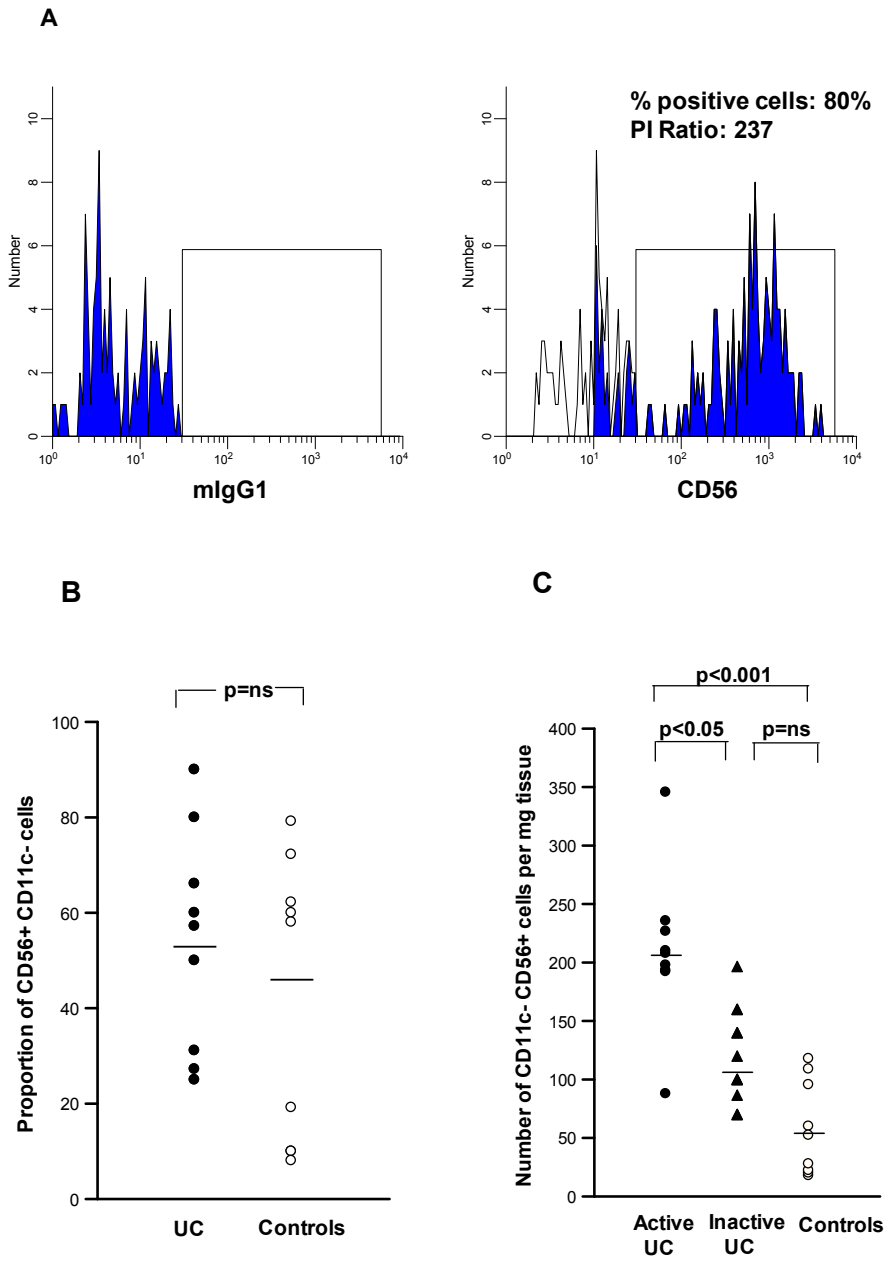


Figure 3.13: Percentage and level of expression of TLR-2 and TLR-4 on CD11c$^-$ cells and CD11c$^+$ DC in UC. This figure shows (A) percentage of staining and (B) level of staining expressed as PIR of TLR-2 and TLR-4 on colonic CD11c$^-$ cells and CD11c$^+$ DC in UC patients (n=30).

3.13 Most Lamina Propria CD11c^{-} HLA-DR^{+} lineage$^{-/dim}$ cells Express CD56 in Ulcerative Colitis

A major subset of the CD11c^{-} HLA-DR^{+} lin$^{-/dim}$ cells stained brightly for the NK cell marker, CD56 **(Figure 3.14A).** A similar proportion was CD56^{bright} in cells extracted from UC and healthy tissue **(Figure 3.14B).** However there was a significant increase in the number of CD11c^{-} CD56^{+} cells extracted from the inflamed colonic tissue of UC patients and a non-significant trend towards increased numbers in the small number of samples of non-inflamed UC tissue examined compared with control tissue **(Figure 3.14C).**



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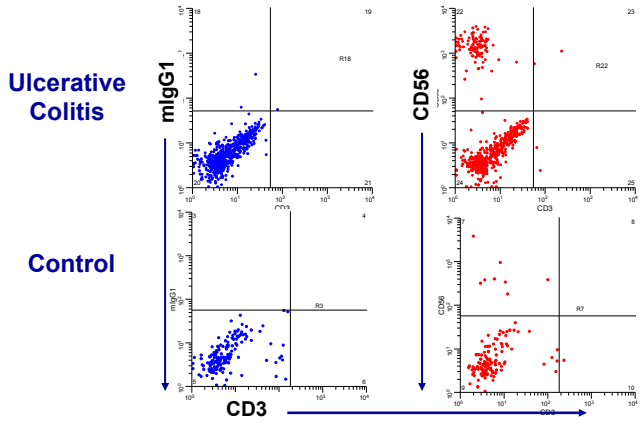


Figure 3.14: Expression of CD56 on colonic CD11c$^-$ cells **(A)** One-parameter histogram showing the expression of CD56 on CD11c$^-$ cells in UC. On the left is the matched isotype control. The PIR represents the intensity of staining after subtraction from the isotype control. Results are representative of 8 experiments; **(B)** The proportion of CD56$^+$ CD11c$^-$ cells in UC and controls; **(C)** The absolute number of CD56$^+$ CD11c$^-$ cells in active UC, inactive UC and controls; **(D)** Expression of CD3 and CD56 on colonic CD11c$^-$ cells in control and UC. Left hand panel (in blue) indicates the isotype control (representative of 3 experiments).

3.14 CD56$^+$ CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ Cells are Scattered throughout the HLA-DR versus Lineage Plot

Backgating of total CD56^{bright} cells in LPMC onto the HLA-DR versus lineage plot demonstrated that only a minority of CD56^{bright} cells were HLA-DR$^+$ and fell within the HLA-DR$^+$ lin$^{-/dim}$ gate. The majority of CD56^{bright} cells were HLA-DR$^-$ and either lin$^-$ or lin^{dim}, presumably representing a mixture of conventional NK cells and NK T cells. This backgating also confirmed that CD56^{bright} cells were scattered throughout the plot and did not represent a population inadvertently captured at the margins of the gate **(Figure 3.15).**

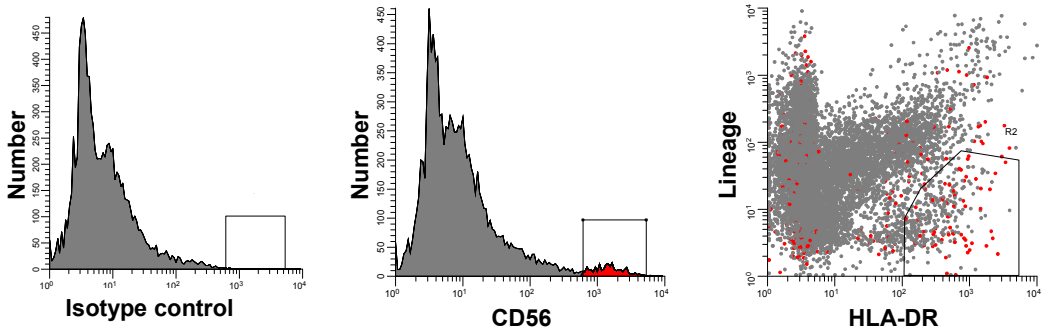


Figure 3.15: Backgating of CD56^{+} CD11c^{-} cells on HLA-DR versus lineage Plot. The left panel shows the isotype control for CD56, middle panel shows the staining of CD56 on CD11c^{-} cells (red area) in a UC patient. Right panel demonstrates backgating of the CD56^{+} CD11^{-} cells on HLA-DR lineage plot (red dots).

We next determined whether these cells expressed other NK cell markers. In active UC, a proportion of lamina propria CD11c^{-} cells expressed CD161 and NKG2D, but the majority lacked expression of NKp30 or NKp44 **(Figure 3.16).** Positive labeling of cells within the lymphocyte light scatter gate was used to provide controls for all antibodies. We were unable to examine co-expression of NK cell markers due to limitation of the four colour flow cytometer.

Overall these data suggest that inflammation in UC is associated with the presence in the colon of a previously undescribed atypical population of cells which shares features of DC and NK cells. In addition a population of CD56^{-} CD11c^{-}HLA-DR^{+}lin$^{-/dim}$ is also increased in number in UC and remains to be further characterized.

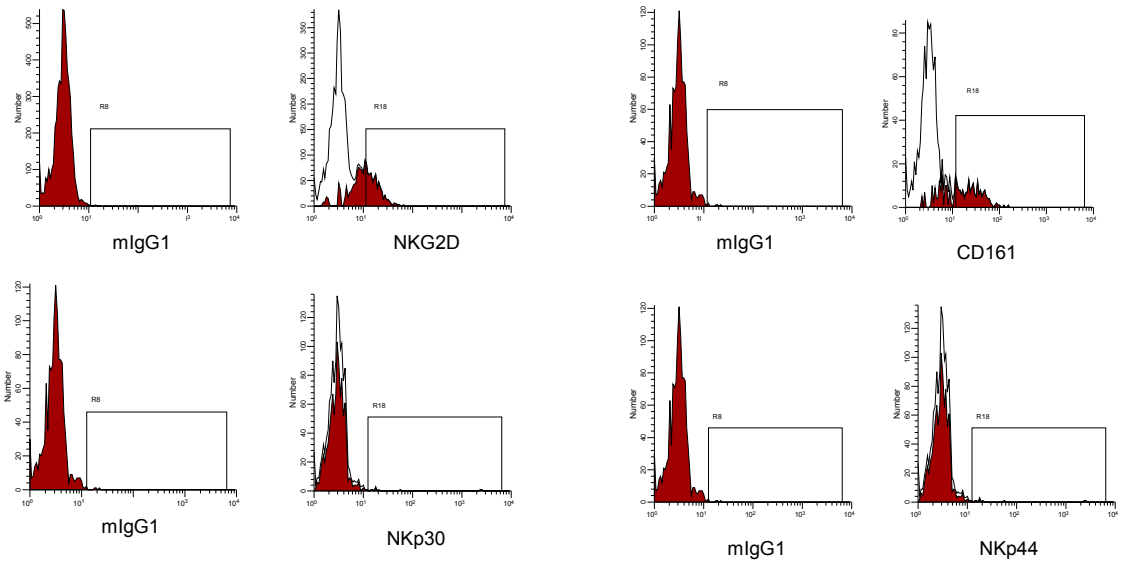


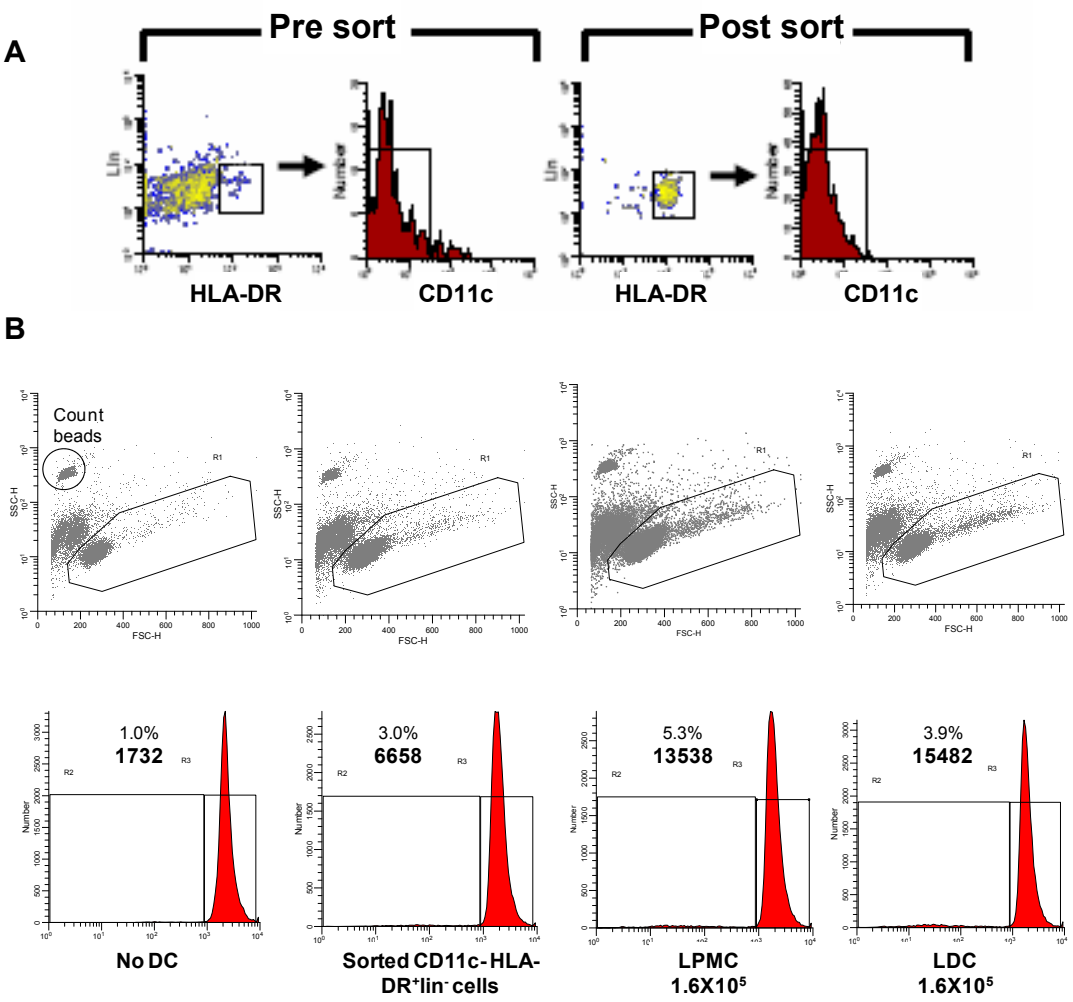
Figure 3.16: Examples of expression of NK cell markers on CD11c$^-$ cells from UC. This figure shows examples of expression of NK cells markers: NKG2D, NKp30, NKp44 and CD161 on CD11c$^-$ cells extracted from UC tissue. Shaded area towards the right of isotype control represents percentage positive cells.

3.15 Functional Attributes of Lamina Propria CD11c$^-$ Cells

3.15.1 Lamina propria CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ cells are Weak Stimulators of Allogeneic T cell Proliferation

The T cell stimulatory capacity of CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ cells was tested in an allogeneic MLR. Sorted intestinal CD11c$^-$ population extracted from UC tissue as shown in **Figure 3.17A** were used to stimulate naive T cells. We also assessed the stimulatory capacity of LPMC (whole cells extracted from UC tissue) and enriched DC population (Low density cells - LDC) obtained from peripheral blood of healthy controls; the latter acts as positive control for our experiment. Naïve CD4$^+$ T cells were used as responder cells in all stimulation experiments. Preparation of enriched DC population or LDC has been described in methods under section 2.2.7. LDC were obtained from PBMC using

nycoprep. There was a dose dependent proliferation stimulated by blood LDC and by an unfractionated LPMC population. Proliferation was also detectable in cultures stimulated by the sorted CD11c$^-$ population (>3x background) although the number of proliferating cells was lower than in cultures stimulated either by LDC or unfractionated LPMC **(Figure 3.17).** Thus, consistent with their low expression of costimulatory molecules, CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ cells were probably weak stimulators of T cell proliferation. However, low numbers of the sorted CD11c$^-$ population precluded a detailed dosimetric analysis that would have dissected this response further.



C

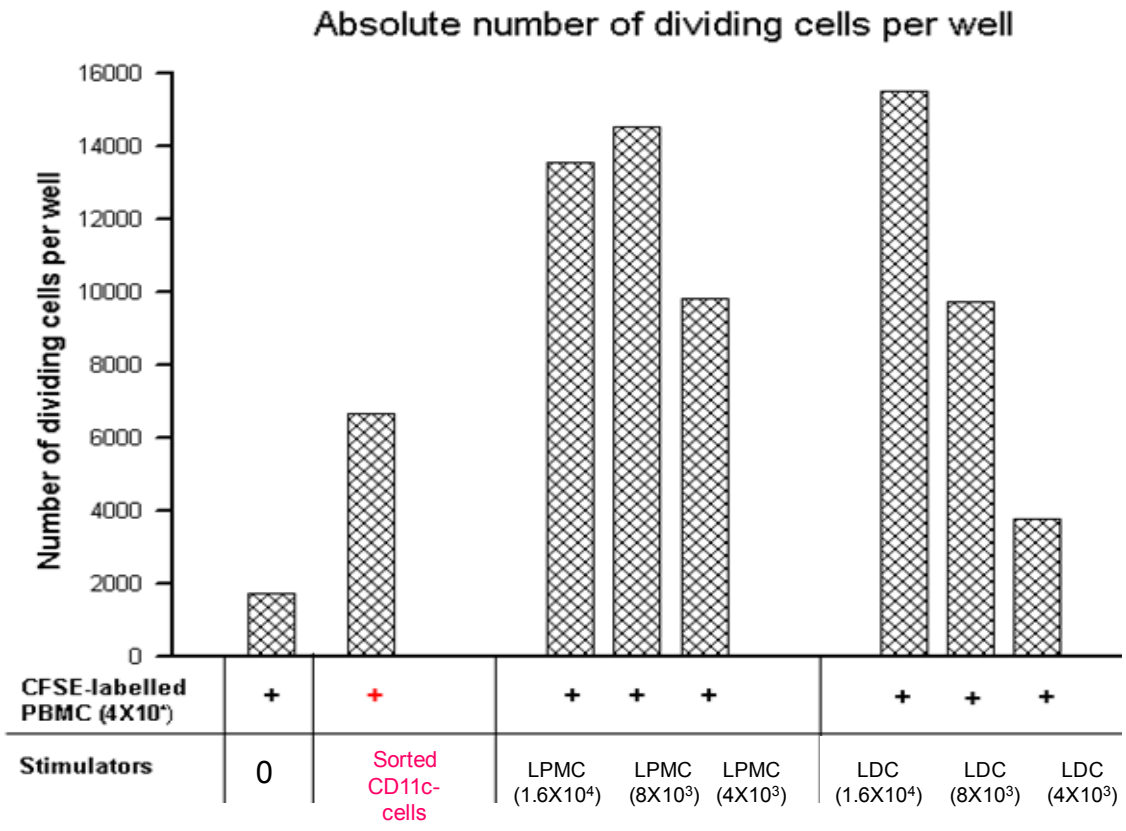


Figure 3.17: Stimulation of allogenic T cell proliferation by sorted colonic CD11c$^-$ cells from inflamed tissue of a UC patient. (A) The two-parameter histograms of HLA-DR and lineage (CD3, CD14, CD16. CD19, CD34) indicate the cells before and after the sorting process. The single-parameter histogram of CD11c indicates the expression of CD11c on these cells, which consist mainly of CD11c$^-$ cells post sort. **(B)** Medium only (No DC), sorted colonic CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ cells, unfractionated LPMC, and blood LDC, were cultured for 5 days in round-bottom well plates with CFSE-labelled CD4$^+$ naïve T cells (Figure representative of 2 independent experiments). Numbers in bold indicate the absolute number of dividing cells per well, percentage figures indicate the proportion of plotted cells that are dividing **(C)** The absolute numbers of proliferating cells per well, by sorted CD11c$^-$ cells, and three different concentrations of LPMC and LDC are shown in this bar chart.

3.15.2 Lamina propria CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ Cells are Poor Spontaneous Producers of IL-12p40 and IL-6

Ongoing intracellular cytokine production of IL-10, IL-12p40, IL-6 and IL-13 by colonic CD11c$^-$ cells was assessed in the absence of exogenous stimulation. We examined these cytokines as previous work from our group showed that DC production of IL-10, IL-12p40 and IL-6 were altered in patients with CD (Hart *et al*., 2005). We also measured IL-13 as lamina propria T cells from UC patients produced greater IL-13 than control cells (Fuss *et al*., 2004). An example of the method used to determine cytokine production has been described in detail in Section 2.2.12 (iii). Compared with the CD11c$^+$ DC, a smaller proportion of CD11c$^-$ cells produced IL-12p40 (CD11c$^-$ 18±9% versus CD11c$^+$ 50±10%; $p<0.05$) in patients with UC. No IL-6 was detected in the CD11c$^-$ cells (-44±19%) when compared with the CD11c$^+$ cells (43±19%; $p<0.05$) **(Figure 3.18).** There was however no significant difference between the proportion of IL-10 and IL-13 producing CD11c$^+$ and CD11c$^-$ cells. In addition, there was no significant difference in the proportion of CD11c$^-$ cells producing IL-10, IL-12p40, IL-6 and IL-13 between patients with UC and healthy controls (data not shown).

We have only assessed the production of IL-10, IL-12p40, IL-6 and IL-13 by CD11c$^-$ cells and future work should involve the proteomic analysis of additional pro-inflammatory cytokines such as IL-1β and IL-8 .

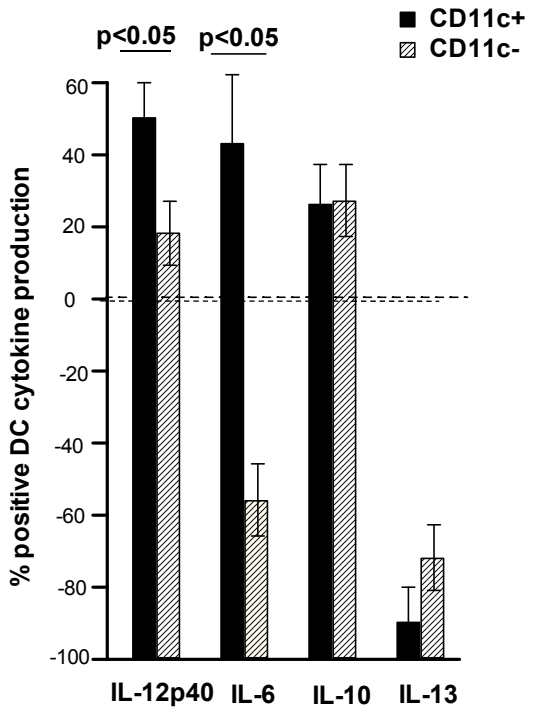


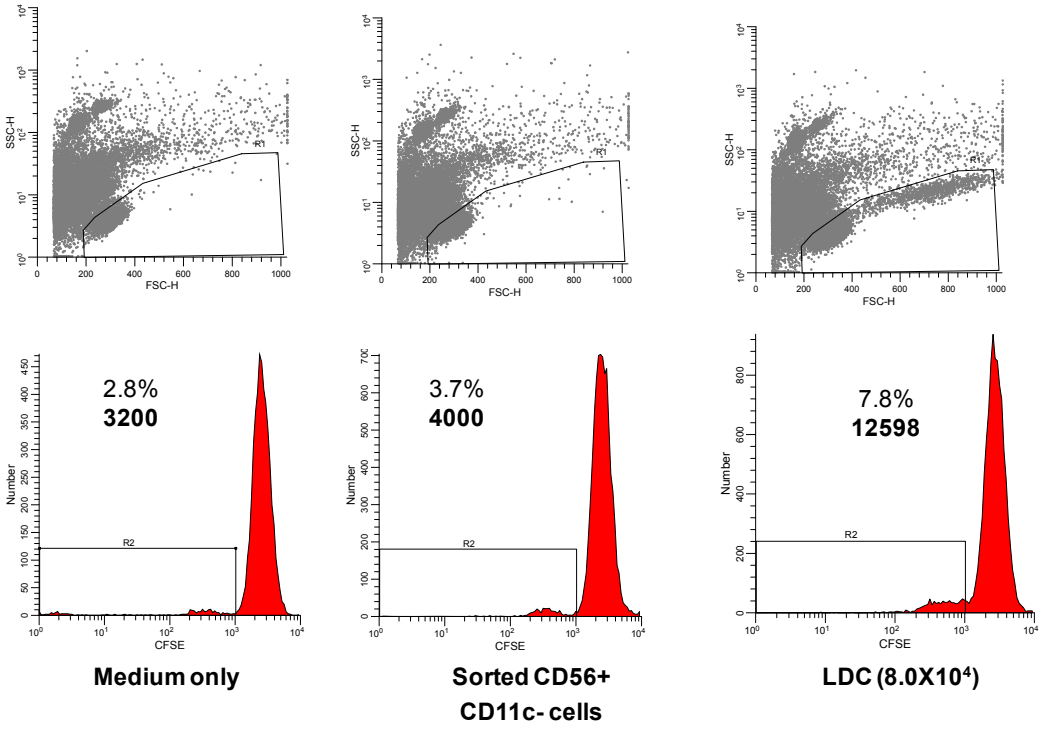
Figure 3.18: Percentage of cytokine production by CD11c$^-$ cells compared with CD11c$^+$ DC in UC. Cytokine production by DC was assessed by intracellular cytokine staining and Super-enhanced D_{max} normalised subtraction. The bar chart shows the mean percentage from pooled data of IL-12p40, IL-6, IL-10 and IL-13 production by CD11c$^-$ cells and CD11c$^+$ DC in UC patients (n=30). When more "positive events" in the control histogram were detected compared with test histogram, subtraction was reversed with the generation of negative events in the test histogram.

3.15.3 Lamina Propria CD56$^+$ CD11c$^-$ HLA-DR$^+$ lin$^{-/dim}$ Cells are Non-Stimulatory

In preliminary experiments, the T cell stimulatory capacity of sorted CD56$^+$ CD11c$^-$ HLA-DR$^+$ lineage$^{-/dim}$ cells was tested in an allogenic MLR. The sorted CD56$^+$ CD11c$^-$ population from colonic tissue of UC patient, and LDC enriched for DC, were used as stimulator cells, and naïve CD4$^+$ T cells as responder cells. There was no proliferation in the medium only culture, and minimal proliferation in cultures stimulated by CD56$^+$ CD11c$^-$ cells. Conversely, proliferation was detectable in cultures stimulated by blood LDC (X2 more than CD56$^+$ CD11c$^-$ culture). The number of proliferating cells was also

lower in cultures stimulated by $CD56^+$ CD11c- cells compared with LDC, but this was comparable with cultures with medium only **(Figure 3.19).** This suggested that $CD56^+$ $CD11c^-$ cells are most likely non-stimulatory. However, low numbers of the sorted population again precluded a detailed dosimetric analysis that would have analysed this response further.

A



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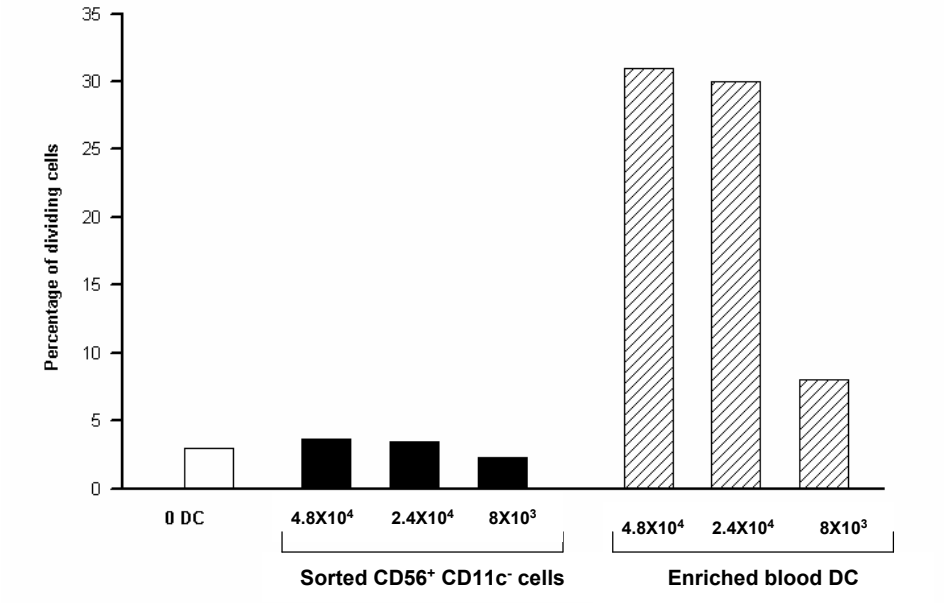


Figure 3.19: Stimulation of allogeneic T cell proliferation by colonic $CD56^+$ $CD11c^-$ cells from UC. (A) Medium only (1st panel), sorted colonic $CD56^+$ $CD11c^-$ $HLA\text{-}DR^+$ $lineage^{-/dim}$ cells (2nd panel), and blood LDC (3rd panel) were cultured for 5 days in round-bottom well plates with CFSE-labelled $CD4^+$ naïve T cells (Figure representative of 2 independent experiments). Numbers in bold indicate the absolute number of dividing cells per well, percentage figures indicate the proportion of plotted cells that are dividing. **(B)** Absolute numbers of proliferating cells per well, by three different concentrations of sorted $CD56^+$ $CD11c^-$ cells and LDC.

3.16 Discussion

We have previously shown that intestinal $CD11c^+$ DC are altered in IBD; and that more $CD11c^+$ DC expressed CD40 and CD86 in CD than controls (Hart *et al*., 2005). The role of intestinal $CD11c^-$ cells falling within the DC gate in IBD remains unclear. Here we have identified increased number of $CD11c^-$ cells in tissues from UC patients compared with controls. Absolute number analysis demonstrated that there was a real increase of $CD11c^-$ cells in the colon of UC rather than a loss of the $CD11c^+$ population. Patients with quiescent (macroscopically normal intestine) and active (macroscopically inflamed intestine) UC were included, and both demonstrated an increase in this subset of cells. Endoscopic and histological intestinal inflammation have been shown to be risk factors for dysplasia and potentially colorectal cancer in patients with long standing UC (Gupta *et al*., 2007; Rutter *et al*., 2004), analysis of $CD11c^-$ cells in apparently quiescent disease required further investigation in this regard.

Increased $CD11c^-$ cells in inflamed UC tissue subsequently reduced in number when inflammation resolved, while number of $CD11c^+$ DC was unchanged. Altogether these data suggested that $CD11c^-$ cells may be markers of intestinal inflammation. In addition,

we established that $CD11c^-$ cells were also increased in CD suggesting that they are associated with intestinal inflammation and not specific to UC. Alterations in colonic $CD11c^-$ cells were unlikely to be secondary to concurrent treatment as patients have been on stable doses of medication although we were not able to exclude the possibilities of the accumulative effects of therapy on these cells.

During inflammation, the composition of cells resident in the intestinal mucosa is enormously altered. Infiltration of large numbers of T cells, monocytes, macrophages and DC into inflamed mucosa has been described in IBD patients (Boirivant *et al*., 1999; Schreiber, 2001; Seegert *et al*., 2001; Zareie *et al*., 2001). Inflamed IBD mucosa contains an increased number of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells, although this increase was significantly lower compared with other inflammatory controls (Maul *et al*., 2005). More mature myeloid DC, that expressed CCR7, have also been found trapped at site of inflammation in CD (te Velde *et al*., 2003).

$CD11c^-$ cells demonstrated some features of DC. Firstly, they expressed MHC class II and had morphological features of DC. The expression of HLA-DR by $CD11c^-$ cells suggested that they may function as APC. $CD11c^-$ cells from UC tissue had a less mature phenotype than their $CD11c^+$ counterparts. Consistent with their lack of co-stimulatory marker expression $CD11c^-$ DR^+ lin^- cells were poor stimulators of proliferative T cell responses. It is conceivable that presentation of antigen by this population in the absence of co-stimulation may lead to the induction of regulatory response but we have yet to examine this possibility.

We examined DC cell surface expression of TLR-2, a receptor for peptidoglycan and lipoproteins from gram positive bacteria (Michelsen *et al*., 2001; Schwandner *et al*., 1999) and TLR-4, which interacts with LPS from gram negative bacteria (Chow *et al*., 1999) and found little expression of these markers on $CD11c^-$ cells. Limited DC cell surface expression of TLR-2 and TLR-4 on $CD11c^-$ cells suggest that they may play a less direct role in bacterial recognition, compared to the $CD11c^+$ cells, although we have not assessed other TLR such as TLR-9.. We studied, in particularly, these TLR as they have been shown to be activated in IBD (Hart *et al*., 2005). Functional studies on the small numbers of human intestinal $CD11c^-$ cells showed that they were poor producers of IL-12p40 and IL-6 but weakly stimulated T cells. The extraction of DC from the human lamina propria can be challenging and low numbers of the sorted population precluded a detailed dosimetric analysis that would have dissected the stimulatory response further. It remains possible that larger numbers of sorted $CD11c^-$ $CD56^+$ cells would reveal the potential stimulatory capacity of these cells.

In peripheral blood $CD11c^-$ $HLA\text{-}DR^+$ $lin^{-/dim}$ cells are predominantly plasmacytoid DC. Here very few $CD11c^-$ cells from UC tissue expressed CD123 and BDCA-2 (Colonna *et al*., 2004), suggesting that they formed a population distinct from blood plasmacytoid DC. Flow cytometry and immunofluorescence staining of sorted $CD11c^-$ cells indicated that majority of the colonic $CD11c^-$ $HLA\text{-}DR^+$ $lin^{-/dim}$ cells were not typical plasmacytoid DC. Control experiments on blood DC confirmed that lack of plasmacytoid markers on colonic DC was not an artefact of enzyme digestion or tissue processing. Permeabilization of colonic DC before labelling did not increase the staining with anti-BDCA2 antibody, arguing against intracellular redistribution of this marker. Middel *et al.* have shown that there were no significant differences in the number or distribution of plasmacytoid DC

between controls and colonic tissue affected by CD (Middel *et al*., 2006). The plasmacytoid DC were randomly distributed within the submucosa and lamina propria and were not associated with T cell clusters, suggesting that plasmacytoid DC from affected tissue of CD are less likely to play a significant role in antigen presentation or T cell stimulation (Middel *et al*., 2006; Neurath *et al*., 2002). Reports of NK cell neoplasms characterised by a $CD3^-/CD4^-/CD56^+/CD13^-/CD33^-$ phenotype, thought to be derived from plasmacytoid DC rather than NK cells have been described (Chaperot *et al*., 2001; Herling *et al*., 2003; Siu *et al*., 2002).

A high proportion of $CD11c^-$ cells from patients with UC stained brightly for the NK cell marker, CD56, and there were elevated numbers of $CD11c^-$ $CD56^+$ cells in UC tissue compared with control tissue. CD56 defines functionally distinct subsets of NK and T cells (Miranti and Brugge, 2002). In humans, NK cells are characterised phenotypically by the presence of CD56 and lack of CD3, phenotypic features which is consistent with the cells described here. However, their low expression of other NK cell markers including NKG2D, NKp30 or NKp44 suggested that they may not represent traditional NK cells.

We found both $CD56^{bright}$ and $CD56^{dim}$ $CD11c^-$ cells in UC tissue, but increased numbers of the former; it is possible that $CD56^{bright}$ cells differentiate to $CD56^{dim}$ cells with resolution of inflammation. In this regard, expansion of $CD56^{bright}$ human NK cells have been described at sites of inflammation (Dalbeth *et al*., 2004). It has been proposed that $CD56^{dim}$ NK cells, upon differentiation from $CD56^{bright}$ NK cells in the periphery, alter chemokine receptor expression and consequently leave inflammatory sites and lymphoid tissue to "re-enter" the peripheral circulation (Chan *et al*., 2007). The physiologic site of

$CD56^+$ $CD11c^-$ cells in the inflamed human mucosa provides a framework for the pursuit of further studies on this novel population.

NK cells have also been reported as the first immune effector cells to arrive at sites of inflammation (Mailliard *et al*., 2005; Mandelboim *et al*., 2001; Martin-Fontecha *et al*., 2004). Increased localization of these cells in inflamed non-lymphoid peripheral organs, including the lungs, liver and intestine suggest that they may stimulate effector T cells (Fehniger *et al*., 2003; Ferlazzo *et al*., 2004; Hanna *et al*., 2003). Other authors have, *in vivo*, reported that NK cells are able to express MHC class II and T cell receptor co-stimulatory molecules. Of interest, activated NK cells with antigen presenting cell-like phenotype, can "fine-tune" T cell activation, when there is direct insult to the peripheral organs (Hanna *et al*., 2004).

Accumulating data also suggest a potential overlap in phenotypic and functional characteristics between NK cells and DC. For instance, in mouse autoimmune diabetes model, a unique regulatory DC subset that express NK specific marker DX5 (bitypic DC/NK cells) but lack basic NK cell functions has been described (Homann *et al*., 2002), whilst Hanna *et al.* showed that human NK cells from tonsils and uterine decidual samples have APC-liked properties and can directly regulate T cell activation (Hanna *et al*., 2004). We have compared our cell population with several recently described NK cells in the intestine, and showed that they shared similar phenotype of $CD3^-$, $CD56^+$ and $CD16^-$. In contrast, our cells are HLA-DR^{bright} and lin^-, and lacked expression of other NK cells markers. We have not examined their production of IL-22. Several studies have reported the presence of intestinal lamina propria NK cells ($CD3^-$, $CD56^+$, $CD16^-$ and $NKp46^+$) that produced IL-22 in the healthy mouse and human tissues (Cella *et al*., 2009; Luci *et al*.,

2009; Mela *et al*., 2007; Sanos *et al*., 2009). To the best of our knowledge only one study has studied NK cells in inflamed Crohn's tissue; these cells were $CD3^-$, $CD56^+$, Lin^-, c kit^+ and secrete IFN-γ (Chinen *et al*., 2007). Our cell population from inflamed and non-inflamed IBD tissue share similar phenotypic characteristics ($CD3^-$, $CD56^+$, $CD16^-$, Lin^-) and weakly stimulate naïve T cells.

In patients with human immunodeficiency virus (HIV) lamina propria and intraepithelial NK cells in the intestine have been reported to have distinct phenotype compared with those in the peripheral blood. The majority of $CD3^-$ $CD56^+$ cells in healthy and HIV-1 infected colonic and duodenal lamina propria lacked CD16 (Mela *et al*., 2007). These intestinal NK cells expressed natural cytotoxicity receptors, NKp44 and NKp46, but lacked CD16, suggestive of their helper functions. The helper functions of lamina propria NK cells have been described by other investigators (Chinen *et al*., 2007; Leon *et al*., 2003). Lamina propria NK cells may differentiate in response to infection or inflammatory mediators.

Recently a $CD56^+$ $NKp44^+$ NK cell subset that produced IL-22 and IL-26 and induced epithelial cell proliferation and IL-10 secretion has been demonstrated in the human small intestine (Cella *et al*., 2009), whereas $NKp46^+$ $CD3^-$ NK cells that co-express RORγt and produced IL-22 in response to signals from commensal microflora, have been identified in the murine intestine (Colonna, 2009; Sanos *et al*., 2009).

Fuss *et al.* found that LPMCs from inflamed tissue of UC patients contain $CD4^+$ cells that express CD161, but lacked expression of invariant NKT cell receptors, and produced increased IL-13 and IL-5 (Fuss *et al*., 2004). In our study, the cell population of interest from inflamed UC tissue were $CD3^-$ and $CD161^-$, they are therefore, by definition, not

conventional NKT cells. The morphology of the $CD11c^-$ cells is more consistent with that of immature DC than T cells.

We speculate that lamina propria $CD56^+$ $CD11^-$ cells may have several functions in UC. Human NK cells with antigen presenting cell-liked features have been shown to regulate T cell activation (Hanna *et al*., 2004), express MHC class II and T cell receptor co-stimulatory molecules *in vivo*., as well as "fine-tune" T cell activation when there is direct insult to the peripheral organs (Hanna *et al*., 2003). When activated, human NK cells express MHC class II and have the ability to present antigens directly and stimulate $CD4^+$ T cell proliferation *in vitro* (Hanna *et al*., 2004). The human intestinal NK cells examined here were weakly stimulatory, but could become stimulatory after appropriate activation. Given that $CD56^+$ $CD11c^-$ cells were poorly stimulatory and few produced cytokine, it is conceivable, although speculative, that they may play a role in intestinal tissue repair in acute UC; or the generation of T regulatory cells. The latter function will need to be determined in further experiments. NK cells have been shown to have a protective role in supporting tissue repair and promoting adaptive immune responses (Martin-Fontecha *et al*., 2004).

One possibility is that $CD11c^-$ cells may be precursors of $CD11c^+$ DC, and that in the gut, $CD11c^-$ cells become $CD11c^+$ DC following stimulation with inflammatory cytokine and / or bacterial components. Alternatively, $CD56^+$ $CD11c^-$ cells may have migrated from peripheral blood to the lamina propria in response to homing signals secreted by epithelial cells under inflammatory conditions. In the peripheral blood human NK cells express $\alpha4\beta7$ which enable them to recirculate to intestinal lamina propria (Perez-Villar *et al*., 1996). $\alpha4\beta7$ is detected on the majority of $CD3^-$ $CD56^+$ lamina propria NK cells and the integrin

αE (CD103) is expressed on only a small proportion of $CD3^-$ $CD56^+$ lamina propria NK cells (Chinen *et al*., 2007; Leon *et al*., 2003).

Unlike $CD11c^+$ $HLA^-DR^+lin^-$ cells, $CD11c^-$ cells did not produce IL-10, IL-12p40, IL-6 or IL-13, and so are less likely to contribute to the pathogenesis of IBD in this way. We hypothesise that the gut microenvironment including its endogenous commensal and pathogenic microbes play a role in controlling NK cell response. Whilst one subset of lamina propria NK cells may act to promote pathogen specific immune responses to enhance Th1 response via the production of IFN-γ, another subset may be involved in innate immunity and have a "repair" function by regenerating the intestinal epithelial barrier. The distinct subset of $CD56^+$ $CD11c^-$ cells described here may be involved in the latter function, to limit the already established inflammation in IBD.

In conclusion, we have reported an increase in a newly-defined distinct subset of immune cells in the inflamed colonic tissue of UC patients. These cells morphologically resemble conventional immature DC, express MHC class II molecules, and are $CD56^{bright}$. *Ex vivo* $CD11c^-$ cells can weakly stimulate naïve T cells. These cells may represent a functionally distinct cell subset in intestinal inflammation, different to previously described NK cells, and their potential role in immunoregulation or tissue repair warrants further investigations.

.

Chapter 4

Cytokine Production by Colonic Myeloid Dendritic Cells in Active Ulcerative Colitis and Crohn's Disease

4.1 Abstract

Introduction: Altered DC function, associated with dysregulated cytokine production, is likely to contribute to the altered response to bacteria that drives inflammation in IBD. We hypothesize that DC function varies between different stages of inflammatory disease and that their function correlates with disease activity and the intestinal microbiota in IBD. We assessed intracellular cytokine production by DC and faecal microbiota in patients with active IBD.

Methods: $CD11c^+$ DC were identified by multi-colour flow cytometry of cells extracted from collagenase digested intestinal tissue from patients with UC (n=28), CD (n=28) and controls (n=12). Spontaneous IL-10, IL-12, IL-6, and IL-13 production by lamina propria DC and lymphocytes was measured by intracellular staining of permeabilised cells in the absence of exogenous stimulation. Intestinal microbiota were analysed by fluorescent in situ hybridisation of fresh faecal samples using a DNA stain to detect total bacterial cells and oligonucleotide probes targeting 16S rRNA of bifidobacteria, bacteroides, *C. coccoides-E. rectale* and *Faecalibacterium praustnitzii.*

Results: In acute UC, a significantly greater proportion of $CD11c^+$ DC produced IL-10 and IL-12p40 than cells from control tissue. Level of staining of IL-10 and IL-12p40 by $CD11c^+$ DC was also significantly greater in UC. In active CD, $IL\text{-}12p40^+$ DC increased and correlated positively with the ratio of pro:anti inflammatory bacteria, namely bacteroides:bifidobacteria. In CD, the proportion of $IL\text{-}6^+$ DC increased and correlated with the Crohn's disease Activity Index and serum C-reactive protein, but negatively with anti-inflammatory *Faecalibacterium praustnitzii.*

Conclusions: Acute UC is associated with increased IL-10 and IL-12p40 production by DC. Bacterially-driven local IL-6 production by intestinal DC may overcome regulatory activity and lead to unopposed effector function and tissue damage in CD. The balance of

pro-inflammatory and immuno-regulatory luminal microbiota is associated with altered mucosal DC function in patients with CD, supporting the concept that intestinal DC function is influenced by the composition of the commensal microbiota.

4.2 Introduction

In Chapter 3, we have shown that human colonic $CD11c^-$ cells are increased in IBD, and we have further characterised this novel population. The numbers of the $CD11c^+$ cells were however not different between IBD patients and controls. We therefore aim to determine whether the functions of $CD11c^+$ cells differ between IBD and controls. Secondly, we wish to assess whether these functional characteristics correlate with disease phenotype and gut microbiota in patients with IBD. Human colonic $CD11c^+$ cells have been previously classified as myeloid DC based on morphology, phenotype and functions (Bell *et al*., 2001). Functionally, these cells displayed endocytic activities, which was intermediate between that of lymphocytes (non-endocytic) and monocytes (highly endocytic), and they were potent stimulators of a primary allogeneic leukocyte reaction upon maturation (Bell *et al*., 2001).

UC and CD are heterogenous disorders. Clinicopathologic and genetic differences between patient subsets may reflect diverse immunological profiles (Baumgart and Carding, 2007). Cytokine profile is likely to be different between the early and late phases of disease progression in IBD (Spencer *et al*., 2002). This hypothesis has been demonstrated in animal models of colitis (Spencer *et al*., 2002). Although early colonic lesions displayed a mucosal cytokine pattern that involved high levels of TNF-α and IL-12, these cytokines were almost undetectable in the late phases of experimental colitis and ileitis. In IL-10-deficient mice, LPMC synthesized large amounts of IL-12 and IFN-γ in early colitis. The production of both cytokines dramatically declined and returned to pre-disease levels in the late phase of colitis, whereas IL-4 and IL-13 production increased progressively from pre-, to early to late disease, indicating that other immune mechanisms

sustained chronic inflammation (Spencer *et al*., 2002). Furthermore a distinct cytokine pattern profile has been detected in early and chronic ileal lesions of adult patients with CD (Desreumaux *et al*., 1997). For example, early ileal lesions of patients with CD were associated with a marked increase of IL-4 mRNA and a decrease of IFN-γ mRNA compared with the normal mucosa of patients with CD or controls, whereas a Th1-type pattern was observed in the chronic ileal lesions. These observations strongly support the concept that disease varies according to its clinical course and stage (Desreumaux *et al*., 1997).

Work from our laboratory has previously shown that in a small group of patients with CD, more myeloid colonic DC produced pathologically relevant cytokines including IL-6 and IL-12p40 than control DC. Conversely, in patients with chronic active UC, there was no difference between IL-12p40 and IL-6 production from myeloid colonic DC, compared with control DC (Hart *et al*., 2005).

Following encounter with antigens, Th lymphocytes differentiate into Th1, Th2 or Th17 effector populations under the influence of the local cytokine milieu and following activation by DC (Maloy and Kullberg, 2008). Strict Th1 polarization has been thought to underlie the pathogenesis of inflammation in CD, supported by high levels of IFN-γ, IL-12 and TNF-α (Fuss *et al*., 1996; Monteleone *et al*., 1997). In contrast, UC was thought to display a tendency towards Th2 polarization with upregulation of IL-5, and the lack of IFN-γ and IL-4. This concept of Th1/CD versus Th2/UC polarization has been challenged by studies that described increased IL-4 and IL-5 in CD (Desreumaux *et al*., 1997), and Th1-related cytokines in UC (Sawa *et al*., 2003; Tsukada *et al*., 2002). Recently, an additional subset, known as Th17 cells, associated with the cytokine IL-23, has further

illustrated the complexity and diversity of effector CD4 $^{+}$ T cells in IBD (Maloy and Kullberg, 2008). Although originally thought to have a deleterious role in autoimmune tissue pathology, the IL-23 / Th17 axis has now been associated with protective immunity at mucosal surfaces (Ahern *et al*., 2008; Maloy, 2008).

We hypothesize that distinct cytokine production by DC favours the dominance of effector responses in human IBD. These responses are influenced by the stage and activity of disease. In the first part of this chapter, we assessed global cytokine production from intestinal tissue which involved examination of production of cytokines from the supernatant of LPMC cultured overnight from patients with IBD, irritable bowel syndrome (IBS) and healthy controls using multiplex ELISA. Diagnosis of patients with IBS were confirmed based on the ROME III criteria for functional bowel disorders (Drossman and Dumitrascu, 2006; Thompson *et al*., 1999).

In the second part of the experiments, we analysed specific cytokines produced by lamina propria myeloid DC from tissues of patients with active IBD and healthy controls. Studies in rodents showed that intestinal bacterial composition changed with colonic inflammation (Lupp *et al*., 2007). In human IBD, faecal and mucosa-associated microbial communities are consistently less diverse with increased temporal instability (Dicksved *et al*., 2008; Martinez *et al*., 2008; Ott *et al*., 2008) and reduced Bacteroidetes and Firmicutes (Frank *et al*., 2007; Swidsinski *et al*., 2008). Altered microbial composition can increase immune stimulation, in particularly by DC. Whether the commensal microbiota influence DC function in patients with IBD remains unknown. Hence, in patients with active CD, we correlated DC cytokine changes with both disease activity and the intestinal microbiota. Patients examined in this chapter represent a well defined group of patients recruited into a

therapeutic intervention trial of probiotics (UC) and prebiotics (CD). Results presented here are data before any therapeutic intervention.

4.3 Aims

- To assess global cytokine production by LPMC from patients with IBD, functional bowel disorders and healthy controls.
- To assess ongoing production of intracellular IL-10, IL-12p40, IL-6 and IL-13 by lamina propria DC in patients with IBD, and to correlate these changes with disease acitivity and intestinal microbiota.

4.4 Patient Characteristics

	No of patients	Age (mean)	Gender Male	Medication	Disease activity
Ulcerative colitis	28	44	12	5-ASA - 18 AZA/6MP - 3 None - 9	Mild to Moderate
Crohn's Disease	28	44	12	5-ASA - 13 AZA/6MP - 21 Steroids - 4 None - 6	Moderate
Irritable bowel syndrome	8	38	2	Nil	-
Controls	12	45	5	Nil	-

5-ASA: 5-aminosalicylic acid
AZA: azathioprine
6-MP: 6-mercaptopurine

Table 11: Baseline characteristics of patients for cytokine analysis

4.5 Amount of Cytokine in Supernatants was not different between Colonic Tissues from Inflammatory Bowel Disease, Irritable Bowel Syndrome and Controls

To obtain a global assessment of cytokine production, ten cytokines (IL-6, IL-8, IL-2, IL-12p70, IL-1β, IFN-γ, TNF-α, IL-10, IL-4, IL-5) were measured from supernatants of tissue cultured overnight in medium from patients with IBD, IBS, and controls, using a Multiple ELISA kit as described in section 2.2.9. **Figure 4.1** showed an example of the measurement of cytokine on flow cytometry. The quantities of cytokine in the supernantant were determined against a standard curve for known concentrations of that cytokine. The amount of cytokine production was corrected for tissue weight.

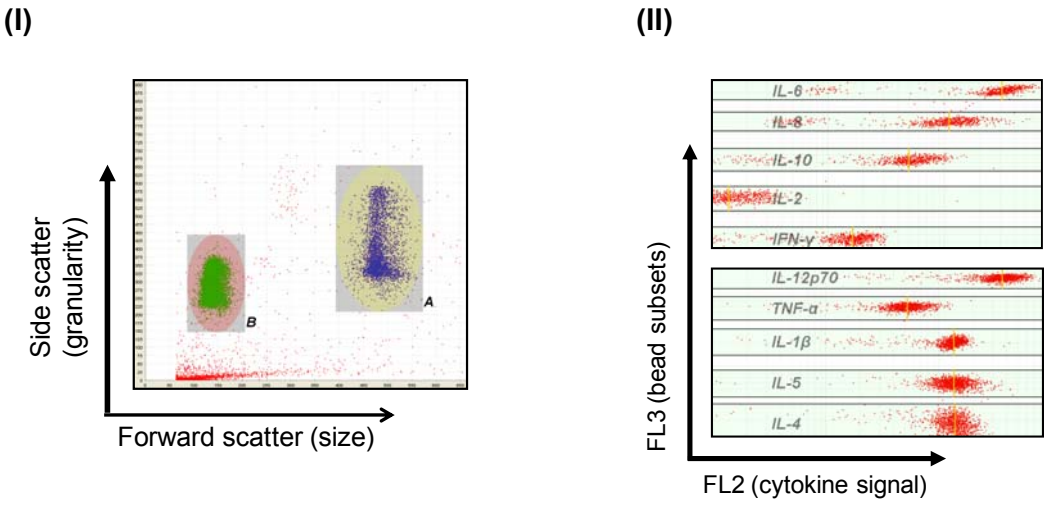


Figure 4.1: Multiplex ELISA of cytokines from supernatant measured by flow-cytometry. (I) Two distinct bead populations ***(A and B)*** were identifiable based on size and granularity; (II) Both bead populations ***(A and B)*** contain 5 different bead subsets with distinct FL3 fluorescence profiles. Each bead subset is coated with a different anti-cytokine antibody. On FL2 signal strength indicates amount of cytokine detected in sample.

In patients with UC, CD, IBS and controls, there were no significant differences between the four groups for any of the cytokines examined. IL-1β, IL-10 and IL-6, were detected in all gut biopsies from patients with UC, CD, IBS and controls. We speculated that these cytokines may be associated with "tissue trauma". However, as this method of cytokine assessment showed no difference between inflammatory bowel disease and control tissues, we proceeded to a more specific approach by assessing the amount of ongoing cytokine produced by DC.

4.6 More Colonic CD11c$^+$ Dendritic Cells Produce IL-10 and IL-12p40 in Acute Ulcerative Colitis than in Controls

The technique used to assess cytokine production by lamina propria DC was initially developed in human peripheral blood and has been described in detail in section 2.2.12 (Bell *et al*., 2001). Spontaneous intracellular cytokine production of IL-10, IL-12p40, IL-6 and IL-13 by colonic DC was assessed in the absence of exogenous stimulation in patients with UC and controls. Proportion of cytokine-positive cells was assessed by intracellular cytokine staining and SED normalized subtraction.

In acute UC, a significantly greater proportion of CD11c$^+$ DC produced IL-10 (median ±SEM; 26±11%) and IL-12p40 (50±10%) than equivalent cells from control tissue where there was no detectable IL-10 (-39±12%; $p<0.05$) and IL-12p40 (-20±14%; $p<0.05$) production by DC. Although not statistically significant, there was a trend towards increased IL-6 production by CD11c$^+$ DC in UC. It is possible that larger sample numbers may produce a significant result. In contrast, CD11c$^+$ DC production of IL-13 did not differ significantly between patients with UC and controls **(Figure 4.2).**

We also assessed the intensity of cytokine staining. The level of cytokine staining expressed as a mean positive intensity ratio (PIR), of IL-10 (UC 2.2 ± 0.5 vs. controls 1.0 ± 0.4; $p<0.05$) and IL-12p40 (UC 1.5 ± 0.2 vs. controls 1.2 ± 0.4; $p<0.05$) by $CD11c^+$ DC was also significantly greater in UC tissue than control tissue **(Figure 4.3).** Conversely, the level of staining did not differ for IL-6 and IL-13 between UC and control.

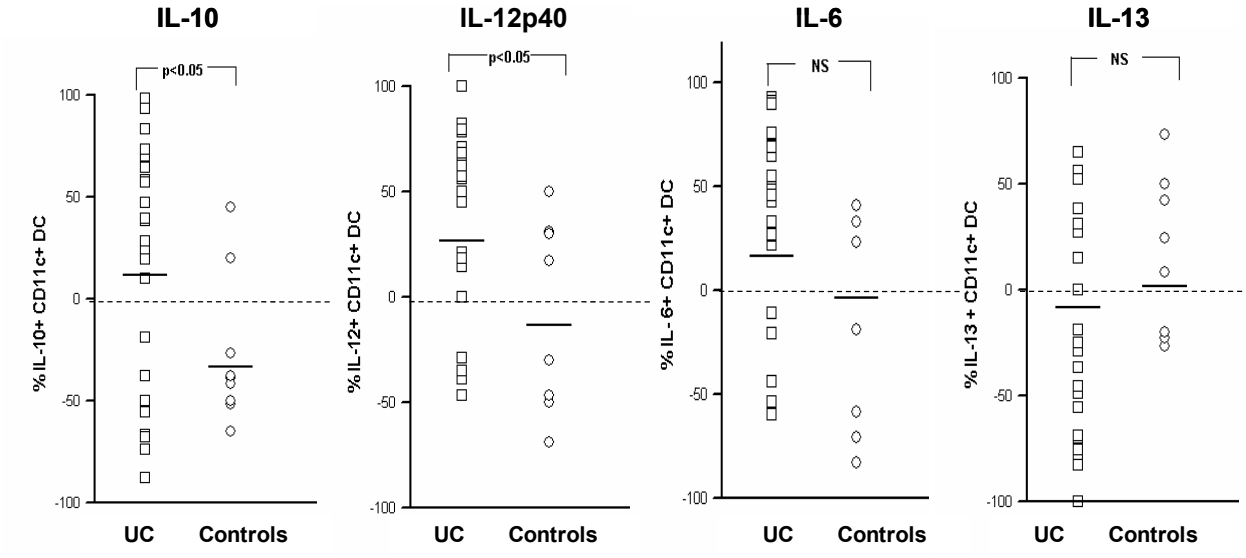


Figure 4.2: Percentage of cytokine production by $CD11c^+$ DC in acute UC and controls. LPMC were cultured in complete medium with and without monensin for 4 hours, surface labelled with monoclonal antibody, washed, fixed and permealised for intracellular cytokine staining. Enhanced normalised subtraction was used to assess the proportion of positively labelled cells (%) in the monensin treated cultures compared with non-treated cultures. By subtracting control histograms (no monensin) from test histograms the accumulation of cytokine was determined. Results are presented as ± cytokine percentage containing cells after subtraction. This method allows the measurement of loss and gain of function. In cases where secreted protein binds back to the surface of cell in the no monensin sample, this sample may contain cytokine positive cells. In such situation the subtraction process can be reversed to generate negative results. In this system, the control sample does not represent zero but represents a reference point on which to quantify the build up of cytokine in our test sample. It is therefore important to quantify the reduction in cytokine production compared with background control as labelling these negative results as zero would be inaccurate. This figure shows the percentage of IL-10, IL-12p40, IL-6 and IL-13

produced by CD11c$^+$ DC in patients with active UC (n=28) and controls (n=10). Results are calculated as median ± SEM. P<0.05 is determined by paired students's t test.

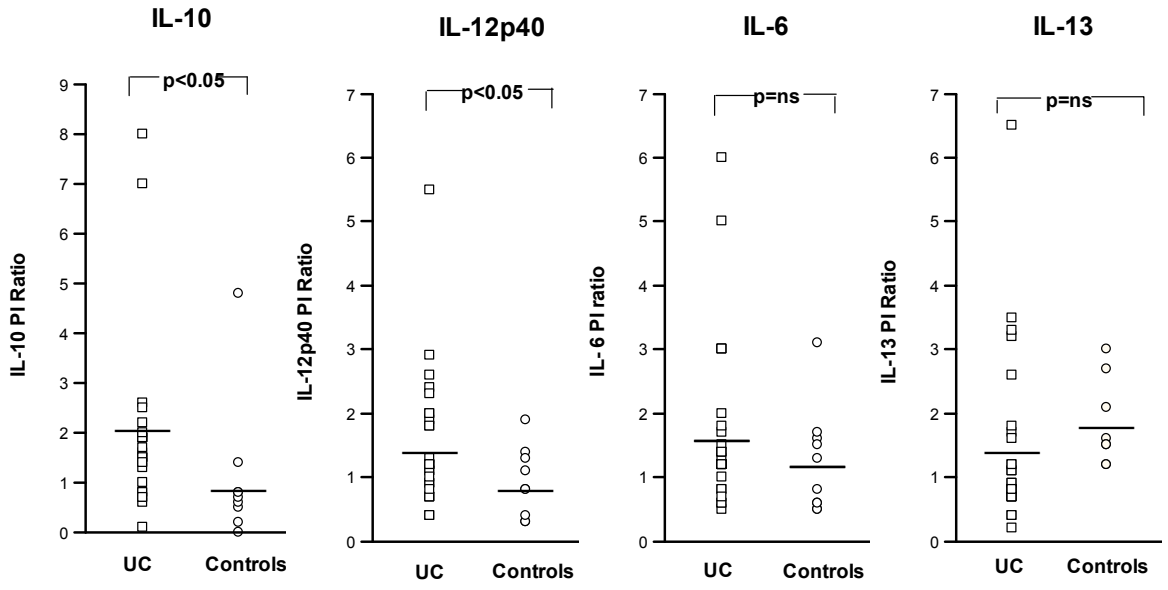


Figure 4.3: Positive Intensity Ratio (IR) of cytokine in acute UC and controls.

LPMC were cultured in complete medium with and without monensin for 4 hours, surface labelled with monoclonal antibody, washed, fixed and permealised for intracellular cytokine staining. Enhanced normalised subtraction was used to assess the level of staining expressed as positive intensity ratio (PIR) of labelled cells in the monensin treated cultures compared with non-treated cultures. This figure shows the PIR of IL-10, IL-12p40, IL-6 and IL-13 produced by CD11c$^+$ DC in patients with active UC (n=28) and controls (n=10).

4.7 More Colonic CD11c$^+$ DC Produce IL-12p40 in Active Crohn's Disease than Controls

We compared the production of intracellular cytokine production by CD11c$^+$ DC in patients with moderately active CD and healthy controls. These results represent the pre-interventional data for patients with moderately active CD who were recruited into a placebo-controlled trial to assess the clinical, immunological and microbiological effects of a high fructo-oligosaccharide diet. Intracellular cytokine production post-treatment is part of an ongoing project. In patients with moderately active CD pre-intervention, significantly greater proportion of CD11c$^+$ DC produced IL-12p40 (48±11%) than equivalent cells from control tissues (-30±11%; $p<0.05$). CD11c$^+$ DC from CD produced IL-10 and IL-6, but there was no significant difference between DC from CD and controls (IL-6: CD 29±8% versus control 23±21%, $p=0.39$; IL-10: CD 34±12% versus controls -27± 12%; $p=0.10$) **(Figure 4.4).** The amount of IL-10, IL-6 and IL-12p40 staining by DC, measured as positive intensity ratio (PIR) was significantly increased in CD compared with controls **(Figure 4.5).**

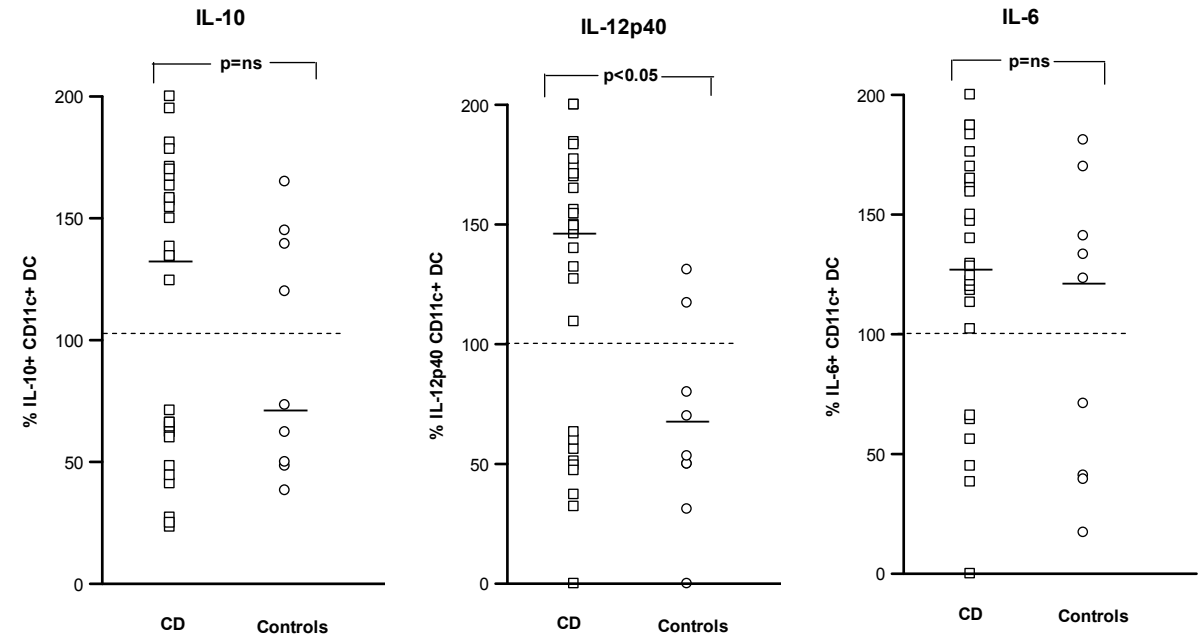


Figure 4.4: Percentage cytokine production by CD11c$^+$ DC in CD and controls

This figure shows the percentage of IL-10, IL-12p40 and IL-6 produced by CD11c$^+$ DC in patients with active CD (n=28) and controls (n=12). Results are calculated as median ± SEM. P<0.05 is determined by paired students's t test.

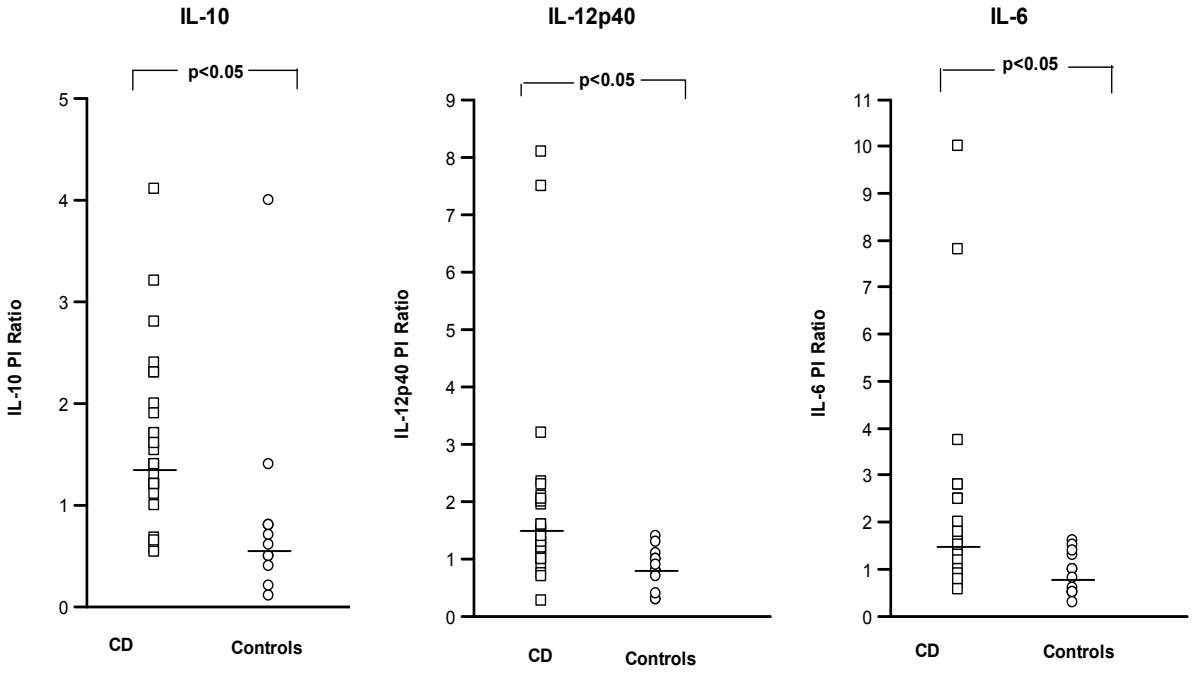


Figure 4.5: Positive Intensity Ratio (PIR) of cytokine in CD and controls

This figure shows the PIR of IL-10, IL-12p40 and IL-6 produced by CD11c$^+$ DC in patients with active CD (n=28) and controls (n=12).

4.8 Correlation of Dendritic Cell Cytokine Production with Crohn's Disease Phenotype and Disease Activity

4.8.1 In Crohn's Disease, Dendritic Cells Produce More IL-12p40 in Colonic Disease than Ileocolonic Disease

For patients with CD, 17 patients had ileocolonic disease and 11 had colonic disease. There was significantly more IL-12p40 production by DC in patients with colonic CD compared with those with ileo-colonic disease (71% ±17 vs. 32%±14; p=0.037) but there was no difference for IL-10 or IL-6 by DC for disease site (data not shown).

4.8.2 In Crohn's Disease, Proportion of IL-6$^+$ Dendritic Cell Correlates Positively with the Crohn's Disease Activity Index (CDAI)

The CDAI is a global measure of activity of CD, mainly in relation to luminal disease. It incorporates eight variables: numbers of stools per day, severity of abdominal pain, general well-being, extraintestinal manifestation, abdominal mass, use of anti-diarrhoeal drugs, haematocrit and body weight. These items yield a composite score ranging from 0 to 600. A score of <150 is generally regarded as reflecting clinical remission, whereas a score above 400 indicates severe disease (Best *et al*., 1976b). The proportion of IL-6$^+$ DC showed positive correlation with the CDAI in patients with CD (Pearson correlation coefficient, r=0.425; p=0.024) **(Figure 4.6A).** Pearson correlation was used for all normally distributed data. Although not statistically significant, the level of IL-6 staining by DC also showed a trend towards positive correlation with the CDAI (r=0.385; p=0.072) **(Figure 4.6B).** Conversely, the proportion and staining of IL-10 and IL-12p40 on DC did not correlate with CDAI (data not shown).

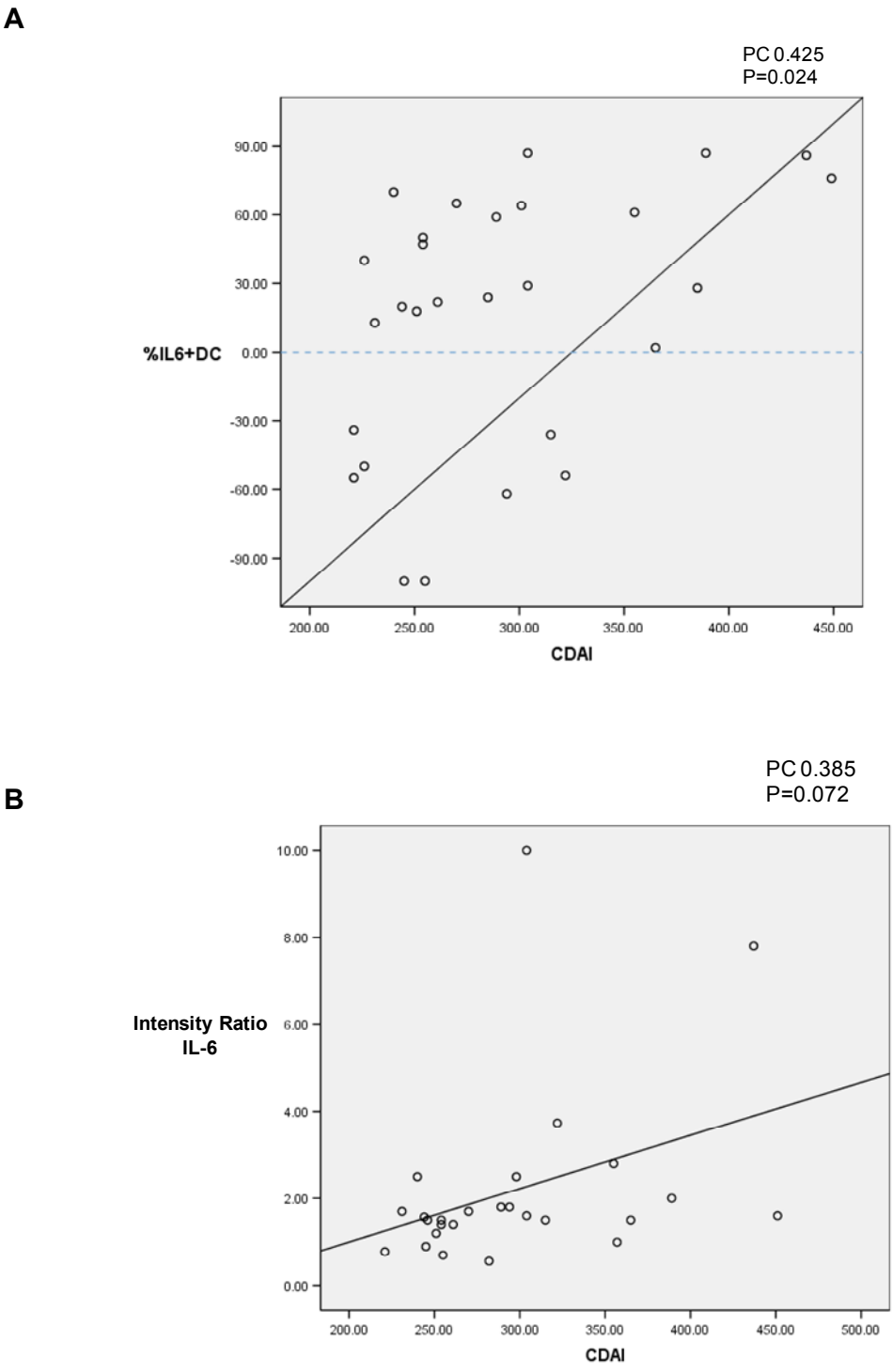
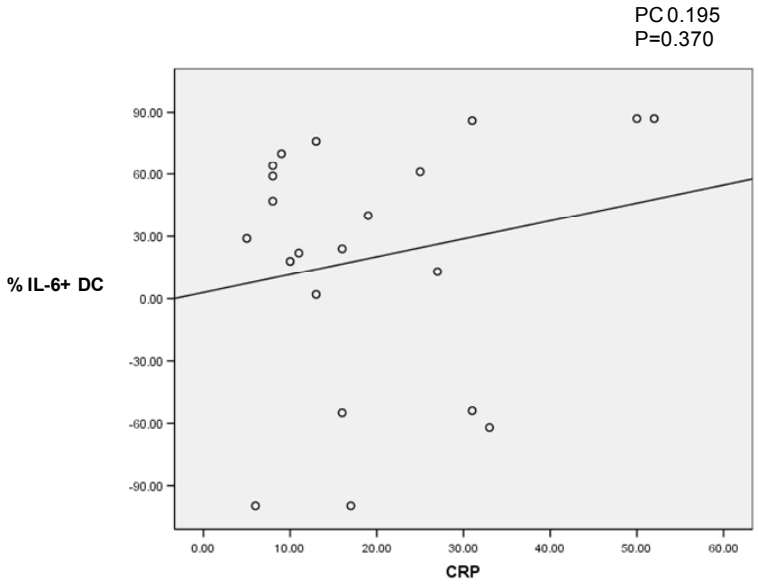


Figure 4.6: Correlation between percentage and intensity of staining of IL-6$^+$ DC and CDAI in patients with CD. Correlation curves between **(A)** the proportion IL-6$^+$ DC and CDAI; **(B)** the positive intensity ratio of IL-6 and CDAI (n=28). PC: Pearson correlation.

4.8.3 In Crohn's Disease, Level of IL-6 Staining on Dendritic Cells Correlates Positively with the Serum C-reactive Protein (CRP)

The level of IL-6 staining on DC, but not the proportion of IL-6^+ DC, correlated positively with the serum CRP in patients with CD (Pearson correlation coefficient, r=0.643; p=0.004) **(Figure 4.7).** In contrast, the level of staining of IL-10 and IL-12p40 on DC did not correlate with CRP (data not shown).

A



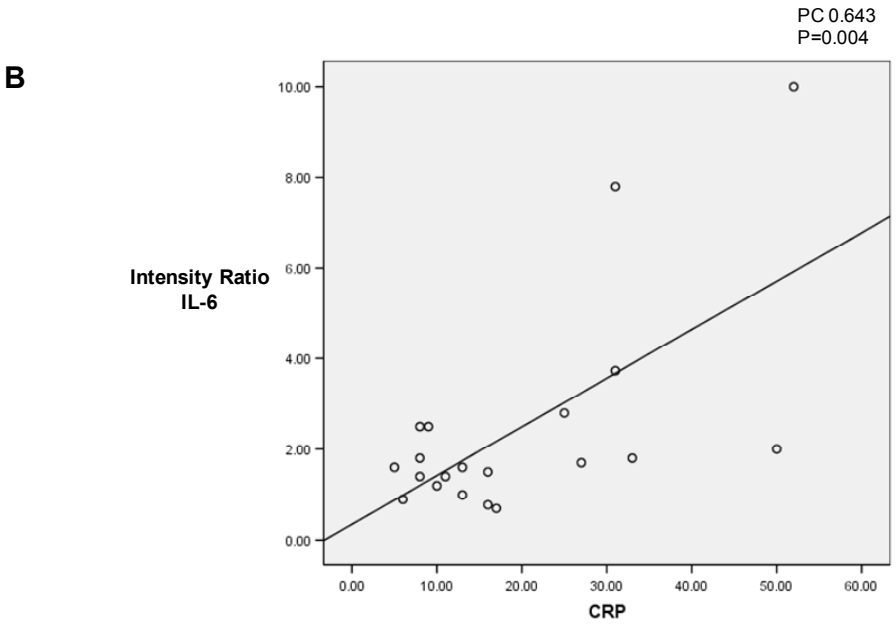


Figure 4.7: Correlation between percentage and level of staining of IL-6$^+$ DC and CRP in patients with CD. Correlation curves between **(A)** the proportion IL-6$^+$ DC and CRP; **(B)** the positive intensity ratio of IL-6 and CRP (n=28).

4.9 Correlation of Dendritic Cell Function with Faecal Microbiota in Crohn's disease

In patients with CD, the ratio of bacteroides:bifidobacteria in faecal samples correlated positively with the level of staining (PIR) of IL-12p40$^+$ DC (Pearson correlation coefficient, r=0.535, p=0.003) **(Figure 4.8A&B).** The proportion of bifidobacteria correlated positively with PIR of IL-10$^+$ DC (r=0.395; p=0.038) **(Figure 4.9).**

IL-6$^+$ DC were detected in 21 of 28 patients with CD. Patients with IL-6$^+$ DC had lower proportions of *F. praustnitzii* (6.4%±1.9) than those without IL-6 producing DC (25.0%±11.0, p=0.013). Thus IL-6$^+$ DC also correlated negatively with the proportion of *F. praustnitzii* (r=-0.50; p=0.008). **(Figure 4.10).**

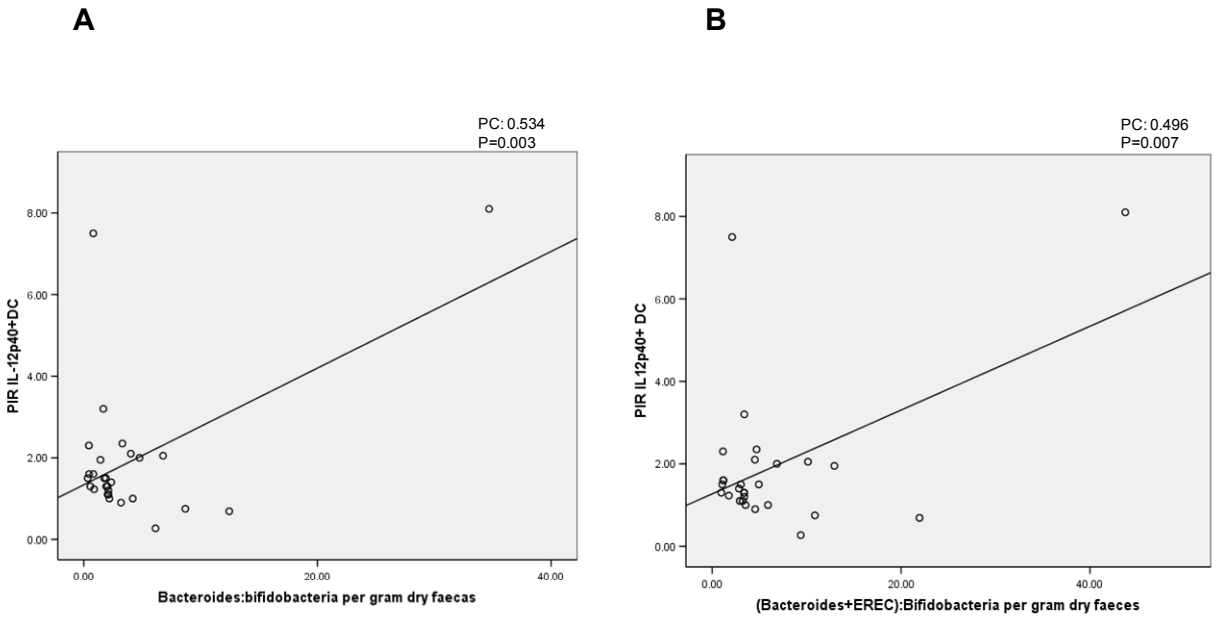


Figure 4.8 Correlation between Bacteroides:bifidobacteria and positive intensity ratio of IL-12p40$^+$ DC. This figure shows correlation curves between the (A) ratio of bacteroides: bifidobacteria and PIR of IL-12p40$^+$ colonic DC; (B) ratio of (bacteroides+EREC): bifidobacteria and PIR of IL-12p40$^+$ colonic DC in patients with CD (n=28). Even when the two outliers with high levels of IL-12p40 staining were removed from analysis, the correlation coefficient and p value remain significant.

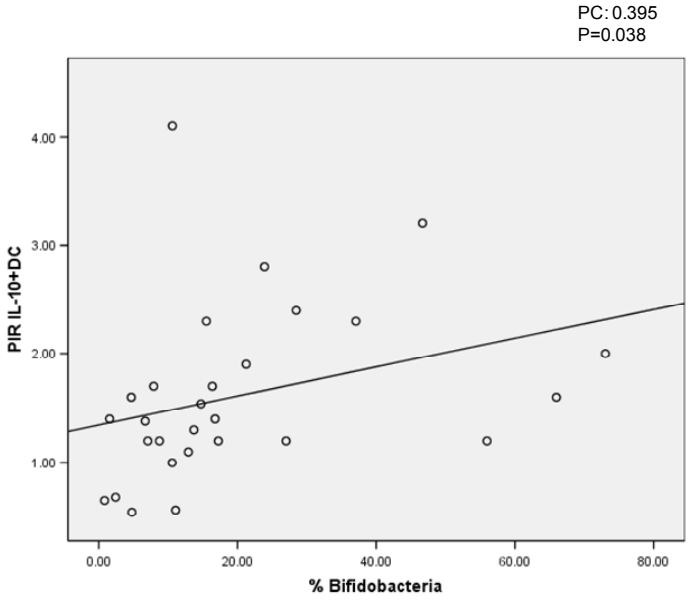


Figure 4.9: Correlation between the percentage of bifidobacteria and positive intensity ratio of IL-10$^+$ DC in CD. This figure shows the correlation curve between the percentage of faecal bifidobacteria and positive intensity ratio of IL-10$^+$ DC in patients with active CD (n=28).

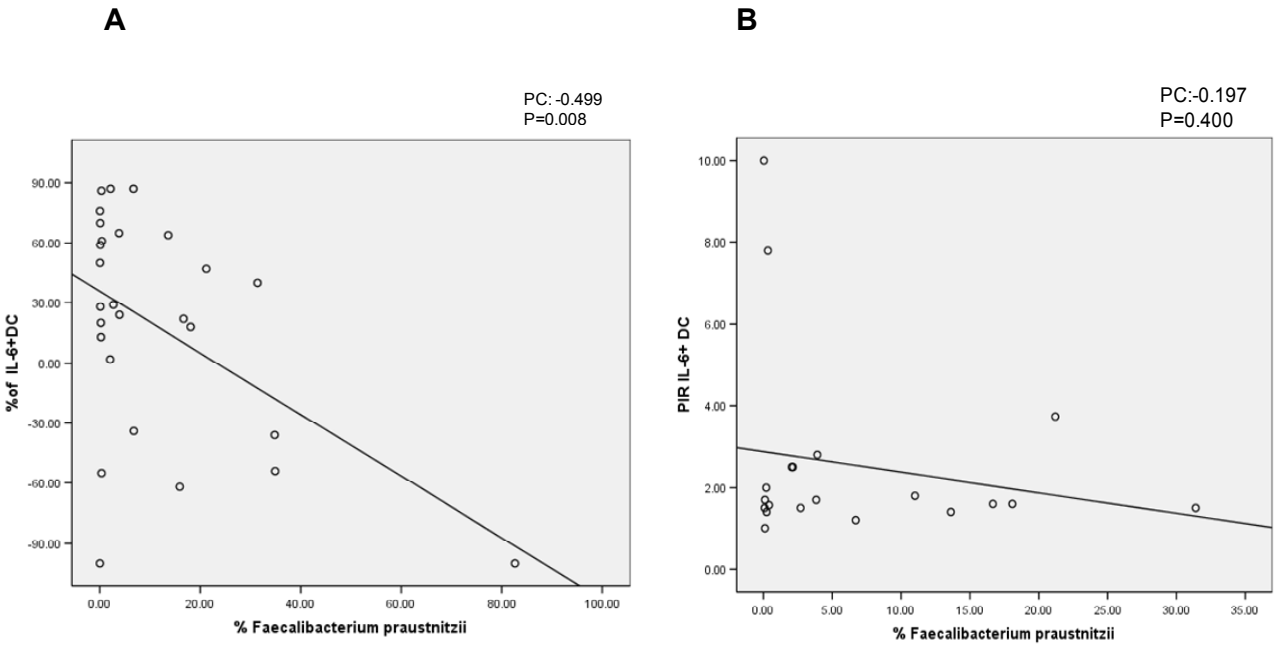


Figure 4.10: Correlation between the percentage of *Faecalibacterium praustnitzii* and percentage and PIR of IL-6$^+$ DC in CD. (A) This figure shows a negative correlation curve between the percentage of faecal *F. praustnitzii* and percentage of IL-6$^+$ DC in patients with active CD (n=28). (B) There was however no significant association between the percentage of *F. praustnitzii* and PIR of IL-6$^+$ DC although there was a trend towards negative correlation.

4.10 Discussion

Cytokine production by human colonic DC is altered in IBD. Here we have shown that in acute UC there was increased production of both IL-10 and IL-12p40 by colonic myeloid DC compared with control DC, whereas in chronic active CD, IL-12$^+$ DC increased, and IL-6$^+$ DC correlated with both the disease activity and serum CRP. In addition, the balance of immunoregulatory and proinflammatory intestinal bacteria was associated with altered DC function in patients with CD. IL-12p40$^+$ DC were associated with pro-inflammatory bacteroides, IL-10$^+$ DC were associated with immunoregulatory bifidobacteria, and IL-6

correlated negatively with anti-inflammatory *F. praustnitzii*. We measured IL-10, IL-12p40 and IL-6 as previous work from our group have shown that production of these cytokines were altered in patients with IBD (Hart *et al*., 2005). We also assessed IL-13 in UC as studies have shown that UC was characterised by Th2 response with increased IL-13 production (Fuss *et al*., 2004; Heller *et al*., 2005b). These data have been achieved with limited numbers of DC extracted from human colonic tissue.

One possible explanation for the raised IL-10 and IL-12p40 in acute UC is that in acute mucosal inflammation, IL-10 and IL-12p40 may act synergistically, and that IL-10 was insufficient to suppress the acute inflammatory response mediated by IL-12. Alternatively, IL-10 at high levels may have a pro-inflammatory effect (Furukawa *et al*., 1999; Santin *et al*., 2000). Synergism between production and effects of Th1 and Th2 cytokines has been described in animal studies (Kanai *et al*., 2006; Wirtz and Neurath, 2007). Cytokines exert their biological functions through Janus tyrosine kinases and STAT transcription factors. STAT3 was most strongly tyrosine phosphorylated in human UC and CD patients as well as in DSS-induced colitis in mice. Suzuki *et al.* showed that in transgenic mice, hyperactivation of STAT3 resulted in severe colitis. In UC, IL-10 may mediate signalling in the inflamed tissue via increased levels of STAT-3 (Suzuki *et al*., 2001).

Both the proportion and amount of staining of IL-12p40^{+} DC were also increased in acute UC. This was in contrast to previous data in patients with ***chronic*** UC which showed no difference in the proportion of IL-12p40^{+} colonic DC when compared with controls (Hart *et al*., 2005). A recent genome-wide association study has implicated the IL-10 gene as an important candidate gene in adult patients with UC. A single nucleotide polymorphishm rs3024505 of the IL-10 gene on chromosome 1q32.1 was associated with UC (Franke *et*

al., 2008); this association has now been extended to the paediatric population with CD (Amre *et al*., 2009), suggesting that defective IL-10 function is likely to be central to the pathogenesis of the UC.

Using quantitative real-time RT-PCR, Melgar *et al*. have shown enhanced IL-10 mRNA levels in T lymphocytes in UC tissue which correlated with disease activity. IL-10 positive cells were scattered in the basal lymphoid aggregates in UC colon and increased number of these cells have been detected in the lamina propria (Melgar *et al*., 2003). In patients with UC, increased IL-10mRNA expression in the serum and colonic tissue have also been described (Kucharzik *et al*., 1995; Niessner and Volk, 1995). *In vitro* activation of human intestinal T cells also induced IL-10 production in lamina propria lymphocytes.

IL-10 is involved in both cell-mediated and humoral immune responses. It has both pro-inflammatory and anti-inflammatory effects. It is also a growth factor for down-regulatory Treg cells (Groux *et al*., 1997). Anti-inflammatory effects of IL-10 include the induction and maintenance of tolerance and appear to be executed through inhibiting the production of pro-inflammatory cytokines by T cells and APC (Fiorentino *et al*., 1991b; Fiorentino *et al*., 1991a). Pro-inflammatory actions of IL-10 have been reported in both man (Furukawa *et al*., 1999; Santin *et al*., 2000) and mice (Balasa and Sarvetnick, 1996; Wogensen *et al*., 1993). For instance, in patients with CD treatment with recombinant human IL-10 induced IFN-γ production (Tilg *et al*., 2002). IL-10 might also induce a pro-inflammatory response instead of the inhibition of intestinal lamina propria lymphocytes in CD (Colpaert *et al*., 2002). Altogether these data suggest that the consequences of IL-10 production are likely to be governed by the local milieu, the type of target cells as well as the concentration of the cytokine.

In animal models of colitis mucosal cytokines differ between the induction and maintenance phases of intestinal inflammation. In a SAMP1/YitFc mouse model of ileitis, the initiation of disease was Th1 mediated with upregulation of IFN-γ and TNF-α which preceded the histologic injury, whereas the establishment of chronic disease was characterised by increased IL-13 and IL-5 mRNA expression (Bamias *et al*., 2005). We measured DC IL-13 production in patients with UC given that non-classical IL-13 producing NK T cells has been associated with UC (Fuss *et al*., 2004). Furthermore, IL-13 has been implicated as a major mediator of intestinal fibrosis (Bamias *et al*., 2005; Wynn, 2003). In an animal model of chronic 2,4,6-TNBS colitis, intestinal fibrosis was dependent on development of an IL-13 response by NKT cells (Fichtner-Feigl *et al*., 2007). In our study there was lack of IL-13 production by DC. It remains possible that DC production of IL-13 may increase if measured in the later stages of UC inflammation, or that the cellular source of IL-13 is from NKT cells and not DC.

Alex *et al*. used a comprehensive serum multiplex cytokine profiling with biometric immunosandwich ELISA to assess the modulation of Th1, Th2, Th17 cytokines in acute and chronic murine models of DSS and TNBS-induced colitis (Alex *et al*., 2008). In TNBS colitis, there was increased IL-12 and IL-17 production in chronic disease. In contrast, DSS colitis switched from a Th1/Th17-mediated acute inflammatory profile with high TNF-α, IL-6 and IL-17, to a predominant Th2-mediated inflammatory response with raised IL-4 and IL-10 and concomitant reduction in TNF-α, IL-6 and IL-17 in the chronic state. Thus distinctive disease-specific cytokine profiles correlated significantly with disease activity and duration (Alex *et al*., 2008).

In patients with CD, an increase in production of pro-inflammatory cytokines has been demonstrated (Fichtner-Feigl *et al*., 2007; Fuss *et al*., 1996). Lamina propria T cells from inflamed CD mucosa showed increased IFN-γ secretion; this was due to an increased number of IFN-γ-secreting $CD4^+$ T cells. In contrast, IL-2, IL-4 and IL-5 production by CD lamina propria T cells was decreased compared with that of control T cells (Fuss *et al*., 1996). In cells from healthy controls, there were little or undetectable cytokine production by colonic DC suggesting a role of DC in maintaining tolerance in such an environment (Harper *et al*., 1996). DC producing IL-12p40 were also prominent in cells from patients with moderately active CD. This is consistent with previous work demonstrating that IL-12p40 is expressed by both colonic DC (Hart *et al*., 2005), and LPMC in CD (Monteleone *et al*., 1997).

Although the frequency of $IL\text{-}6^+$ DC were not different between subjects, the level of IL-6 staining by DC was increased in CD, which correlated with both disease activity and a systemic marker of inflammation, CRP. We have correlated DC cytokine production with CDAI, CRP and faecal microbiota. When making multiple comparisons between several variables, it is important to adjust the threshold and apply the Bonferroni correction, as discussed under methods in section 2.2.14.

Therefore $IL\text{-}6^+$ DC may have significance in the systemic inflammatory response. IL-6 has multiple well-characterised functions during acute phase response and B cell differentiation. IL-6-deficient mice were resistant to autoimmune diseases (Ohshima *et al*., 1998; Okuda *et al*., 1998), and were less susceptible to colitis (Suzuki *et al*., 2001). Jones *et al*. recently showed higher serum IL-6 concentrations in CD patients with more severe endoscopic disease activity (Jones *et al*., 2008). IL-6 plays a major role in T cell activation

in vitro and *in vivo* due to its ability to overcome suppression mediated by Treg. Pasare *et al.* demonstrated that the production of IL-6 by DC in response to TLR ligation during infection was critical for T cell activation by overcoming the suppressive effects of $CD4^+$ $CD25^+$ Treg cells (Pasare and Medzhitov, 2003).

The cytokine network in IBD is a complex, dynamic system and there is likely to be interplay between various cytokines. We used multiplex ELISA to assess the overall inflammatory infiltrate in IBD and the interplay between various cytokines. These included Th1 cytokines (IL-1b, IL-12p70, IL-6, IL-8, TNF-α and IFN-γ) and Th2 cytokines (IL-4, IL-5 and IL-10). In patients with IBD, IBS, and controls, cytokines associated with "tissue trauma", IL-1β, IL-10 and IL-6, were detected in gut biopsies from all subjects. The source of these cytokines could be from macrophages, DC or T cells.

CD has been considered to be a typical Th1 disease, characterised by excess IFN-γ production in the inflamed intestine (Fuss *et al*., 1996; Kobayashi *et al*., 2008; Matsuoka *et al*., 2004), whereas UC has been traditionally referred to as a "Th2- like" or "mixed" phenotype as this disease does not exhibit clearly distinct phenotype (Heller *et al*., 2005a; Okazawa *et al*., 2002). Since this work has been completed, Th17 cells, which exclusively produces the pro-inflammatory cytokine IL-17 has been proposed to play a role in the pathogenesis of murine model of colitis and human IBD (Annunziato *et al*., 2007; Annunziato *et al*., 2008; Fujino *et al*., 2003; Nielsen *et al*., 2003; Zhang *et al*., 2006). IL-23 can promote IL-17 production by Th17 cells (Mangan *et al*., 2006).

We have focused on IL-12 composed of the p40 and p35 subunits in our experiments; IL-12 shares the p40 unit with IL-23; IL-23 is composed of p40 and the subunit p19

(Oppmann *et al*., 2000). The functional role of IL-12 has been reevaluated given that earlier studies which used antibodies directed against the IL-12/23p40 subunit, was also part of the IL-23 heterodimer. Mouse models of intestinal inflammation have since demonstrated a more important role of IL-23 than IL-12 in driving inflammation. Hue *et al.* showed that IL23p19 antibodies suppressed chronic intestinal inflammation in T cell deficient *Helicobacter hepaticus*-infected RAG knockout mice (Hue *et al*., 2006). In a transfer colitis model, recipient mice deficient in IL-23p19 and IL-12/IL-23p40 were protected against colitis, whilst mice deficient in IL-23p19 developed colitis (Kullberg *et al*., 2006). IL-23 also play a role in human IBD (Duerr *et al*., 2006; Elson *et al*., 2007; Fuss *et al*., 2006; Kobayashi *et al*., 2008; Neurath, 2007; Schmidt *et al*., 2005). Upregulation of IL23p19 mRNA was found in the colonic mucosa of UC and CD patients (Kobayashi *et al*., 2008; Schmidt *et al*., 2005) and lamina propria macrophages from CD patients produced large amounts of IL-23 (Fuss *et al*., 2006). Kobayashi *et al*. recently demonstrated that recombinant IL-23 enhanced IL-17 production by lamina propria $CD4^+$ T cells in UC, but had a lesser effect on lamina propria $CD4^+$ T cells in CD (Kobayashi *et al*., 2008). The mucosal IL-23p19 expression levels correlated with IL-17 in UC and IFN-γ in CD, suggesting that IL-23 may play important roles in controlling the differential Th1/Th17 balance in both UC and CD (Kobayashi *et al*., 2008). We have not assessed IL-23 or IL-17 in our patients as the importance of these cytokines in intestinal inflammation came about after this work has completed.

A separate T cell-derived cytokine, IL-21, has been reported to be increased in IBD. IL-21 plays a role in controlling Th17 cell responses in both DSS colitis and TNBS-relapsing colitis. IL-21-deficient mice were largely protected against both colitides and failed to up-

regulate Th17-associated molecules during gut inflammation, hence suggesting a role for IL-21 in controlling Th17 cell responses (Fina *et al*., 2008).

DC directly interact with trillions of non-invasive intestinal bacteria *in vivo*, and their functions are likely to be influenced by distinct bacteria subsets (Christensen *et al*., 2002; Zeuthen *et al*., 2006). Distinct commensals confer different properties to *in vitro*-generated DC. Fink *et al.* showed that freshly isolated DC from murine Peyer's patches, MLN and spleen, matured similarly in response to bacterial stimuli but intestinal DC demonstrated tissue-dependent functions (Fink and Frokiaer, 2008). For instance, MLN and Peyer's patches DC produced mainly IL-10 and little IL-12 and IFN-γ in response to *E. coli* and *B. longum*, but MLN DC produced more IFN-γ and less IL-10 in response to *L. acidophilus* (Fink and Frokiaer, 2008). Other *ex vivo* studies showed that DC from the human colon lamina propria produced IL-10 but no IL-12 upon exposure to entero invasive *Salmonella* (Rimoldi *et al*., 2005) and the probiotic mixture VSL#3 (Hart *et al*., 2004c). Thus individual strains of commensal bacteria have varying immunomodulatory properties and can polarize the distinct immune response.

We have shown that in patients with active CD, IL-12p40 production by DC was associated with the proinflammatory bacteria, bacteroides and EREC, whereas IL-10 secreted by DC correlated with bifidobacteria. These data suggest that resident commensal bacteria in patients with IBD can influence intestinal DC function *in vivo*. The phylum Firmicutes, particularly the species *F. praustnitzii,* were consistently reduced in patients with IBD and infectious colitis (Sokol *et al*., 2009b; Swidsinski *et al*., 2008). *F. praustnitzii* can be considered an immunoregulatory bacteria; *In vitro*, PBMC stimulation by *F. praustnitzii* reduced IL-12 and IFN-γ production and enhanced IL-10 secretion. In

TNBS colitis, administration of live *F. praustnitzii* or its supernatant decreased the severity of colitis (Sokol *et al*., 2008). We demonstrated that patients with CD who had undetectable IL-6 production had more *F. praustnitzii* than those with IL-6^+ DC. We speculated that *F. praustnitzii* may create an anti-inflammatory environment by suppressing IL-6 production by DC *in vivo.* Alternatively, in patients with CD who had altered *F. praustnitzii,* intestinal DC activated by microbial stimuli blocked the suppressive effects of $CD4^+$ $CD25^+$ Treg cells and that this process was dependent on IL-6 production (Pasare and Medzhitov, 2003). Upon *in vivo* stimulation with *E. coli*, MLN and Peyer's patches DC produced TGF-β which concomitantly increased IL-6, possibly leading to the induction of Th17 cells, and the enhancement of epithelial barrier function (Weaver *et al*., 2006). Human DC exposed to commensal-related bacteria in the absence of inflammatory signals, upregulated costimulatory molecules, produced IL-1β and IL-6. DC also converted naive $CD4^+$ T cells into hyporesponsive T cells that displayed suppressor function (Baba *et al*., 2008). Furthermore, when DC were selectively depleted in mice before the initiation of DSS-induced colitis, the disease was more severe, compared with that of DC-intact mice (Qualls *et al*., 2009). Increased IL-6 expression in colon tissues correlated positively with increased colitis severity in DC-depleted mice (Qualls *et al*., 2009). Consistent with our data, regulation of IL-6 production may contribute to DC-mediated control of intestinal inflammation, which is associated with the severity of disease.

Our current work from human tissue suggests that distinct DC cytokine production is likely to be influenced by the disease stage and activity in CD and UC. Resident commensal bacteria also play specific roles in modulation DC functions in intestinal inflammation.

Chapter 5

The Effects of Therapy on Intestinal Dendritic Cells Phenotype and Function in Inflammatory Bowel Disease

5.1 Abstract

Introduction: In UC gut bacteria drive the inflammatory process. Bacterial recognition and T cell responses are shaped by intestinal DC; the therapeutic effects of probiotic bacteria may relate to their modulation of intestinal DC function. We aimed to assess the effect of probiotics and steroids in acute UC, clinically and in relation to their effects on colonic DC *ex vivo*.

Methods: Rectal biopsies were obtained from patients with active UC before and after treatment with the mixed probiotic preparation VSL#3, corticosteroids or placebo, and from healthy controls. Myeloid colonic DC were identified from freshly isolated lamina propria cells using multi-colour flow cytometry. Surface expression of activation markers, CD40, CD86, pattern recognition receptors, Toll-like receptor (TLR)-2 and TLR-4, and spontaneous intracellular IL-10, IL-12p40, IL-6, and IL-13 production by DC were measured.

Results: Ten of 14 patients on VSL#3 responded to treatment compared with 5 of 14 on placebo ($p=0.064$). In ***VSL#3***-treated patients TLR-2 expression on DC decreased ($p<0.05$), IL-10^{+} colonic DC increased and IL-$12p40^{+}$ DC decreased ($p<0.005$). In contrast, in patients on ***placebo*** TLR-2 expression, and IL-12p40 and IL-6 levels were increased (all $p<0.05$). Corticosteroid treatment was associated with significant increased IL-10 and reduced IL-12p40 production by colonic DC.

Conclusions: In acute UC, treatment with the probiotic mix VSL#3 and corticosteroids is associated with altered "favourable" intestinal DC function, inducing increased levels of regulatory cytokines and down-regulation of pro-inflammatory cytokines.

5.2 Introduction

UC, a common chronic relapsing inflammatory disorder of large bowel, results from inappropriate mucosal immune response to indigenous microbiota and luminal antigens in genetically susceptible individuals. Intestinal microbiota in patients with active UC is altered compared with that of healthy individuals (Cummings *et al*., 2003; Swidsinski *et al*., 2002). Manipulating gut flora, using probiotics, may correct and/or prevent pro-inflammatory signals in UC. VSL#3, which is a combination of eight different probiotic bacterial strains, is effective in maintaining remission and preventing development of pouchitis (Gionchetti *et al*., 2000; Gionchetti *et al*., 2003; Mimura *et al*., 2004). In an open-label study (Bibiloni *et al*., 2005), and a recent placebo-controlled trial published in abstract form (Makharia *et al*., 2008), VSL#3 is effective in the treatment of patients with mild to moderately active UC, with a response rate of 50 to 70 percent. Two components of VSL#3, *S salivarius* subspecies *thermophilus* and *B. infantis,* have been detected in biopsies, demonstrating that these bacteria reach the diseased bowel (Venturi *et al*., 1999).

The ability of intestinal DC to sample and respond to gut bacteria, suggests that they may be targets for probiotic bacteria. The effects of probiotic bacteria on DC are likely to be central in immunomodulation by these bacteria (Ng *et al*., 2009). *In vitro* the VSL#3 mixture, in particular its bifidobacterial components, is a potent inducer of IL-10 in DC from both blood and intestinal biopsies, and inhibits generation of Th1 cells. VSL#3 also diminishes proinflammatory effects of LPS by decreasing LPS induced production of IL-12p40 in DC while maintaining IL-10 production (Hart *et al*., 2004c). It remains to be established whether the *in vivo* administration of probiotics, such as VSL#3, in the setting

of resident microbiota and active mucosal inflammation, results in the modulation of gut DC.

It has been generally accepted that corticosteroids are highly effective and the most frequently used immunosuppressive agents to induce remission in patients with moderate to severely active UC, and in patients who have failed 5-ASA. The immunosuppressive effects of steroids have been ascribed mainly to the suppression of T cell activation (Liberman *et al*., 2009). Subsequent work has shown that steroids can regulate DC maturation and immune function *in vitro* and *in vivo* suggesting that these mechanisms may play a role in preventing overstimulation of the immune system (Moser *et al*., 1995). Whether corticosteroids modulate such DC functions, and thus contribute to its efficacy in patients with UC has not been studied.

We studied the effects of therapeutic intervention with bacterial modification (probiotics) and standard immunosuppression (corticosteroids) on intestinal DC function in patients with UC. We included a control group of patients who have received placebo treatment. We correlated changes in DC function with therapeutic efficacy.

5.3 Hypothesis

Therapeutic manipulation with probiotic bacteria or conticosteroids induces immunoregulatory DC responses in patients with active UC.

5.4 Aims

- To investigate the effects of probiotics on DC function *in vivo* in patients with UC
- To assess the effects of corticosteroids on DC function *in vivo* in patients with UC

5.5 Patient Characteristics

Patients with active UC were recruited at one specialist hospital, as part of a multi-centre, double blind, placebo-controlled therapeutic trial evaluating the effectiveness of the probiotic preparation, VSL#3, for the treatment of mild to moderately active UC. Twenty-eight patients with acute UC were included in this laboratory sub-study. Twelve healthy controls with no history of bowel disease were studied as controls.

Baseline characteristics of all patients, including concurrent medical therapy in the UC patients, are shown in **Table 12.** Diagnosis was made using clinical parameters, radiographic studies, endoscopic and histologic criteria.

Disease activity was assessed using the UC disease activity index: UCDAI, range of scores 0 (no active disease) to 12 (maximum disease) (Singleton, 1987). Patients were eligible if they had an UCDAI of 3 to 8 and were symptomatic for less than four weeks. Tissue samples were from macroscopically inflamed rectal mucosa and inflammatory activity was confirmed by histological evaluation (Section 2.2.16).

Patients were on stable doses of medication. Exclusion criteria included the use of steroids within the last 4 weeks; use of antibiotics within the last 2 weeks; change in dose of 5-

aminosalicylate acid (ASA) within the last 4 weeks; change in dose of rectal 5-ASA or steroids within 7 days prior to study entry, or the use of probiotic preparations during study. Rectal therapy was not permitted.

	VSL#3	Placebo
Male/female	4/10	7/7
Mean age, year (range)	45 (21-70)	42 (28-57)
Disease duration, years (range)	6 (1-16)	9 (1-27)
Disease extent		
Distal	7	8
Extensive	2	1
Total	5	5
Mean UCDAI, range	7 (4-8)	6 (5-8)
Concomitant medications (%)		
Mesalazine	7 (50)	11 (79)
AZA/6-MP	1 (7)	1 (14)
None	7 (50)	2 (14)

Mesalazine: Stable for at least 4 weeks
AZA/6MP: Stable for at least 12 weeks

Table 12: Baseline characteristic of patients with acute UC treated with VSL#3 or placebo

5.6 Modulation of Intestinal DC by Probiotic bacteria VSL#3 *in Vivo* in Acute Ulcerative Colitis

5.6.1 More Patients treated with VSL#3 had a Clinical Response Compared with Patients on Placebo

Fourteen patients were randomized to VSL#3 and 14 to placebo. Ten of 14 patients on VSL#3 responded to treatment compared with five of 14 on placebo (p=0.064, Fisher's Exact Test). Three and six patients from the VSL#3 and placebo group, respectively,

withdrew from the trial within one week due to lack of therapeutic efficacy or worsening disease and were treated with oral corticosteroids. In the ***VSL#3*** group, intention to treat analysis, that is including those who withdrew within the first week of treatment, demonstrated that remission (UCDAI≤2) was achieved in 7 (50%) patients, clinical response in a further 3 (21%) patients, no response in 3 (21%) patients, and 1 (7%) patient did not have the final sigmoidoscopy assessment. In patients who had ***placebo***, remission was seen in 5 (36%), response in 0 (0%) and no response in 9 (64%). **Figure 5.1** shows the clinical response of treated patients by treatment arm.

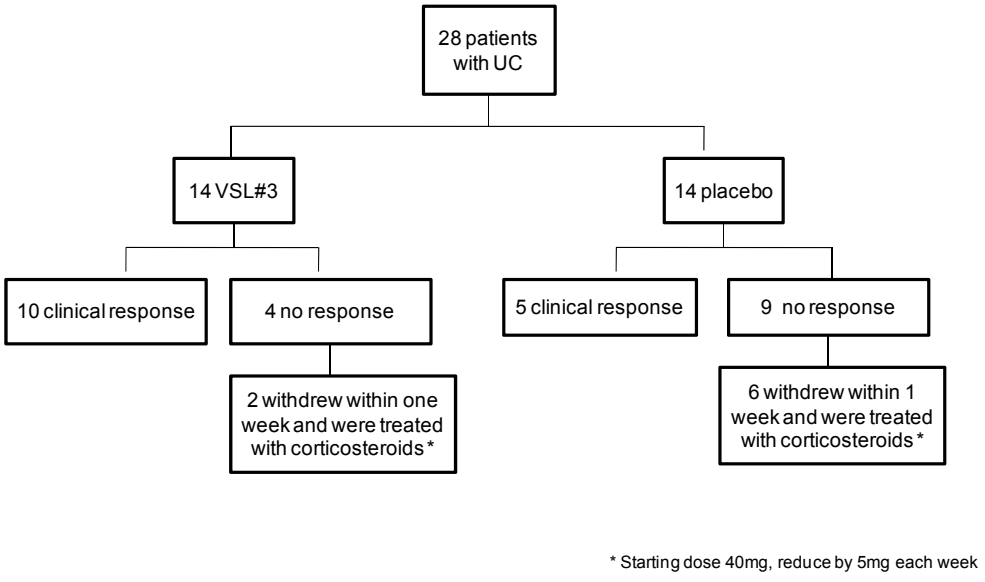


Figure 5.1: Clinical outcome of patients with UC treated with VSL#3, placebo and corticosteroids. In patients who were treated with VSL#3, two of four patients did not have a response after one week and were treated with prednisolone 40mg daily (reducing by 5 mg each week) over a course of eight weeks. In patients who had placebo, six of nine patients did not have a response and were treated with prednisolone after one week.

5.6.2 VSL#3 Downregulates TLR-2 Expression on CD11c$^+$ Colonic Dendritic Cells

Expression of co-stimulatory molecules (CD40 and CD86) and TLRs (TLR-2 and TLR-4) was assessed on lamina propria CD11c$^+$ DC before and after probiotic or placebo treatment. We assessed these markers as they have been shown to be activated in patients with IBD from our previous work (Hart *et al*., 2005). Two measures of expression were determined on these cells: the proportion of DC expressing a given marker and the intensity of staining or positive intensity ratio (PIR), which represents the amount a given marker is expressed on positive cells. TLR-2 expression was significantly reduced in patients treated with VSL#3 (30±7% to 4±3%; p=0.04) **(Figure 5.2A).** As there was no significant difference between TLR-2 expression on DC in the VSL#3 or placebo group before treatment, this suggested that reduction in TLR-2 post VSL#3 was unlikely to be an artifact due to higher TLR-2 expression in the VSL#3 group prior to therapy. However, the median value for percentage TLR2-expressing DC in the pre-treated VSL#3 group was approximately twice that of the placebo group, hence there is a possibility that the significant reduction in TLR-2 expression post-VSL#3 treatment was an artefact. There were no significant differences in CD40, CD86 or TLR-4 expression after VSL#3 treatment **(Figure 5.2A).**

In patients on placebo, there were no significant changes in DC expression of CD40, CD86, TLR-2 and TLR-4 **(Figure 5.2B).**

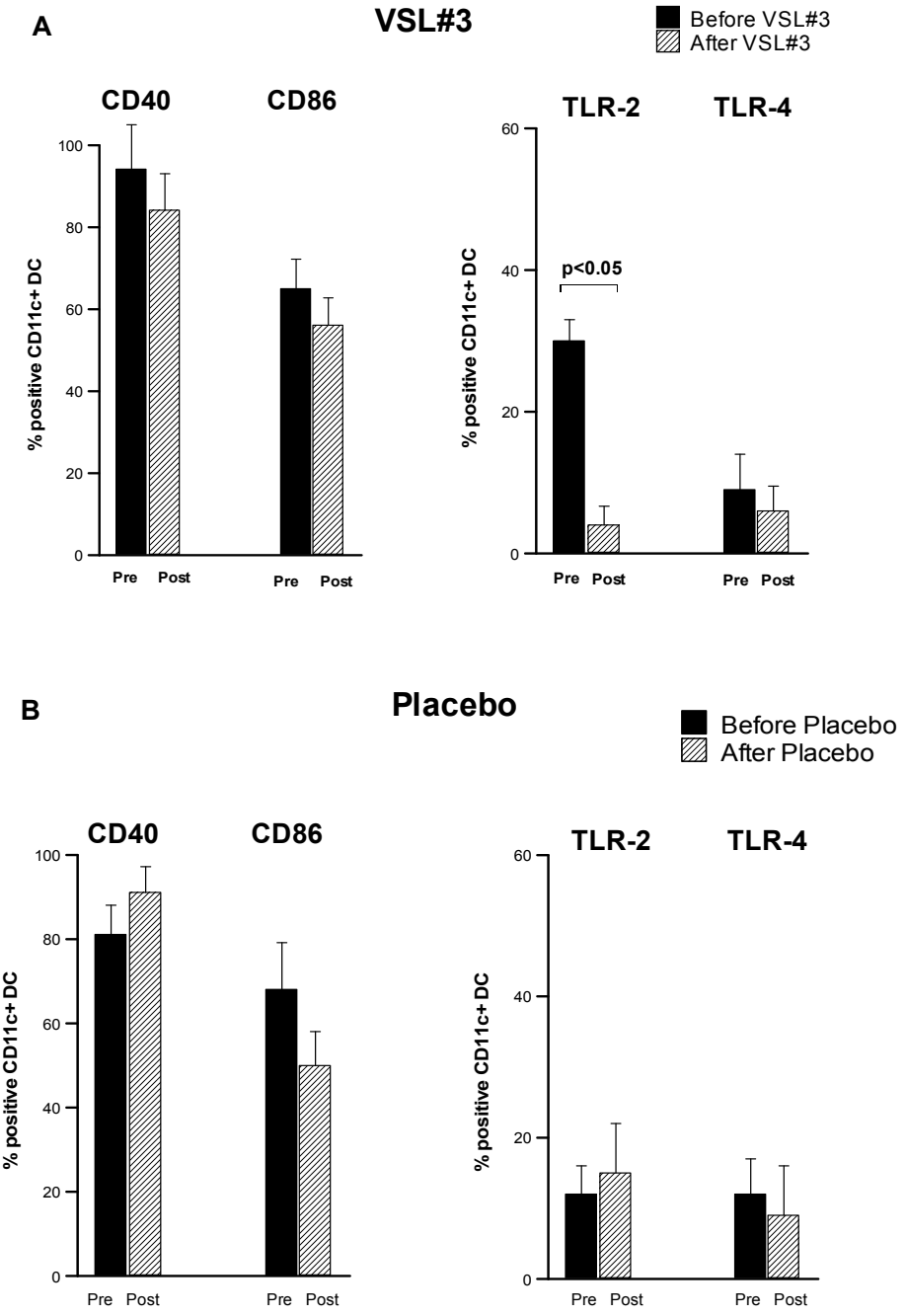


Figure 5.2: Co-stimulatory molecule and Toll-like receptor expression by colonic CD11c$^+$ DC after VSL#3 or placebo treatment. (A) The proportion of colonic CD11c$^+$ DC expressing CD40, CD86, TLR-2 and TLR-4 in acute UC after VSL#3 treatment (n=10); **(B)** The proportion of colonic CD11c$^+$ DC expressing CD40, CD86, TLR-2 and TLR-4 in acute UC after placebo (n=9).

5.6.3 VSL#3 Increases IL-10 and Inhibits IL-12p40 Production by CD11c$^+$ Colonic Dendritic Cells

Cytokine production by DC extracted from the lamina propria of colonic tissue of patients with UC was assessed at two time points: (i) prior to the start of therapeutic intervention and, (ii) eight weeks post probiotic or placebo. The methods used for all cytokine data have been described in section 2.2.12. There was significantly greater IL-10 production (10 ±17% to 56 ±17%; p=0.005) and decreased IL-12p40 production (62±9% to -2±14%; p=0.005) by colonic DC following VSL#3 treatment **(Figure 5.3).** For patients who had placebo, there were no significant changes in the proportion of cytokine producing DC for IL-10, IL-12p40, IL-6 or IL-13 **(Figure 5.4).**

The level of staining (PIR) of IL-12p40 was significantly lower after VSL#3 treatment (2.1±0.2 to 1.0±0.4; $p<0.05$), although the baseline IL-12p40 staining was higher in the VSL#3 group than placebo group. In contrast, there were no significant changes in percentage or level of staining in IL-6 or IL-13 production by DC after VSL#3. In patients treated with placebo, there was a significant increase in the level of staining for IL-12p40 and IL-6 on CD11c$^+$ DC **(Figure 5.5).**

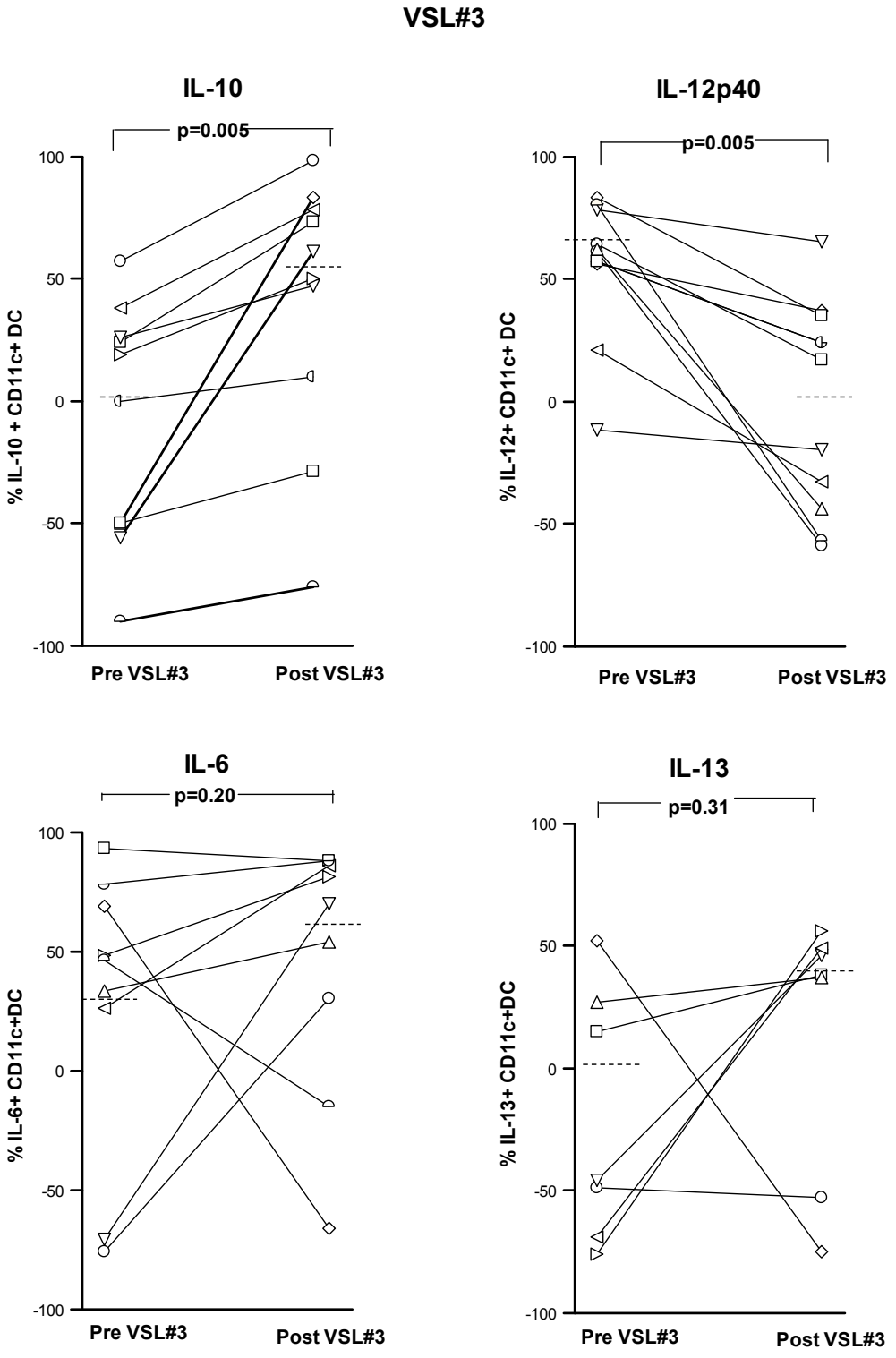


Figure 5.3: Cytokine production by colonic DC before and after treatment with VSL#3. Paired samples of colonic DC from patients with acute UC, obtained before and after VSL#3 treatment were stained for the presence of intracellular IL-10, IL-12p40, IL-6 and IL-13. The proportion (%) of cytokine positive DC is shown in this figure (n=10).

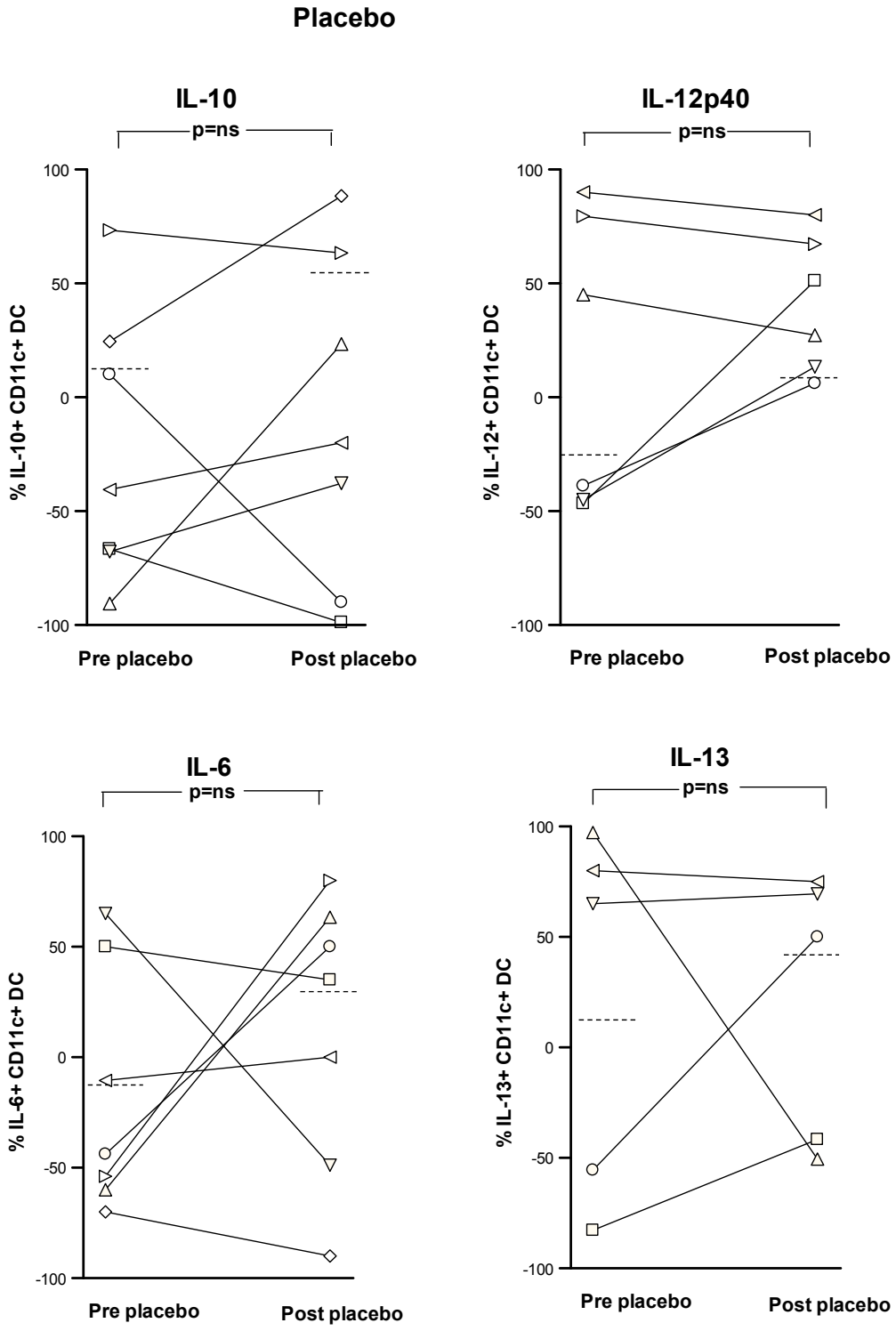


Figure 5.4. Cytokine production by colonic DC before and after treatment with placebo. Paired samples of colonic DC from patients with acute UC, obtained before and after placebo treatment, were stained for the presence of intracellular IL-10, IL-12p40, IL-6 and IL-13. The proportion (%) of cytokine positive DC is shown in this figure (n=7).

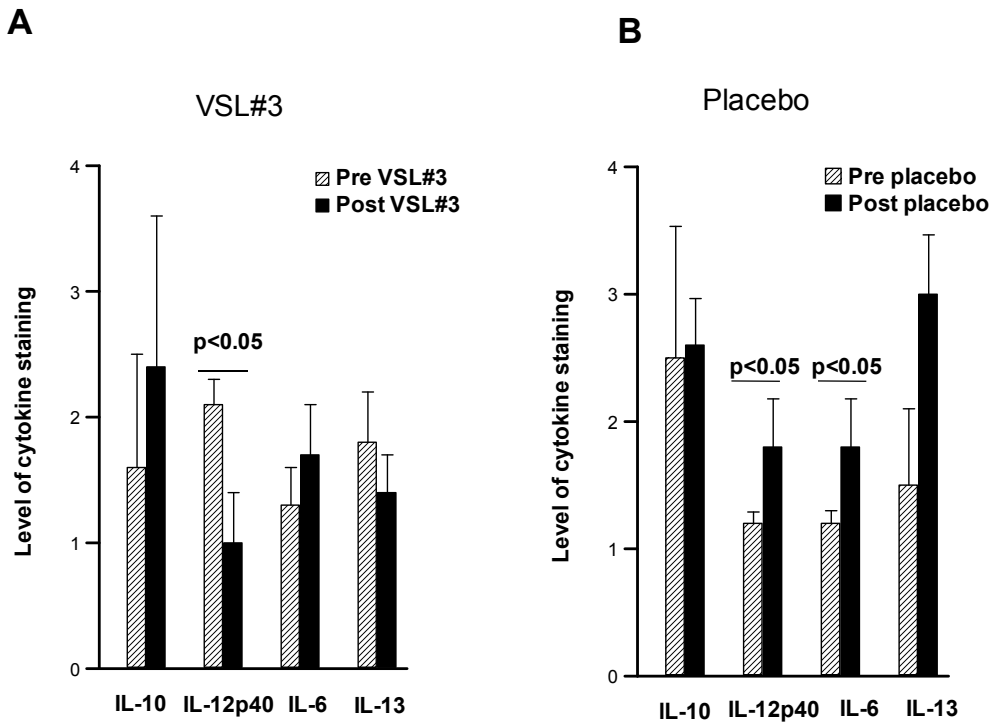


Figure 5.5: Positive intensity ratio of cytokine staining before and after VSL#3 or placebo. **(A)** Level of cytokine staining in patients who were treated with VSL#3 (n=10); **(B)** Level of cytokine staining in patients who received placebo (n=9).

5.7 Modulation of Intestinal Dendritic Cells by Oral Corticosteroids *in Vivo* in Acute Ulcerative Colitis

5.7.1 Corticosteroids did not Affect the Expression of Co-stimulatory Molecules and Toll-like Receptors on Colonic Dendritic Cells

In a subgroup of patients who were treated with eight weeks of corticosteroids (starting dose oral prednisolone 40mg daily and reduced by 5 mg each week until zero; n=8) changes in colonic DC phenotype and function after steroid therapy were assessed. As shown in figure 5.1, two and six patients from the VSL#3 and placebo group, respectively,

withdrew from the trial within one week due to lack of therapeutic efficacy or worsening disease and were treated with oral corticosteroids.

There was no difference in the proportion and levels of staining of DC expressing co-stimulatory molecules or TLRs before and after steroids.

5.7.2 Corticosteroids Enhance IL-10 and Inhibit IL-12p40 Production by CD11c$^+$ Colonic Dendritic Cells

There was a significant reduction in colonic CD11c$^+$ DC production of IL-12p40 after treatment with corticosteroids (p=0.012). Conversely, IL-10 production by CD11c$^+$ DC increased significantly after steroid therapy (p=0.032) **(Figure 5.6).** In addition, mean PIR for IL-12p40 decreased from 1.7 to 1.0, whilst mean PIR for IL-10 increased from 1.3 to 1.8 (p=0.04). There were no significant changes in CD11c$^+$ DC IL-6 or IL-13 production after corticosteroids.

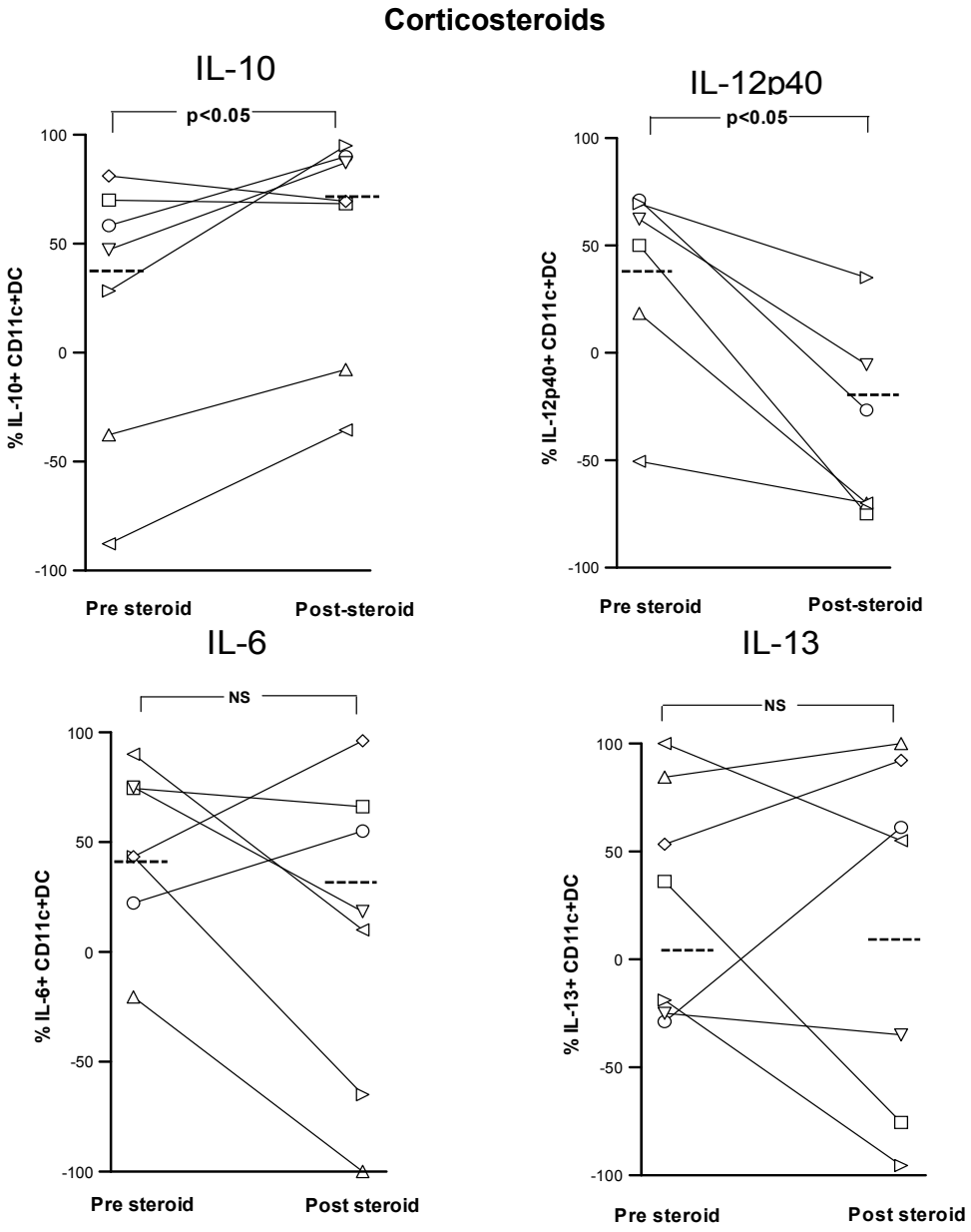


Figure 5.6: Cytokine production by colonic dendritic cells before and after treatment with corticosteroids. Paired samples of colonic DC from patients with acute UC, obtained before and after steroid treatment, were stained for the presence of intracellular IL-10, IL-12p40, IL-6 and IL-13. The proportion (%) of cytokine positive DC is shown in this figure.

5.8 Correlation of Disease and Histological Activities with Dendritic Cell Functions

5.8.1 No Correlation between Ulcerative Colitis Disease Activity Index and Changes in Dendritic Cell Phenotype or Functions

There was no significant correlation between patients' UCDAI score (median 6; range 4-8) at baseline and the baseline measurement of percentage positive cells for surface phenotypic markers or intracellular cytokines.

Group	Variable	Correlation Coefficient	P-value
All patients	Il-10	-0.36	0.13
	Il-12	-0.05	0.84
	Il-6	0.09	0.69
	Il-13	-0.05	0.85
	CD40	0.10	0.68
	CD86	-0.41	0.11
	TLR2	-0.15	0.56
	TLR4	-0.14	0.61

Table 13: Association between the UCDAI scores at baseline and the percentage of positive cells at baseline

5.8.2 Lack of Change in Tissue Histologic Activity Index Post VSL#3 or Corticosteroid Treatment

Patient's pre and post treatment tissue was scored for histological inflammatory activity. In all treated patients, there was no significant change in mean histologic activity index (VSL#3: 1.6 to 1.8; p=0.41; placebo 1.9 to 1.2; p=0.098; steroids 2.0 to 1.0; p=0.17). In 9 of 10 patients, inflammatory infiltrate remained in tissue post VSL#3.

Eight patients were treated with prednisolone (two and six patients were initially randomised to VSL#3 or placebo, respectively, but withdrew due to lack of clinical response). In patients on steroids altered cytokine profile occurred irrespective of resolution of inflammatory cell infiltrate. In five patients who had steroids, an inflammatory cell infiltrate was still present on histology (score 1-3) but changes were detected in cytokine profiles.

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5.8.3 Unchanged Inflammatory Cell Activities in Supernatants Measured by Multiplex ELISA of Cytokines Post VSL#3 and Corticosteroid Treatment

In patients who were treated with oral VSL#3 or steroids, cytokines including IL-10, IL-8, IL-6, IL-4, IL-5, IL-1β, TNF-α, and IL-12p70 were detected in the supernatant measured in overnight tissue cultures. There was no significant difference in the amount of cytokines (in pg/ml/mg) in tissue before and after treatment (data not shown). The unchanged inflammatory cell activities in supernatants measured by ELISA, together with the lack of histological change post treatment, suggest that changes in DC cytokine production are unlikely to result from resolution of inflammatory cell infiltrates, and rather are likely to reflect altered cell function.

5.9 Clinical Outcome and Dendritic Cell Function in VSL#3-treated Patients

We explored whether there was any relationship between the change in percentage of cytokine produced by DC and clinical response in patients treated with VSL#3, but there was no association. Furthermore there were only two patients who did not achieve a clinical response and therefore numbers were too small to make any meaningful statistical

analysis. In **Figure 5.7,** open symbols represent patients with a clinical response or remission and red symbols and lines represent patients with no clinical response.

We also compared cytokine changes in all patients (VSL#3 treated or placebo) who improved clinically post treatment (n=13) with cytokine changes in those who did not (n=6). There were no significant differences in any measured parameter between the two groups; observed changes in DC profile may therefore relate to effects of treatment rather than to disease resolution alone. There were also no differences between patients in the placebo treated group who achieved remission and those who had no clinical response in DC changes for surface markers or cytokines.

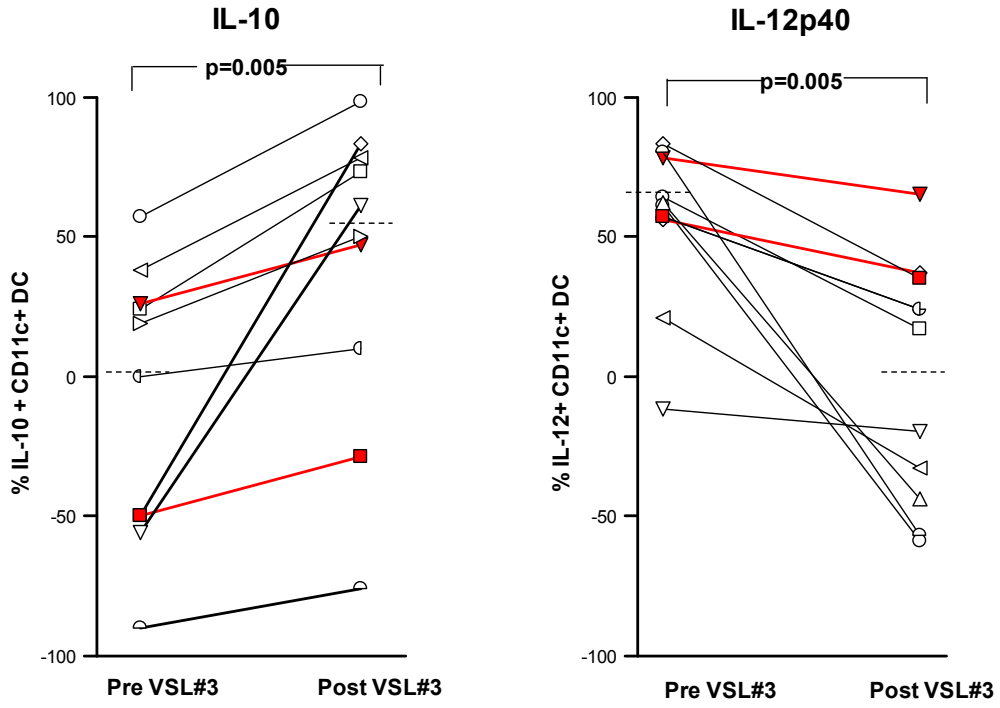


Figure 5.7: Clinical outcome and dendritic cell cytokine production in VSL#3-treated patients. This figure shows changes in DC production of IL-10 and IL-12p40 after VSL#3 treatment in patients with a clinical response (defined by a reduction in >50% UCDAI) and in those without a clinical response**.** ***Open symbols*** represent patients with a clinical response or remission and ***red symbols and lines*** represent patients with no clinical response.

5.10 Discussion

Patients with UC treated with oral VSL#3 had increased IL-10 and reduced IL-12p40 production by colonic DC, these effects were not seen in placebo-treated patients. In this subset of trial patients, more patients on VSL#3 compared with placebo also had a clinical response to treatment and clinical response was associated with beneficial DC function in most patients. Despite limited numbers of DC isolated from human tissue, we have demonstrated that treatment with oral VSL#3 and corticosteroids, but not placebo, modulated human colonic DC towards a regulatory/anti-inflammatory profile, suggesting that probiotic and steroid effects on DC are likely to be central to their therapeutic effects in patients with UC.

DC represent only around 0.2% of viable cells from human gut biopsies and numbers of DC are therefore limited; our development of techniques to identify, purify and study their functions were central to work in this chapter (Bell *et al*., 2001). DC are likely to be pivotal in mediating the balance between effector and regulatory lymphocyte responses with different outcomes influenced by exposure of DC to microbial products. Immature DC from non-intestinal sites expressed low levels of CD40 but this activation marker was up-regulated when DC underwent maturation or activation as occurred in inflamed mucosa of patients with IBD (Baumgart *et al*., 2004; Hart *et al*., 2005). We specifically measured TLR-2 and TLR-4 as previous work from our laboratory has shown that colonic myeloid DC have enhanced expression of TLR-2 and TLR-4 in IBD, especially in inflamed mucosa of patients with CD(Hart *et al*., 2005).

DC respond differentially to different types of microbes via PRR (Bauer *et al*., 2001; Kadowaki *et al*., 2001; Takeuchi *et al*., 1999). Soluble factors such as cytokines or

bacterial products can modulate TLR expression. In UC, after probiotic treatment, downregulation of TLR-2 can be a direct or indirect effect. In murine models of colitis, the anti-inflammatory effect of probiotics may be mediated via TLR-9 (Rachmilewitz *et al*., 2004), and possibly via TLR-2 as shown here. Downregulation of TLR-2 by DC after VSL#3 may reflect reduced inflammation after treatment, although this effect was not seen with resolution of inflammation after placebo; or it could be secondary to downstream local immune modulation or recruitment of un-activated cells from blood. The signalling of VSL#3 via TLR-2 on DC resulted in IL-10 production. TLR-2 knockout mice displayed defective production of IL-10 (Ferreira *et al*., 2007). In addition, *Candida albicans* induced immunosuppression through TLR2-derived signals that mediated increased IL-10 production and survival of Treg cells (Netea *et al*., 2004). It has also been shown that DC expression on TLR resulted in a non-inflammatory response (Rescigno and Matteoli, 2008). For instance, freshly isolated lamina propria murine DC showed little induction of IL-12 in response to TLR ligation, but was associated with constitutive IL-10. Thus, IL-10 may maintain lamina propria DC in a partially non-responsive state to TLR ligation (Monteleone *et al*., 2008).

Different probiotic bacteria act through multiple and contemporaneous pathways rather than by a single common mechanism. Current findings that i*n vivo* treatment with VSL#3 of patients with acute UC induced IL-10 and downregulated IL-12p40 production in colonic DC are in line with previously published *in vitro* data that VSL#3 is a potent inducer of IL-10 by blood and lamina propria DC (Hart *et al*., 2004c). These mechanistic studies were performed in a subgroup of patients who were part of a larger clinical trial; results of the larger trial assessing clinical effects of VSL#3 in acute UC will be presented separately at a future date.

IL-10 and IL-12p40 produced by DC exert largely opposite immunomodulatory effects. IL-12p40 is a Th1 polarising cytokine that induces IFN-γ production by T cells and NK cells (Heufler *et al*., 1996), whereas IL-10^{+} DC elicit a Th2 response to suppress IL-12 production and consequently IFN-γ.

In patients with pouchitis, VSL#3 increased pouch tissue IL-10, and reduced proinflammatory cytokines IL1-α, IFN-γ, and TNF-α to untreated levels(Ulisse *et al*., 2001). These beneficial immune changes seen *in vivo* provide at least a partial explanation for efficacy of VSL#3 in pouchitis (Ulisse *et al*., 2001). IL-10 is likely to be important in the maintenance of immune homesotasis in the gut. The delivery of IL-10 to the intestine using bacterial or viral vectors appeared to ameliorate established inflammation in animal models of colitis. However, anti-IL-10 antibody has limited efficacy in the treatment of patients with CD (Schreiber *et al*., 1995), but have not been formally tested in patient with UC.

As described in chapter 4, studies have identified IL-23 which shares the p40 subunit with IL-12, as a mediator of intestinal inflammation (Hue *et al*., 2006; Uhlig *et al*., 2006). Our assay for intracellular cytokine does not currently allow us to distinguish whether IL-12p40 reflects biologically active IL-12 or IL-23 but effects of probiotic bacteria on DC IL-12p40 production may impact upon IL-23 production. IL-23 is produced by activated myeloid cells including DC following bacterial stimulation (Becker *et al*., 2003), or via CD40 signalling (Uhlig *et al*., 2006). Becker *et al.* showed that DC from IL-23p19 deficient mice produced elevated levels of IL-12, and that IL-23 downregulated IL-12 expression upon TLR ligation. The *in vivo* blockade of IL-12p40 in IL-23p19 deficient

mice protected the mice from colitis. These data suggested a cross-regulation of IL-12 expression by IL-23, at least, in T cell-dependent colitis (Neurath, 2007).

Studies in human IBD showed increased IL-12 and IL-23 expression in the intestinal tissue of patients with CD (Fuss *et al*., 2006; Schmidt *et al*., 2005). Treatment with an anti-IL-12/23p40 antibody (directed against the p40 subunit of IL-12 and IL-23) revealed high remission and response rates in patients with active CD compared with placebo (Mannon *et al*., 2004). Ustekinumab, a similar antibody against IL-12/23p40 was effective in patients with active CD who had previously failed infliximab (Sandborn *et al*., 2008). Future trials assessing the efficacy of an anti-IL-23p19 antibody in human intestinal inflammation will help to clarify whether treatment success seen with IL-12/23p40 antibody was specific to neutralising the Th17-specific IL-23 subunit.

Several groups have demonstrated that therapies for IBD modulate DC functions (Hart *et al*., 2005; Mannon *et al*., 2009; Thomas *et al*., 2009). We have previously shown a precedent for therapy altering colonic DC in CD patients treated with anti-TNF-α antibody, infliximab, which downregulated CD40 expression on colonic DC (Hart *et al*., 2005). Probiotics may also have an indirect effect via APC in the gut; human monocyte-derived DC matured in the presence of *Lactobacillus rhamnosus* resulted in both reduced T cell proliferation and IL-2, IL-4 and IL-10 production (Braat *et al*., 2004a).

Saccharomyces bourladii, a probiotic yeast preparation, has demonstrated clinical efficacy in clinical trials; Thomas *et al.* showed that this probiotic yeast preparation may exhibit part of its anti-inflammatory activity through modulation of DC phenotype function and migration. *In vitro* culture of human monocyte-derived myeloid DC with *S. Bourladii*

supernatants led to reduced expression of CD40, CD80 and CCR7 on DC, increased IL-10, and reduced IL-6 and TNF-α production (Thomas *et al*., 2009). In mice *Lactobacillus acidophilus* strain L-92 induced apoptosis of antigen-stimulated T cells by by modulating DC function *in vitro* and *in vivo* (Kanzato *et al*., 2008). Furthermore, Mannon *et al.* have recently demonstrated that the clinical benefit from granulocyte-colony stimulating factor (G-CSF) treatment in CD is accompanied by an increase in plasmacytoid DC ($CD123^+$), a DC phenotype associated with regulatory responses, in the lamina propria of inflamed colonic mucosa; the increase in numbers of these cells were restricted only to patients who had a clinical response. Treated patients also had a significant increase in $IL\text{-}10^+$ circulating memory T cells (Mannon *et al*., 2009).

We have shown increased concentrations of both total faecal microbiota, and bifidobacteria associated with increased IL-10 by colonic DC, an observation consistent with effects of bifidobacteria on DC observed *in vitro* (Ng *et al*., 2008). Treatment of patients with CD with pre-biotic fructo-oligosaccharide increases total gut bacteria and bifidobacteria in patients with clinical improvement, together with a trend towards increase $IL\text{-}10^+$ DC (Lindsay *et al*., 2006). Altogether these data suggest that commensal microbiota, even without administered probiotics, may modulate intestinal DC function.

Individual strains of within VSL#3 display distinct and diverse immunomodulatory effects, arguing against a common bacterial component such as peptidoglycan mediating their effects. *In vitro,* the most marked anti-inflammatory effect was shown by the bifidobacteria species which upregulated IL-10 production by DC and reduced expression of CD40 and CD80 (Hart *et al*., 2004c). Whether individual strains of bacteria may similarly exert immunomodulatory effects on *ex vivo* DC in IBD patients is currently

unknown. Ibnou-Zekri *et al.* showed that two strains of Lactobacillus that exhibit similar *in vitro* growth, survival and adherence properties had different colonization patterns and resultant host immune responses at the mucosal and systemic levels (Ibnou-Zekri *et al*., 2003).

Corticosteroids, in the form of dexamethasone or prednisolone, have been known to affect DC function. *In vitro* steroids inhibit DC production of IL-12p70 and TNF-α, and corticosteroid-exposed DC generate IL-10 producing regulatory T cells (Piemonti *et al*., 1999b; Woltman *et al*., 2000). In one study, dexamethasone selectively down-regulated expression of co-stimulatory molecules on viable DC *in vitro*, and reduced their immunostimulatory properties, whereas *in vivo*, a single injection of dexamethasone results in impaired antigen presenting function; this finding correlated with reduced numbers of splenic DC (Moser *et al*., 1995).

Human monocyte-derived DC cultured with dexamethasone showed a higher endocytic activity, a lower APC function, and a lower capacity to secrete cytokines than untreated cells. Dexamethasone cultured-DC also has the ability to partially block terminal maturation of already differentiated DC (Piemonti *et al*., 1999b; Piemonti *et al*., 1999a). In patients with myasthesnia gravis, an autoimmune neuromuscular disease, oral prednisolone treatment prevented LPS-induced maturation of monocyte-derived DC by inhibiting the up-regulation of costimulatory molecules, limiting production of IL-12 and IL-23, and enhancing IL-10. $CD4^+$ T cells cultured in the presence of tolerogenic DC were hyporesponsive and can suppress autologous $CD4^+$ T cell proliferation(Luther *et al*., 2009). Here, we have demonstrated for the first time in a defined group of patients with acute UC, the beneficial effects of oral steroid therapy on *ex vivo* colonic DC suggesting

that one mechanism by which corticosteroids ameliorated intestinal inflammation was via modulation of DC. Corticosteroids appeared to share similar effects to those of VSL#3 on DC function *in vivo*.

Changes in DC with therapy may be from sampling of bacteria by DC or via bacterial interactions with other cells. It is unlikely to be secondary to resolution of inflammation as beneficial cytokine changes were seen even when post-treatment tissue still contained inflammatory cells (by histological criteria). Inflammatory cell infiltrates are slow to resolve after treatment of acute colitis, despite symptomatic and macroscopic mucosal improvement, reinforcing the potential for longer term effects on overt inflammation of changes in DC. It is unlikely that other existing medications played a significant role in cytokine changes observed in our experiments as these patients have remained on stable medication before and throughout the study.

In conclusion, we have shown effects of medical therapy on DC function in acute UC, providing evidence that exogenously administered bacteria influence gut inflammatory activity via effects on gut DC. Clinical improvement in patients treated with VSL#3 but not placebo was associated with significant increase in ongoing production of IL-10 and decrease in IL-12p40 and TLR-2 expression in gut DC. Corticosteroids had similar effects. Accumulating data suggest that properties of DC may be fundamental to modulation of inflammation in IBD.

Chapter 6

Expression of Gut Homing Markers on T Cells and Human Colonic Dendritic Cells

6.1 Abstract

Introduction: Recruitment of lymphocytes to sites of intestinal inflammation is central to the inflammatory process in IBD, and appears to be heavily influenced by specialized gut DC. Intestinal DC imprint homing molecules on T cells that they activate. In the mouse, the ability to imprint gut homing is confined to $CD103^+$ DC. *In vitro* colonic DC from UC induce higher levels of β7 integrin on T cells than do DC from healthy tissue. To test whether DC imprinting is dysregulated in human IBD, the expression of CD103 on colonic DC, and the expression of tissue-specific homing receptors on dividing T-cells, were examined in patients with UC.

Methods: Multi-colour flow cytometry was used to measure the (1) expression, including the proportion and level of staining, of CD103 on colonic DC in patients with UC, and (2) expression of homing markers on $CD4^+$ naive T-cells following activation by lamina propria mononuclear cells (LPMC).

Results: In UC, the proportion and level of staining of $CD103^+$ colonic DC were significantly lower in UC compared with equivalent cells from controls, due to a genuine loss of $CD103^+$ cells rather than an increase in $CD103^-$ cells. Compared with myeloid DC population, $CD103^+$ cells were infrequent among $CD11c^-$ and lymphocyte populations. There was no difference in the proportion of $CD103^+$ DC in paired samples of tissue from ileum and colon of UC. Whole LPMC from UC tissue were highly stimulatory, and subsets of responding T-cells expressed high levels of β7 and CCR4, but little CLA and CCR9.

Conclusions: In UC, colonic DC expressing CD103 were significantly reduced possibly as a result of increased migration from the tissues. A substantial proportion of T cells activated by colonic LPMC expressed the homing molecules, β7 and CCR4, but not CLA

and CCR9. Alterations in the number and phenotype of $CD103^+$ DCsuggest dysregulation of the homing process in UC; this process may serve as a valuable therapeutic target.

6.2 Introduction

Mucosal immune responses are compartmentalised. T cells that are activated in lymphoid tissue draining intestinal sites home selectively back to the gut by upregulating homing markers including α4β7 (Berlin *et al*., 1993; Butcher and Picker, 1996). This process is tightly controlled by multiple signals (Mora *et al*., 2003; Stagg *et al*., 2002). In mice, homing of T cells is imprinted by the activating DC; only T cells activated by intestinal DC become $\alpha4\beta7^{hi}$ and $CCR9^{+}$ (Hart *et al*., 2004b). Altered DC recruitment or local DC modulation in inflammatory disease may lead to dysregulated imprinting of lymphocytes. In IBD, there are changes in numbers and functions of gut homing β7 and $CCR9^{+}$ populations (Hart *et al*., 2004b; Papadakis *et al*., 2001). Furthermore, levels of β7 are higher on T cells activated by colonic DC from UC patients compared with controls (Rigby *et al*., 2006). β7 pairs with the integrins, αE (CD103) and α4. In mice, αE is expressed by mucosal DC and lymphocytes, and the ability to imprint CCR9 and α4β7 resides in a population of MLN and intestinal lamina propria DC that express the integrin CD103 (Johansson-Lindbom *et al*., 2005); this integrin may indicate a population of DC that has received local conditioning signals (Jaensson *et al*., 2008). However, the ability to induce CCR9 on T cells is not a property of all $CD103^{+}$ DC found in many mucosal tissues and LN, but a selective property of MLN and small intestinal lamina propria $CD103^{+}$ DC (Benson *et al*., 2007; Coombes *et al*., 2007; Mucida *et al*., 2007; Sun *et al*., 2007). $CD103^{+}$ DC are found in lymphoid tissue which drains non-intestinal epithelial surfaces but the imprinting properties of these cells remain to be defined.

A population of intestinal DC characterised by CD103 has the ability to induce the de novo differentiation of naive T cells into $CD4^{+}$ $CD25^{+}$ $Foxp3^{+}$ Treg via a TGF-β- and RA-

dependent mechanism (Coombes *et al*., 2007). This population is presumed to be recruited from the lamina propria (Johansson-Lindbom and Agace, 2007; Sun *et al*., 2007). Thus, in the steady state intestinal RA-producing $CD103^+$ DC, possibly conditioned by local environmental signals, may favour the generation of gut trophic Treg that limit the development of chronic intestinal inflammation. The impact of inflammation upon these DC imprinting pathways is currently unknown.

DC populations in tissue in steady state are likely to be functionally distinct from those present in inflammation (Brenan and Puklavec, 1992; Kilshaw and Karecla, 1997), but currently little is known about how these 'inflammatory DC' imprint homing or how dysregulated imprinting may contribute to human IBD. In addition, in man, the role of CD103 expression on DC in the control of lymphocyte homing and the effects of inflammation have not been explored.

6.3 Hypothesis

Imprinting of lymphocyte homing by lamina propria mononuclear cells and DC is altered in inflammatory bowel disease.

6.4 Aims

- To investigate the expression of CD103 on colonic DC from UC and healthy controls.
- To assess expression of tissue-specific homing receptors on T-cells activated by whole LPMC in UC patients.

6.5 Human Intestinal Lamina Propria Dendritic Cells Express CD103

The integrin CD103 ($\alpha_E\beta_7$) is expressed by a proportion of DC isolated from the MLN and colonic lamina propria of normal mice (Johansson-Lindbom *et al*., 2005). In a murine model, CD103 is expressed by the majority (64 to 80%) of the $CD11c^+$ MHC class II^+ lamina propria DC (Annacker *et al*., 2005; Johansson-Lindbom *et al*., 2005). To assess CD103 expression by colonic DC in human, LPMC were obtained from biopsy tissue and $CD11c^+$ $HLA\text{-}DR^+$ $lineage^{-/dim}$ myeloid DC were identified by multi-colour flow cytometry.

In healthy controls, CD103 expression was detectable within the lamina propria $CD11c^+$ $HLA\text{-}DR^+$ $lineage^{-/dim}$ population (37±3%), and to a much lesser extent within the $CD11c^-$ $HLA\text{-}DR^+$ $lineage^{-/dim}$ population (6±3%; $p<0.05$). As expected, a small population of lymphocytes also expressed CD103 in controls (8±1%).

The level of expression of CD103 by $CD11c^+$ DC (7.3±1.2) was not significantly different from the level of expression by lymphocytes (6.7±2%), but was significantly higher than those of $CD11c^-$ cells (3.5±0.7; $p<0.05$).

6.6 CD103 is Expressed on Dendritic Cells from Ileal and Colonic Tissue

We next compare expression of CD103 in ileal and colonic tissue. In three healthy controls with endoscopically and hisologically normal mucosa, paired samples were taken from the ileum and colon and CD103 expression was assessed. There was no significant

difference in the proportion of DC expressing CD103 in the ileum compared with the colon **(Figure 6.1).**

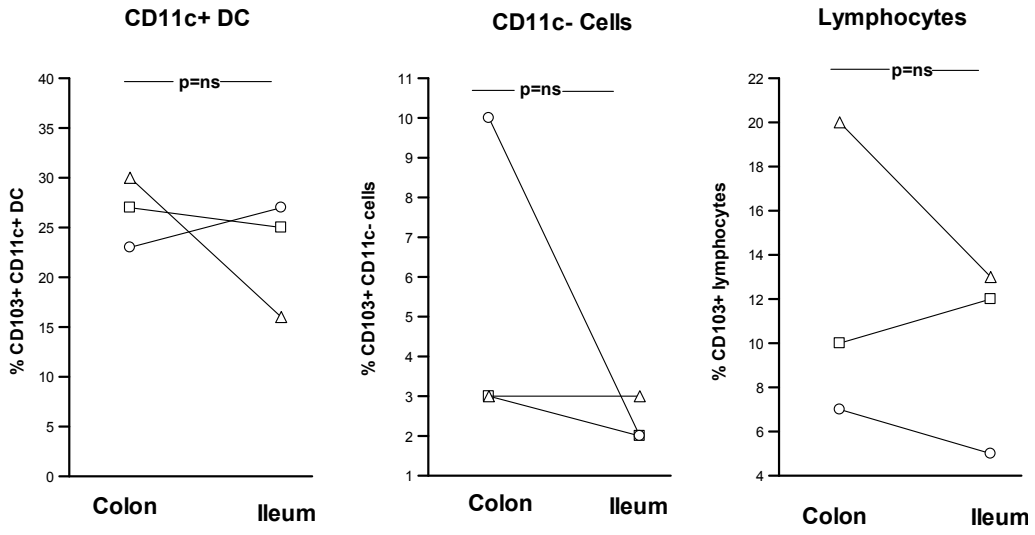


Figure 6.1 CD103 expression on DC and lymphocytes in ileal and colonic tissue of controls. The proportion of $CD103^+$ $CD11c^+$ DC, $CD103^+$ $CD11c^-$ DC and $CD103^+$ lymphocytes is shown in ileal and colonic tissue of healthy controls. Paired samples (from ileum and colon) were taken from 3 healthy controls. Lines link samples taken from the same individual.

6.7 Fewer Colonic Dendritic Cells from Ulcerative Colitis Express CD103

The proportion of lamina propria $CD11c^+$ DC and $CD11c^-$ cells expressing CD103 was significantly lower in cells from UC patients (22±5% versus 37±3% for $CD11c^+$ DC, $p<0.05$; 0% versus 6±3% for CD11c- cells, $p<0.05$; **Figure 6.2**) than controls.

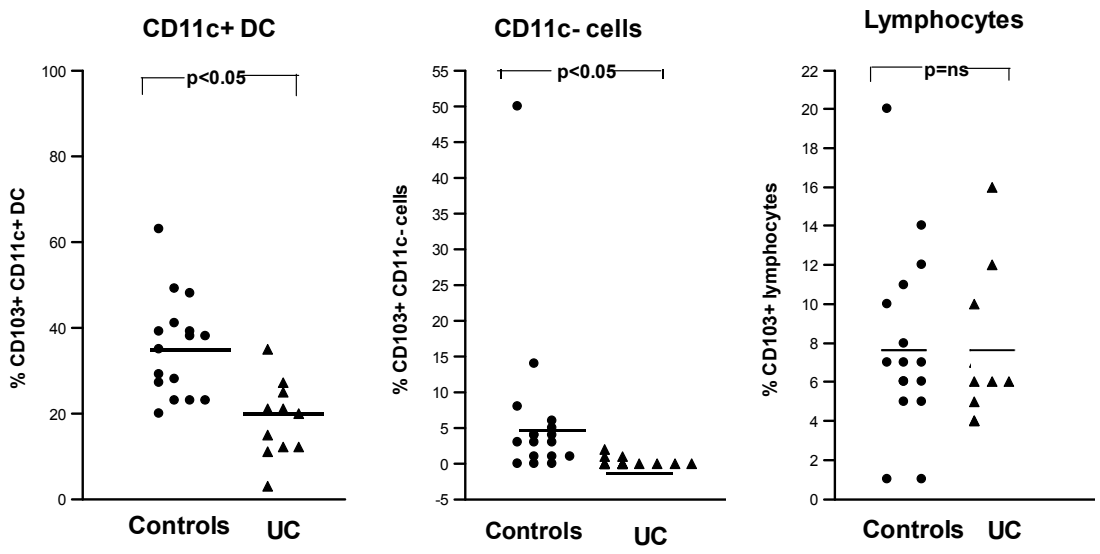


Figure 6.2: Proportion of CD103 expression on lamina propria DC and lymphocytes in controls and UC. The figure shows the proportion of $CD103^+$ $CD11c^+$ DC, $CD103^+$ $CD11c^-$ cells and $CD103^+$ lymphocytes in healthy controls (n=15) and patients with UC (n=11). All samples were taken from colonic tissue. Horizontal line represents mean value.

This difference was due to a genuine loss of $CD103^+$ cells rather than an increase in the $CD103^-$ population because the absolute number of $CD103^+$ $CD11c^+$ DC isolated from colonic tissue was lower for UC patients than controls. (UC 22±9 per mg tissue versus control 47±9 per mg tissue; $p<0.05$; **Figure 6.3**). In contrast, the numbers of $CD103^-$ cells did not differ significantly between UC patients and controls. The number and proportion of $CD103^+$ $CD11^-$ cells and $CD103^+$ lymphocytes did not differ between UC patients and controls.

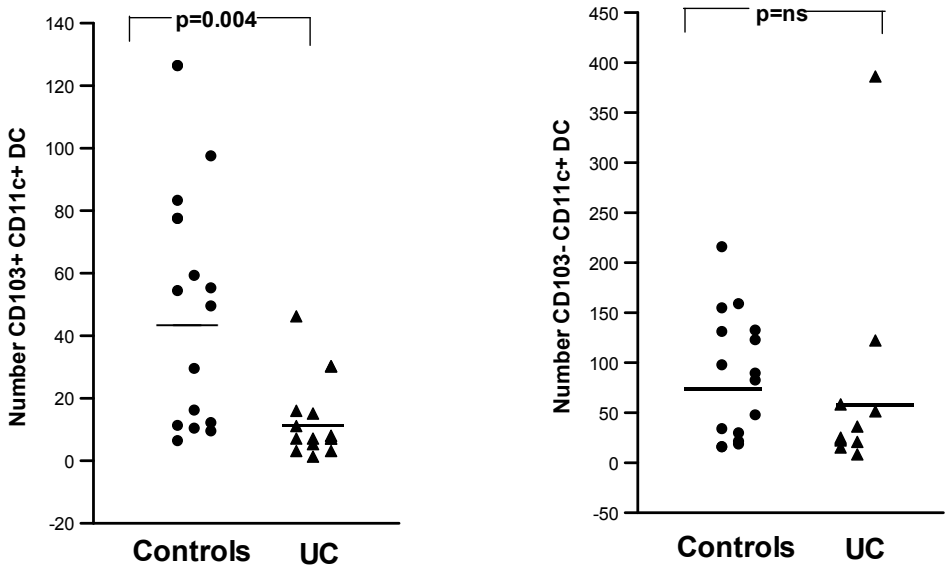
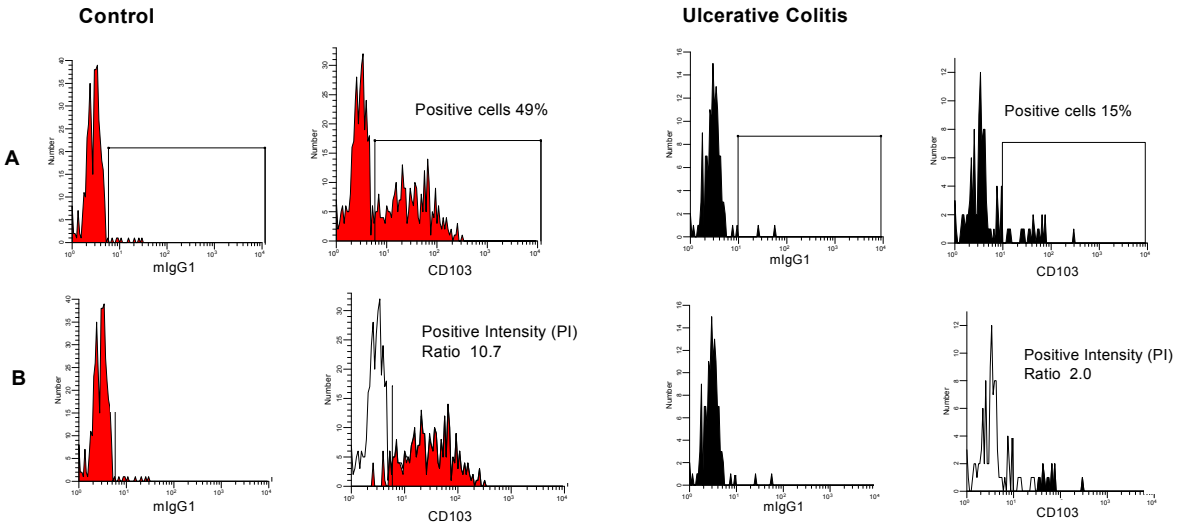


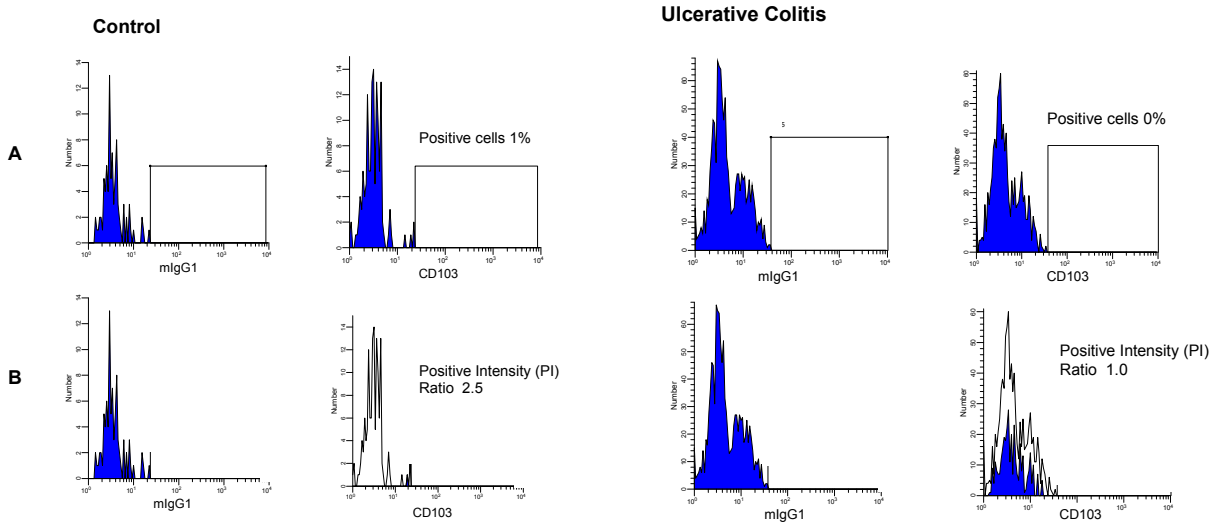
Figure 6.3: Absolute number of CD103$^+$ CD11c$^+$ DC and CD103$^-$ CD11c$^+$ DC in controls and UC. The number of CD103$^+$ and CD103$^-$ CD11c$^+$ DC per milligram tissue from inflamed UC tissue and normal control tissue are shown (UC=15, controls=11).

Examples of expression of CD103 on lamina propria CD11c$^+$ DC, CD11c$^-$ cells and lamina propria lymphocytes in controls and UC are shown in **Figure 6.4**. In addition, CD11c$^+$ DC and CD11c$^-$ cells from UC tissue both expressed lower levels of CD103 than equivalent cells from control tissue (Positive intensity Ratio; PIR= 4±0.8 versus 7±1.3 for *CD11c$^+$ DC*, $p<0.05$; PIR=1±0.2 versus 3.0±0.7 for *CD11c$^-$ cells*, $p<0.05$; **Figure 6.5**). CD103 levels on lymphocytes were not significant between UC and controls.

CD11c+ DC



CD11c⁻cells



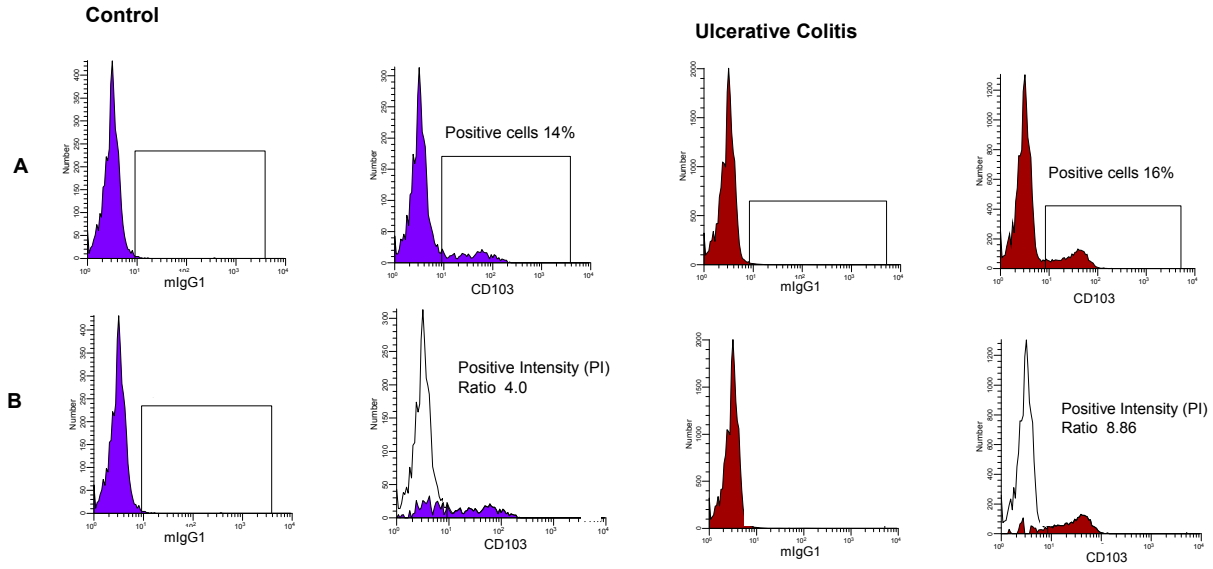


Figure 6.4: Examples of expression of CD103 on lamina propria DC and lamina propria lymphocytes in controls and UC. Flow cytometric representative examples showing CD103 expression on LP $CD11c^{+}$ DC, $CD11c^{-}$ cells and lymphocytes from inflamed mucosa of a patient with active UC and normal tissue of a control subject. For each cell type, the top panel **(A)** shows the proportion positive cells. All events falling within the box region exceed isotype control staining intensity and are regarded as positively labeled cells. In the bottom panel **(B)**, the shaded part of the histogram (PIR) indicates the level of staining of CD103 after subtraction of staining with the isotype control. We analysed a total minimum of 2000 events for each cell subset.

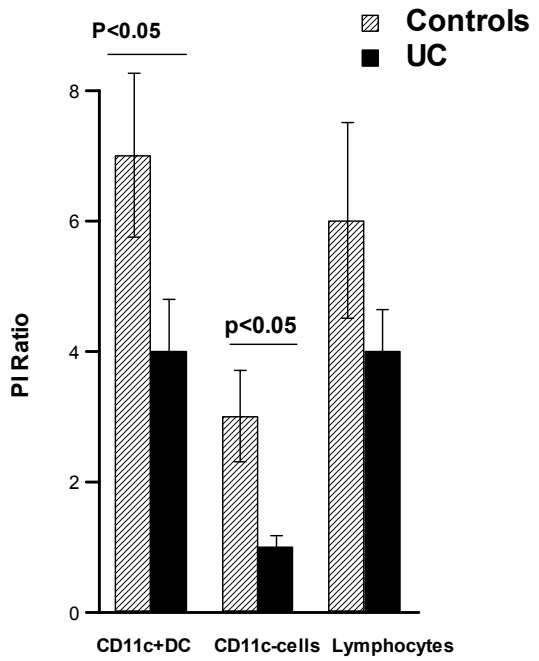


Figure 6.5: Level of CD103 expression (PIR) on DC and lymphocytes in controls and UC. The level of CD103 staining observed on $CD11c^+$ DC, $CD11c^-$ cells and lymphocytes was measured relative to the aurofluorescent properties of an unlabelled bead population (n=10 each patient group).

6.8 In Ulcerative Colitis, Whole Lamina Propria Mononuclear Cells Stimulate Naive T Cells to Express B7

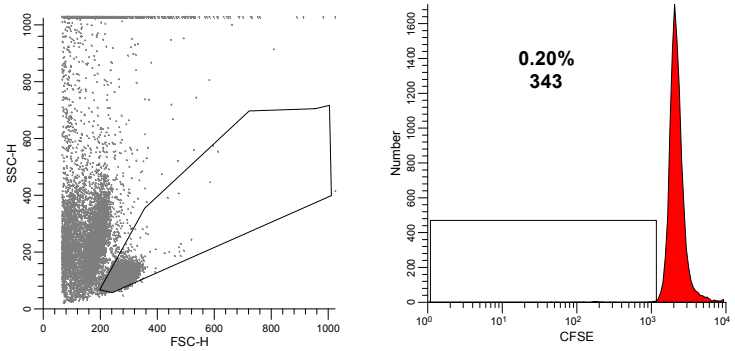
The influence of LPMC upon T cell expression of homing and migration molecules was assessed in a series of stimulation experiments. We used whole LPMC instead of sorted DC as it was practically difficult to extract DC for stimulation experiments due to the small numbers present in the human lamina propria. Whole LPMC were extracted from inflamed UC tissue as described in methods (Section 2.2.2). CFSE-labelled T-cells ($4x10^5$/well) were incubated for 5 days in round-bottom 96-well microtitre plates with LPMC ($8x10^4$/ well or graded concentrations). Similar concentrations of LDC from peripheral blood of a healthy control were also cultured stimutaneously with naive T cells.

Cells were recovered and $CFSE^{lo}$ proliferating cells were identified and quantified by flow cytometry. The expression of gut homing (β7, CCR9) and skin homing (CCR4, CLA) markers was measured on proliferating T cells. The proportion of dividing T cells were greatest when stimulated by the highest concentration of LPMC (LPMC concentration $8X10^4$; proportion dividing T cells = 50%) compared with stimulation by lower concentrations of LPMC (LPMC concentration $4X10^4$; proportion dividing T cells =15%) **(Figure 6.6).**

β7 was consistently expressed on T cells activated by whole LPMC from UC. LPMC induction of β7 was comparable to that achieved when using peripheral blood LDC as the stimulus (mean proportion of $\beta7^+$ $CD4^+$ T cells: *LMPC-stimulated*, 61±4%; *LDC-stimulated*, 58±6%). Approximately 25 percent of dividing T cells stimulated by LPMC and LDC expressed CCR4 (mean proportion of $CCR4^+CD4^+$ T cells: *LMPC-stimulated*, 25±2%; *LDC-stimulated*, 25±2%; n=2). Examples of expression of homing markers on dividing cells are shown in **Figure 6.7.** There was a dose dependent expression of β7 and CCR4 on proliferating T cells stimulated by LPMC. Conversely, proportion of T cells expressing of β7 and CCR4 reduced as the concentration of LDC increased **(Figure 6.8).**

Approximately 15 percent of proliferating T cells expressed CLA when stimulated by LDC but CLA expression was almost undetectable on T cells stimulated by LPMC. Overall there was little expression of CCR9 on T cells stimulated by both LDC and LPMC **(Figure 6.8).**

A


SSC-H
FSC-H
0.20%
343
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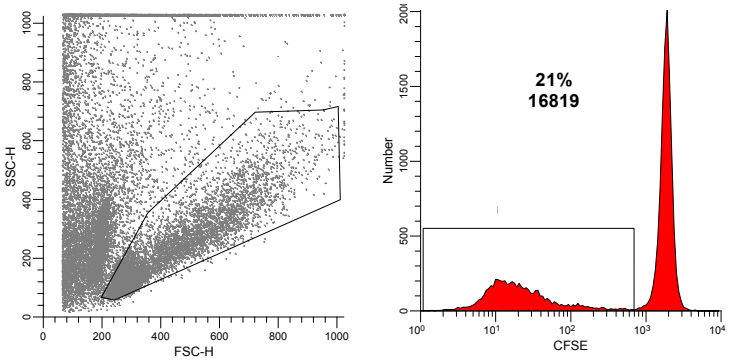
CFSE

B


SSC-H
FSC-H
21%
16819
Number
CFSE

C

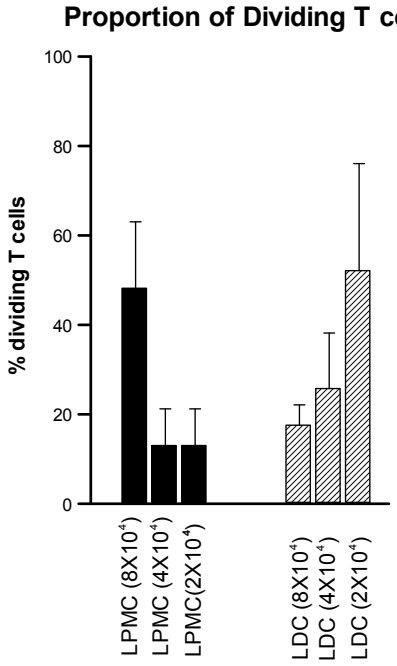
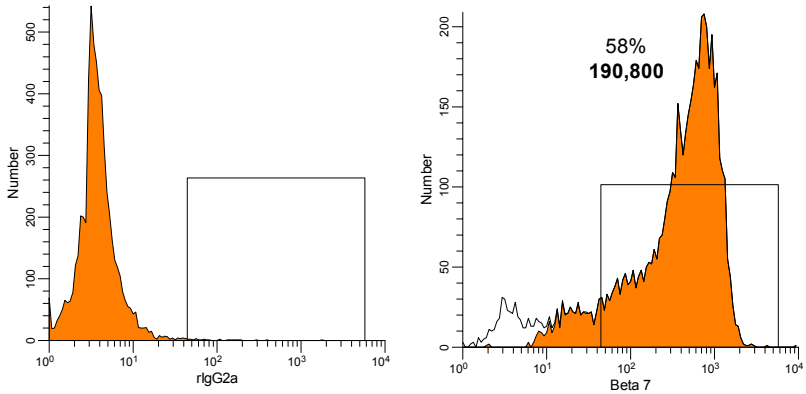

Proportion of Dividing T cells
% dividing T cells
LPMC (8X10⁴)
LPMC (4X10⁴)
LPMC(2X10⁴)
LDC (8X10⁴)
LDC (4X10⁴)
LDC (2X10⁴)

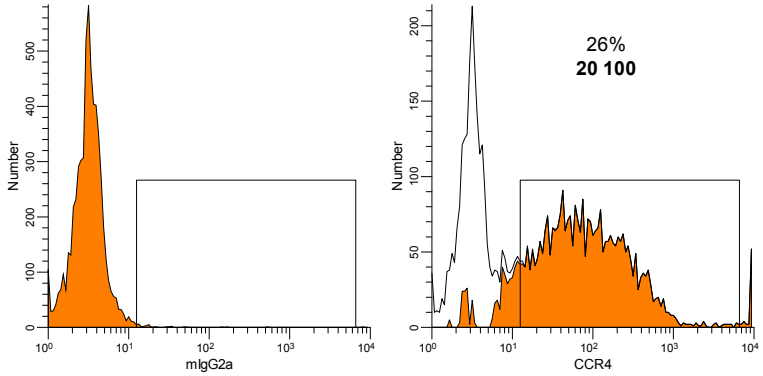
Figure 6.6: Stimulation of naive CD4$^+$ T cells by LPMC from UC patient

LPMC extracted for UC tissue were cultured for 5 days in round bottom well plates with CFSE-labelled naive T cells. Responder cells were cultured with medium alone; **(A)** and with LPMC; **(B)** % represents proportion of dividing cells and figure in bold represents absolute number of dividing cells. Figure **(C)** indicates the cumulative percentage of T cells plotted that are dividing when stimulated by LPMC and LDC (enriched DC from peripheral blood of healthy control) of different concentrations. Results were pooled data from two experiments.

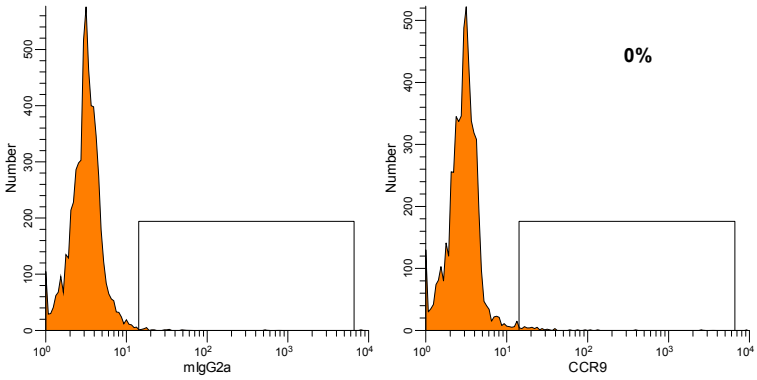
A



B



C



D

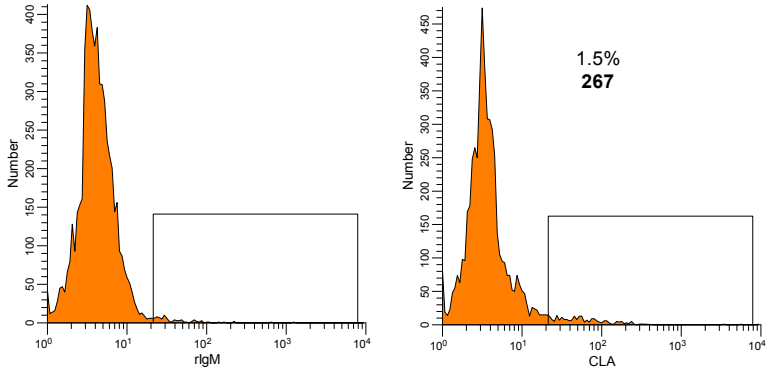


Figure 6.7: Examples of expression of homing markers on proliferating T cells stimulated by LPMC from UC; (A) β7 (B) CCR4 (C) CCR9 and, (D) CLA.

Numbers in bold indicate absolute number of dividing cells per well. Relative to an isotype control**,** % figure indicate the proportion of dividng T cells expressing the given marker.

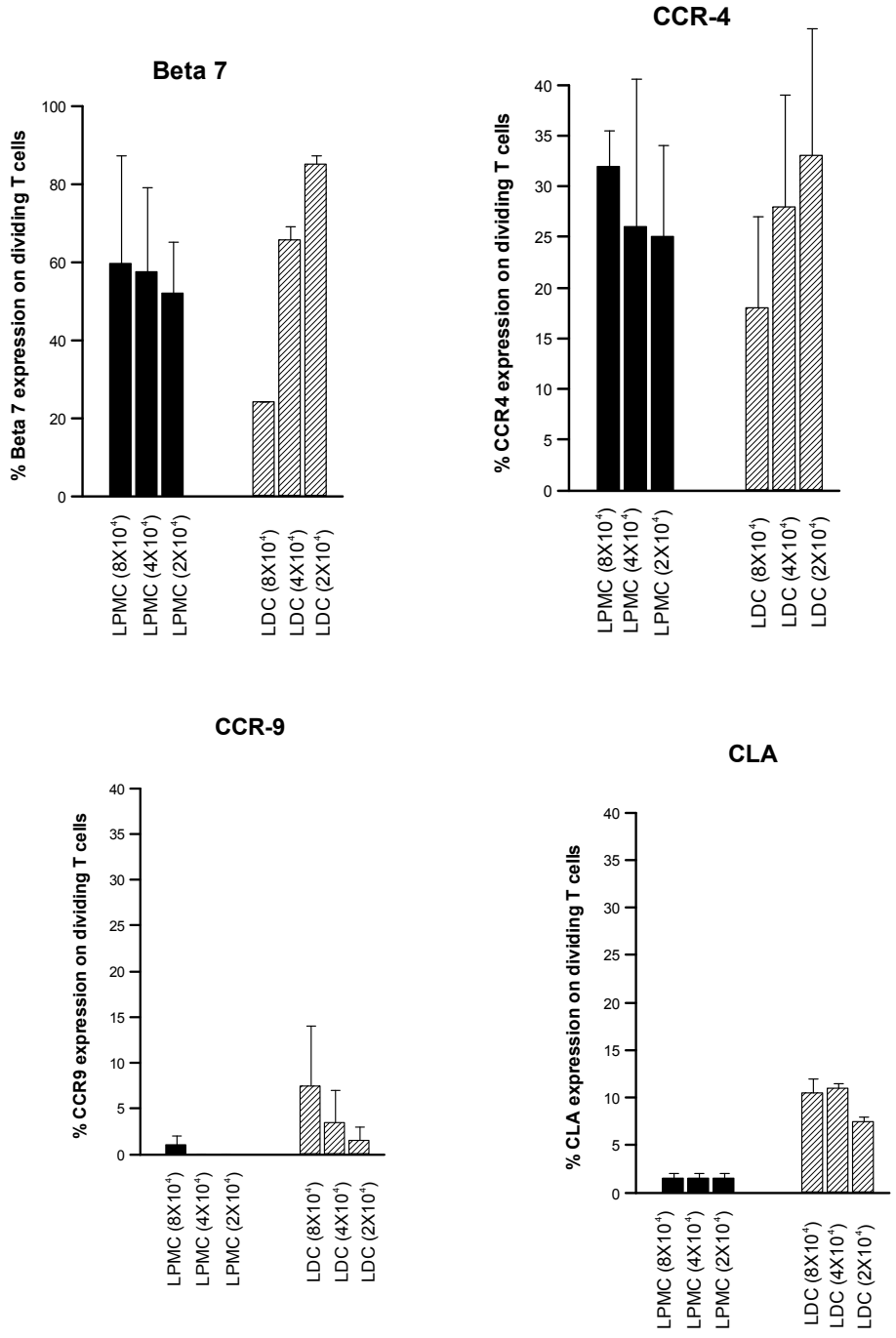


Figure 6.8: Cumulative percentage of expression of β7, CCR4 , CCR9 and CLA on $CD4^+$ T cells activated by LPMC from UC patients and LDC from healthy controls.

Three different concentrations ofLPMC ($8X10^4$, $4X10^4$, $2X10^4$) obtained from UC tissue and LDC ($8X10^4$, $4X10^4$,$2X10^4$) from peripheral blood of healthy controls were cultured for 5 days in round-bottom well plates with CFSE-labelled naive T cells. Dividing cells were labelled for homing markers. This figure shows the proportion of dividing T cells that express β7, CCR4, CCR9 and CLA (results were pooled data from 2 experiments).

6.9 Discussion

In the first part of this chapter, CD103 is expressed on human colonic DC and there is reduced $CD103^+$ myeloid DC in the colon of UC patients compared with control tissue. In mice, the ability to imprint a gut homing phenotype resides in a $CD103^+$ DC population found in the lamina propria of the small intestine and in gut-associated lymphoid tissue (Jaensson *et al*., 2008). This specialised DC population has been shown to enhance Foxp3 T cell differentiation, upregulate RA receptor and induce α4β7 and CCR9 on responding T cells (Jaensson *et al*., 2008). $CD103^+$ lamina propria DC appeared to be derived from circulating DC precursors but were less likely to be from lamina propria CD103 intermediate DC. Importantly, $CD103^+$ DC with similar phenotype and functional properties have been identified in human MLN (Eksteen *et al*., 2008; Eksteen *et al*., 2009; Jaensson *et al*., 2008). The ability of these cells to selectively induce CCR9 seems to be maintained by $CD103^+$ MLN DC isolated from small bowel Crohn's patients. These data suggest that human intestinal lamina propria DC may represent a novel target for regulating human intestinal inflammatory diseases.

In humans CD103 was expressed by almost 40 percent of colonic lamina propria $CD11c^+$ DC from healthy donors but only on approximately 20 percent of DC from patients with UC. There was indeed a true loss of $CD103^+$ DC from the UC colon rather than a dilutional effect due to the recruitment of additional $CD103^-$ DC based on absolute number analysis. Together with the enhanced T-cell expression of β7 integrin in the presence of migratory 'walk-out' DC from UC patients (Rigby *et al*., 2006), we speculated that the loss of $CD103^+$ DC from the colon in UC may be secondary to increased migration of $CD103^+$ DC out of the tissues. Murine studies suggest that $CD103^+$ DC were indeed migratory (Bedoui *et al*., 2009; Siddiqui and Powrie, 2008). Bedoui *et al.* showed that

dermal CD103$^+$ DC were the migratory subset most efficient at processing viral antigens into the MHC class I pathway, potentially through cross-presentation of skin-derived self antigens (Bedoui *et al*., 2009). It will be valuable to confirm this theory by assessing CD103 expression on "walk out" DC cultures. Nonetheless this possibility has been supported by a recent study showing an increase in CD103$^+$ DC in the lymph nodes of IBD patients (Jaensson *et al*., 2008).

Intestinal CD103$^+$ DC, but not DC at other anatomical sites express key enzymes involved in the oxidative metabolism of retinol. In mice MLN and small intestinal lamina propria CD103$^+$ DC efficiently induced enhanced Foxp3 T cell differentiation, RA receptor signaled and generated CCR9$^+$ α4β7$^+$ gut tropic T cells *in vitro* (Jaensson *et al*., 2008; Johansson-Lindbom *et al*., 2005). Additionally, CD103$^+$ intestinal DC, but not their CD103$^-$ counterparts, expressed RALDHs and were able to generate RA from retinal (Lampen *et al*., 2000). In CCR7$^-$ deficient mice, total DC numbers were reduced in the MLN but there was a greater reduction in the CD103$^+$ DC population than other DC populations (Jang *et al*., 2006; Johansson-Lindbom *et al*., 2005; Worbs *et al*., 2006).

Recent data showed that human intestinal epithelial cells promoted the differentiation of tolerogenic DC to drive the development of adaptive Foxp3$^+$ Treg cells. This process was not seen in patients with CD. Human MLN CD103$^+$ DC that expressed CCR7 were able to drive Treg differentiation, and were likely to represent a lamina propria-derived migratory population (Iliev *et al*., 2009b).

CD103$^+$ DC have emerged as a functionally specialised DC in various tissues. In the skin, only the CD103$^+$ subset, but not langerhan cells and classical dermal cells, can efficiently

present antigens of herpes simplex virus type 1 to naive $CD8^+$ T cells, suggesting that $CD103^+$ DC were the main migratory subtype that have the ability to cross-present viral and self antigens (Bedoui *et al*., 2009).

In small bowel CD, $CD103^+$ DC were present in the MLN and these DC induced CCR9 on responding T cells (Jaensson *et al*., 2008). αEβ7 is thought to be important for intraepithelial lymphocyte (IEL) localization or function. In α E-/- mice, intestinal and vaginal IEL numbers were reduced, consistent with the known binding of αEβ7 to E-cadherin expressed on epithelial cells. Lamina propria T lymphocyte numbers also diminished, and αEβ7 was involved in the expansion/recruitment of TCR $\alpha\beta^+$ $CD8^+$ IEL following microbial colonization (Hart *et al*., 2004b; Papadakis *et al*., 2001).

In the second part of the experiments, we used freshly isolated LPMC from UC patients to stimulate naive T cells and assessed the expression of homing molecules on proliferating T cells. β7 integrin expression on responder T cells was observed when stimulated by LPMC, and the proportion of β7 expression was comparable to that achieved when using peripheral blood LDC as the stimulus. Furthermore, CCR4, a receptor implicated in homing to the skin, but not CLA, was also up-regulated on T cells stimulated by LPMC from UC tissue. We have yet examined the expression of these markers on control tissue in parallel and the direct comparison of T cell expression of homing molecules cultured with LPMC from UC tissue and control tissue requires further investigation. The enriched blood DC were obtained from donors different from donors of LPMC cells. Future experiments will concentrate on comparison of stimulation by both LPMC and LDC from UC patients to avoid patient variability.

Nonetheless, previous experiments using colonic DC or "walk-out" cells from tissue enriched for DC from UC patients have shown that these cells stimulated a strong, dose-dependent, proliferative response in allogeneic, naïve $CD4^+$ T-cells, and they expressed a higher proportion of $\beta7^+$ than blood DC. In addition the levels of $B7^+$ were significantly higher on T-cells activated by colonic DC from UC tissue than control colonic tissue, or on T-cells activated in parallel by blood DC (Rigby *et al*., 2006).

We have shown that both blood and LPMC stimulated naïve T cells to express high levels of β7. However only T cells stimulated by blood expressed CLA; this observation may be secondary to the failure of LPMC to upregulate skin homing molecule.

The observation that LPMC induced dividing T cells to express a skin homing marker CCR4 is plausible. A complex pattern of marker co-expression irrespective of the tissue source of the DC added to the culture has been previously observed. Our group has previously shown that dividing T cells expressing B7, CLA, CCR4 and low levels of CCR9 have been detected irrespective of whether stimulating DC were derived from the gut, skin or sputum (Rigby *et al*., 2006). In that study, the majority $\beta7^+$ population stimulated by colonic DC in UC patients could be subdivided into $CCR4^+$ and $CCR4^-$ cells, as well as CLA^+ and CLA^- subset. CLA and CCR4 also appeared to be predominately reciprocally expressed.

In the mouse, the chemokine CCL25 and its receptor CCR9 plays a selective role in effector T-cell homing only to the small intestinal mucosa. In this regard, it contrasts to α4β7/MAdCAM-1 that facilitate recruitment of effector T cells into the small and large intestine (Svensson *et al*., 2002). CCR9 is expressed by a subset of $\alpha4\beta7^+$ T cells in the

human peripheral blood, by most of human and murine small intestinal T cells, and by the minority of colonic cells (Zabel *et al*., 1999). Its ligand CCL25 is constitutively expressed by small intestinal epithelial cells but not colonic cells (Wurbel *et al*., 2000).

In summary, these data show dysregulated DC imprinting of lymphocyte homing in patients with UC, and it is likely that a better understanding of the regulation of these processes may help facilitate targeting of these pathways for future therapy.

Chapter 7

General Discussion and Future Research

7.1 General Discussion

In the first part of the thesis, intestinal DC populations from patients with IBD were altered compared with that from healthy controls suggesting that they may play a role in the inflammatory process. This is a follow-up from previous work assessing the characteristics of DC in the intestine of patients with IBD. Our group has previously characterised the $CD11c^+$ DC and showed that they were activated in IBD. In this study we characterised the $CD11c^-$ cells in the human intestine in both IBD and healthy controls. The proportion of these cells was increased in UC compared with controls. $CD11c^-$ cells were unlikely to be macrophages or activated lymphocytes as under the electron microscope they had morphology of DC and phenotypically they expressed putative DC markers but not CD14 or CD16. Consistent with their low expression of costimulatory molecules, $CD11c^-$ $HLA\text{-}DR^+$ $lin^{-/dim}$ cells were probably weak stimulators of T cell proliferation. $CD11c^-$ cells also appeared to be poor producers of IL-12p40 and IL-6 and weak activators of T cells. Based on these functional properties, we speculate that this population may function in immune regulation and tissue repair, although this functional relevance needs further investigations (discussed under future work). DC have been reported to have repair or angiogenic properties (Fields *et al*., 2003). For instance, the production of serine proteases by damaged cells promotes DC maturation and tissue repair (Riboldi *et al*., 2005). Myeloid DC activated in the presence of LPS, IL-10 and calcitriol produce vascular endothelial growth factor (VEGF) which leads to production of potent angiogenic cytokines (Riboldi *et al*., 2005).

$CD11c^-$ cells showed morphological and functional features of immature DC. Abundant numbers of $langerin^+$ immature DC have been identified in the subepithelial space of IBD

tissue (Kaser *et al*., 2004). Langerin is a marker for immature DC involved in antigen presentation. Immature DC can mount non-responsiveness to specific antigens. This may be important in the resolution of intestinal inflammation. In some autoimmune disorders DC non-responsiveness to antigen that cross-react with self antigen (auto-antigen) play a critical role (Knight *et al*., 2002) in immune homeostasis. For example in patients with type-1 diabetes mellitus there was failure of DC "proper" maturation which resulted in insufficient stimulatory effects on the proliferation of suppressor T cells (Jansen *et al*., 1995).

An "immature DC" milieu is likely to favour a Th2 response with the induction of anti-inflammatory cytokines and the downregulation of pro-inflammatory cytokines during the resolution phase of inflammation, and therefore promoting tissue remodelling and repair (Gratchev *et al*., 2001; Takahashi *et al*., 2004). These data emphasize that DC may also have a secondary role in reinforcing and maintaining tissue repair, in addition to the controlling the passage of antigens and/or pathogens via the tissues.

$CD11c^-$ cells expressed high levels of CD56 but did not exhibit phenotypic markers of plasmacytoid DC. The acquisition of CD56, a distinct adhesion molecule on $CD11c^-$ cells, suggest that this population might have been recruited to sites of inflammation; we found both $CD56^{bright}$ and $CD56^{dim}$ $CD11c^-$ cells in the UC tissue, but increased numbers of the former; it is possible that $CD56^{bright}$ cells differentiate into $CD56^{dim}$ cells with resolution of inflammation. The physiologic site of $CD56^+$ $CD11c^-$ cells in the inflamed human mucosa provides a framework for the pursuit of further studies on this novel population. Human NK cells with antigen presenting cell-like features regulated T cell activation (Hanna *et al*., 2004), expressed MHC class II and T cell receptor co-stimulatory molecules

in vivo., as well as "fine-tuned" T cell activation, when there was direct insult to the peripheral organs (Hanna *et al*., 2003).

The increased of $CD11c^-$ cells in quiescent or inactive UC may be a marker of subclinical inflammation. Both macroscopic and histological intestinal inflammation in IBD is a risk factor for the development of dysplasia and colorectal carcinoma (Rutter *et al*., 2004). Further exploration of the potential of $CD11c^-$ cells as a biomarker for microscopic inflammation in patients with a long- standing UC will be of interest. Here we used patients with CD as our positive controls. The increase of these cells in both UC and CD suggest that they are likely to be associated with inflammation instead of disease-specific. Although $CD11c^-$ $CD56^+$ cells may not be unique to UC, specific modulation of this population may lead to new therapeutic approaches. Further clarification of the role of these cells in mucosal immunity will better our understanding of pathological intestinal inflammation and could identify new targets for gut specific-intervention.

The methods established in our laboratory have allowed us to identify and phenotype freshly isolated lamina propria DC from small numbers of intestinal biopsies obtained at endoscopy with minimal manipulation (Bell *et al*., 2001). These experiments were technically challenging due to the limited numbers of DC extracted from each biopsy. Intestinal DC derived from human colonic tissue were sorted on flow cytometry; small numbers of DC were then assessed by immunofluorescence and used in stimulation experiments. In all experiments, epithelial DC were removed using EDTA and the lamina propria DC were studied. Recently, there has been growing evidence of the importance of epithelial cells in barrier function in inflammatory disorders, therefore these cells may be targets for future work. Intestinal immune homeostasis is likely to be regulated by the

interaction between epithelial cells and DC (Rimoldi *et al*., 2005); several investigators have shown that commensal bacteria can interact with epithelial cells to induce inhibitory signals (Artis, 2008; Kelly *et al*., 2004; Neish *et al*., 2000). PRR, like TLR-9, has been shown to be expressed on the cell surface of epithelial cells to induce partial activation of inflammatory response (Lee *et al*., 2006). The local environment, in particularly, intestinal epithelial cells may also play a role in shaping DC function (Rimoldi *et al*., 2005). Thus it would be of interest to extract human intestinal epithelial cells during steady state and intestinal inflammation, and study their interplay with DC in maintaining immune homeostasis.

In the second part of the thesis, we studied spontaneous production of cytokines in IBD. DC have the ability to produce cytokines without stimulation, and that these data reflect more accurately their functions than when stimulated with LPS. There was an increased in ongoing IL-10 production by DC in acute UC. Although the initial events leading to IL-10 production may be a response to inflammation aimed at down-regulating the acute inflammatory response, IL-10 may also function to exacerbate the disease in this milieu (Furukawa *et al*., 1999; Santin *et al*., 2000). The observation of elevated IL-10^{+} DC in acute UC is likely to have therapeutic implications. Recent genome wide association scanning has demonstrated a susceptibility gene specific for UC related to IL-10 (Franke *et al*., 2008; Silverberg *et al*., 2009; Zhang *et al*., 2008). Although two large earlier clinical trials with IL-10 treatment in CD patients have shown limited therapeutic effects and bell-shaped dose–response curves (Fedorak *et al*., 2000; Schreiber *et al*., 2000), IL-10 may be a potential therapeutic target for UC although this idea has not been formally tested.

The traditional concept of UC as a strict Th2-mediated disease (Berrebi *et al*., 2003), has been recently challenged. The infiltrate in UC evolves during the course of disease; early in the disease there was an increase in both Th1 (TNF-α) and Th2 (IL-4) cytokines produced by intestinal homing $B7^+$ homing memory cells, whereas disease progression was characterised by increased activated NKT cells that produced IL-13 and IL-5 (Fuss *et al*., 2004). We have shown that colonic DC produced increased amount of both Th1 and Th2-related cytokines, IL-12p40 and IL-10 in UC. In contrast, CD was characterised by increased IL-6 production by DC which correlated with the severity of disease activity and CRP levels; IL-6 secreted by DC in CD may render pathogen-specific T cells refractory to the suppressive activity of T regulatory cells (Pasare and Medzhitov, 2003). "Walk out" cells from UC tissue also demonstrated secretion of both Th1 and Th2-related cytokines .

In the third part of the thesis, we studied, for the first time, the *in vivo* immunological effects of a clinically active probiotic mixture, VSL#3. The intestinal bacteria flora contributes significantly to the pathogenesis of IBD, along with genetic susceptibility and mucosal immune dysregulation. Probiotics are likely to become an integral component of the therapeutic armamentarium of IBD in combination with traditional anti-inflammatory and immunosuppressive agents. Clinical results of the randomised placebo-controlled trial of VSL#3 are currently being analysed and will be presented separately.

Almost all data in the literature of probiotics were derived from *in vitro* studies and animal models. Studies in animal models of colitis and experimental studies with probiotics are characterized by a high level of heterogeneity, and in most currently available animal models, disease largely occurs as a result of experimental manipulation. In patients with UC, the probiotic mixture VSL#3 induced IL-10 production by intestinal DC. Such effects

were not seen in placebo-treated patients. It is increasingly apparent that different probiotic bacteria act through multiple and contemporaneous pathways rather than by a single common mechanism. Ibnou-Zekri *et al*. have highlighted that the activity of probiotic strains *in vitro* may not parallel similar *in vivo* behaviour. In their study, two strains of Lactobacillus that exhibited very similar *in vitro* growth, survival and adherence properties showed distinct differences in colonization patterns and resultant host immune responses at both the mucosal and systemic levels (Ibnou-Zekri *et al*., 2003). In contrast we have demonstrated consistent *in vitro* and *in vivo* mechanistic data of the effects of probiotics on human DC, suggesting an important mechanism by which these bacteria appear to mediate resolution of inflammation. *In vitro* probiotics increased IL-10 production and inhibited IL-12p40 secretion by DC (Hart *et al*., 2004c). Even such consistent *in vitro* and *in vivo* mechanistic data, however, only correlate with the clinical outcome in some patients. Some of our IBD patients were on immunomodulators. These drugs change DC function. However due to the stringent criteria of patient recruitment in the trial, existing medication were used at stable doses for at least three months, suggesting that changes in DC were likely to be the true effects of probiotics and less likely to be contributed by effects of other concurrent medication.

The positive effects of VSL#3 on intestinal inflammation have been tested in animal models and humans. In DSS-induced colitis, mice fed with VSL#3 showed decreased tight junction protein expression, and increased apoptotic ratio, hence providing protection to epithelial barrier (Mennigen *et al*., 2009). VSL#3 also upregulated mucosal alkaline sphingomyelinase activity in the intestine of IL10-knock out mice with colitis and in humans with UC (Soo *et al*., 2008).

Up to now, much of the information on DC subsets at this time is derived from cells isolated *ex vivo*. Most studies of DC function to date have relied either on purification of cells from tissues, which detach them from thir complex microenvironment, or on studies of cells produced in culture, which is far from physiological. It will therefore be valuable to manipulate these DC subsets *in vivo* as well as to determine their consequences for tolerance and immunity, in both health and inflammation. One way of doing so is to selectively deliver antigen to a DC subset, as can now be done with dying cells that selectively target the $CD8^+$ subset of mouse DC (Liu *et al*., 2002; Schulz and Reis e Sousa, 2002). The next leap in this front will involve techniques which allow one to label and track subpopulations of DC, for instance by using two-photon microscopy. Future advances that allow for the studies of the genome and proteome of small numbers of DC will provide us with a more precise analysis of the states of DC differentiation and functions in animal models.

One key element in the pathogenesis of intestinal inflammation in IBD is increased leukocyte recruitment from the circulation to target intestinal tissue. This is a highly regulated process that depends on sequential interactions with a series of adhesion molecules (Butcher and Picker, 1996). In patients with UC, DC imprinting of T-cells with a colonic homing phenotype is altered; colonic DC stimulate more $CD4^+$ T cells to express the gut homing integrin β7, and induce higher levels of β7, than do equivalent cells from the healthy colon (Rigby *et al*., 2006). Enhanced β7 expression may result in increased T-cell migration to the colon contributing to the local inflammatory process, and this may relate to disease pathogenesis. T cells activated in the absence of DC but with exposure to stimulating antibodies in the presence of RA can also upregulate β7 expression (personal communication N Gellatly).

In the mouse MLN, CD103 expression defined a subset of functionally specialised and lamina propria-derived DC (Annacker *et al*., 2005; Coombes *et al*., 2007). In CCR7 deficient mice, $CD103^+$ DC were reduced in MLN but not in the lamina propria. This observation would suggest that $CD103^+$ MLN DC were derived from the lamina propria and that these cells were responsible for the generation of gut-trophic T cells in the MLN. Although earlier work has suggested a role of MLN environment in DC imprinting, DC were imprinted with the ability to generate gut-trophic T cells prior to entry into MLN. For example, small intestinal lamina propria DC can generate $\alpha4\beta7^+$ $CCR9^+$ T cells while $CD103^-$ MLN DC cannot (Annacker *et al*., 2005). This observation indicates that the MLN environment is not essential for imprinting DC with the ability to generate gut-trophic T cells (Johansson-Lindbom *et al*., 2005). This discrepancy may relate to the production of RA, presumely by $CD103^+$ DC, which within the MLN are sufficient to act in trans to generate $\alpha4\beta7^+$ T cells by $CD103^-$ DC (Agace, 2006). We have shown that less $CD103^+$ DC were identified in the lamina propria of UC patients, compared with control tissue. Whether this relates to increased migration to MLN requires further investigation. To take this observation further, one would explore whether "walk-out" cells express CD103, and secondly, separate CD103 populations and their assess their stimulatory capacity.

RA is required for the development of gut homing phenotype. It also acts as a co-factor for TGF-β dependent $FoxP3^+$ regulatory T-cell development (Coombes *et al*., 2007; Sun *et al*., 2007) Thus, RA-producing gut DC may contribute to the generation of regulatory T cells targeted to intestinal tissue. In light of these data it will be important to examine the production and the activation of TGF-β by colonic DC from UC patients and healthy subjects and to determine the functional properties of the gut-targeted T-cells generated.

Our data showed that LPMC from inflamed UC tissue stimulated naive T cells to express β7 and CCR4. In contrast, CCR9 was not detectable on dividing T cells activated by colonic LPMC in UC. The role of CCR9 and CCL25 in mediating homing to the colon is ill-defined. Kunkel *et al.* showed that a proportion of $CD4^+$ $CD8^+$ T cells isolated from the human colon expressed CCR9. However, CCL25 expression was not expressed on colonic tissue in patients with CD (Papadakis *et al*., 2001). Moreover entry of T cells into the murine colon during "steady state" or tissue inflammation was not dependent on CCR9 or CCL25 (Hosoe *et al*., 2004).

In IBD gut inflammation can trigger inflammation at extraintestinal sites. Up to 40 percent of patients with IBD develop skin and liver manifestations of their disease. Most of these extraintestinal manifestations such as pyoderma gangrenosum, erythema nodosum, non-erosive arthropathy and uveitis have been linked to disease relapses and improve on disease resolution (Orchard *et al*., 1998; Orchard *et al*., 2002). It will be of interest to compare the imprinting of a skin homing phenotype by intestinal DC or LPMC from patients with UC and CD, with and without extra-intestinal skin manifestations, to explore whether inappropriate targeting of T-cells to the skin might contribute to disease at these distant sites.

7.2 General Conclusions

In the first part of the thesis, the phenotypic and functional properties of human intestinal lamina propria DC have been described. In healthy controls, lamina propria cells were predominantly myeloid DC. In patients with IBD, we identified an increase in an inflammation-associated population of $CD11c^-$ cells that expressed MHC class II, had morphological features of DC on electron microscopy, and proportionally expressed increased NK cell marker, CD56, but did not belong to the plasmacytoid DC lineage. These cells reduced in number after inflammation resolved. We speculated that they may play a role in immune regulation or tissue repair **(Chapter 3).**

In the second part of the thesis, we assessed the functional activities of myeloid DC during different stages of inflammatory bowel disease, and explored their relationship with intestinal microbiota in patients with IBD. In patients with acute UC, more colonic myeloid DC produced both the regulatory cytokine IL-10 and the pro-inflammatory cytokine IL-12p40 than DC from healthy controls, whereas in patients with chronically active CD, there were increased colonic IL-$12p40^+$ DC, and elevated IL-6 and IL-12p40, compared with controls. In CD, level of IL-6 on DC correlated with the severity of disease acitivity and serum CRP. The ratio of bacteroides:bifidobacteria in faecal samples also correlated positively with pro-inflammatory IL-$12p40^+$ DC staining. The proportion of bifidobacteria correlated positively with IL-10^+DC staining. IL-6^+ DC also correlated negatively with the proportion of *F. prausnitzii.* These data support the concept that intestinal DC function is influenced by the composition of the commensal microbiota and that in patients withactive CD, the balance of proinflammatory and regulatory bacteria was associated with altered DC function **(Chapter 4).**

We next hypothesise that probiotic bacteria with clinically evident benefits in IBD modulate the immune system, at least in part, via effects on DC *in vivo*. In patients with acute UC treated with the probiotic mixture VSL#3, TLR-2 expression on DC significantly decreased, DC production of IL-10 was enhanced and IL-12p40 was reduced. These effects were not seen in patients treated with placebo. Oral corticosteroid treatment was associated with increased IL-10 and reduced IL-12p40 production by colonic DC **(Chapter 5).** Thus therapeutic effects in IBD may, in part, relate to modulation of DC function.

In the last part of the thesis, we showed that in patients with active UC, colonic DC expressing the integrin, CD103, were significantly reduced compared with controls. LPMC from inflamed UC tissue stimulated naive T cells to express β7 and CCR4 but not CLA or CCR9. Altogether the data suggest dysregulation of the gut homing process in patients with UC **(Chapter 6).**

7.3 Future Work

Data presented in the first part of the thesis highlighted a striking increase in a unique population of inflammation-associated cells in the tissue of IBD patients that share features of both DC and NK cells. Studies in the future will involve the investigation of the role of these cells in the inflammatory response in IBD. Indeed NKT cells have been implicated in chronic mucosal inflammation in animal models of colitis (Fuss *et al*., 2004). Distinct subsets of NKDC in mucosal inflammation have also been described *in vitro*. There is a possibility that *in vitro* $CD11c^-$ cells may become $CD11c^+$ DC (or vice versa)

following stimulation with inflammatory cytokines and or bacterial products/components. It will be of interest to determine whether $CD11c^+$ and $CD11c^-$ cells are distinct lineage or whether they have been derived from the same precursors. The examination of the functional response of $CD11c^-$ cells to TLR ligands may provide further insight into their role in bacterial recognition and intestinal inflammation. Human $CD56^+$ $NKp44^+$ cells present in the Peyer's patches and lamina propria have been shown to secrete IL-22 and IL-17. These cells expressed CCR6 which promoted leukocyte homing to the intestinal mucosa, as well as CCL20, the ligand for CCR6, suggesting that they can also promote their accumulation in the intestine (Colonna, 2009). Unlike typical NK cells, they did not release perforin and IFN-γ. Future experiments to delineate the functions of our described cells would involve assessing their expression for CCR6 and their ability to produce IL-22.

The observation that $CD11c^-$ cells expressed CD56 raised the possibility that they may function in the killing of target cells (eg. MHC-class 1 targets). Lastly, IFN-γ and TGF-β are known to be produced by DC and it will be important to assess whether this unique population of cells are major sources of these regulatory cytokines. Other cytokine to be tested include TNF-α. The techniques employed here to assess the effects of $CD11c^-$ cell stimulation on conventional T cell populations could also be extended to examine cytokine production and killing activities by this population. Experiments as such are however challenged by the small numbers of human intestinal DC.

More clinically relevant would be to investigate the role of these cells in IBD-associated dysplasia. These cells were present during macroscopic inflammation but may also represent early markers of microscopic inflammation, given that they were also increased

in non-inflamed inactive UC tissue. It will be useful to assess whether this is a truly recruited mucosal subset or a local DC population, as such, examining the expression of α4β7 or CD103 on freshly isolated cells. *Ex vivo* stmulation by TFG-β for the induction of mucosal homing markers would help further characterise the functions of these cells. Stimulation with TLR ligands of $CD56^+$ $CD11c^-$ cells would allow us to understand the "low responsiveness". Lastly, whether $CD11c^-$ cells are influenced by other anti-inflammatory therapies which target PPAR-γ receptor such as mesalazine warrants further investigations.

In patients with UC, we observed two distinct groups of patients in terms of IL-10 production by DC, a group with IL-10^+ DC and a second group with no detectable IL-10^+ DC. We speculated that this difference may relate to differences in the genetic profile of these patients. Hence further characterisation of phenotype and genotype of patients with IBD will be important. For instance, the assessment of cytokine production by DC in UC and CD in patients with either IL-10 genetic susceptibility or NOD2/CARD 15 mutation and those without these genetic variants will be important.

There has been growing interest in other members of the IL-12 family, and their role in IBD. IL-23 is produced by activated myeloid cells including DC following bacterial stimulation (Becker *et al*., 2003), or via CD40 signalling (Uhlig *et al*., 2006). IL-27 is a heterodimeric cytokine composed of p28 and Epstein-Barr virus-induced gene 3 (EBI3). *In vitro* IL-27 is produced by activated monocytes and DC. Apart from inducing proliferation of naive $CD4^+$ T cells, it synergises with IL-12 for IFN-γ production. Our assay for intracellular cytokine did not currently allow us to distinguish whether IL-12p40 reflects biologically active IL-12 or IL-23. Effects of probiotic bacteria on DC IL-12p40

production may impact upon IL-23 production. Future work should clarify whether IL-12p40 in DC reflects IL-12, IL-23 and/or IL-27.

Altered microbial composition and function in IBD result in increased immune stimulation, epithelial dysfunction, or enhanced mucosal permeability. DC function was influenced by the resident intestinal microbiota. Investigating the signals from pro or anti-inflammatory bacteria that dictate functions of DC *in vivo* would be important. In addition, host genetic polymorphisms most likely interact with functional bacterial changes to stimulat immune responses that result in chronic tissue injury. Identification of these susceptibility genes and microbial alterations in individual patients could lead to selective targeted interventions.

The most clinically significant area of research described in this thesis is the *in vivo* immunoregulatory effects of VSL#3 on DC function in patients with UC. Understanding probiotic action may permit modulation of the immune system, both locally and systemically, and to offer novel and useful means to modulate host immunity for protection from, or treatment of IBD. *In vitro* not all lactobacillus and bifidobacterium species are equally beneficial; each may have individual mechanisms of action that is dependent on host characteristics. Different bacteria may have dominant effects in different genetic backgrounds and in diseases which vary in their pathogenesis. Thus optimal use of various probiotics may depend on identifying patient subsets by genetic, phenotypic, stool microbiologic or serologic criteria. Apart from live organisms, DNA derived from probiotic bacteria has now been shown to enhance IL-10 production by DC, possibly via a TLR-9 dependent manner. These data suggest that probiotic bacterial DNA

alone may represent a means of modulating DC function; this hypothesis requires further testing in patients with IBD.

In the healthy intestine, commensal bacteria in the form of probiotic bacteria have the ability to induce regulatory cytokines by intestinal DC thus contributing to a regulatory environment. Similar observations have been described in the inflamed intestine in IBD. Future experiments aimed at measuring cytokine production including IL-10, IL-12p40, TGF-β and IL-4, by naive T cells following stimulation by intestinal DC in both health and inflammation will provide insight into whether intestinal DC stimulate regulatory or inflammatory responses under different conditions.

An extension to our data should involve experiments looking at the effects of standard immmunosuppressants (AZA/6MP), and 5-ASA, on *ex vivo* DC function. 5-ASA mediates its an-inflammatory effects via PPAR-γ. It will be interesting to explore DC as therapeutic targets in such a scenario. Such data will provide novel mechanisms into the effects of drugs on *ex vivo* DC phenotype and function, so that treatments can be more targeted in such patients.

The homing process in IBD has gained immense interest in exploring disease pathogenesis. Apart from studying lamina propria DC, future experiments should involve the isolation of epithelial cells for their expression of CD103. In mice, CD103 appeared to be expressed predominantly by intraepithelial cell. $CD8^+$ effector T cells acquired CD103 subsequent to the entry into epithelial tissues, a process that was dependent on TGF-β (El-Asady *et al*., 2005; Ericsson *et al*., 2004; Lefrancois *et al*., 1999). Human intraepithelial cells promoted the differentiation of tolerogenic DC to drive the development of adaptive

Foxp3$^+$ Treg cells. It is possible that CD103 is induced following interaction between lamina propria DC and epithelial cells. We have shown for the first time that CD103 is expressed on human lamina propria DC and numbers of these cells are reduced in UC. The ability of CD103$^+$ DC to generate small intestinal-trophic T cells suggests that DC imprinting may occur simultaneously to their acquisition of CD103. This process is potentially modulated by factors released by epithelial cells or their local surrounding environment(Iliev *et al*., 2009a; Rescigno *et al*., 2008). In this regard, the putative role of epithelial cells in the generation of α4β7 gut-trophic T cells merits further studies. For example, DC can be conditioned with intraepithelial cells isolated from healthy or IBD donors and analyzed for their ability to induce α4β7$^+$ T cells and Treg cell differentiation. Separated CD103$^+$ and CD103$^-$ DC from human MLN can also be used to stimulate Treg development, with and without RA or TGF-β. DC can also be assessed for their expression of chemokine receptors such as CCR7 to assess for migratory properties.

We have performed some preliminary experiments but failed to identify sufficient epithelial DC for phenotypic analysis although epithelial lymphocytes were detectable; the latter demonstrated consistent expression of CD103. Future studies should focus on optimisation and refinement of methods used to extract epithelial DC from human intestinal tissue, and thus study on their expression of CD103. In the mouse, intestinal CD103$^+$ DC expressed ALDH1a2, a key enzyme which is responsible for β7 expression on T cells. In human, it is unclear whether colonic CD103$^+$ DC function in similar ways, and whether CD103$^+$ DC induce β7$^+$ T cells similarly between colonic DC from inflamed and non-inflamed IBD tissues.

We have cited evidence throughout this thesis describing the importance of the interaction between the intestine as an immune priviledged site and its interaction with the intestinal microbiota. Recent work has focused on the bacteria-host interaction showing that the host, a component of its microbiota, and probiotic bacteria can adapt their substrate utilization in response to one another. A better understanding of the host bacteria-to-immune cell interactions in the GI tract will provide potential mechanisms of disease pathogenesis and how we could manipulate the GI microenvironment. A picture is also emerging of the various roles of DC in immune homeostasis and bacteria recognition in the healthy intestine and inflammatory state. As our knowledge of these specialized cells expand, manipulating and targeting them for the treatment of intestinal inflammatory diseases will become possible.

7.4 Ongoing Work

The ingestion of the prebiotic, fructo-oligosaccharide increases luminal bifidobacteria and improve clinical response in patients with CD (Lindsay *et al*., 2006). A multi-centre placebo-controlled trial of fructo-oligosaccharide in the treatment of CD is in progress and is close to completion. It has provided us with the opportunity to assess *ex vivo* functional properties of DC before and after ingestion of prebiotics. The immunological data will be available when the study is completed and unblinded. We have analysed the pre-treatment data on DC functions in patients with CD *(Chapter 4)* and the post-intervention data will be analysed once patients recruitment has completed.

PUBLICATIONS

Original Papers (Related to Thesis)

SC Ng, S Plamondon, H Omar-Al-Hassi, N English, N Gellatly, MA Kamm, SC Knight, AJ Stagg. A novel population of human $CD56^+$ $HLA\text{-}DR^+$ colonic lamina propria cells is associated with inflammation in ulcerative colitis. *Clinical Experimental Immunology* 2009; 158(2): 205-218.

SC Ng, S Plamondon, MA Kamm, AL Hart, HO Al-Hassi, T Guenther, AJ Stagg, SC Knight. Immunosuppressive effects via human intestinal dendritic cells of probiotic bacteria and steroids in the treatment of acute ulcerative colitis *(Submitted to Inflammatory Bowel Disease 2009).*

SC Ng, RJ Rigby, AL Hart, N Gellatly, S Plamondon, N McCarthy, H Omar Al-Hassi, MA Kamm, SC Knight, AJ Stagg. Altered imprinting of lymphocyte homing by colonic dendritic cells in human ulcerative colitis *(Submitted to Gut 2009).*

SC Ng, G Arslan Lied, F Sandhu, MA Kamm, T Guenther, N Arebi. Predictive value and clinical significance of myenteric plexitis in Crohn's disease. *Inflammatory Bowel Disease* 2009; 15(10):1499-507.

SC Ng, N Arebi, MA Kamm. Medium term results of oral tacrolimus treatment in refractory inflammatory bowel disease. *Inflammatory Bowel Diseases* 2007;13(2): 129-134.

Review Articles

SC Ng, B Gazzard. Advances in sexually-transmitted gastrointestinal infections. *Nature Clinical Practice Gastroenterology and Hepatology* 2009; 6(10): 592-607.

SC Ng, AL Hart, MA Kamm, AJ Stagg, SC Knight. Mechanisms of action of probiotics: Recent advances. *Inflammatory Bowel Disease* 2009;15(2): 300-10.

SC Ng, MA Kamm. Therapeutic strategies for the management of ulcerative colitis. *Inflammatory Bowel Disease* 2008;15(6):935-50.

SC Ng, MA Kamm. Review article: new drug formulations, chemical entities and therapeutic approaches for the management of ulcerative colitis. *Alimentary Pharmacology and Therapeutics* 2008;28(7):815-29.

SC Ng, MA Kamm. Management of post-operative Crohn's disease. *American Journal of Gastroenterology* 2008;103(4):1029-1035.

Book Chapter

Basic Physiology: Intraluminal Defensins. **SC Ng**, AL Hart. Probiotics: Clinical Guide Chapter 1: section 2 (in press).

Abstracts and Posters

SC Ng, NE. McCarthy, S Plamondon, JL Benjamin, CRH Hedin, A Koutsoumpas, AL Hart, MA Kamm, A Forbes, K Whelan, SC Knight, JO Lindsay, AJ Stagg
Expression of Toll-liked receptor-4 and production of interleukin-6 by intestinal dendritic cells correlate with disease activity and C-reactive protein in active Crohn's disease (Accepted for poster presentation at Gastro 2009 London).

SC Ng, JL Benjamin, A Koutsoumpas, NE. McCarthy, CRH Hedin, S Plamondon, CL Price, AL Hart, MA Kamm, A Forbes, SC Knight, JO Lindsay, K Whelan, AJ Stagg
Altered intestinal dendritic cell function in patients with active Crohn's disease is associated with an imbalance in pro-inflammatory and anti-inflammatory faecal microbiota (Accepted for poster presentation at Gastro 2009 London).

SC Ng, NE McCarthy, S Plamondon, MA Kamm, AJ Stagg, SC Knight. Distinct cytokine patterns identified from multiplex profiles of patients with inflammatory bowel diseases, functional bowel disorders and controls. *Gastroenterology* 2009 (136) (suppl 2): S1750; Digestive Disease Week, Chicago, 2009.

SC Ng, NE McCarthy, S Plamondon, MA Kamm, AJ Stagg, SC Knight. TNF-α production and differential release of Th1/Th2 cytokines detected in colonic tissues of inflammatory bowel disease patients but not controls. *Journal of Crohn's and Colitis* 2009(3): S134 (P316); European Crohn's and Colitis Organisation Meeting, Hamburg, 2009.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Altered imprinting of lymphocyte homing by colonic dendritic cells in human ulcerative colitis. *Journal of Crohn's and Colitis* 2009(3): S132 (P309); European Crohn's and Colitis Organisation Meeting, Hamburg, 2009.

SC Ng, S Plamondon, MA Kamm, AJ Stagg, SC Knight. Oral probiotic bacteria, VSL#3, is associated with down-regulation of pro-inflammatory cytokine production by gut dendritic cells in patients with ulcerative colitis. *Immunology* 2008:OP172. British Society of Immunology, Glasgow, 2008.

SC Ng, NE McCarthy, S Plamondon, MA Kamm, AJ Stagg, SC Knight. TNF-α production and differential release of Th1/Th2 cytokines from gut biopsies is associated with "trauma response" in inflammatory bowel disease but not controls. *Immunology* 2008: OP57. British Society of Immunology, Glasgow, 2008.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Dysregulated gut homing in patients with ulcerative colitis is aoosicated with altered CD103 (Alpha-E) Expression by Human Colonic Dendritic Cells. *Immunology* 2008:4.7. British Society Immunology, Glasgow, 2008.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Probiotic VSL#3, but not placebo, is effective in acute colitis and associated with down-regulated inflammatory intestinal dendritic cells. *Gut* 2008(58); suppl 2:OP222.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Intestinal dendritic cells in ulcerative colitis have altered homing characteristics (CD103-alpha E) expression: A valuable therapeutic target? *Gut* 2008(58); suppl 2: OP045.

SC Ng, S Plamondon, HO Al-Hassi, N English, N Gellatly, MA Kamm, SC Knight, AJ Stagg. A novel population of $CD56^+$ $HLA\text{-}DR^+$ colonic lamina propria cells is associated with inflammation in ulcerative colitis. *Gastroenterol 2008:T1217;* Digestive Disease Week, San Diego, 2008. *Gut* 2008; 57 (suppl 1):374; British Society of Gastroenterology, Glasgow, 2008. *Journal of Crohn's and Colitis* 2008; 2(1):P281; European Crohn's and Colitis Meeting, Lyon, 2008.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Intestinal dendritic cells in ulcerative colitis have altered CD103 (α-E) expression: in support of dysregulation in gut-homing. *Gastroenterology 2008:T1218;* Digestive Disease Week, San Diego, 2008. *Gut* 2008;57 (suppl 1):375; British Society of Gastroenterology, Glasgow, 2008. *Journal of Crohn's and Colitis* 2008; 2(1):P265; European Crohn's and Colitis Meeting, Lyon, 2008.

SC Ng, A Koutsoumpas, CR Hedin, NE McCarthy, S Plamondon, MA Kamm, SC Knight, A Forbes, JO Lindsay, K Whelan, AJ Stagg. Production of IL-10 by colonic dendritic cells is associated with higher concentrations of faecal microbiota in patients with active Crohn's disease. *Gastroenterology* 2008:W1201; Digestive Disease Week, San Diego, 2008. *Gut* 2008;57 (suppl 1):377; British Society Gastroenterology, Glasgow, 2008. *Journal of Crohn's and Colitis* 2008; 2(1):P302; European Crohn's and Colitis Meeting, Lyon, 2008.

SC Ng, S Plamondon, HO Al-Hassi, MA Kamm, SC Knight, AJ Stagg.
Corticosteroids increase IL-10 and inhibit IL-12p40 production by Intestinal Dendritic Cells in Acute Ulcerative Colitis: Novel Mechanisms of Therapy. *Gastroenterology 2008:T1162;* Digestive Disease Week, San Diego, 2008. *Gut* 2008;57 (suppl 1):095; British Society Gastroenterology, Glasgow, 2008. *Journal of Crohn's and Colitis* 2008; 2(1):P262; European Crohn's and Colitis Meeting, Innsbruck, 2008.

SC Ng, S Plamondon, AL Hart, MA Kamm, SC Knight, AJ Stagg. Effective probiotic treatment (VSL#3), but not placebo, in acute ulcerative colitis is associated with down-regulation of inflammatory intestinal dendritic cells. *Gastroenterology* 2008:M1202; Digestive Disease Week, San Diego, 2008. *Gut* 2008;57 (suppl 1):096; British Society Gastroenterology, Glasgow, 2008. *Journal of Crohn's and Colitis* 2008; 2(1):P266; European Crohn's and Colitis Meeting, Lyon, 2008.

A Koutsoumpas, CRH Hedin, **SC Ng,** MA Kamm, JD Sanderson, SC Knight, AJ Stagg, A Forbes, JO Lindsay, K Whelan. The concentration and proportion of key bacterial groups of the faecal microbiota are different between smokers and non-smokers with active crohn's disease. *Gastroenterology* 2008;T1162; Digestive Disease Week, San Diego, 2008.

A Koutsoumpas, CRH Hedin, **SC Ng,** MA Kamm, JD Sanderson, SC Knight, AJ Stagg, A Forbes, JO Lindsay, K Whelan. Smokers with active Crohn's disease have differences in the concentration and proportion of key bacterial groups of the faecal microbiota. *Gut* 2008; 57 (suppl 1):400; British Society of Gastroenterology, Glasgow, 2008.

SC Ng, S Plamondon, HO Al-Hassi, MA Kamm, SC Knight, AJ Stagg.
Increased gut plasmacytoid dendritic cells in acute ulcerative colitis – Key mediators of immunity and inflammation? *Gastroenterology* 2007;132(4) (Suppl 2): A561; Digestive Disease Week, Washington, 2007.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Intestinal cytokine production by dendritic cells in acute ulcerative colitis – an immunoregulatory role? *Gastroenterology* 2007;132(4) (Suppl 2):A700; Digestive Disease Week, Washington, 2007.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Accumulation of plasmacytoid dendritic cells in the intestine of acute ulcerative colitis. *Gut* 2007;56 (Suppl 2): 357; British Society Gastroenterology Meeting, Glasgow, 2007.

SC Ng, S Plamondon, HO Al-Hassi, MA Kamm, SC Knight, AJ Stagg. Increased colonic dendritic cells in acute ulcerative colitis – Key mediators of immunity and inflammation? *Journal of Crohn's and Colitis* 2007. Suppl 1(1): P185; European Crohn's and Colitis Meeting, Innsbruck, 2007.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Intestinal cytokine production by dendritic cells in acute ulcerative colitis. *Journal of Crohn's and Colitis* 2007. Suppl 1(1): P186; European Crohn's and Colitis Meeting, Innsbruck, 2007.

SC Ng, S Plamondon, HO Al-Hassi, MA Kamm, SC Knight, AJ Stagg. Increased Gut Plasmacytoid Dendritic Cells in Acute Ulcerative Colitis – Key mediators of Immunity and Inflammation? British Society of Immunology, Glasgow, Feb 2007; European Crohn's and Colitis Meeting, Innsbruck. February 2007; *Gastroenterology* 2007;132(4) (Suppl 2): A561; Digestive Disease Week, Washington, 2007.

SC Ng, S Plamondon, HO Al-Hassi, MA Kamm, SC Knight, AJ Stagg. Increased colonic dendritic cells in acute ulcerative colitis – Key mediators of immunity and inflammation? Falk Symposium, Dresden, 2007.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Corticosteroids increase IL 10 and inhibit IL-12p40 production by intestinal dendritic cells in acute ulcerative colitis – A novel mechanism of therapy. Falk Symposium, Dresden, 2007.

SC Ng, S Plamondon, H O Al-Hassi, MA Kamm, SC Knight, AJ Stagg. Increased colonic dendritic cells in acute colitis: key mediators of immunity and inflammation? *Gut* 2007; 56 (suppl III):A51:213.

SC Ng, S Plamondon, H O Al-Hassi, MA Kamm, SC Knight, AJ Stagg. Corticosteroids increase IL-10 and inhibit IL-12p40 production by intestinal dendritic cells in acute ulcerative colitis – a novel mechanism of therapy. *Gut* 2007; 56 (suppl III): A51:214.

Oral Presentations (National and International)

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Probiotic VSL#3, but not placebo, is effective in acute colitis and associated with down-regulated inflammatory intestinal dendritic cells. 16th United European Gastroenterology Week October 2008, Vienna.

SC Ng, S Plamondon, MA Kamm, SC Knight, AJ Stagg. Intestinal dendritic cells in ulcerative colitis have altered homing characteristics (CD103-alpha E) expression: A valuable therapeutic target? 16th United European Gastroenterology Week, October 2008, Vienna. (TRAVEL GRANT AWARDED)

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SC Ng. Gut, bugs and dendritic cells. Northwest London Gut Club June 2008

SC Ng, S Plamondon, HO Al-Hassi, MA Kamm, SC Knight, AJ Stagg.
Corticosteroids increase IL-10 and inhibit IL-12p40 production by intestinal dendritic cells in acute ulcerative colitis: Novel mechanisms of therapy. British Society Gastroenterology Meeting, March 2008, Birmingham.

SC Ng, S Plamondon, AL Hart, MA Kamm, SC Knight, AJ Stagg. Effective probiotic treatment (VSL#3), but not placebo, in acute ulcerative colitis is associated with down-regulation of inflammatory intestinal dendritic cells. British Society Gastroenterology Meeting, March 2008, Birmingham.

S.C.Ng, S.Plamondon, HO Al-Hassi, MA Kamm, SC Knight, AJ Stagg. Increased colonic dendritic cells in acute colitis: key mediators of immunity and inflammation?
United European Gastroenterology Week, October 2007, Paris (TRAVEL GRANT AWARDED).

SC Ng, S Plamondon, HO Al-Hassi, MA Kamm, SC Knight, AJ Stagg. Corticosteroids increase IL-10 and inhibit IL-12p40 production by intestinal dendritic cells in acute ulcerative colitis – a novel mechanism of therapy. United European Gastroenterology Week, October 2007, Paris.

SC Ng. Modulation of the intestinal microbiota in inflammatory bowel disease. St Mark's Hospital Grand Round, December 2007.

Awards and Travel Grants

2009	Rustgi International Travel Award (by American Gastroenterology Association)
2009	Invited to Young Clinician's Programme in Gastro 2009 (London)
2008	"Yakult Research Travel Award Prize Winner" (Abstract presented at Digestive Disease Week 2008, San Diego)
2007	Travel Grant for one of the best oral presentations at the United European Gastroenterology Meeting, Paris 2007
2007	"Yakult Travel Award" (1st Runner-up abstract presented at Digestive Disease Week 2007, Washington)

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