

The modulation of Human Dendritic Cells by Glucagon Like Peptide - 2

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by

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The candidate confirms that the work presented in this report is the result of
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Abstract

Glucagon-like peptide-2 (GLP-2) is a pleiotropic peptide secreted in the human intestine with known intestinotrophic properties beneficial in conditions like short bowel syndrome (SBS); a condition characterized by malabsorption of both fluid and nutrients. Left untreated, SBS can lead to dehydration, malnutrition, and weight loss. Teduglutide, a long acting analogue of GLP-2, has been used in multiple clinical studies to elucidate its trophic properties. Murine studies however have also shown that GLP-2 inhibits pro-inflammatory cytokines raising the possibility of an anti-inflammatory property and its potential use in intestinal inflammatory conditions; in particular inflammatory bowel disease (IBD). Dendritic cells (DC) play a central role in the initiation and regulation of the immune system. They bridge the innate and adaptive immune systems and are unique in their ability to activate naïve T cells as well as dictate the type of T-cell immunity.

We hypothesized that GLP-2 has an immunomodulatory role and exert this action via DC. Toxic effects of GLP-2 peptide on human DC *in-vitro* have not previously been experimented. Our experiments showed that GLP-2 did not have a toxic effect on DC at 1pM, 1nM and 1μM concentrations and hence we were subsequently able to look at the effects of GLP-2 on human DC phenotype and function. Using whole blood and intestinal biopsies from healthy volunteers, we obtained a population of enriched low density cells (LDC) which offered a novel and 'physiological' model for DC. These cells

were labelled with appropriate fluorochromes and assayed by a flow cytometer.

We established that DC incubated overnight with GLP-2 had a reduced intensity ratio of HLA DR and an increased expression of CD14 in a dose dependent way compared with controls. The expression of co-stimulatory molecule CD86 was also higher in the treated DC. This phenotypic change suggests that GLP-2 modulated DC into an immature state although still able to stimulate T-cell proliferation. Ongoing cytokine production of IFN- γ and IL-12 from healthy blood DC was inhibited by GLP-2 however only cytokine production of IFN- γ from intestinal lamina propria DC was inhibited. These findings suggest that GLP-2 may induce a 'homeostatic' or 'immuno-tolerant' state and block Th1 cytokines in DC.

Functional experiments confirmed that GLP-2 modulated DC enhanced T cell proliferation although this occurred only with intestinal DC. GLP-2 conditioned DC also functionally affected the cytokine profile of T cells by reducing the cytokines IFN- γ in both human blood and intestinal DC and IL-12 in only the latter. Hence our human DC *in-vitro* findings mirror some of the results found in murine studies showing GLP-2 effects on blocking Th1 cytokines. The results suggest that GLP-2 has an immunoregulatory effect and that the mechanism of action may possibly involve direct effects on DC. GLP-2 therefore is able to modulate DC characteristics and function leading to future application as an immunotherapy for inflammatory diseases.

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Awards and Travel Grants

October 2011	Shire Innovation Fund for SpRs UEGW Travel Bursary Abstract submission to UEGW 2011 Stockholm
June 2011	ASNEMGE/EAGE 8th Summer School of Gastroenterology - Shortlisted to participate
January 2011	ASNEMGE Young Investigator Meeting Shortlisted to participate
1995-2000	MB BCh BAO distinctions awarded in both preclinical and clinical years

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Abbreviations

APC	Antigen Presenting Cell(s)
ASA	Aminosalicylic Acid
AZA	Azathioprine
$\beta 7$	Beta 7 Integrin
Bx	Biopsy
Bx-SN	Biopsy Supernatant
CCR	CC Chemokine Receptor
CD	Crohn's Disease
CDAI	Crohn's Disease Activity Index
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
CLA	Cutaneous Lymphocyte Antigen
CLP	Common Lymphoid Progenitors
CLM	Common Myeloid Progenitors
DC	Dendritic Cell(s)
DC-SIGN	Dendritic cell specific ICAM-3 grabbing non-integrin
DNA	Deoxyribonucleic Acid
DPP-IV	Dipeptidyl Peptidase-IV
DSS	Dextran Sulphate Sodium
DTT	Dithiothretol
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
ELISA	Enzyme linked immunoabsorbent assay

FACS	Fluorescence Assisted Cell Sorter
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
Foxp3	Forkhead Box Transcription Factor p3
FSc	Forward Scatter
GALT	Gut associated lymphoid tissue
GI (tract)	Gastrointestinal (tract)
GLP-1	Glucagon like peptide-1
GLP-2	Glucagon like peptide-2
GLP-2R	Glucagon like peptide-2 receptor
GM-CSF	Granulocyte-macrophage Colony Stimulating Factor
GRS	Graded Response Score
HBSS	Hank's Balanced Salt Solution
HLA	Human Leucocyte Antigen
HSC	Haematopoietic Stem Cells
HPN	Home Parenteral Nutrition
Hr	Hour(s)
IBD	Inflammatory Bowel Disease(s)
ICAM	Intracellular Adhesion Molecule
IEC	Intraepithelial cell(s)
IEL	Intraepithelial lymphocyte(s)
IF	Intestinal Failure
IFN	Interferon
Ig	Immunoglobulin(s)
IGF-1	Insulin like growth factor-1

IL	Interleukin
IR	Intensity Ratio
L.	Lactobacillus
Lin	Lineage cocktail of monoclonal antibody (CD3, CD14, CD16, CD19 and CD34)
LDC	Low Density Cell(s) (enriched for blood DC)
LP	Lamina Propria
LPS	Lipopolysaccharide
mDC	Myeloid Dendritic Cell
min	Minute(s)
M cell	Microfold cell
MACS	Magnetic Cell Sorting
MadCAM-1	Mucosal Addressin Cell Adhesion Molecule -1
MALT	Mucosa Associated Lymphoid Tissue
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIF	Macrophage Inhibitory Factor
Min	Minutes
MLN	Mesenteric Lymph Node
MI	millilitres (s)
μM	Micromolar
MLR	Mixed Leucocyte Reaction
MNC	Mononuclear Cells
MoDC	Monocyte-derived Dendritic Cell(s)
MP	Mercaptopurine

NFκB	Nuclear factor kappa-light chain enhancer of activator B cells
NK	Natural Killer Cell
nM	Nanomolar
NOD	Nucleotide Binding Oligomerisation Domain
PAMPs	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell(s)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell(s)
PeCy5	Phycoerythrin-cyanin 5.1 Conjugate (Cy-chrome/PC5)
PFA	Paraformaldehyde
PI	Propidium Iodide
PS	Phosphatidylserine
PI ratio	Positive Intensity Ratio
PN	Parenteral Nutrition
PPAR	Peroxisome Proliferator Activated Receptor
PRRs	Pattern Recognition Receptors
pM	Picomolar
RA	Retinoic Acid
RANK	Receptor Activator of NFκB
RPMI	Roswell Park Memorial Institute
RORγt	Retinoic acid-related Orphan Receptor
RT	Room Temperature (ambient)
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
TNF α	Tumour Necrosis Factor α
TNBS	Trinitrobenzene sulfonic acid
TSLP	Thymic Stromal Lymphopoetin
U	Units
UC	Ulcerative Colitis
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive Intestinal Peptide
Vs	Versus (compared with)

Chapter 1

General Introduction

Introduction to GLP-2 and Dendritic cells

Glucagon like peptide-2 (GLP-2), a potent intestinal peptide growth factor secreted from the human intestine has garnered significant interest in the last few decades amongst scientists and physicians. This is in light of its wide variety of potential biological functions in treatment of gastrointestinal (GI) – related disorder, such as short bowel syndrome (SBS), inflammatory bowel disease (IBD), chemotherapeutically induced GI mucositis and NSAID induced enteritis. This interest has generated recent drug development with therapeutic potentials. Teduglutide, a long acting analogue of GLP-2, has been used in multiple clinical studies to elucidate its trophic properties and is now the first orphan drug for the treatment of SBS.

The main focus of this thesis is to investigate the possible immunological effects of GLP-2, primarily on dendritic cell (DC) function, which is thought to be fundamental in the inflammatory process. DC is distinct from other immune cell types because they possess unique abilities to initiate the primary immune response and determines whether an active or tolerogenic immune response occurs. Another property of DC is their ability to imprint homing properties on T cells (effector cells) that they stimulate, in order to localise immune responses to a specific tissue. Augmenting the phenotype and function of DC could be an important therapeutic target to modulate the inflammatory pathway.

Hence, apart from knowledge of GLP-2, an understanding of DC origin, identification, functionality as well as role in inflammatory bowel disease in relation to GLP-2 is essential and key in this thesis.

1.1 Dendritic Cells

Dendritic cells (DC) are professional antigen presenting cells (APC) that play an essential role in the initiation and regulation of the immunity. They originate from haematopoietic stem cells (HSC) in the bone marrow and are situated in all tissues particularly at sites of pathogen entry (skin, nose, lungs, stomach and intestines). DC are a complex, heterogenous population of cells and are widely distributed. Although they constitute a small proportion of the overall mononuclear cell population, they play a disproportionate role in orchestrating the immune response.

DC are distinct from other professional APC (B-cells and macrophages) because they possess the unique ability to initiate the primary immune response, generating immunological memory. They act as immune sentinels or 'gate-keepers', constantly patrolling the peripheral tissues and responding to a spectrum of environmental cues. DC expresses microbial pattern recognition receptors (PRR) and once DC encounter an antigen, PRR activates DC, enabling them to migrate to secondary lymphoid organs and initiate the adaptive immune response. DC can determine whether an active or tolerogenic immune response occurs to a particular antigen hence whether an inflammatory (e.g., against invading pathogens) or tolerogenic (e.g.,

against commensal bacterial antigens) immune response predominates (Banchereau and Steinman, 1998; Bell et al., 1999; Hart, 1997; Steinman, 2012). This pivotal position at the interface between innate and adaptive immunity allows DC to shape the immune response to a vast range of antigenic challenges.

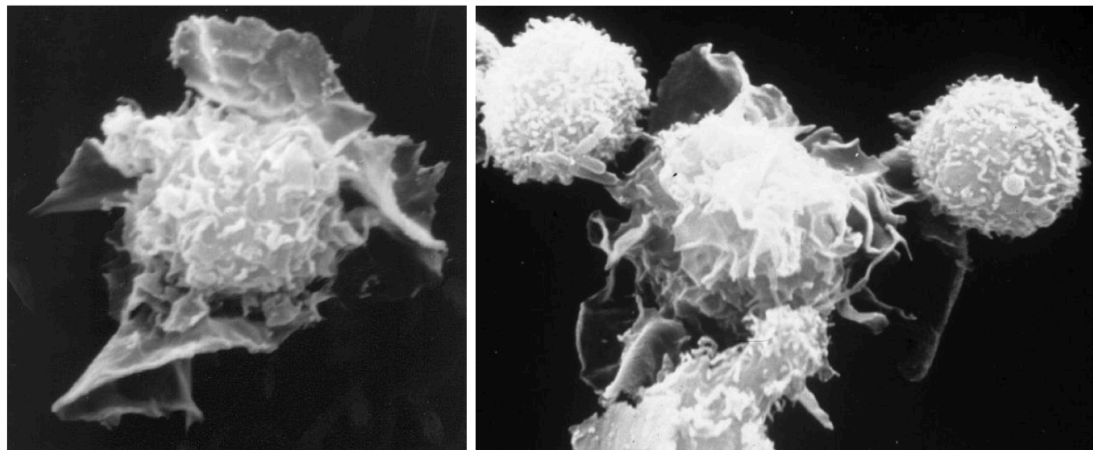
The 'gate-keeper' role of DC is dependent on several intricate innate properties:

- 1) **Acquisition of antigen in the peripheral tissues**, such as the lamina propria of the gut, through special mechanisms for antigen capture and processing e.g. endocytosis. DC recognise microbial antigens through PRR, such as Toll-like receptors (TLR), or respond to a spectrum of non-microbial stimuli including cytokines that indicate danger or tissue damage (e.g. TNF α) (Stagg et al., 2003).
- 2) **Capacity for rapid differentiation and maturation** enabling DC to present antigen, expressing high levels of several molecules on the cell surface such as MHC class I and II molecules, accessory molecules CD40, CD80, CD86 and early activation markers such as CD83.
- 3) **Migration to local lymphoid tissues** from specific tissue compartments e.g. lamina propria of the gut to mesenteric lymph nodes (MLN) and Peyer's patches.
- 4) **Interaction of naïve effector T-cells** to initiate and determine adaptive immune responses. The profile of DC-derived cytokines directs the polarization of T cell Th1/ Th17 and Th2 phenotypes. Production of IL-12 and IL-23 by DC promote a Th1/Th17 response, which

predominates in inflammatory conditions like Crohn's disease, whereas IL-4 and IL-10 contribute to a Th2 or T regulatory cell response (Steinman & Banchereau, 2007).

1.1.1 Dendritic Cell Origin

The first description of DC was in 1868 by the medical student Paul Langerhans. His 'Langerhans cell' was initially observed in gold-stained human skin epidermis and demonstrated dendritic-like projections in their cytoplasm (Langerhans, 1868). These cells were subsequently identified as a unique subset of DC containing specific cytoplasmic Birbeck's granules (Birbeck et al., 1961). In 1973, Steinman and Cohn then discovered and characterised an unusual 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. This included large nuclei, a lack of features suggestive of active endocytosis and cytoplasm bearing 'pseudopods' or 'dendrites' of various forms, sizes and numbers; they were therefore named 'Dendritic Cells' (Steinman & Cohn, 1973), although Brigid Balfour coined the term 'veiled cells', referring to their veil-like rather than dendritic morphology at electron microscopy (Figure 1.1).



a)

b)

Figure 1.1 Dendritic Cell Morphology by Electron Microscopy.

a) Scanning electron micrographs of 'veiled' human dendritic cells (work done by Brigid Balfour and Stella Knight). b) DC 'veils' interacting with lymphocytes (Tyndall, Knight et al., 1983).

Steinman and Cohn subsequently showed that DC expressing major histocompatibility complex (MHC) class II molecules were required for antigen presentation and were major stimulants in the mixed lymphocyte response (MLR) (Steinman et al., 1983). It was later demonstrated that DC also existed in humans (Van Voorhis et al., 1982). Multiple DC subsets have now been identified in mice and humans that can be characterised by distinct phenotypes and functional activities (Ardavin, 2003; Shortman & Liu, 2002; Mann ER et al., 2013).

1.1.2. Dendritic Cell Lineages and Subsets in Humans

DC constitute a relatively small proportion of immune cells in blood and peripheral tissues (0.5-1%). They do not express a single specific marker that identifies the cells as DC. Hence, DC can be difficult to identify. DC characterisation in humans is however possible through a process of exclusion. This procedure is done by elimination of cells expressing non-DC cell surface markers using flow cytometry. DC express MHC Class II (HLA-DR), but this histocompatibility antigen is also expressed on other APC such as B-cells and macrophages. However, DC do not express a set of lineage markers such as CD3 (T cells), CD 14 (monocytes), CD16 (macrophages), CD19 (B-cells), or CD34 (progenitor/ stem cells) (Bell et al., 2001). Therefore DC can be classified as HLA-DR positive but lineage marker negative. Molecules involved in maturation, co-stimulation, antigen uptake, microbial recognition, migration and also cytokine production are all used to phenotypically distinguish DC subset.

Identification of Subsets in Dendritic Cell populations

DC are produced from haematopoietic stem cells within the bone marrow. Common lymphoid progenitors (CLP) or common myeloid progenitors (CMP) are derived from differentiation of these stem cells within the bone marrow. Progenitors of DC in bone marrow migrate via the blood stream and home to peripheral tissues where they encounter several essential growth factors such as GM-CSF, IL-4, IL-5, TNF- α , TGF- β , and IL-3 secreted by various cell types

including endothelial cells, keratinocytes and fibroblast in the microenvironment. Such growth factors determine the fate of the progenitors to differentiate into multiple subsets of DC described both in mice as well as human populations, e.g. immature Langerhans DC, intestinal DC or plasmacytoid DCs (pDC) (Ardavín, 2003; Shortman and Liu, 2002). These subsets are characterised by expression of cell surface markers such as CD11c, CD4, CD8 α and CD11b to name a few (Shortman and Liu, 2002).

The subsets of mature DC freshly isolated from tissue within humans are not as well studied as mouse DC. Human DC subtypes have previously been mainly defined using peripheral blood, although tissue data is also available primarily from immunohistological analysis of DC populations in human tissues. The ability to generate DC *in-vitro*, led to an expansion of research on DC but it is important to note that differences exist between *ex-vivo* and *in-vitro* (Osugi, 2002; Mann ER et al., 2013).

There are two distinct subsets of DC found in humans, conventional CD11c⁺ 'myeloid' DC and the CD11c⁻ 'plasmacytoid' or 'lymphoid' DC. These DC subsets recognize different microbial pathogens through specific receptors, which in turn induce different types of innate and adaptive immune response (Figure 1.2) (Shortman, 2002).

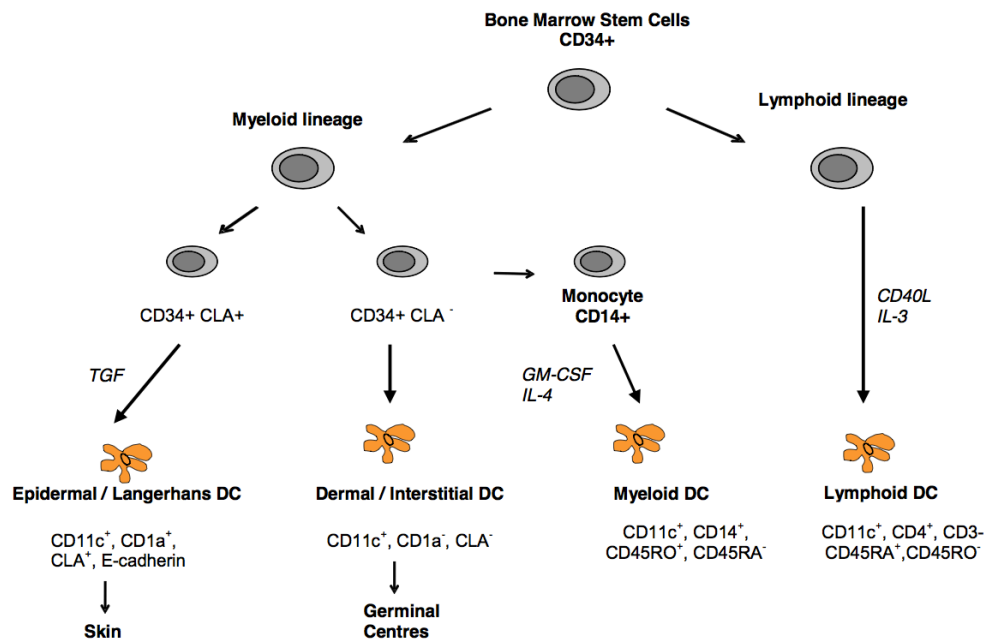


Figure 1.2. Dendritic cell lineage.

Differentiation of CD34⁺ stem cells into epidermal (Langerhans) DC, dermal/interstitial DC and 'myeloid' and 'lymphoid' DC.

Myeloid DC CD 11c⁺

Myeloid DC (mDC) in humans are monocyte derived and require GM-CSF for their survival and maturation. Within the blood, there are low levels of co-stimulatory markers (Sallusto, 1994) and these cells have a high phagocytic capacity suggesting that mDC are immature DC. mDC express CD11c⁺, which is a $\beta 2$ integrin that can act to mediate adhesive leukocyte interactions with other cells (Córbi, 1997). mDC also express other myeloid markers such as CD11b, CD13, CD33, CD1c and CD141 as well as expressing high levels of GM-CSF receptors. CD45RO is also expressed, with little expression of CD45RA. They express low levels of IL-R3⁺ critical for development of pDC.

Final maturation to CD14⁻, CD83⁺, CD86⁺ DC expressing high levels of surface MHC class II is achieved by stimulation with proinflammatory cytokines such as tumour necrosis factor- α (TNF α) or microbial products such as lipopolysaccharide (LPS) (Han, 2009). Monocyte derived DC can be derived *in-vitro* from monocytes in the presence of GM-CSF and IL-4 (Sallusto, 1994).

Plasmacytoid DC CD11c⁻

Human pDC were initially recognised by their plasma cell-like morphology and unique surface phenotype (CD4⁺, IL-3R⁺, CD11c⁻). Originally called interferon-producing cells due to production of type 1 interferons (IFNs), they are found in blood and many lymphoid tissues (Shortman and Liu, 2002). IFNs produced by pDC directly inhibit viral infections and also activate antiviral functions of other cells, such as mDC, natural killer (NK) cells and B-cells. Distinct factors regulate the survival and differentiation of CD11c⁻ DC precursors; these cells are critically dependent on interleukin 3 (IL-3) and CD40L for maturation (Grouard et al, 1997). pDC do not express or express very low levels of CD11c and GM-CSF, but rather express high levels of other lymphoid markers such as CD123, CD303 and CD304 (Ardavín, 2001).

Functionality of DC subsets

It has been proposed that different DC subsets direct specific immune responses, such as polarising a Th1/Th2 response or immunotolerance. For example, human myeloid DC derived from monocytes (MoDC) in the laboratory are associated with elevated IL-12 production and the generation of IFN γ stimulating proliferation of Th1 cells (Rissoan et al., 1999). pDC are functionally characterised by the production of large amounts of type-1 interferons (IFN α / β) in response to viral antigens, stimulating IFN γ producing T-cells and a Th1 type immune response, but are also capable of generating Th2 responses (Siegal et al., 1999; Cella et al., 2000).

This outline of DC function is likely to present a gross oversimplification of DC capabilities since there appears to be a considerable degree of plasticity in their responses, which is dependent on several factors including local environmental factors such as DC lineage, maturation status and numbers; danger and inhibitory cytokine exposure and modulation by microbial factors or other environmental cues. Hence, DC are likely to display tissue specific specialisation that enables them to function appropriately in diverse settings.

1.1.3 Dendritic cell Maturation

DC are able to evolve from immature, antigen-capturing cells to mature, antigen-presenting, T cell-priming cells. They are able to convert antigens into immunogens and express molecules such as cytokines, chemokines, co-stimulatory molecules and proteases to initiate immune responses. The T-cell mediated immune responses can vary to include tolerogenic or inflammatory Th1, Th2 or Th17 responses, depending on the specific DC lineage and maturation stage, in addition to the activation signals received from the surrounding microenvironment.

The ability of DC to regulate immunity is dependent on DC maturation. A variety of factors can induce maturation following antigen uptake and processing within DCs. These factors include bacterial-derived antigens such as LPS or whole bacteria, inflammatory cytokines, ligation of select cell surface receptors and viral products. The process of DC maturation involves a number of phenotypical and functional changes. These changes can commonly involve redistribution of major histocompatibility complex (MHC) molecules from intracellular compartments to the DC surface, down-regulation of antigen internalisation, an increase in the surface expression of co-stimulatory molecules, morphological changes with formation of dendrites, cytoskeleton re-organization, secretion of chemokines, cytokines and proteases, and surface expression of adhesion molecules and chemokine receptors (Shin 2006).

Immature DC reside in peripheral tissues and in the absence of further stimulation remain in an immature state, with low expression of co-stimulatory molecules and cytokines (Mahnke, 2005) (Kubach, 2005). These cells possess high phagocytic activity and if they encounter tissue injury, captured antigens will become activated (Hart, 1997). It is this encounter with antigens that induces the maturation of immature DC to functional APC. With DC maturation there is less expression of the receptors involved in antigen uptake and higher expression of molecules required for T cell activation or priming such as MHC, CD40 and CD86; thus enabling T cell signalling via CD28 on T cells (Caux, 1994).

The level of expression of the co-stimulatory molecules CD40 and CD86 are higher in activated DC. These DC surface molecules engage with their respective ligands on T-cells, in addition to MHC class II/ T cell receptor interaction. One of the major DC functions regulated by CD40 ligation is cytokine production leading to downstream production of IL-12p and IL-6. CD40 ligands are also expressed on B-cells and DC/B-cell interaction via CD40 ligand plays a role in B-cell proliferation as well as regulation of B cell homeostasis (Ma 2009). Similarly, CD86 upon DC-T-cell interaction is able to induce IL-2 production and T-cell proliferation (Freeman, 1993).

1.1.4 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. 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; these immature cells present self-antigens for the induction and maintenance of self-tolerance (Steinman et al., 2000; Steinman et al., 2003). 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 naïve T cells takes place. This pathway is mediated by the interaction between PRRs, such as TLR, nucleotide-binding sites and leucine-rich repeat containing receptors (NLR), retinoic acid inducible gene-I (RIG)-like receptor on DC (Lavelle et al., 2010), and pathogen-associated molecular patterns (PAMPs) present on the surface of microorganisms (Kaisho and Akira, 2003). Some DC populations may enter lymph nodes directly from the circulation whereas others pass through non-lymphoid tissues and in doing so may transport antigens from the periphery to lymph nodes. When migrating from the 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).

Different chemokines receptors are important for the process of DC migration. CCR2 is involved in 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 DC to the lamina propria. 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 $\alpha 4\beta 7$ integrin and CCR9 on T cells that they activate resulting in homing to the small intestine (Mora et al., 2003; Stagg et al., 2002). More on lymphocyte homing will be discussed later in this chapter.

1.1.5 Stimulation of Adaptive Immunity

DC can contribute to the expansion and differentiation of most classes of lymphocytes. 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. $CD4^+$ T cells known as T helper (Th) cells can be divided into two functional subsets based on their cytokine production (Mosmann et al., 1986). These two pathways of differentiation are known as Th1 and Th2.

Th1 cell differentiation

The Th1-Th2 hypothesis was based on the observation that each subset of CD4⁺ Th cells produces different patterns of cytokines and induces different effector functions. Th1 differentiation has been characterised by the production 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 cytotoxicity in NK cells and CD8⁺ T cells (Mosmann et al., 1986). The production of IL-12 by DC drives a Th1 response (Macatonia et al., 1995). IL-12 is a heterodimeric cytokine composed of 2 disulphide-linked subunits, p35 and p40. IL-12p40 expression is generally thought to be restricted to cells with phagocytic activity whereas IL-12p35 is expressed ubiquitously at low levels (Trinchieri, 2003). The p40 subunit's production is regulated and induced by products of bacteria, parasites, viruses and fungi in DC as well as monocytes, neutrophils and macrophages. DC secrete IL-12 in large quantities once stimulated through their cell surface receptors and antigens. Ligands of Toll like receptors that induce IL-12 release include lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan and bacterial (CpG) DNA. IL-12 then subsequently leads to release of IFN- γ after the induction of NK cells and T cells. This effect on NK and T cells is enhanced synergistically by IL-19 (Yashimoto et al., 1998). Also, the combination of IL-12 and IL-18 continue to stimulate IFN- γ and IL-12 production from APCs. The cytokine combination of IL-12 and IFN- γ also serve to inhibit Th2 cell differentiation as well as inhibiting the production of

cytokines involved in Th2 pathway differentiation; IL-4, IL-5 and IL-13 (Agnello et al., 2003).

Two other known IL-12 related cytokine members IL-23 and IL-27 have also been described. IL-23 is a heterodimer of p19 and p40 subunits and it induces proliferation of memory T cells (Oppmann et al., 2000). Like IL-12, IL-23 induces IFN- γ production in both T cells and DC, and may promote the expansion of CD4⁺ T cells that produce IL-17 (Langrish et al., 2005). IL-27 consists of p28 and EB13 subunits and it induces the proliferation of naïve T cells (Pflanz et al., 2002). Both IL-23 and IL-27 are produced by DC (Smits et al., 2004).

Th2 cell differentiation

Th2 cells drive humoral immunity directed towards clearing extracellular pathogens most notably parasitic infections as well as inflammatory allergic reactions. They stimulate B cells to produce immunoglobulin (IgG)-E and enhance the maturation of eosinophils and the degranulation of mast cells and basophils. The production of IL-4 after stimulation by antigens influences naïve T cells to differentiate through the Th2 pathway. Also involved in the initiation of Th2 differentiation is the thymic stromal lymphopoietin (TSLP). TSLP is an epithelial cell-derived cytokine implicated in promoting type 2 inflammation through effects on both innate and adaptive immune cells and is also known to activate basophils. TSLP-activated DCs prime naïve T cells to produce IL-4 and other cytokines linked to the Th2 profile (Soumelis et al.,

2002). TSLP also induces DCs to produce Th2-attracting chemokines such as CCL-17 and CCL-22 (Perros et al 2009).

IL-4 acts to optimize the molecular requirements for Th2 cell differentiation via the IL-4/ STAT 6 pathway leading to an increased expression of the transcription factor GATA3. GATA3 is an important regulator of Th2 cell phenotype, stabilizing it by inhibiting IFN- γ production (Ferber et al., 1999) and downregulating IL-12R β 2 expression in a manner independent of IL-4 (Ouyang et al., 1998). GATA3 is expressed in small amounts by naïve T cells but this is markedly upregulated during Th2 cell differentiation. STAT6, additionally, controls the expression of IL-4 locus control region (Ansel et al., 2006). *In vitro* studies have also identified the involvement of STAT5, which is activated early in the pathway after T cell receptor engagement induces IL-2 production that then activates STAT5. This activation of STAT5 coupled with the early production of GATA3 seen after T cell receptor activation induces IL-4 production. The IL-4 produced then affects the IL-4R/STAT6 pathway to upregulate GATA3 (Paul, 2010).

Th17 cell differentiation

The relatively recent discovery of a further Th cell lineage Th17 cells have shifted thinking away from the previously described Th1/Th2 paradigm. Their primary role in health appears to be clearance of pathogens not adequately handled by Th1 or Th2 cells (Korn et al., 2009). Pathogens as diverse as the Gram-positive *Propionibacterium Acnes*; the Gram-negative *Citrobacter*

Rodentium, *Klebsiella Pneumoniae*, *Bacteroides* and *Borelia* species; the acid-fast *Mycobacterium Tuberculosis*; and fungi-like *Pneumocystis Carinii* and *Candida Albicans* can all trigger strong Th17 response (Korn et al., 2009). This cell lineage develops from naïve T cells in the presence of TGF- β and IL-6 and has contrastingly been described as both an immunoregulatory and pro-inflammatory cytokine respectively. The effects of the Th17 response have been viewed as pro-inflammatory given the secretion of cytokines including IL-17a, IL-17f, IL-21, IL-22, IL-6 and TNF- α and its regulation by IL-10 (Yosef et al., 2013)(Olsen et al., 2011). There is also data supportive of anti-IL17a antibody therapies in psoriasis, rheumatoid arthritis and uveitis (Hueber et al., 2010).

There is however suggestion that Th17 have some plasticity to their function, with an ability to stop production of IL-17a as well as express T-bet, which is a regulator in Th1 cell differentiation and IFN- γ (Symons et al., 2012). There also appears to be a variation in the function of Th17 signatory cytokine IL-17a. A mouse transfer model of colitis has shown that IL17a -/- T cells induce a more aggressive disease phenotype compared with the wild type cell population, implying a protective effect of IL17a in this model (O'Connor et al., 2009).

The key transcription factor involved in the process is retinoic acid-related orphan receptor (ROR γ t), with its importance being demonstrated in ROR γ t negative mice (Ivanov et al., 2006). Similarly, there is markedly decreased IL-17 expression in vitro in T cells that lack ROR γ t (Ivanov et al., 2007). The

induction of ROR γ T is dependent on STAT3, which in turn is activated by IL-6, IL-21 and IL-23 (Korn et al., 2009). STAT1 activation acts to inhibit the development of Th17 cells and is activated via IL-27 and IFN- γ pathway (Batten et al., 2006; Stumhofer et al., 2006). The important role of the STAT proteins in the differentiation and regulation of Th17 cells is further demonstrated by the inhibitory role that IL-2 dependent STAT5 expression has on these cells (Laurence et al., 2007).

1.2 Intestinal Dendritic Cells and Immune Regulation

The gastrointestinal (GI) tract is in constant contact with a diverse and dynamic luminal environment that contains large numbers of commensal bacteria and a variety of pathogens. In this highly antigenic environment, the ability to maintain immune tolerance to commensal bacteria or self-antigens and the ability to mount effector responses to invading pathogens is a key feature of the gut immune system. Mucosal DC located at the intersection between the innate and adaptive immune systems are likely to be central to this process. They constantly survey the microbial environment, coordinate immune responses to danger signals (Niess and Reinecker, 2005), and prime naïve T cells to control overwhelming infections or tissue inflammation (Nagler-Anderson, 2001; Steinman et al., 2003).

There is a general consensus that DC in the gut are generally hyporesponsive (Coomes JL et al., 2008) maintaining immune tolerance in the gut by generation of tolerogenic T-cell responses towards food antigens and

commensal microbiota, preventing unnecessary inflammation and hypersensitivity. However, when this process is dysregulated, inflammation ensues and this is considered a probable pathway of which inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) occurs (Strober et al., 2007). 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 (Chirido et al., 2005; Hart et al., 2005). 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 the organised lymphoid tissue. This include Peyer's patches and mesenteric lymph nodes (MLN) (where the induction of T and B cell response occurs) and within generalised tissues interspersed with effector cells, which include the lamina propria and intestinal epithelium (Iwasaki and Kelsall, 2001; Maric et al., 1996). Recently, a previously unrecognised network of DC that respond to microbial stimuli and upregulated CD80 and CD86 *in vivo* have been identified within the muscular layer of the mouse intestine (Flores-Langarica et al., 2005).

In the mouse, three subsets of CD11c⁺ DC have been described in the MLN and Peyer's patches: CD11b⁺CD8 α ⁻ DC in the subepithelial dome, CD11b⁻CD8 α ⁺ in the interfollicular regions and CD11b⁻CD8 α ⁻ (double negative) subsets in both areas (Iwasaki and Kelsall, 2000; Iwasaki and Kelsall, 2001). The murine spleen also contains three main CD11c⁺DC subsets which include

CD11b⁺CD4⁺, CD11b⁺CD4⁻ and CD11b⁻CD8α⁺ cells. CD11b⁺ and CD11b⁻ populations, but not CD8α⁺ DC, have been described within the murine small intestinal lamina propria. The former appears to be the predominant subset in the terminal ileum. Lamina propria DC that express the integrin CD103 have been identified in the mouse intestine (Annacker et al., 2005; Johansson-Linbom et al., 2005).

Similar to other tissues, intestinal DC is a heterogeneous 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 mouse intestine, the most extensive DC system populates the lamina propria of the small and large intestine. Many different cell types in the gut mucosa express CD11c and HLA-DR/ MHC Class II, which various groups use as a basis of DC characterization; however, other studies have indicated that most of these cells are not genuine DC (Persson EK et al., 2010, Varol C et al., 2010). DC expressing CX3-chemokine receptor 1 (CX3CR1), a molecule essential for ability of cells to extend transepithelial projections into the gut lumen (Niess et al., 2005), have been described in mice, but these cells expressed CD14 (Rescigno et al., 2009, Varol C et al., 2009). Several laboratories exclude CD14⁺ cells in their criteria used to identify DC. Indeed, DC 'exclusion ' or lineage cocktail comprises CD3, CD14, CD16, CD19, CD34 and CD56 and is used to identify DC by excluding T-cells, B-cells, monocytes, macrophages and stem cells, then selecting HLA-DR⁺ cells as putative DC.

In the human colon, lamina propria DC are characterised by their expression of CD83 and dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN), and they have the phenotype of CD11c⁺Lin⁻ with an immature state and low TLR-2 and -4 expression compared to their blood counterparts. These are largely CD11c⁺ myeloid cells with few identifiable pDC (CD11c⁻CD123⁺) (Bell et al., 2001; Hart et al., 2005).

It has been thought that the main site of induction of mucosal immunity occurs in the Peyer's patches due to their close proximity with the intestinal lumen and specialized M cells however recent evidence suggests that the Peyer's patches may not be essential for the induction of oral tolerance or active immunity (Mowat, 2003). In the absence of the Peyer's patches, MLN are critical for the tolerance induction (Guegemann et al., 1998; Spahn et al., 2002). 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 microorganisms.

1.2.1 Sampling of Luminal Antigens by Intestinal Dendritic Cells

DC are present as immature cells with high phagocytic activity within the 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, 1991).

Within the GI tract, several pathways have been described by which DC can sample and process specific antigens or pathogens. The main route for microorganisms or macromolecules to gain access to the mucosal immune system is via specialized epithelial cells, known as M cells. 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 (Kraehenbuhl and Neutra, 2000). 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 also 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. Macrophages in close proximity were however not involved in this process. These data suggests 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 (Geissman et al., 2001; Heystek et al., 2002). M cells covering the Peyer's patches also 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 Rotavirus, CD11c⁺CD11b⁻CD8⁻CD4⁻ DC in the Peyer's patches have been described to 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 1970's through electron-microscopic (EM) studies of rat ileum (Collan, 1972; Rescigno et al., 2001b; 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.,

2001b). The formation of transepithelial dendrites depends on CX3CR1. These lamina propria DC may include subsets that express IL-12p40 and IL12p19, 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 1.3 shows an electron microscopy (EM) picture of a human lamina propria colonic DC sending processes into the intestinal lumen for sampling of antigen (APRG EM).

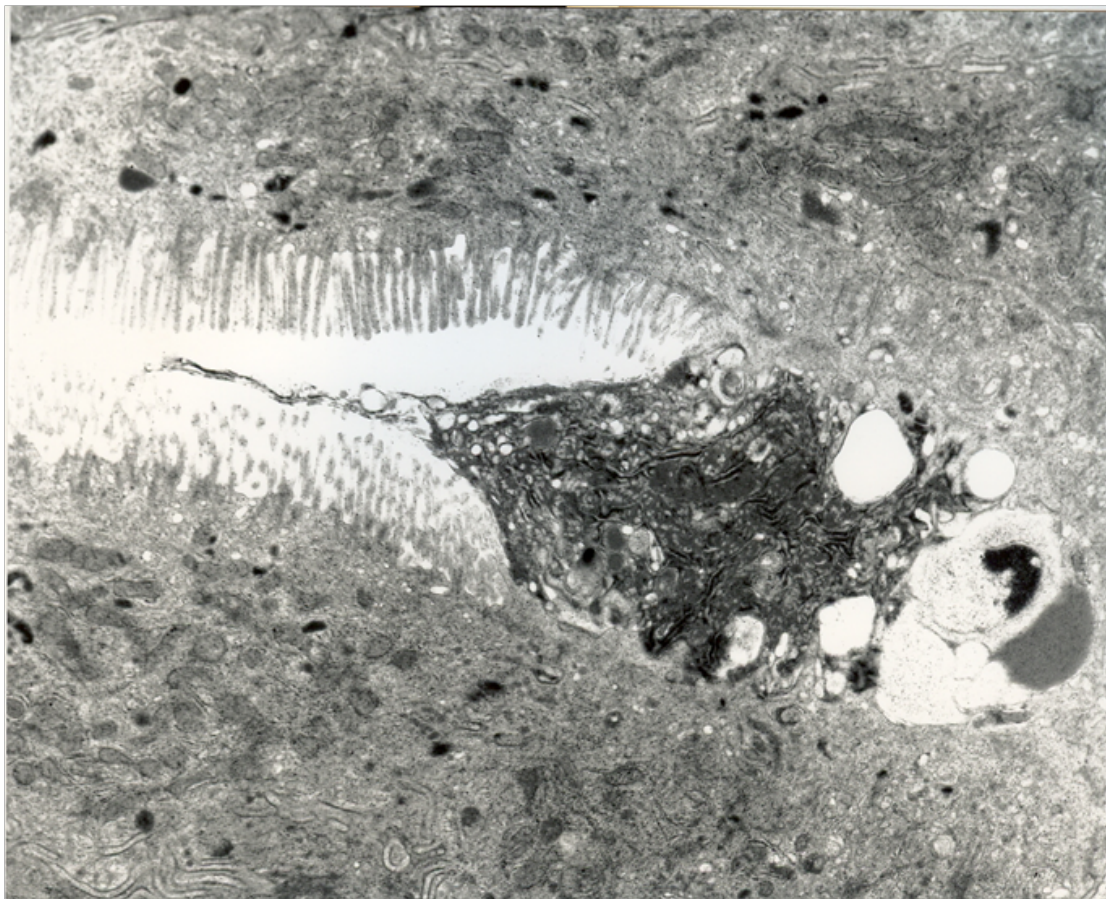


Figure 1.3. Electron Microscopy (EM) of a human colonic DC sampling antigen.

This EM picture shows antigen sampling by human lamina propria colonic DC from a control subject (Courtesy of Mr Nicolas English, APRG).

1.2.2. Induction of Tolerance and Active Immunity by Intestinal Dendritic Cells

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. This process of migration occurs at a faster rate when DC are activated. However, the nature of DC migration 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). DC which migrate from the intestine in the steady state, are paradoxically able to induce strong inflammatory responses from naïve T cells, despite their role in the maintenance of oral tolerance. Intestinal lymphatic DC from the thoracic ducts of rats stimulated strong proliferative responses, induced secretion of INF- γ and proliferation of FoxP3-positive lymphocytes (Milling et al., 2009; Hadis et al., 2011).

Earlier studies have shown that 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. This process involves the interaction between commensal bacteria and distinct DC populations, both in the periphery and local mucosal tissue.

Several studies have demonstrated that DC tolerance can be achieved by the downregulation of CD80 and CD86 (Steinman et al., 2003), the interactions between DC and novel co-stimulatory molecules such as CD200 and CD200R (Gorczynski et al., 2005), and the signalling through novel receptor ligand interactions (Howne et al., 2000). In addition, induction of tolerance can be achieved by the control of T cell proliferation through release of immunosuppressive cytokines, IL-10, TGF- β and INF- γ (Levings et al., 2001; Levings et al., 2002; Yamagiwa et al., 2001, Saraiva et al., 2010), and the production of metabolites such as indoleamine 2,3 deoxygenase (IDO) (Grohmann et al., 2000; Matteoli et al., 2010).

After oral administration, antigen is transported via afferent lymphatic by DC into the draining MLN, which is obligatory for the induction of oral tolerance (Worbs et al., 2006). The majority of DC entering the MLN largely originates from the lamina propria suggesting that these cells are 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 pDC induces differentiation of 'T regs' like cells that release IL-10 and IL-4 after DC maturation with CpG (Bilsborough et al., 2003), whereas bone marrow and spleen derived pDC 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 produce IL-10 whereas CD11c⁺CD8α⁺ DC and CD11c⁺CD11b⁻CD8α⁻ DC produce IL-12p70 following stimulation with CD40L (Iwasaki and Kelsall, 2001; Kellermann and McEvoy, 2001). The data therefore suggests that the induction of tolerance versus active immunity may be complex and is dependent on the tissue microenvironment, the types of DC signalling events and response to microbial stimuli.

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

1.2.3. Intestinal Dendritic Cells and Microbial Interactions

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. The ability of DC to sample luminal bacteria suggests that they play a critical role in the surveillance of pathogens in the gut environment. 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

bacteria. For instance, when 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).

Human CD4⁺ T cells from the lamina propria of healthy intestinal tissue exhibits reactivity to commensal bacteria (*Enterobacter*, *E. Coli*, *Enterococcus* species) as well as to the pathogen, *Salmonella Typhimurium*. Depletion studies suggested that bacteria-specific CD4⁺ T cell activation and proliferation were dependent on intestinal DC, which themselves exhibit a pro-inflammatory cytokine profile upon bacterial stimulation (Howe et al., 2009).

Activation and maturation of DC occurs when DC recognise PAMPs on the surface of bacteria by way of PRR on their surface. It is unclear how PRR monitoring and response distinguish between abundant normal microbiota and the rare pathogen. It is thought that the difference between pathogen and commensal can be due to the acquisition of a single plasmid. This has resulted in the current designation of PRR ligands as microbial associate molecular patterns (MAMPs) as opposed to the previous more limited term PAMP (Neish, 2008).

The best characterised of all PRR are the TLRs. At least 11 members of the TLR family have been described and their respective microbial products reported (Akira et al., 2001). TLR-2 expressed by CD11c⁺ DC and its ligands is required for recognition of gram-positive cell wall components, including lipoproteins and peptidoglycans (Kadowaki et al., 2001). TLR-4 recognises

lipopolysaccharide (LPS) from *E. Coli* (Takeuchi et al., 1999); TLR-5 recognises flagellin from gram-negative bacteria, and TLR-9 recognises bacterial DNA and it is expressed by pDC and B cells.

It has also been recognised that there is receptor specific spatial distribution that helps to discriminate between pathogen and commensal bacteria. TLR-2 and -4 are expressed on the surface of DC and macrophages; TLR-5 is found on the basolateral surface of epithelium; TLR-9 is restricted to the cytoplasmic vacuolar compartments whereas nucleotide-binding oligomerization domain 2, (NOD2), is detected intracellularly.

In the mouse, pDC and CD4⁺ DC express TLR-7 but lack TLR-3 expression (Doxsee et al., 2003). In human, mDC express TLR-1 to -6 and TLR-8 (Liu, 2005), whereas pDC exclusively express TLR-7 and TLR-9 (Krug et al., 2001a; Stagg et al., 2003).

DC express NOD1, which recognises muramyl-tripeptides from gram negative bacteria, and NOD2, common to peptidoglycans of all bacteria species. 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. NOD2 is a key cytoplasmic sensor of intracellular bacterial peptidoglycan and a mediator of innate immunity. Enteric pathogens can alter intestinal DC function. For example, *Heligmosomoides Polygyrus* infection induces DC activation and IL-10

expression, which impair host protection against *Citrobacter Rodentium* infection, resulting in an enhanced bacterial infection and the 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.

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. DC-SIGN is implicated to play a role in the induction of various responses mediated by DC. It is a c-type lectin expressed mainly on DC and recognizes mannose- and fructose- containing glycans that are present on endogenous and on microbial or viral surface. Konstantinov et al. have shown that the major S layer protein, SlpA, of *L. Acidophilus* NCFM is the first probiotic bacterial DC-SIGN ligand identified that is functionally involved in the modulation of DC and T cells functions (Konstantinove et al., 2008).

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

1.2.4 Regulation of Lymphocyte Homing by Intestinal Dendritic Cells

Lymphocytes continuously migrate around the body and meet with antigens via APC. 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. In the intestine, lymphocytes primed in MLN draining intestinal sites have the propensity to home back to the intestine. Two homing molecules promote specific homing to the gut: $\alpha 4\beta 7$ which is attracted to its ligand, mucosal addressin cell adhesion molecule (MAdCAM-1), expressed on high endothelial venules, 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). In a recent murine study, a subset of CCR9⁺ pDC from small intestine suppressed development of intestinal inflammation in mice (Mizuno et al., 2010), linking CCR9 expression with immune tolerance. The study showed that CCR9^{lo} DC were potent stimulators of naïve T-cells and it hypothesised that this DC fraction may have a more pathogenic nature compared with its CCR9^{hi} counterpart.

Specific homing pathways also exist for other organs. For example, 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).

Retinoic acid (RA) is required for the induction of $\alpha 4\beta 7$ and CCR9 by mucosal lymphocytes. RA binds to intracellular retinoic receptors, which activates the transcription of genes encoding $\alpha 4\beta 7$ and CCR9 (Figure 1.4).

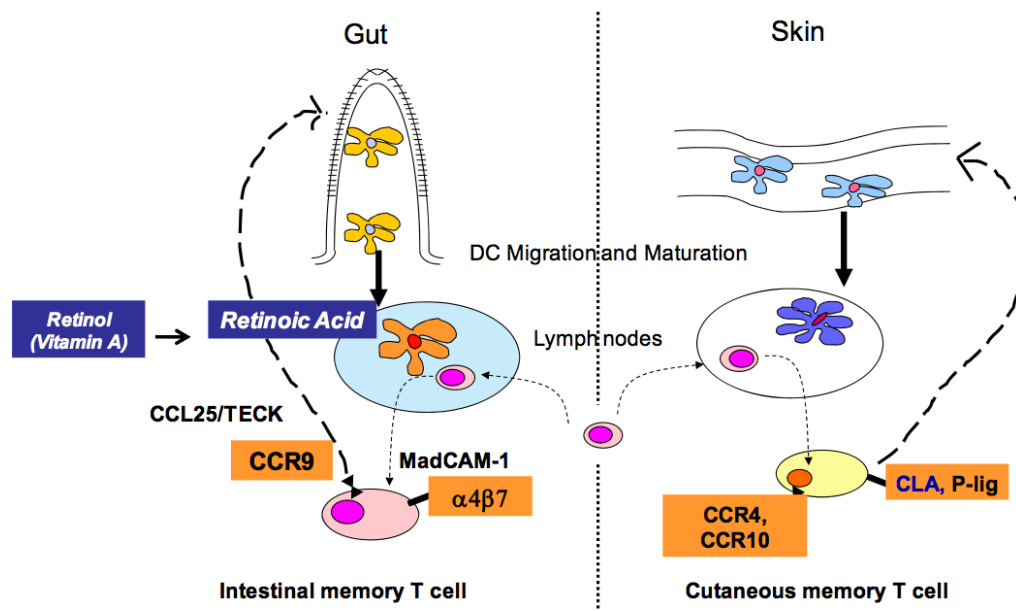


Figure 1.4. Control of lymphocyte homing by dendritic cells.

DC in gut-related lymphoid organs imprint T cells with gut-homing specificity by providing retinoic acid during antigen presentation. MLN-DC and PP-DC produce retinoic acid from retinol (vitamin A), and imprint gut-homing specificity on T cells upon activation. The imprinted T cells express both $\alpha 4\beta 7$ and CCR9, which binds to MAdCAM-1 and CCL-25, respectively, and migrate into the small intestinal tissue (left panel). Conversely, DC in the 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. Several groups have shown that DC imprint the expression of homing molecules on T cells that they activate (Stagg et al., 2003; Johansson-Linborn et al., 2003; Mora et al., 2003). Work from our laboratory has demonstrated that in mice DC from MLN, but not from peripheral lymph nodes, induce $\alpha 4\beta 7$ on T cells, thus targeting T cells back to intestinal tissue *in vivo* (Stagg et al., 2002). Murine intestinal DC from Peyer's patches, MLN and the lamina propria induce $\alpha 4\beta 7$ 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 addition to their key role in regulating tolerogenic and immunogenic responses, DC have a critical role in the generation of tissue trophic effector T cell subsets. In the mouse, the ability to generate CCR9⁺ $\alpha 4\beta 7$ ⁺ gut homing T cells appear to be confined to a functionally distinct subset of MLN DC that express the integrin α chain CD103, and DC derived from this sites are more potent at generating gut trophic effector T cells than MLN DC. Hence, this implies that DC are imprinted with the ability to generate gut trophic T cells before entering MLN. In contrast to this, CD103⁻ MLN DC are incapable of generating gut trophic T cells. CD103 (αE) is the α chain of the integrin $\beta 7$; it is expressed on the majority 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).

Commensal bacteria can also directly influence the gut-homing capacity of leucocytes (Kunii et al., 2011). Work from our laboratory, recently identified a peptide secreted by *Lactobacillus Plantarum*, called STp due to the abundance of serine and theonine residues within its sequence. This peptide was present in the healthy human colon but missing from the gut in UC patients. It induced regulatory properties on human gut DC *in vitro* as well as had enhanced ability to imprint skin-homing properties on stimulated T-cells (Bernardo et al., 2012). It was hypothesised that STp has a role in gut homeostasis that is missing in UC, one potential mechanism being enhancing DC ability drive effector T-cell responses away from intestinal sites.

The mechanism and specific signals involved in DC imprinting appear to be dependent on vitamin A. The vitamin A metabolite, retinoic acid (RA) can be endogenously produced, or 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 'T reg' play a central role in the maintenance of immunological haemostasis and tolerance in the gut. Most studies of these include naturally occurring population of CD4⁺CD25⁺Foxp3⁺ T reg cells that develop in the thymus. Similar to conventional T cells, 'T reg' have homing

receptors allowing migration into specific tissues and DC are thought to play an important role in the generation of 'T reg' cell responses. In particular $\alpha 4\beta 7$ is induced by 'T reg' when exposed to DC from MLN but not from peripheral lymph nodes, and RA appears to be central to this induction of $\alpha 4\beta 7$ 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 'T reg' cells (Coombes et al., 2007; Sun et al., 2007). The CD103⁺ DC population displays an enhanced ability to generate 'T reg' compared with their CD103⁻ counterparts *in vitro*, and this ability is inhibited by RA receptor (RAR) antagonist (Benson et al., 2007).

Eckburg et al. found that CD103⁺ DC induced Th17 T cell differentiation *in vitro* can be inhibited by addition of an RAR antagonist to the culture medium (Eckburg et al., 2005). Moreover, the addition of high concentrations of RA inhibited Th17 cell differentiation, whereas addition of low doses of RA promoted Th17 cell differentiation. Thus, DC-derived RA can act as a co-factor for the generation of FoxP3⁺ regulatory cells and, dependent upon the prevailing cytokine milieu and levels of RA signalling, influence the balance between generation of regulatory cells and effector Th17 cells.

1.2.5 Differences in DC phenotype and function throughout the gut (differences between small bowel and colon).

The GI tract is in constant contact with numerous commensal microbiota and food antigens hence it is necessary to have different mechanisms at play to generate an immune tolerance to prevent constant inflammation in a healthy gut. The colon and ileum have different functions as mentioned earlier, with the colon having the main function of water and salt extraction and the ileum being one of absorption of nutrients and minerals in food. Also, the load of bacteria in the ileum ranges from 10^4 to 10^8 CFU/mL; however, the colon is much more heavily colonised with a bacterial load of 10^{11} to 10^{12} CFU/mL (Simon GL et al., 1986, Quigley EM et al., 2006). Despite these differences both in function and bacterial colonisation, there are few studies that distinguish between properties of immune cells in these compartments.

Recent data from our laboratory have shown that there are specific functional differences between gut DC derived from colon compared to ileum (Mann EM et al., 2015). Regulatory properties of gut DC and their ability to imprint specific homing profiles on T-cells are dependent on their anatomical location within the gut. There is a lower proportion of DC producing pro-inflammatory cytokines (TNF- α and IL- β) in the human colon compared with the ileum and colonic DC exhibit an enhanced ability to generate CD4⁺FoxP3⁺IL-10⁺ T cells (T-reg). Also, a greater proportion of colonic DC express the lymph-node-homing marker CCR7 along side enhanced endocytic capacity for bacterial sampling; this difference was most striking in CD103⁺Sirp α ⁺ DC.

Colonic DC also appear to be more tolerogenic compared to ileal DC with enhanced expression of inhibitory receptor ILT3 (although this expression was mainly restricted to CD103⁺ DC). There is also a difference between colonic and ileal DC abilities to imprint homing properties on stimulated T cells (CCR 9 for small bowel homing and CCR 4 for skin homing). Ileal DC had an enhanced ability to generate CCR9⁺ (β7⁺) T cells compared with colonic DC which had an enhanced ability to imprint CCR4 on T cells. This differences in ileal and colonic DC imprinting may have implications in IBD with tissue compartmentalisation and also have bearing on how GLP-2 modulates DC inflammation within each of the compartments.

1.3. Role of Dendritic Cells in Inflammatory Bowel Disease

Microbial colonisation has an effect on the instruction and regulations of the gut immune system (Guarner et al., 2006). Abnormal interactions between the gut microbiota and the mucosal immune system are key in the development of chronic intestinal inflammation (Asquith et al., 2010). Crohn's disease (CD) and ulcerative colitis (UC), collectively termed inflammatory bowel disease (IBD), results likely 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 Commeli, 2007; Baumgart and Carding, 2007; Sartor, 2006).

In IBD, DC are likely to be of fundamental importance. Animal models of colitis have provided strong evidence that the interaction between 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). Approximately half of the colonic DC are CD11b⁺ whilst the remaining half were CD11b⁻B220⁺ pDC. In human IBD studies, activated DC has been shown to accumulate at sites of intestinal inflammation (Bell et al., 2001; Silva MA, 2009), comprising phenotypically heterogeneous populations of DC (Verstige et al., 2008).

One of the best characterised animal models involves the transfer of CD45RB^{high} CD4⁺ T cells into SCID (severe combined immunodeficiency) mice which results in chronic intestinal lesions as those seen in human IBD (Malstrom 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 inhibit the accumulation of CD134L⁺ DC suggesting that regulatory T cells may function, in part, to prevent DC activation. Moreover, blocking CD132-CD134L interactions ameliorated the colitis, reduced T cell proliferation and the numbers of α4β7⁺ T cells in the MLN.

DC are 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 (Mottet et al., 2003).

CD25⁺CD4⁺ T cells have been found to proliferate between clusters of CD11c⁺ DC in the MLN and inflamed colon. These results suggest that DC are important in driving T cell responses during inflammation (Krajina 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. It is most likely that pathogenic T cell responses inducing IBD are primed or re-stimulated in junctional CD4⁺ T cell/ DC aggregates (Leithauser et al., 2001).

There are also studies showing 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 produce higher levels of IL-12p40, IL-23p19 and IL-10 (Becker et al., 2003).

IL-23 is a heterodimer that shares the p40 subunit with IL-12. IL-23 consists of IL-23p19 and IL12p40 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. 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.

Qualls et al. demonstrated that resident DC can suppress the severity of acute DSS colitis and that 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), 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 investigations have shown an increase 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 is an increase in CD83⁺ and DC-SIGN⁺ lamina propria DC, which may produce IL-12 and IL-18 during intestinal inflammation (Velde et al., 2003). Infiltration of CD83⁺CCR7⁻ DC have been reported in the subepithelial dome; these cells internalize 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 are possible precursors of DC, have been identified

as a potential source of TNF- α . There are increase 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 are 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 also 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 tissue (Vuckovic et al., 2001), whereas Baumgart et al. have demonstrated that patients with active IBD lack 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, is enhanced on lamina propria DC of patients with CD and UC. In CD, more colonic DC produce pro-inflammatory cytokines, IL-6 and IL-12p40, than controls. In addition, treatment of patients with CD 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 increase number of CD83⁺ and CD86⁺ lamina propria cells, most likely DC, which produce 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 contains

numerous basal aggregates consisting of lymphocytes and CD80⁺ dendritiform cells that most likely represent activated DC (Yeung et al., 2000). DC generated *in vitro* from peripheral blood monocytes show increase immuno-stimulatory capacity, and produce 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, are increased in lamina propria of UC patients (Christ et al., 1998). UC is also associated with an atypical Th2 response mediated by non-classical NKT cells producing IL-12 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 in the regulation of human intestinal immunity.

1.4 Glucagon Like-Peptide 2.

Glucagon like peptide-2 (GLP-2), a potent intestinal peptide growth factor has specific trophic properties in the gut and is pivotal in the regulation of mucosal morphology, function and integrity. Daniel J. Drucker first described these properties in 1996 and since, the body of research investigating GLP-2 has grown exponentially, giving light to its central role in intestinal physiology in

both health and disease (Drucker DJ et al., 1996). In the last decade, it has become evident that GLP-2 has a therapeutic role in the treatment of short bowel syndrome (SBS) with a good safety profile (Wallis K et al., 2009; Yazbeck R et al., 2010).

SBS is a malabsorption disorder that occurs when part of the small intestine is missing (congenital short bowel) or has been surgically removed due to pathology. Complications from nutritional malabsorption occur and cause significant comorbidities. Main causes of SBS include Crohn's disease, volvulus, ischemia, injury from trauma, tumours, necrotizing enterocolitis (premature newborn), bypass surgery to treat obesity and surgical resection of disease or damaged small bowel.

The effects of GLP-2 resulting in higher absorptive capacity of the intestine allowed patients with SBS less dependence on parenteral nutrition, therefore improving nutritional status and quality of life. This would be a 'first in class' treatment for patients with SBS (Hornby PJ et al, 2011).

Recently, through a number of murine studies, GLP-2 has shown promise in reducing mucosal inflammation and maintaining mucosal integrity, leading to the concept that it may have therapeutic applications in conditions like IBD, chemo and radiation-induced mucositis as well as NSAID-induced enteritis. More studies, both murine and in humans are needed to see if these findings will translate into clinical practice. Through this thesis, we therefore wish to explore the possible immunomodulatory properties of GLP-2 at a cellular level

and explore the possible mechanisms by which GLP-2 could potentially do this.

1.4.1 Synthesis, Secretion and Degradation

Synthesis

Glucagon like peptide-2 is a 33 amino acid peptide with an estimated molecular mass of 3766.16 (Hartmann B et al., 2000). GLP-2 is co-encoded within the proglucagon gene along with glucagon like peptide -1 (GLP-1), which in mammals, gives rise to a single mRNA transcript that is expressed in the alpha (α) cells of the endocrine pancreas, in the enteroendocrine L cells of the intestine and in the hypothalamus and brainstem of the central venous system (CNS) (Baggio LL et al., 2004; Drucker DJ et al., 1988; Drucker DJ et al., 1989). Proglucagon mRNA is translated into a single 160 amino acid precursor protein, producing several biologically active proglucagon-derived peptides via tissue-specific post-translational processing. In the pancreatic α cells, proglucagon is cleaved by prohormone convertase (PC)-2 to form glucagon, the major glucagon fragment and intervening peptide (IP)-1. In the GI tract and in the brain, the processing of proglucagon, which is operated by PC1/3, results in GLP-1, GLP-2, IP-2, oxytomodulin and glicentin formation (Figure 1.5).

Much of the understanding of GLP-2 biology and function has been discovered in tandem with Glucagon like peptide-1 (GLP-1). GLP-1 is

synthesised in the same manner as GLP-2 but has mainly an incretin effect and this has led to development of its analogues being directed towards treatment of endocrine abnormalities and type 2 diabetes.

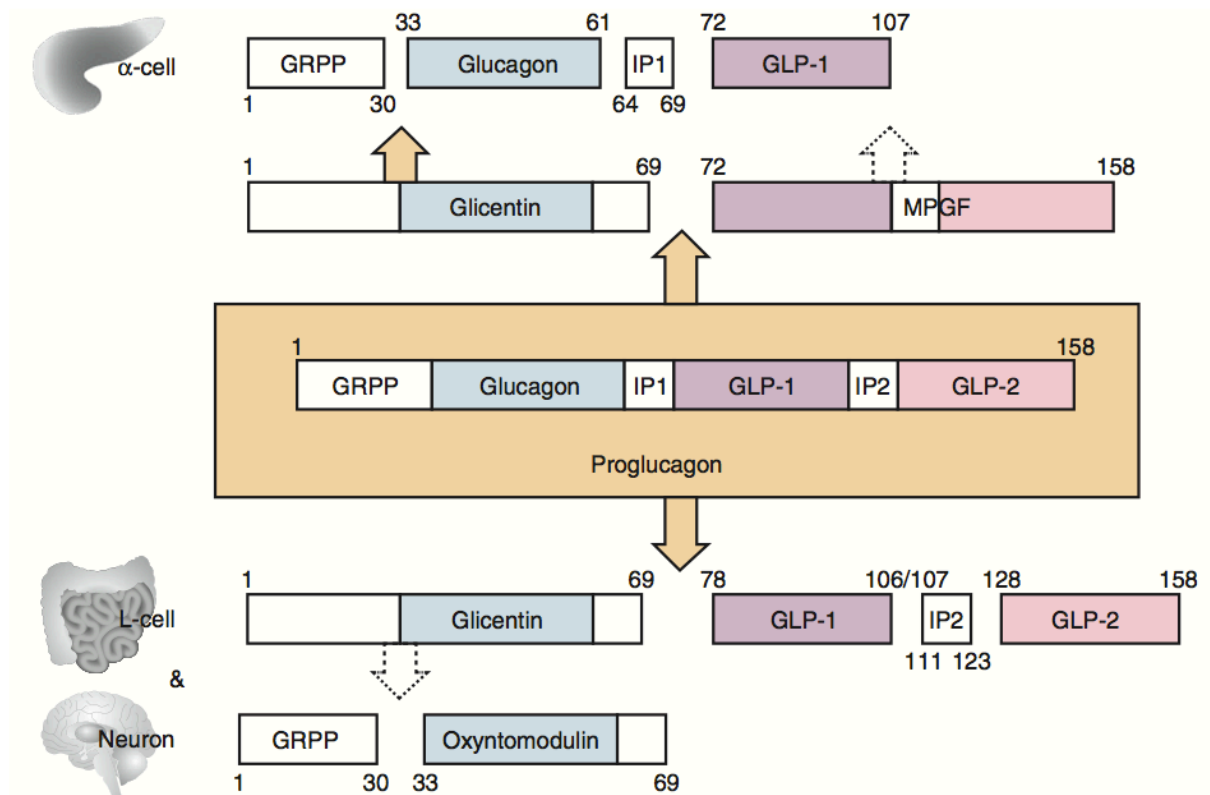


Figure 1.5 Structure of proglucagon and glucagon-like peptide 2.

The proglucagon-derived peptides, derived from post-translational processing of proglucagon, in both pancreas and intestine are indicated.

Abbreviations: GLP-1, glucagon-like peptide 1; GRPP, glicentin-related pancreatic polypeptide; IP-1 and -2, intervening peptides 1 and 2; MPGF, major proglucagon-derived fragment.

Secretion

Biologically active GLP-2¹⁻³³ is secreted by enteroendocrine L cells, most of which are located in the distal ileum and colon. The chief stimulus for intestinal secretion of GLP-2 is the ingestion of nutrients, including glucose, fatty acids and dietary fibre (Brubaker PL et al., 1986). Protein has no effects. GLP-2 is secreted in a biphasic pattern, with an early peak followed by a longer second phase after ingestion of nutrients. It is likely that the early phase of GLP-2 secretion is due to the stimulation of the L cells by various neural and endocrine factors, in contrast with the second or late phase, which is caused by direct stimulation of intestinal L cells by digested nutrients (Xiao Q et al., 1999). After ingestion of nutrients, plasma levels of GLP-2 increase 2- to 5-fold, depending on the size and nutrient composition of the meal. The peptide diffuses across the subepithelial lamina propria to activate afferent nerves and/or enter the circulation; thus they may act as paracrine agents as well as endocrine hormones (Figure 1.6).

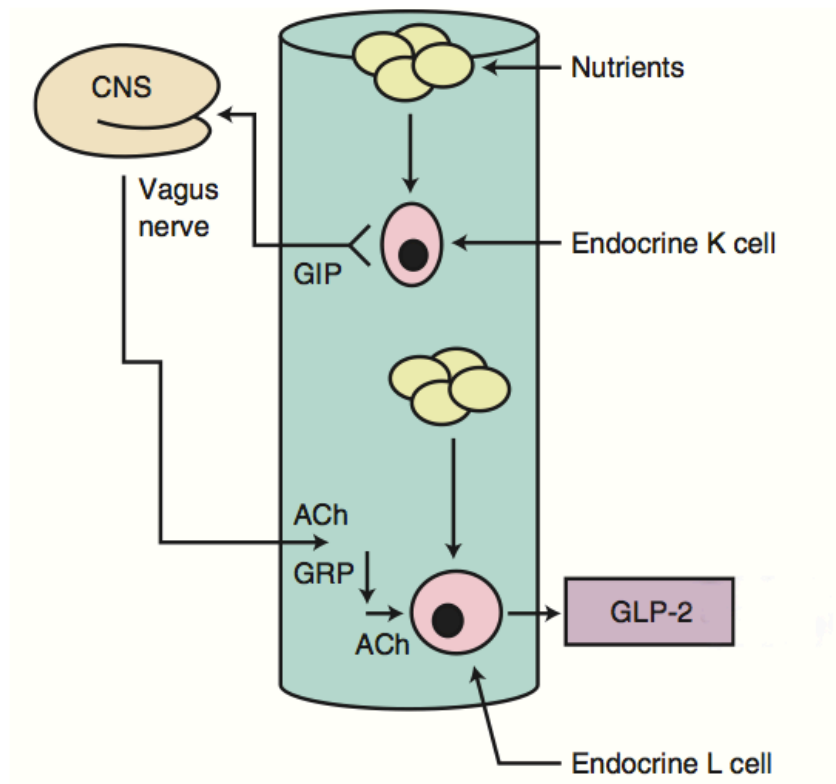


Figure 1.6. Direct and indirect effects of nutrients on secretion of GLP-2.

Entry of nutrients into the proximal small intestines initiates an early peak of secretion mediated through the vagus nerve. The afferent component of this neural loop is activated by ingested nutrients either directly or through release of an enteroendocrine hormone, such as GIP from the K cells. Vagal efferent fibres then stimulate the distal L cells through a pathway that likely involves both ACh and GRP within the enteric nervous system. Further aboral movement of the nutrients down the lumen of the small intestine stimulates a second, later peak of GLP-2 secretion through direct effects on L cells.

Abbreviations: Ach, acetylcholine; CNS, central nervous system; GIP, glucose-dependent insulinitropic polypeptide; GLP-1, glucagon-like peptide 1; GLP-2, glucagon-like peptide 2; GRP, gastrin releasing peptide. (Reprinted with permission – Katharina Wallis PhD Thesis 2009)

The mechanisms by which nutrients induce the release of peptides from the enteroendocrine cells have not been fully elucidated. Currently little is known about the cellular/secretion mechanism responsible for GLP-2 but is felt to be analogous to that of GLP-1. One mechanism that has been described involves enteroendocrine cell activation to release these peptides and is mediated by cellular uptake and intracellular metabolism of glucose. This triggers peptide exocytosis via ATP-sensitive potassium-channel closure, depolarisation and calcium channel activation, similar to insulin secretion (Reimann F et al., 2002).

Another suggested mechanism involves downstream mediating effects of GLP-2 (Dubé PE et al., 2007) (Figure 1.7). For example, vascular endothelial growth factor [VEGF] and transforming growth factor- β (TGF- β) have been linked to GLP-2 induced wound repair (Bulut K et al., 2008), insulin-like growth factor-1 (IGF-1) appears to be essential for GLP-2 induced intestinal epithelial proliferation and nitric oxide [NO] might be a key mediator in GLP-2 induced up-regulation of intestinal blood flow (Guan X et al., 2006) Also, the release of vasoactive intestinal peptide (VIP) from enteric neurons mediates the anti-inflammatory effects of GLP-2 (Sigalet DL et al., 2007).

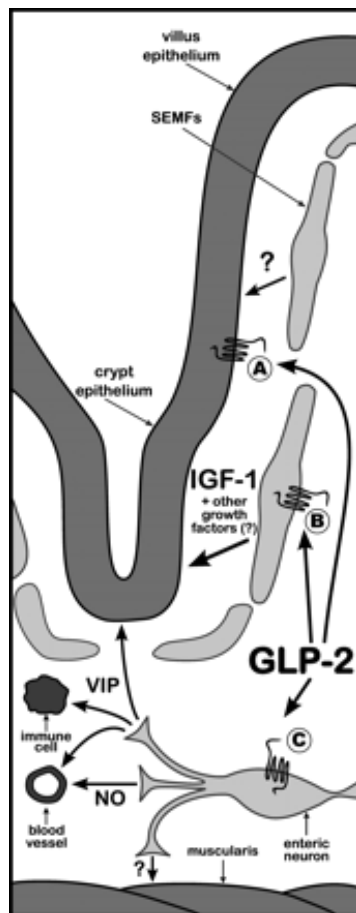


Figure 1.7. Proposed model for the indirect mechanisms of glucagon-like peptide-2 (GLP-2) action in the intestine.

Expression of the GLP-2R in intestinal endocrine cells (A), intestinal SEMFs, (B) and enteric neurons (C) suggests that GLP-2 acts indirectly to produce its diverse actions in the intestine. IGF-I is critical for the ability of GLP-2 to induce intestinal growth and activate crypt cell proliferation. GLP-2-mediated enteric neuronal signaling enhances intestinal blood flow through a mechanism involving NO production and has anti-inflammatory actions through VIP [16]. (Reprinted with permission – Katharina Wallis PhD Thesis 2009). Abbreviation: GLP-2R, Glucagon-like peptide-2 receptor; SEMFs, subepithelial myofibroblasts; IGF-I, insulin-like growth factor-1; NO, nitric oxide; VIP, vasointestinal polypeptide.

Degradation

GLP-2 is degraded quickly through cleavage of N-terminal histidine and alanine by the ubiquitously expressed proteolytic enzyme dipeptidyl peptidase-IV (DPP-IV), resulting in the generation of biologically inactive GLP-2³⁻³³ (Figure 1.8) The importance of DPP-IV in the regulation of GLP-2¹⁻³³ has been demonstrated *in vitro* and *in vivo* using animal models (Meier JJ et al., 2006). Bioactive GLP-2¹⁻³³ has an apparent half-life of 7 min in humans (Hartmann B et al., 2000). Once in the plasma, the kidney provides the major route of clearance for GLP-2.



Figure 1.8. The amino acid sequence of human GLP-2 (amino acids 1-33) and its site of cleavage by the peptidase dipeptidyl peptidase 4 (DPP-IV) (arrow) to GLP-2 (amino acid 3-33) are shown (Drucker D et al., 1999).

Prolongation of half-life

To date, there are two different successful strategies in mitigating the issue with the short half-life of GLP-2. The first is to use the mimetics of GLP-2 that are resistant to inactivation by DPP-IV, thus prolonging and enhancing the effect of the hormone. Teduglutide (GATTEX; NPS Pharmaceuticals, Bedminster, NJ, USA) was developed by replacing alanine with glycine in position 2 of GLP-2, providing a molecule with a half-life of 3-4 h.

The second strategy involves inhibition of DPP-IV therefore prolonging the effect of endogenously secreted glucagon like peptides (GLP-1 and GLP-2). Drugs like vidagliptin (Galvus; Novartis Pharmaceuticals, East Hanover, NJ, USA) and sitagliptin (Januvia; Merck & Co., Whitehouse Station, NJ, USA) that are specific DPP-IV inhibitor for GLP-1 are currently used for treatment of non-insulin dependent diabetes.

1.4.2 Role of GLP receptors

Like glucagon, the action of GLP-2 and GLP-1 are mediated through class 2 G-protein-coupled receptors. These receptors are distinct and specific for either GLP-2 or GLP-1, despite sharing the conserved properties of their class.

GLP-2 receptor (GLP-2R) has been cloned from the stomach, small bowel and hypothalamus cDNA libraries (Munroe DG et al., 1999). GLP-2R expression is restricted to the GI tract and the CNS, with limited expression in the lung, cervix and vagal afferents, although cardiac expression in rats has been reported recently (Angelone T et al., 2012).

The exact cellular localization of the GLP-2R in the gut in early studies has been a source of controversy. GLP-2R has been reported in enteroendocrine cells, enteric neurons and subepithelial myofibroblast. However, in the murine GI tract, the GLP-2R is expressed exclusively in neurons and myofibroblasts and is not present at the mucosal level (Bjerknes M et al., 2001). It is now generally accepted that the above three cell types express GLP-2R in the intestine.

The relatively high prevalence of the GLP-2R in the gut might explain why, to date, GLP-2 mediated effects have been observed almost exclusively in the GI tract (Sinclair EM et al., 2005). Because the GLP-2R is expressed in the subepithelial myofibroblasts and in the enteric nervous system as well as human enteroendocrine cells, and not on the crypt cells or enterocytes

themselves, it has been proposed that GLP-2 exerts its actions on the mucosa via intermediate effectors derived from GLP-2R-expressing cells (Yusta B et al., 2000) as mentioned earlier.

Determining how GLP-2 produces its biological effects, which mediators are involved and how these mediators interact is an area of intense research.

1.4.3 Physiological effects of GLP-2

GLP-2 was first discovered as an intestinotrophic factor in 1996; today, it is recognised as a hormone that influences multiple functions specifically in the GI tract. The main biological effects of GLP-2 are related to the regulation of energy absorption and maintenance of mucosal morphology, function and integrity of the intestine (Drucker DJ et al., 1996). However, in considering the actions of GLP-2, it is important to note that this peptide has been found to exhibit different actions in different species (i.e. rodents, pigs and humans). As mentioned earlier, over the last decade, it has become evident that GLP-2 (and its analogues) play a growing role as a therapeutic in the treatment of short bowel syndrome (SBS) with a good safety profile based on its specific effects on fluid absorption. (Wallis K et al., 2009; Yazbeck R et al., 2010). The role of GLP-2 on absorption of fluid will be discussed in more depth at the end of the chapter.

Intestinotrophic Effects

A key beneficial effect of GLP-2 on the gut is its ability to increase intestinal growth owing to the enhancement of crypt cell proliferation and inhibition of apoptosis, resulting in expansion of villus height (Figure 1.9). GLP-2 appears to act through intestinal IGF-1 to induce intestinal growth and crypt cell proliferation (Figure 1.10).

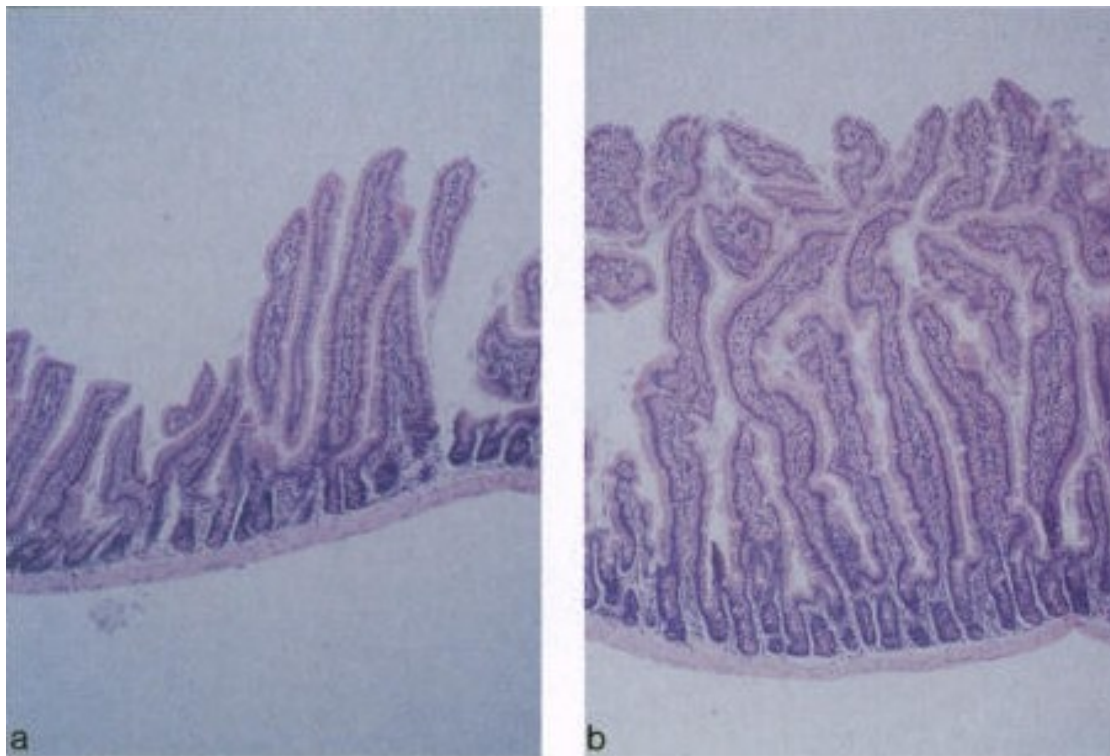


Figure 1.9: Effect of GLP-2 on murine small intestine.

Histological appearance of small intestine epithelium from control (a) and GLP-2-injected (10 days) (b) mice. (Reprinted with permission – Katharina Wallis PhD Thesis 2009)

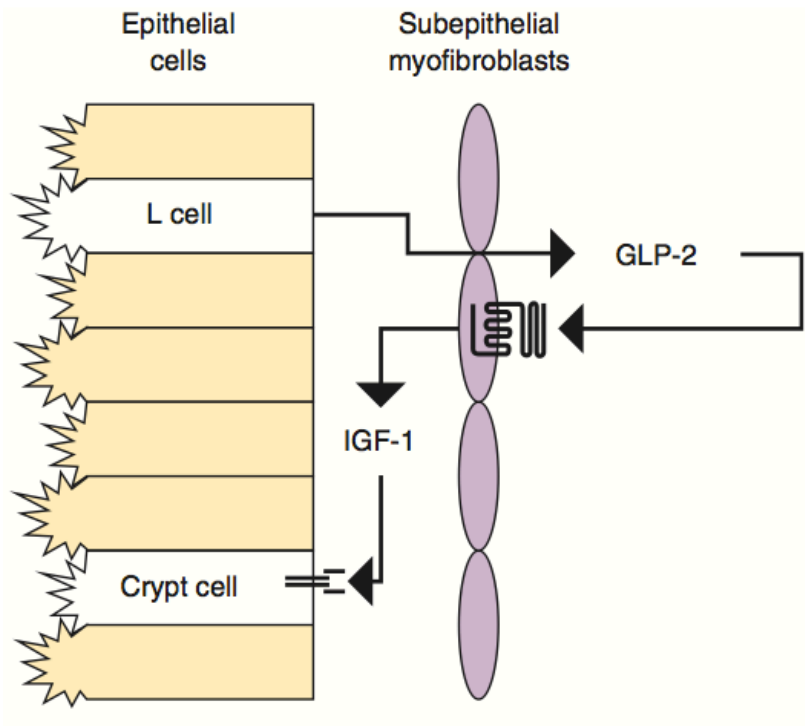


Figure 1.10. Schematic representation of interactions between GLP-2 and IGF-1 in the regulation of intestinal growth.

After secretion by the intestinal L cell into the circulation, GLP-2 activates the G protein coupled GLP- 2 receptor in the subepithelial myofibroblast cells, which subtend the epithelium as a syncytium. This leads to release of IGF-1, which then acts in a paracrine fashion on the tyrosine kinase IGF-1R expressed in the proliferative compartment of the crypt (Dube PE et al., 2008).

However, the mechanisms through which GLP-2 affects the epithelium in an IGF-1 dependent manner have not been fully explained. Studies in murine intestinal subepithelial myofibroblasts suggest that the phosphatidylinositol 3 kinase/ Akt pathway may be implicated in the stimulatory effects of GLP-2 (Leen JL et al., 2011). These findings provide further evidence that IGF-1

produced by intestinal subepithelial myofibroblast cells play a key role in the intestinotrophic effects of GLP-2.

A number of studies have demonstrated that exogenously administered GLP-2 is trophic for the small intestine and, to a lesser extent, the colon. Administration of exogenous GLP-2 to rats during and after massive bowel resection augmented adaptive growth in the residual small intestine without compromising endogenous GLP-2 production and secretion (Koopman MC et al., 2008). Sustained administration of GLP-2 is necessary for intestinal adaptation, and benefits are lost when exogenous GLP-2 is discontinued. Similar trophic and functional responses to exogenous GLP-2 administration are seen in adult patients in whom the terminal ileum and colon have been resected (Jeppesen PB et al., 2001; Jeppesen PB et al., 2005). Adaptive responses are impaired in these individuals, who have limited meal-stimulated GLP-2 secretion due to removal of GLP-2 secreting L cells. Treatment with GLP-2 improved intestinal function and nutritional status in these patients.

The association between GLP-2 and intestinal growth/adaptation is most evident in a variety of pathological conditions, including post-resection intestinal adaptation, coeliac disease, parenteral nutrition-induced intestinal atrophy and IBD.

Mucosal Integrity

GLP-2 maintains mucosal integrity by enhancing intestinal barrier function and decreasing transcellular and paracellular epithelial permeability (Benjamin MA et al., 2000). GLP-2 enhances barrier function within the setting of experimental food allergy, stress or diabetes, reducing the uptake of antigen, the secretory response and the number of inflammatory cells (Cameron HL et al., 2005; Cameron HL et al., 2003; Hadjiyanni I et al., 2009). The effects of GLP-2 in increasing barrier function have been confirmed in non-obese diabetic and ob/ob obese murine models. Administration of prebiotic to ob/ob mice induces GLP-2-dependent upregulation of tight junction proteins zonulin-1 and occludin (Cani PD et al., 2009).

Mucosal Immunity

The protective and reparative effects of GLP-2 have also been studied in the context of IBD. Circulating concentration of bioactive GLP-2 were elevated in patients with IBD with active disease compared to healthy controls (Xiao Q et al., 2000) indicating an innate adaptive mechanism to the intestinal injury associated with IBD. Animal investigations have shown that mucosal GLP-2 concentrations are decreased in areas of colonic inflammation (Schmidt PT et al., 2000).

Evidence of GLP-2 potential anti-inflammatory properties surfaced when Sigalet et al looked at the use of GLP-2 in rats with trinitrobenzene sulfonic

acid (TNBS) - induced ileitis or dextran sulphate sodium (DSS) - induced colitis (Sigalet DL et al., 2007). GLP-2 treatment resulted in significant reduction in inflammatory cytokines such as IL-1 β , INF- γ and TNF- α along with reduction in neutrophil activity. This effect has been demonstrated in rat models of ileitis and colitis. GLP-2 treatment, given either immediately or after inflammation, significantly reduced body weight loss, mucosal inflammation indices, inflammatory cytokine levels and inducible NO synthase expression. These effects may be mediated by activity of VIP, which is produced by the enteric nervous system and known to act as an anti-inflammatory agent, because co-administration of selective antagonist for VIP blocked the actions of GLP-2. Notably, the anti-inflammatory activity of GLP-2 was not associated with an increase in the rate of crypt cell proliferation. Instead, crypt cell proliferation and apoptosis within crypts in inflamed tissues were reduced. These findings support a potential additional neural mechanism of action for GLP-2, with therapeutic implications distinct from its role in promoting crypt cell proliferation.

Subsequently, Ivory *et al* confirmed the anti-inflammatory actions of GLP-2 via use of IL-10 knockout (*IL-10^{-/-}*) mouse model and established that this immunomodulatory effect was IL-10 independent (Ivory CPA et al., 2008). GLP-2 altered the mucosal response of inflamed intestinal epithelial cells and macrophages by activation of the SOCS-3 pathway, which antagonizes the IL-6 mediated increase in STAT 3 signalling. The study also confirmed that the anti-inflammatory actions of GLP-2 were IL-10 independent.

ZP1848 and ZP1846 are GLP-2 mimetics recently developed by Zealand Pharma to enhance intestinal repair and attenuate inflammation. More specifically, ZP 1848 is a GLP-2R agonist that is currently in clinical development for the treatment of Crohn's disease (Skarbalienė J et al., 2011). ZP1846 is a GLP-2 peptide analogue that consistently stimulated growth of small intestinal mucosa in mice and decreased the incidence and severity of chemotherapy-induced diarrhoea in rats (Petersen YM et al., 2006).

Energy Absorption

GLP-2 exerts numerous other actions within the GI tract to promote energy absorption. It increases the uptake of luminal nutrients, including sugars and lipids, by augmenting the activity and the expression of nutrient transporters (Hsieh J et al., 2006) and by enhancing the expression of different enzymes involved in digestion. The major clinical benefit shown to date in adult patients is an increase in fluid and electrolyte absorption. In clinical studies, administration of GLP-2 or the degradation-resistant analogue teduglutide has been shown to slightly improve intestinal absorption, as indicated by increases in faecal wet weight (i.e. the measure of fluids and faeces excreted in bowel or ostomy output) and other indices of nutritional status (i.e. absorption of energy, macronutrients and electrolytes) in patients with SBS, even though differences were small and many did not achieve statistical significance (Jeppesen PB et al., 2001).

GLP-2 also increases mesenteric blood flow, thus providing another mechanism to facilitate digestion and absorption of nutrients (Bremholm L et al., 2009). GLP-2 has also been shown to inhibit gastric acid hypersecretion (Wodjdemann M et al., 1999) and intestinal chloride secretion (Baldassano S et al., 2009).

Gastric motility

The effects of GLP-2 and GI motility remain controversial. In animal models, GLP-2 has been demonstrated to reduce antral motility in pigs and decrease fundic tone in mice, leading to an increase of stomach capacity (Amato A et al., 2009). Results regarding the ability of GLP-2 to suppress gastric motility in humans are conflicting, with GLP-2 either having no influence or slowing gastric emptying (Jeppesen PB et al., 2001; Nagell CF et al., 2004). The discrepancies in results may be due to the differences in methodologies used to assess emptying or due to the type of test meal administered (low-calorie liquid meal vs high-calorie solid meal). The effects of GLP-2 on gastric emptying and fundus tone indicate that GLP-2 could influence feeding behavior. However, it is noted that the satiety effect is much more potent with GLP-1.

1.4.4 Clinical use of GLP-2

Short bowel syndrome (SBS) is defined by a combination of symptoms and signs that occur after extensive surgical resection of the intestine. This highly disabling condition is characterized by malabsorption of both fluid and nutrients and, left untreated, can lead to dehydration, malnutrition, and weight loss. The term intestinal failure (IF) applies when an adequate balance of nutrients and water cannot be maintained without dietary support. IF often remains a short-term problem in the postoperative period. However, a small number of patients will require long-term parenteral nutrition (PN) or, in selected cases, intestinal transplantation. Such patients will typically have less than 100 cm of small bowel leading to an end-stoma or less than 50 cm connected to a functioning colon. Although PN has revolutionized IF treatment, it has a significant impact on quality of life and carries considerable risks, mainly hepatic failure, central vein thrombosis, and recurrent sepsis, all of which will reduce life expectancy. Survival following intestinal transplantation is still inferior to that of long-term PN due to the high incidence of graft rejection and other postoperative complications (Pironi L et al., 2008).

To date, the management of SBS or other types of intestinal failure focuses primarily on supplementation of nutrients, fluid and electrolytes via long-term PN and supportive medical management via use of agents that reduce secretion (H₂ receptor blockers, proton pump inhibitors, and octreotide) and motility (codeine, opium, lomotil, and loperamide)(Nightingale J et al., 2006). Hence, a major unmet need exists for treating patients with intestinal failure.

There has been much interest in GLP-2 as a target for SBS-associated intestinal failure. Preclinical studies in animal models of SBS have shown beneficial effects of GLP-2, consisting of increased body weight, restored absorptive capacity of the bowel, improved adaptive growth of the residual bowel, increased villus and mucosal height and improved mucosal antioxidant capacity (Liu X et al., 2006; Scott RB et al., 1998; Washizawa N et al., 2004) (Figure 1.11). Administration of GLP-2 improved nutrient absorption and nutritional status in SBS patients with colectomy, who have normal GLP-2 fasting levels but do not show a post-prandial physiologic increase of the peptide. However, the limiting factor in clinical use of GLP-2 as mentioned previously is the short half-life in circulation (6-7 min); consequently, several DPP-IV resistant GLP-2 analogues have been developed, of which teduglutide has obtained FDA approval.

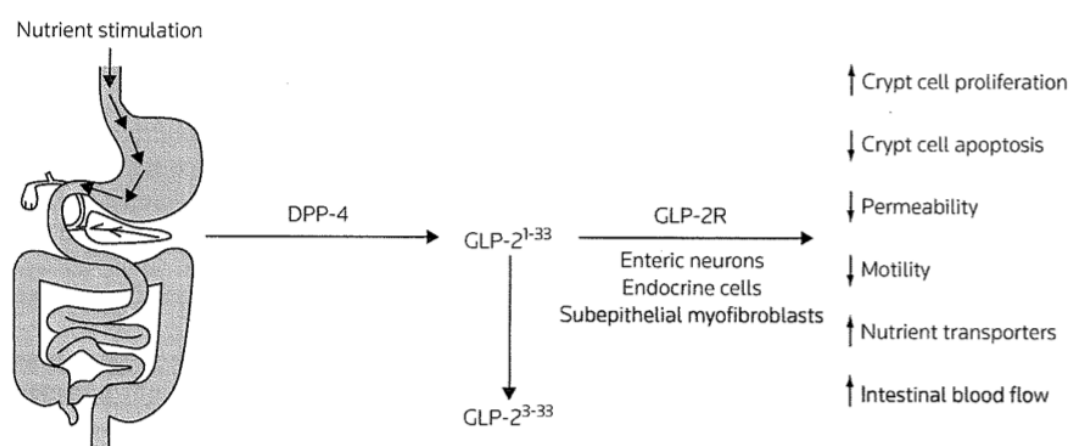


Figure 1.11. The secretion, regulation and biological activity of GLP-2.

Moreover, exogenous GLP-2 analogues may be beneficial in treating other gut-related diseases, such as mucosal damage resulting from radiation, chemotherapy and non-steroidal anti-inflammatory drugs (NSAIDS) usage (Booth C et al., 2004; Boushey RP et al., 1999; Boushey RP et al., 2001). Although teduglutide may have therapeutic benefits at different stages of intestinal disease, the greatest therapeutic efficacy has been observed when the peptide is given before induction of gut injury. In mice with radiation-induced mucositis, for example, teduglutide increased intestinal weight, crypt size, villus height and crypt stem-cell survival when given before irradiation. However, in experimental murine NSAID-induced enteritis, teduglutide improved histological evidence of the disease with a decrease in neutrophil infiltration, whether administered before, concomitant with, or after indomethacin. Consistent with the general mucosal cytoprotective actions of the peptide, findings from a pilot study suggested the potential effectiveness of teduglutide for inducing remission and mucosal healing in patients with active moderate to severe Crohn's disease (Buchman AL et al., 2010)

Teduglutide [h(Gly2) GLP-2, ALX-0600] substitutes glycine in place of alanine in the key second position of the peptide, resulting in resistance to DPP-IV degradation and a longer biological half-life. Teduglutide is expected to be the first orphan drug for the treatment of SBS. In an open-label 21-day study in 16 patients with SBS, teduglutide doses ranging between 0.03 and 0.15mg/kg/d subcutaneous (SC) decreased faecal wet weight and faecal energy excretion and increased wet weight absorption, urine weight and urinary sodium excretion (Jeppesen et al., 2005). These effects were reversed over a 3-week

post-treatment follow-up period. The changes in excretion and absorption were associated with increased villus height, crypt depth and mitotic index in the jejunum, and no changes in these mucosal proliferation indices in the colon.

In the pivotal phase III study (004 study), 83 SBS patients received placebo, teduglutide 0.05mg/kg/d or teduglutide 0.1mg/kg/d SC for 24 weeks and it was shown that the 0.05mg/kg/d group was superior to placebo in achieving a >20% reduction in parenteral fluid volume need and in obtaining a graded response score (a response evaluation taking into account magnitude and duration of reductions in parental fluid need) (Jeppesen PB et al., 2011). Response of similar magnitude of the 0.1mg/kg/d group did not reach statistical significance, probably because of higher baseline values in this group. Oral fluid intake was significantly decreased in the 0.1mg/kg/d group and statistically significant increases in body weight occurred in the two teduglutide dose groups compared with placebo. Sixty-five patients opted to enter an open-label, 28 week extension study (O'Keefe et al., 2013) which showed that patients who received 1 year continuous teduglutide treatment, the mean reduction of weekly parenteral support volume was 4.9L/week equivalent to a 52% reduction from baseline levels.

A second phase III study (020 study) with similar design elements (Figure 1.12) – 24 weeks prospective, randomized, double blind, placebo-controlled, parallel-group, multi-national and multicenter study was conducted in the US, Canada and Europe (Jeppesen et al., 2012). Adults with SBS (>18 years of

age) due to diverse causes (e.g. Crohn's disease, vascular disease, volvulus, injury and other) who were dependent on parenteral support for at least 12 months and at least three times per week were recruited. After an optimization and stabilization period of 16 weeks to achieve a urine output of 1-2L per day, 43 patients were randomized to a 0.05mg/kg/day dose of teduglutide and 43 patients received placebo. The proportion of teduglutide-treated patients achieving a 20-100% reduction of parenteral support at week 20 and 24 was statistically significantly higher compared to placebo (27 out of 43 patients, 62.8% versus 13 out of 43 patients, 30.2%; $p=0.002$). At week 24, teduglutide treatment resulted in a 4.4L/week (32%) reduction in parenteral support volume from a pretreatment baseline of 12.9L/week while maintaining oral fluid intake, urine production and body weight constant throughout the study. Placebo-treated patients had average parenteral support reductions of 2.3 ± 2.7 L/week (21%) at week 24, but they significantly increased their oral fluid intake by 1.6 ± 3.6 L/week ($p<0.009$) in order to maintain urine production constantly. In patients completing the study, 21 patients treated with teduglutide (54%) versus nine on placebo (23%) achieved at least 1-day reduction in parenteral support administration ($p=0.005$).

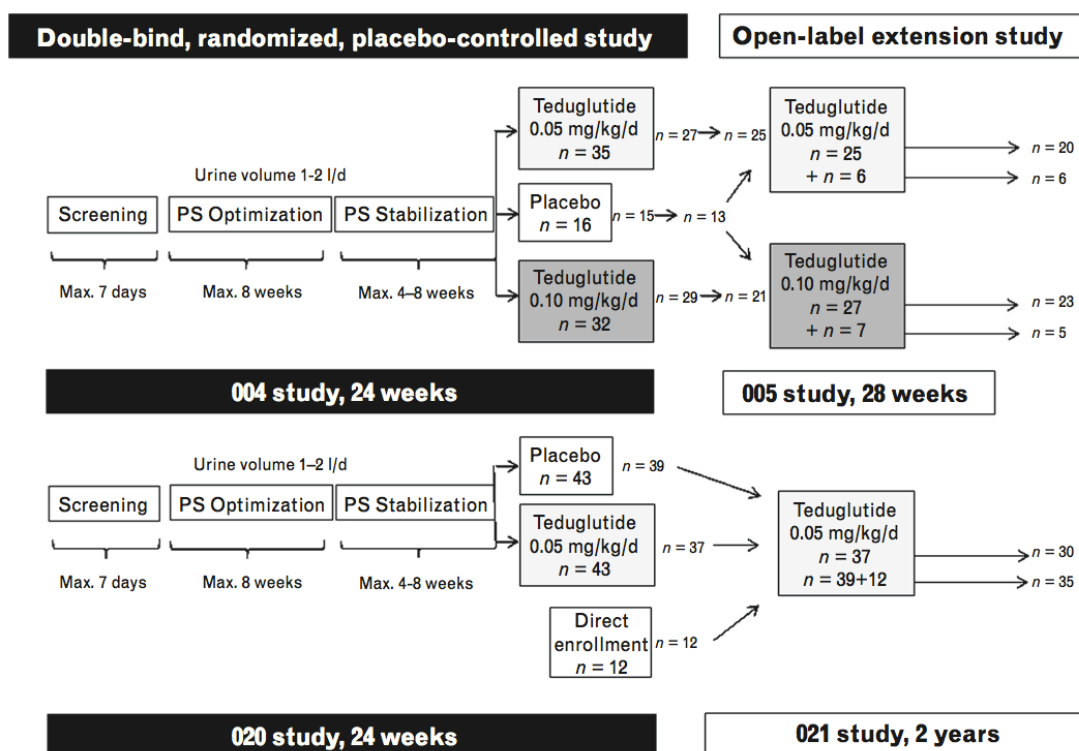


Figure 1.12. Design of the phase 3 placebo-controlled trials of teduglutide in adult patients with SBS-IF.

SBS-IF, short bowel syndrome-associated intestinal failure.

Following the 020 study, a long-term 30-month 021 extension study was carried out where teduglutide was showed to result in addition, clinical meaningful reductions in parenteral support volume from 12.9 L/week at baseline to 4.9L/week after 30 months of treatment. Independence from parenteral support was achieved in 13 patients included in the 020 and 021 studies. In general, teduglutide was well tolerated with the distribution of discontinuation of treatment due to adverse events being similar between patients given teduglutide and placebo. The adverse event profile was generally consistent with the underlying disease condition and the known mechanism of action of teduglutide: the most frequently reported adverse

were gastrointestinal-related (Table 1.1) (NPS Pharmaceuticals). In the 020 study, the overall parenteral support reductions were associated with increases in QoL. Teduglutide significantly improved the scores of 9 of 17 individual items of SBS-QoL scale from baseline at week 24. Effects were not significant compared to placebo (Jeppesen PB et al., 2013).

Adverse reaction	Placebo (n=59) [n (%)]	Teduglutide (n=77) 0.05 mg/kg/day n (%)
Abdominal pain	16 (27)	29 (38)
Upper respiratory tract infection	8 (14)	20 (26)
Nausea	12 (20)	19 (25)
Abdominal distension	1 (2)	15 (20)
Vomiting	6 (10)	9 (12)
Fluid overload	4 (7)	9 (12)
Flatulence	4 (7)	7 (9)
Hypersensitivity	3 (5)	6 (8)
Appetite disorders	2 (3)	5 (7)
Sleep disturbances	0 (0)	4 (5)
Cough	0 (0)	4 (5)
Skin hemorrhage	1 (2)	4 (5)
Patients with stoma		
Gastrointestinal stoma complication ^a	3 (14) ^a	13 (42) ^a

Table 1. Adverse reactions in at least 5% of teduglutide-treated SBS patients and more frequent than placebo in studies 004 and 020.

^aPercentage based on 53 patients with a stoma (n 1/4 22 placebo, and n 1/4 31 teduglutide 0.05 mg/kg/day).

Teduglutide (Gattex) is now approved for use in treatment of patients with short bowel syndrome-associated intestinal failure by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

1.4.5 Safety and tolerability of GLP-2

In human studies, GLP-2 and GLP-2 analogues and agonist have been generally well-tolerated, with the incidence of adverse effects similar to that of placebo-treated subjects. Because GLP-2Rs are found predominantly in the GI tract, to date GLP-2 associated GI adverse effects have been observed in clinical trials. In a 2-year open-label study with teduglutide in 76 SBS patients with intestinal failure, treatment was well tolerated, with the major adverse events being gastrointestinal (22% mainly abdominal pain, distension, nausea and vomiting) (Schwartz LK et al., 2011). No neutralizing antibodies have been reported in published clinical trials.

The potential for carcinogenesis or promoting the growth of subclinical malignancies is a concern with use of GLP-2 or its analogues. The proliferative actions of GLP-2 in the GI tract have been demonstrated to occur in a regulated manner in normal tissue. Findings that GLP-2R mRNA is present in human intestinal carcinoid tumours suggest that GLP-2 has the potential to stimulate the proliferation of neoplastic tissue. However, there has been no evidence of dysplasia or malignancy reported with the use of GLP-2 in humans. Indeed, a recent report suggests that human colon cancer has less expression of GLP-2R protein than the surrounding noncancerous tissue (Bengi G et al., 2011). However, in preclinical models in which a known carcinogen was first used to induce a malignancy, GLP-2 may promote tumourigenesis (Thulesen J et al., 2004). In studies in which a known GI carcinogen was given first to stimulate malignant changes, GLP-2 enhanced

the growth of polyps and tumours; administration of a long acting GLP-2 analogue (Teduglutide) or GLP-2 itself promoted the growth of dimethylhydrazine-induced colonic polyps – tubular adenomas confined to the colonic mucosa- in mice. Although the neoplasms were not cancerous, malignant transformation may occur in time. A recent report documented an increase in dysplasia with GLP-2 in two novel models of inflammation-associated colon cancer. In rats fed the carcinogen 2-Amino-1-methyl-6-phenylimidazo [4,5-b] pyridine and a high-fat diet, 2 of 9 (22%) rats receiving hGly²- GLP-2 developed intestinal cancer compared with 0 of 7 (0%) control rats. In the other set of experiments, mice with chronic dextran sodium-sulphate induced colitis were administered azoxymethane to promote development of colon cancer. Among mice that received control injections, 56% exhibit high-grade dysplasia or colon cancer compared with 64% of mice that received hGly²- GLP-2 and 46% of mice that received a GLP-2 antagonist (Trivedi S et al., 2012).

Although there have been no safety signals of malignancy in the clinical trials for teduglutide, it remains unclear what impact such analogues will have in the long term.

Summary

Given the trophic and immunological effects of native GLP-2 and teduglutide, and the preferential location of GLP-2 receptors within areas of the intestine often affected in patients with IBD, more studies looking into the immunomodulatory actions of GLP-2 would contribute to future translation studies 'from bench to bedside'.

1.5 Hypothesis

We hypothesise that GLP-2 exerts its immunomodulatory actions via dendritic cells that are central in orchestrating the intestinal inflammatory process.

1.6 Aims

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

1. To assess the cellular toxicity of GLP-2 in culture, in preparation for *in vitro* experiments with human DC.
2. To determine the effects of GLP-2 *in vitro* on the maturation and co-stimulatory phenotype of human DC.
3. To determine the effects of GLP-2 *in vitro* on the endocytosis of human DC.

4. To determine the effects of GLP-2 *in vitro* on the cytokine profile on human DC.
5. To determine the effects of GLP-2 *in vitro* on the functional phenotype, stimulatory potency for allogeneic T cells on human blood DC and intestinal DC and T cell cytokine profile.

Chapter 2

General Materials and Methods

2.1 General Materials

2.1.1 Ethical Approval

Ethics Committee and Competent Authority Approvals

The study protocols and associated laboratory investigations of dendritic cell immune regulation in human blood and intestinal biopsy material was secured to investigate “The Effects of GLP-2 on the immune system” and “Dendritic Cell Immune Regulation” using human blood and intestinal biopsy material (Brompton, Harefield & NHLI Research Ethics Committee, Ref. 09/H0708/78 and Brent and Harrow Research Ethics Committee, Ref. 05/Q0405/71). Written informed consent was obtained from all participants, patients and healthy volunteers.

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

2.1.2 Materials

Human Blood Samples

Venesection was performed on participants, patients and healthy volunteers according to the study protocol. Informed consent (EC No.09/H0708/78 and 05/Q0405/71) was obtained prior to peripheral blood sampling. Blood samples for *in vitro* laboratory investigations were collected in 10ml lithium heparin vacutainer tubes (BD Biosciences) and processed within 1 hour of venesection in the Antigen Presentation Research Group (APRG) laboratory.

2.1.2 Human Intestinal Samples

Colonic biopsies were obtained from healthy controls at routine colonoscopy from healthy patients. These patients had macroscopically and histologically normal intestines, and had been referred with symptoms of rectal bleeding or change in bowel habit. Exclusion criteria included inflammatory bowel disease or other inflammatory conditions (e.g. sarcoidosis, tuberculosis, diverticulitis), any current cancer of any type, previous bowel cancer or polyposis syndromes.

Written informed consent was obtained from all patients before the procedure (EC numbers as above). Between eight to ten mucosal biopsies (40- 60 mg of tissue) were taken per patient from the **ileum & colon**. All patients had received either full bowel preparation with magnesium citrate and senna or

macrogol (Klean-Prep). The biopsies were collected in complete medium and transported immediately to the laboratory on ice for processing. All experimental procedures were started within an hour of taking the mucosal biopsies.

2.1.3 Culture Media

Culture Medium

For the culture of both peripheral blood mononuclear cells (PBMCs) and low density cells (LDCs), cells were suspended in RPMI 1640 Dutch modification (Sigma Aldrick Co. Ltd, Irvine, UK) supplemented with 2mM L-glutamine, 100ug/ml streptomycin, and 100units/ml penicillin.

Complete Medium

During prolonged processing or culture of intestinal biopsy and resection specimens, cells were suspended in RPMI-1640 Dutch Modification medium supplemented with 10% foetal calf serum (FCS, Harlan Laboratories), 2mM L-glutamine, 100ug/ml streptomycin, 100 units/ml penicillin (Sigma, Poole, England). For culture of intestinal biopsy samples an additional 25ug/ml gentamicin (Sigma) was added.

2.1.4 Glucagon Like Peptide-2 (GLP-2)

Human GLP-2 (1-33) trifluoroacetate salt with a molecular formula of $C_{165}H_{254}N_{44}O_{55}S$ (estimated 3766.16g molecular mass; 1mg) (BACHEM AG; Hauptstrasse 144, Switzerland H-5662) was diluted in 5uL (35%) ammonia and divided into aliquots of 10mM. Calculated concentrations of $10^{-12}M$ (1pM), $10^{-9}M$ (1nM) and $10^{-6}M$ (1μM) were obtained by dilution in sterile water within each experiment.

2.1.5 Buffers

FACS buffer

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

MiniMACS buffer

MiniMACs Buffer contains PBS 500ml, 0.37g EDTA (2mM) and 2.5g (0.5%) Bovine Serum Albumin (BSA, Sigma, Germany). This was used for washing and labelling cells in the MiniMACSTM magnetic cell separation system (Miltenyi Biotec, Germany).

10x Binding Buffer

10x Binding Buffer (Abcam, UK) was supplied pre-formulated and consisted of 0.1 M Hepes, pH 7.4, 1.4 M NaCl 25 mM CaCl₂ was diluted with distilled water to a working concentration of 1x Binding Buffer. This binding buffer contains an optimal concentration of calcium required during cell labelling for calcium-dependent binding of Annexin V to externalised phosphatidylserine (PS) on cell surfaces.

2.1.6 Reagents

5-carboxyfluorescein-diacetate-succinimidyl ester (CFSE)

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

Dithiothreitol (DTT)

DTT (Sigma-Aldrich) is a strong reducing agent and was added to calcium- and magnesium- free HBSS to remove mucus, faeces and debris from human colonic biopsies.

Ethylenediaminetetraacetic acid (EDTA)

EDTA (Sigma, Dorset) is a chelating agent; it chelates metal ions to prevent cell clustering. It was added to calcium- and magnesium-free HBSS to remove epithelial cells from human colonic biopsies.

Leucoperm A and B

Leucoperm A (Serotec, UK, 100µl per 50µl whole blood/ per tissue) was used as a fixative and contains paraformaldehyde. Leucoperm B (Serotec, UK, 100µl per 50µl whole blood/ per tissue) was used to permeabilise cells and is detergent based.

Ficoll- Paque

Ficoll-PaqueTMPlus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) is a polysaccharide dissolved in an aqueous solution and creates a density gradient at centrifugation for separation of peripheral blood mononuclear cells (PBMC) from whole blood.

FITC-Dextran

FITC-Dextran (Sigma) was used to determine phagocytic capacity of cultured DC by addition to culture at a concentration of 1mg/ml followed by demonstration of fluorescence upon cellular internalisation using Flow Cytometry.

Flow Count Beads

Flow-count™ fluorospheres (Coulter Immunotech, High Wycombe, UK) were used as a reference population during flow cytometry analysis of human cells for the calculation of absolute cell numbers.

Foetal calf serum (FCS)

FCS (TCS cellworks, Buckingham, UK) was added to cell culture media to provide growth factors for cells. FCS also blocks non-specific binding during monoclonal antibody labelling. Stored in aliquots at -80°C.

Hanks' balanced salt solution (HBSS)

Calcium- and magnesium-free HBSS (Gibco BRL, Paisley, Scotland) was used to wash tissue suspension from human colonic biopsies.

Monensin

Monensin (Sigma, UK) was prepared in ethanol at a stock concentration of 3mM and stored at -70°C. Monensin was further diluted and added to cell cultures to give a final concentration of 3µM. This concentration inhibits intracellular protein transport and causes newly synthesized cytokine to be trapped within the Golgi apparatus of cells.

Nycoprep

NycoPrepTM1.068 (PROGEN Biotechnik GmbH, Germany) was used to isolate low-density cells (LDC) enriched for DC from cell culture suspensions by creating a centrifugal density gradient to separate LDC from the non-adherent fraction of PBMC or colonic “walkout” cells following overnight culture. LDC were used as a source of DC for mixed leucocyte reactions (MLR) with allogeneic T-cells. This was an alternative to a metrizamide density gradient, exposure to which can inadvertently decrease the expression of CD14 by monocytes through stimulation and alter the properties of antigen presenting cells (Kabel et al., 1989).

Paraformaldehyde (PFA)

Paraformaldehyde (BDH chemicals, Poole, UK) was used as a fixative by addition (100-400mL) to cells post antibody-labelling. Labelled cell suspensions could then be stored at 4°C in the dark until flow cytometry acquisition within 48 hours. A stock solution of 1% PFA was created by dissolving in saline (0.85%) with slow heating to 60°C at pH 7.0 – 7.4 and was stored at 4°C.

Propidium Iodide (PI)

Propidium Iodide (Sigma, Poole) is a fluorescent compound that penetrates necrotic and late apoptotic cells. Live cells, or cells with intact cell membranes

do not allow passage of this fluorescent compound and hence it is excluded. Stock solution of 1 mg ml^{-1} was diluted 1:20 in PBS to create a $50\mu\text{g/ml}$ working stock concentration in the Annexin V apoptosis assay. PI was stored, protected from light, at 4°C .

Sodium azide

Sodium azide (Sigma, MO, USA) is an inorganic compound that prevents capping and shedding of monoclonal antibodies used for flow cytometric analysis of cells. Sodium azide (0.02%) is one of the constituents of FACS buffer.

Trypan blue

Trypan blue (Sigma, Poole) is a diazo dye used to selectively colour dead cells and tissue. Live cells, or cells with intact cell membranes are not coloured; hence the dye exclusion method was used to count live cells. Trypan blue was used at a working concentration of 0.4%.

2.1.7 Antibodies

Conjugated antibodies

The following monoclonal antibodies (mAb) and their isotype-matched controls were obtained from the same manufacturer (Table 2)

Table 2. Flurochrome-conjugated antibodies

Abs	Clone	Conjugated	Isotype	Manufacturer
Specificity		Flurochrome	Control	
Annexin V		FITC	N/A	BD Bioscience
B7 integrin	FIB504	FITC/PE	rlgG2a	BD Pharmigen
CCR 4	205410	PE	mlgG2b	R&D Systems
CCR10	314315	APC	rlgG2a	R&D Systems
CD3	UCHT1	FITC/PE	mlgG1	BD Biosciences
CD8	SK1	FITC/APC	mlgG1	BD Biosciences
CD11c	KB90	FITC/PE	mlgG1	Dako Cytomation
CD14	RM052	FITC/PE	mlgG2b	Beckman Coulter
CD40	LOB7/6	FITC/PE	mlg2a	Serotec
CD86	24F	FITC/PE	mlgG1	BD Biosciences
CLA	HECA-452	FITC	rlgM	BD Biosciences
DC-Sign	120507	PE	mlgG2b	R&D System
HLA-DR	G46-6	FITC/APC	mlgG2a	BD Pharmigen
IFN- γ	D9D10	PE	mlgG1	Serotec
IL10	JES3-19F1	APC	rlgG1	BD Bioscience
IL12	Ber-ACT8	APC	mlgG1	BD Bioscience
IL17a	41802	PE	mlgG1	R&D Systems
TLR 2	TLR2.3	FITC/PE	mlg2a	Serotec
TLR 4	HTA 125	FITC/PE	mlg2a	Serotec

mlgG1	X40	FITC/PE	n/a	BD Biosciences
mlgG1	679.1Mc7	PECy5	n/a	Beckman Coulter
mlgG2a	X39	FITC/APC	n/a	BD Biosciences
mlgG2a	G155-178	PE/PeCy5	n/a	BD Pharmigen
rlgG2a	LODNP-16	PE/PeCy5	n/a	Beckman Coulter
rlgG2a	54447	APC	n/a	R&D systems
mlgG2b	133303	PE/APC	n/a	R&D systems
mlgG2b	27-35	PECy5/APC	n/a	BD Pharmigen
rlgM	R4-22	FITC/Biotin	n/a	BD Biosciences

Dendritic Cell Lineage exclusion cocktail

DC lineage cocktail is an optimised, pre-formulated cocktail of PE Cy-5 conjugated anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD34 and anti-CD56 used to exclude non-DC cell lineage at multi-colour flow cytometry. Lineage exclusion is important as there is some cross-reactivity with monocytes and B-cells when staining with CD11c+ / BDCA1+ alone (Personal communication: H.Omar, APRG).

Immunomagnetic beads and cell separation columns

Immunomagnetic beads (Miltenyi Biotec, Germany) conjugated to monoclonal antibodies (MAbs) specific for cell surface antigens (e.g. CD14, HLA-DR, CD3, CD19) were utilised in conjunction with LD magnetic columns to separate cells expressing these markers from a cell suspension using the MiniMACS

apparatus (Miltenyi) system. In a specially designed magnetic field, the immunomagnetically targeted cell is acted upon by well defined magnetic force, while the non-targeted cells are acted upon by a weak diamagnetic force operating in the opposite direction. As a result of this magnetic force, the cells can either be directed in a specific direction or retained in the magnetic field (positive cell fraction), thus facilitating the separation of the targeted from non targeted cells (negative cell fraction). To separate the targeted cell population from cell mixture, there are two main approaches in immunomagnetic separation: positive selection and negative depletion. In positive selection, the target cell population is magnetically labeled and collected in the positive fraction (either collected in an outlet flow or removed after being retained by the device) after running through the separation system. In negative depletion, the desired cells are not immunomagnetically labeled; in contrast the undesired ones are magnetically labeled and subsequently removed. The choice of the approaches depends on a range of factors including the concentrations of the desired cells, the final purity desired, and the commercial availability of magnetic reagents as well as the operation convenience.

2.3 Methods

2.3.1 Separation of Peripheral Blood Mononuclear Cells from Whole Blood

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood over a Ficoll- Paque density gradient. Whole blood was diluted 1:1 in RPMI- 1640 Dutch Modification medium and layered onto the meniscus of 10ml Ficoll-Paque in 50ml plastic tubes (BD Falcon), taking care not to break the interface. This was centrifuged (2000rpm (550g) / 30 minutes / 20°C) without application of the centrifuge brake. PBMC were harvested from the Ficoll- cell interface (Buffy coat layer) with a Pasteur pipette.

PBMC were washed in complete medium (for subsequent culture) or FACS buffer (for immediate antibody labelling), centrifuged (1800rpm / 10min / 20°C), decanted of supernatant and re-suspended as appropriate. This washing step was repeated again (centrifuged at 1500rpm / 5 minutes / 20°C). Ficoll gradient separation yielded approximately $2-4 \times 10^6$ PBMC per ml of whole blood obtained. The total number of mononuclear cells in suspension was calculated by the Trypan blue exclusion viability count.

2.3.2 Viable cell Counting

The Trypan blue dye exclusion method was used to count cells following density gradient separation for addition to culture or the Mixed Leucocyte

Reaction in appropriate numbers. Viable cells exclude the blue dye from their cytoplasm and are recognisable at light microscopy. A known volume of cell suspension (usually 50mL) was diluted (1:5) in culture medium containing Trypan blue (1:5) and dye-excluding cells counted using an improved Neubauer haemocytometer.

The Neubauer Haemocytometer is commonly used to determine cell density (concentration) when in suspension. This glass slide consists of a rectangular indentation that creates a chamber of known volume. This chamber is engraved with a laser-etched perpendicular lines creating a grid of a known area. The raised edges of the haemocytometer hold the glass coverslip 0.1 mm off the marked grid. This gives each square in the grid a defined volume (i.e. $1\text{mm}^2 = 100\text{nL}$). Cell suspension is placed on the chamber under the coverslip, and resultant capillary action completely fills the chamber with the sample. The number of cells in the chamber can be determined by counting cells under a light microscope. The total number of cells per ml can be discovered by simply multiplying the total number viable cells found in a 1mm^2 haemocytometer grid by 104.

2.3.3 Isolation of Low-Density Cells from PBMC

Low density cells enriched for DC were isolated by culturing PBMC for 24-hours at 4×10^6 cells per ml (5ml) in T25 (25cm^2) plastic flasks (Falcon) containing complete medium (37°C in a humidified atmosphere of 5% CO_2). Non-adherent cells were removed by gentle agitation and aspiration of the

suspending media. Low-density cells (LDC) were then separated from the non-adherent cell suspension by density gradient centrifugation over NycoPrep 1.068 at 1700rpm for 15 minutes. LDC were harvested from the NycoPrep interface which was enriched for DC at 30-80%.

2.3.4 Preparation and Processing of Intestinal Tissue

Human intestinal biopsies were obtained at routine colonoscopy. Biopsies were collected in complete medium [RPMI 1640 Dutch modification (Sigma-Aldrich, Dorset, UK) supplemented with 100U/ml penicillin/streptomycin, 25µg/ml L-glutamine and 10% foetal calf serum] with gentamicin (25 mg/l) on ice.

Our laboratory has optimized the walk out technique from mucosal tissue samples in which cells such as DC spontaneously migrate from the tissue sample into the culture media, thereby avoiding destructive and prolonged tissue digestion with collagenase (Bell et al., 2001). The “walkout” technique offered the ability to extract adequate cell numbers for phenotyping and has been favoured over tissue digestion for several reasons: 1) There were concerns over the adverse effects of collagenase on cells during prolonged tissue biopsy digestion (Personal communication Dr Hafid Omar, APRG); and 2) LDC could be pre-conditioned in complete media containing GLP-2 whilst in situ within the biopsy tissue during the walkout process, likely with a limited degree of continued exposure to the gut tissue microenvironment.

Overnight “walkout” cultures were incubated overnight in a humidified incubator at 37°C, gassed at 5% CO₂, in 24-well flat-bottomed plates (Falcon, Becton Dickinson, USA) containing 1mL complete medium with and without GLP-2 at various concentrations. Walkout cells from the individual wells of each GLP-2 conditioning were pooled after separation from the culture medium and remnant biopsy material removed using a cell filter (Beckton Dickinson). Walkout cells were then either labelled with directly conjugated antibodies for phenotypic analysis of gut DC or, following isolation of low-density cells over a NycoPrep gradient, used to stimulate T-cells in the MLR.

2.3.5 Preparing and Culturing MoDC

MoDC were differentiated in culture from CD14⁺ monocytes isolated by positive selection from PBMC using the following protocol.

PBMCs were prepared from peripheral whole blood (50ml) of healthy adult donors over a Ficoll gradient. PBMC were washed twice by suspension in ice cold Mini-MACS buffer and centrifuged (1500rpm / 5 minutes / 40C). Supernatant was decanted, leaving about 300 ml of cell suspension in the residual buffer. Thirty microlitres (30mL) of CD14⁺ immunomagnetic microbeads (MiltenyiBiotec, Germany) were added to the re-suspended cells and incubated on ice for 30 minutes. Afterwards, the cells were again washed twice in mini-MACS buffer as above. Following decanting of the supernatant, cells were again re-suspended in the residual volume of cell buffer. CD14⁺ cells were then separated from the PBMC suspension by positive selection

separation on Mini-MACS columns using the VarioMACSTM Separation System (MiltenyiBiotec, Germany).

The Mini-MACS column, magnet was pre-cooled to 4°C and assembled. The mini-MACS column was flushed through with 2ml of cold mini-MACS and eluent discarded. PBMCs in residual buffer were then added to the column followed by 0.5ml of cold mini- MACS buffer. This eluent contained cell suspension depleted of CD14+ cells and was discarded. CD14+ cells adherent to the column were washed out by removing the column from the magnetic field and adding mini-Macs buffer (1ml). This was forced through the column using the plunger provided. This eluent contained the purified CD14+ cells (monocytes) and was retained.

The CD14+ cell suspension was washed with mini-MACS buffer as above and centrifuged at 1400rpm / 5 minutes / 40C. After removing the supernatant, the cells were re-suspended in 4mls of complete medium and centrifuged at 1500rpm / 5 minutes / 20°C. This was in order to wash out EDTA from the cells before the cell culture. After removing the supernatant, the cells were re-suspended in complete medium (1 ml) and a cell count taken in a Neubauer's chamber.

The CD14+ cells were diluted in complete medium and cultured in wells of a 24-well plate at a density of 500,000 cells per ml per well. The final culture medium contained complete medium supplemented with 0.1µg/ml Granulocyte macrophage colony stimulating factor (GM-CSF, Promega) and

50U/ml IL-4 (Promega). On day 4 of the cell culture, 0.5 ml of medium was removed from the top portion of each well (cells are adherent and lay at the bottom of the wells) and replaced with 0.5ml of complete fresh medium containing twice the concentrations of GM-CSF and IL-4. Therefore, after mixing the medium in each well, the final cytokine concentration was replenished (GM-CSF: 0.1mg/ml/well, IL-4: 50 U/ml/well). On day 5, the cells (now monocyte-derived dendritic cells, MoDC) were gently re-suspended in the culture medium using a Pasteur pipette.

The cell culture was assayed using flow cytometry on day 6 to confirm differentiation of monocytes into MoDC, demonstrating loss of surface CD14 expression but positivity for HLA-DR. MoDC were then simply identified according to the FSC:SSC scatterplots (Figure 2.1).

For stimulation experiments, MoDC were cultured for an additional 24 hours at a concentration of 200,000 cells/ml, either in basal culture (internal control for each experiment) or after supplementation with GLP-2 at final concentrations of 10^{-6} M, 10^{-9} M and 10^{-12} M. Each condition was assayed in at least 3 independent experiments (triplicate).

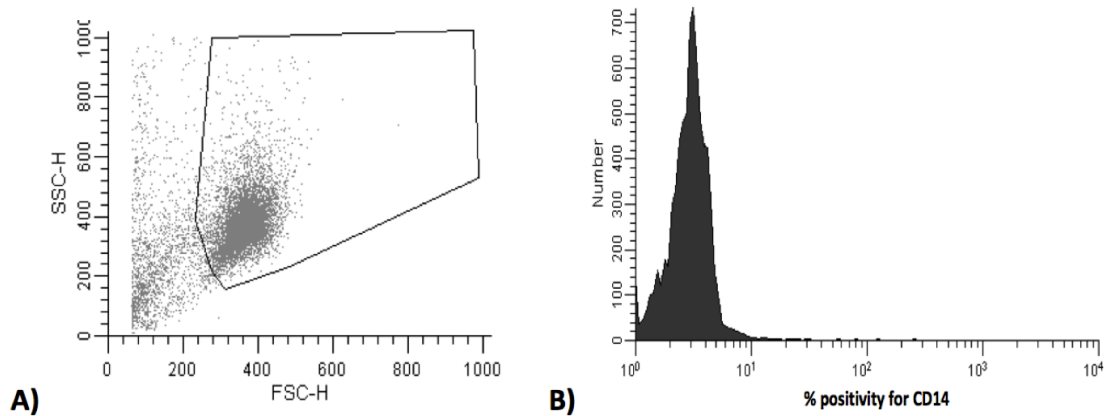


Figure 2.1 Identification of MoDC flow cytometry.

A) Viable cell FSC:SSC plot (Gated); B) Gating on viable cell population (Gated) reveals cells are MoDC (negative for DC-exclusion cocktail expression CD14 expression).

2.3.6 DC Endocytosis Assay

FITC-dextran (1mg/ml) was added to each culture condition for the final two hours of culture. Immediately after the addition of FITC-Dextran, half of the labelled cell suspension volume (500ml) was transferred into a new tube and incubated at 4°C for two hours, while a parallel tube was incubated at 37°C. Therefore, at physiological temperature DC were able to phagocytose the FITC-labelled dextran after differential conditioning in the presence of GLP-2 or basal medium. In contrast, cell metabolism was arrested in those cells incubated at 4°C; hence these cells were used as an internal negative control of the assay during flow cytometry. DC were cultured for 24 hours at a concentration of 200,000 cells/ml, either in basal culture (internal control of each experiment) or after supplementation with GLP-2 at final concentrations

of 10^{-6} M, 10^{-9} M and 10^{-12} M. Each condition was assayed in at least 3 independent experiments (each in triplicate).

2.3.7 Cytokine Labelling

For intracellular cytokine labelling, paired cultures of gut DC, 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% CO₂ in air. Cells were labelled for surface markers for 20 minutes on ice, fixed with 50µL leucoperm A and permeabilized with 100µL leucoperm B 5 µL of anti-cytokine antibody (IL-10-APC, IL-12-APC, IL-17-PE, IFN- γ -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.

2.3.8 Mixed Leucocyte Reaction

Preparation of T-cells for Mixed Leucocyte Reaction

PBMC were isolated from healthy donor blood (50ml) and suspended in 5ml complete medium before counting. Approximately 2×10^6 PBMC were removed and kept on ice. The remaining PBMC were washed twice by suspension in MiniMACs buffer with centrifugation (1400rpm / 5 minutes /

4°C). Supernatant was discarded and PBMC were re-suspended in the residual buffer (~200mL).

T-cells were then separated from the PBMC suspension using negative selection to deplete CD19, CD14 and HLA-DR cells (including monocytes, B-lymphocytes and antigen-presenting cells such as macrophages and dendritic cells). Anti-CD19, anti-CD14 and anti-HLA-DR microbeads (30uL each) were added to the cell suspension and incubated on ice for 20 minutes. In the meantime, the magnetic cell sorting system (VarioMACSTM Separation System - Miltenyi Biotec) was set-up and columns washed through with MiniMACS buffer.

PBMCs incubated with the microbeads were added directly to the column and allowed to run through, followed by 4 ml of cold mini-MACS buffer. This eluent contained T-cells depleted of CD19, CD14 and HLA-DR cells which were retained. In order to increase T-cell purity, a new depletion column was set-up and the T-cell eluent run through again, before washing twice in cold PBS with centrifugation (1200rpm / 5 minutes / 4°C). Supernatant was decanted and the T-cell pellet re-suspended in 3ml PBS.

Five microlitres of stock CFSE (100uL) was suspended in 10ml PBS (CFSE1). Three millilitres (3ml) of this solution was then diluted in a further 3ml PBS (CFSE2). This was added to the T-cell suspension, mixed and left for 1 minute 30 seconds. Immediately after this time period, the T-cell / CFSE suspension was vigorously mixed by shaking and left for a further 1 minute 30

seconds. Foetal calf serum (6ml) was then added to quench free CFSE. The CFSE- labelled T-cells were then washed twice by adding complete media and centrifugation (1200rpm, 5min). Supernatant was decanted and the T-cell pellet re-suspended in 1ml of complete medium. A Trypan blue cell count was then performed to determine T-cell concentration.

Mixed Leucocyte Reaction (MLR)

DC-rich low density cells from intestinal biopsies or blood were pre-conditioned by culture for 24-hours in 5ml polystyrene round-bottom (FACS) tubes (BD Falcon™) containing 500,000 cells in 1ml complete medium with or without GLP-2 (10^{-6} M, 10^{-9} M and 10^{-12} M). Cells were pooled into respective control and GLP-2 groups and washed in complete medium by centrifugation twice (1400 rpm for 5 minutes).

Graded numbers of pre-conditioned LDC were added at concentrations equating to 0%, 1%, 2% and 3 % of the T-cell population (4×10^4 T-cells /well) to stimulate CFSE-labelled allogeneic T-cells in 96-well round-bottom plates made up to 200uL complete medium per well and incubated at 37°C in a humidified atmosphere of 5% CO₂ for 5 days.

Proliferation of T-cells leads to reduced CFSE-fluorescence in dividing cells compared to the original non-divided CFSE-labelled T-cell population. Consequently, homing marker expression by a specific proliferating T-cell

population, in response to stimulation by pre- conditioned LDC, could be determined.

2.3.9 Annexin Apoptosis Assay

Cells undergoing the early phases of apoptosis express phospholipid phosphatidylserine (PS) on outer layer of their cell membranes. Annexin V is a Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS. Annexin can be conjugated with a flurochrome (FITC). Cells in late apoptosis cannot exclude the dye propidium iodide (PI). These properties enable the identification of cells undergoing early and late apoptosis (Vermes et al., 1995). PBMC were diluted in complete medium at $1 \times 10^6/\text{ml}$. PBMC or LDC were then incubated with complete medium or complete medium containing GLP-2 at several concentrations (10^{-6}M , 10^{-9}M and 10^{-12}M) for time periods 4, 24 and 48 hours.

Upon completion of culture, cells were washed twice in cold PBS with centrifugation (1700rpm / 5minutes / 4°C). Following decanting of supernatant, the cell pellet was re- suspended in 1x Binding buffer. One hundred microlitres (100 μl) of each cell suspension were removed to a series of 5ml polystyrene round-bottomed tubes (BD Falcon TM). Cells were labelled with 5ml Annexin V– FITC and 10ml Propidium Iodide per tube as appropriate and incubated for 15 minutes at room temperature in the dark. After incubation, 400 μL of 1x binding buffer was added per tube. Samples were then immediately acquired by flow cytometry without online compensation.

2.3.10 Identification of Cell Populations and Phenotype

Conjugated Antibody Labelling

Cell suspensions were washed into FACS buffer before labelling in 5ml polystyrene round- bottom tubes (BD Falcon™) and placed on ice in the dark for 20 minutes. To reduce non- specific antibody binding, 15 mL FCS was added to each labelled tube prior to labelling. Cells were then washed twice into FACS buffer (1400 rpm for 5 minutes at 4°C). For labelling with directly conjugated antibodies, cells were fixed at this point with 1% PFA. In the absence of a directly conjugated antibody, for example there is no APC-labelled CLA conjugate, an addition step was required prior to fixation. For indirect labelling, the cells were first incubated with the required biotinylated antibody (CLA-biotin), washed and then re- incubated with Streptavidin conjugated to the appropriate flurochrome dye (e.g. Streptavidin-APC). Unoccupied binding sites were blocked with the addition of 15 mL FCS before adding antibody at each labelling step. Cells were then washed twice into FACS buffer and then fixed in 1% PFA. Fixed cells were stored at 4°C prior to acquisition on the flow cytometer, within 48 hours.

Flow cytometry and Data Analysis

Flow cytometry acquisition

Flow cytometry allows simultaneous multiparametric analysis of the physical characteristics of thousands of cells per second, as they pass through a one or more focused laser beams whilst in suspension. As the cells pass through the beams, they disrupt and scatter light in two different planes; the degree of scatter in each plane is characteristic of the cells' size (forward scatter/FSC) and granularity (side scatter/SSC). Monoclonal antibodies conjugated to fluorescent dyes and attached to cells can be detected by colour specific detectors (channels) of the flow cytometer. The machine registers the fluorescence generated as cells pass through it, enabling analysis of phenotypic properties of the cells.

Cell data were acquired using a 4-colour FACSCalibur flow cytometer (Becton-Dickinson Biosciences) capable of detecting the flurochrome dyes FITC, PE, PECy5 and APC which correspond to 4 detector channels for specific wavelengths (FL1, FL2, FL3 and FL4 respectively). CellQuest software (Becton-Dickinson) was used for partial online compensation prior to cell acquisition, and generation of listmode data files. Total compensation and analysis of flow cytometry listmode data was carried out offline using WinListTM software (Verity Software House, Maine).

Partial online compensation required the use of 5 compensation tubes containing PBMC either unlabelled or single-labelled with fluoro-chrome-conjugated mAb to CD3⁺ or CD8⁺ (1 tube for each fluoro-chrome: FITC, PE, PECy5 and APC). The compensation tube containing unlabelled PBMC cell suspension was initially used to set the amperes and voltages on the machine, based on the cells' background or auto-fluorescence. First the FSC:SSC plot was adjusted using the FSC gain and range and SSC voltage to place the cell population of interest in the centre of FSC vs SSC dot plot and to eliminate most of the fine subcellular debris.

Data was acquired partially compensated online during flow cytometer acquisition. For partial online compensation, a region was drawn around the lymphocyte region on the FSC versus SSC plot and the cell suspensions labelled with each fluoro-chrome conjugated CD3⁺ or CD8⁺ mAb were analysed. Compensation involved adjusting on the flow cytometer so that autofluorescence of the unlabelled cells runs in the middle of the first decade on the four decade log scale (between 100 and 101). This was repeated for all fluorescence parameters (FL1, FL2, FL3). Samples were then acquired and saved as listmode data files.

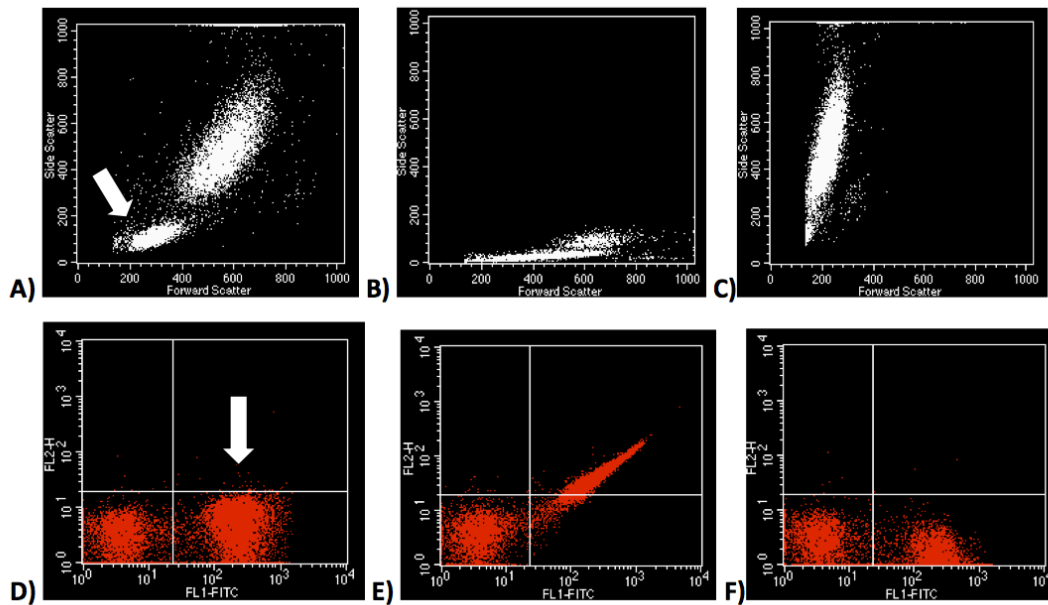


Figure 2.2 CellQuest screenshots of FSC:SSC plot during Data Acquisition Plot set-up.

A) Correctly set scatter plot showing lymphocyte region (arrow), B) SSC too low and C) FSC too low. CellQuest screen shots during partial compensation of FL1 using FITC- CD8⁺ labelled lymphocytes prior to data acquisition: D) Correctly compensated, showing background autofluorescence (white arrow) and fluorescence due to FITC antibody labelling, E) Under Compensated and F) Over Compensated.

WinList™ Analysis

Completion of compensation

Compensation was completed offline using compensation toolbox on the WinList™ software program, using lymphocyte populations labelled with mAbs specific for T-cells. Each individual experiment had specific compensation settings, saved as compensation files.

Additional offline compensation of acquired data was necessary to remove artefact arising as a result of background cellular fluorescence and “spill-over” of fluorochrome fluorescence in the 2nd log across the different channel wavelengths, particularly between neighbouring fluorochrome channels i.e. PE(FL2) needed to be compensated from FITC(FL1) and PE-Cy5(FL3).

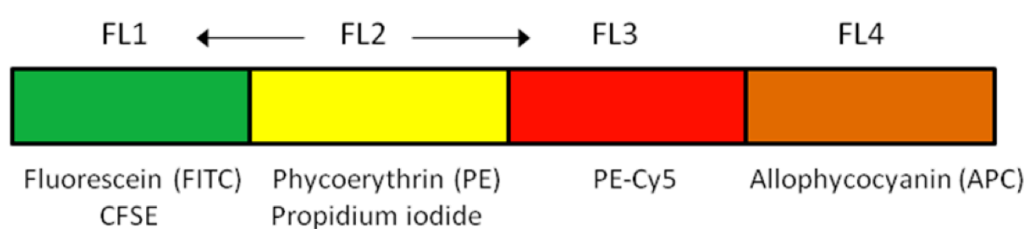


Figure 2.3. Compensation involved drawing a region around the recognised lymphocyte population of the FSC versus SSC histogram plot.

Single-colour cell samples labelled only with either anti- CD8 or anti-CD3 were evaluated for each colour channel (FL1 - 4).

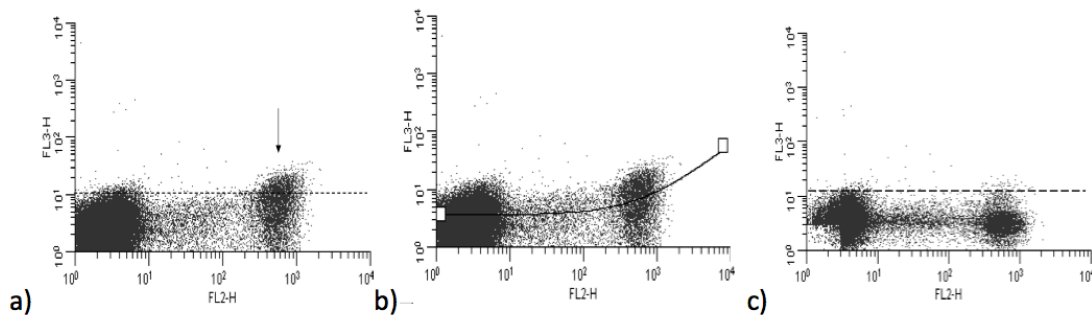


Figure 2.4 Winlist offline compensation.

PE fluorescence in channel FL2: PE- CD8⁺ mAb labelled lymphocytes show the PE signal fluoresces a) mostly in the FL2 channel, but bleeds over into the 2nd log decade (>101) of the FL3 channel, b) Correction of compensation parameter and c) Correct and complete compensation with minimal spill over.

Gating and Subtraction of Isotype Control

Region Method of Subtraction (RMS)

The percentage of cells expressing a given surface marker was measured by determining the proportion of fluoro-chrome-conjugated mAb stained cells demonstrating increased fluorescence beyond that due to staining with an isotype matched control antibody. Measurement of this difference in fluorescence can be achieved by 2 distinct methods during Winlist analysis: 1) Region gating and 2) Enhanced normalised subtraction.

A region is drawn that excludes irrelevant, isotype matched control mAb or non specific staining (Figure 2.5a). All cell events that fall within this region exceed isotype control staining fluorescent intensity and are regarded as positively labelled cells. Hence, in the cell sample labelled with the mAb of interest, any fluorescence detected within this region (beyond the distribution of the isotype staining) is a positively labelled cell (Figure 2.5b). The WinListTM software calculates the proportion of cells in any given region relative to that of the cell population on which it is gated. In this manner, regions can be drawn on single parameter histograms representing positive and negative cell populations, e.g. CD11c⁺ and CD11c⁻ DC, for putative mDC and pDC populations respectively. When using the region gating method, the level of staining was quantified as the mean fluorescence intensity (MFI).

This method discounts all events that fall within the distribution of the control histogram, more accurately delineates cell subpopulations based on surface antigen expression and reduces impact of differences in fluorochrome (antibody binding and free fluorochrome variation between batches of antibody).

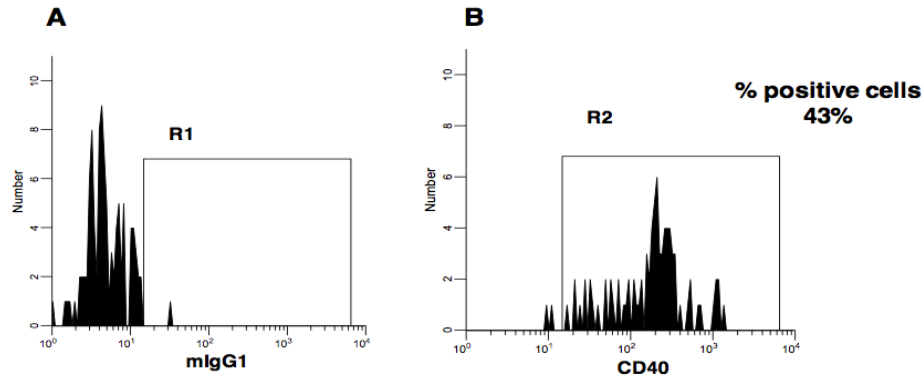


Figure 2.5: Cell surface labelling by region gating.

A region is drawn that excludes non-specific staining or isotype matched control monoclonal antibody staining. The same region is applied to the phenotype staining histogram (B). All events that fall within this region and exceed isotype control staining were 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 accurately delineates subpopulations based upon high surface antigen expression.

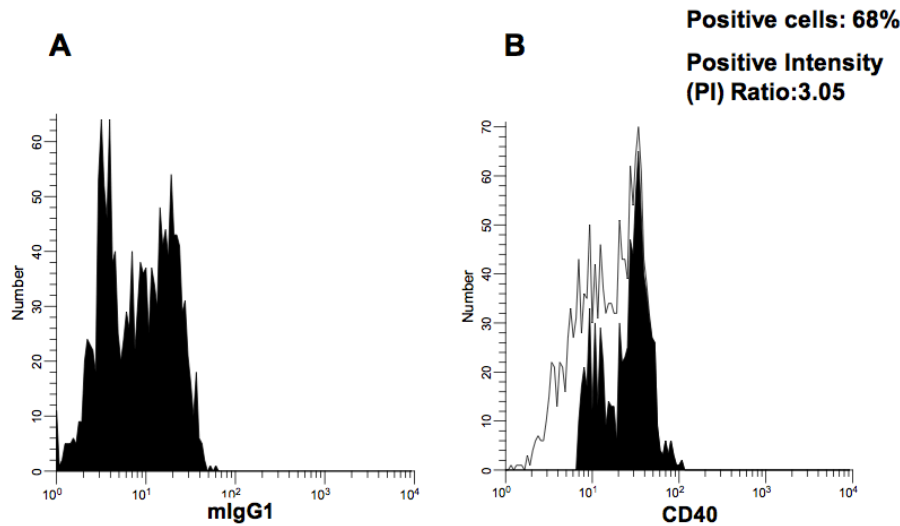


Figure 2.6: Positive Intensity Ratio.

(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 intensity ratio (PI Ratio) 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 PI ratio compared the relative intensities of positive events in a test sample (B) compared with all events in control distributions (A).

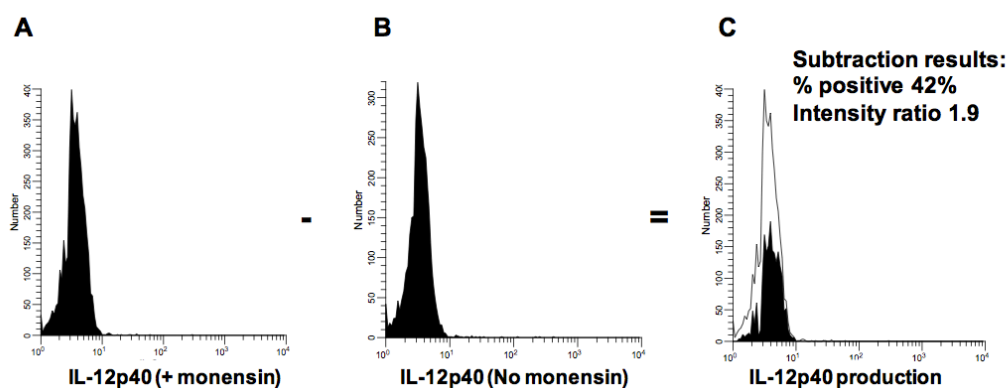


Figure 2.7: Intracellular cytokine production by gut DC measured with Enhanced Normalised Subtraction (ENS).

Detection of low amounts of ongoing intracellular cytokine production in DC & T-cells were measured by Enhanced Normalised Subtraction (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. The IL12 in the sample without monensin acted as a reference sample so that when more “positive events” in this control histogram were detected compared with the monensin histogram, subtraction was reversed indicating loss cytokine.

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.*, 2003) (Figure 2.7).

The percentage of cytokine-positive cells was determined by superenhanced Dmax (SED) normalized subtraction. Normalized cumulative histograms of staining of cells cultured without monensin (control) 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); 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. The subtraction technique allows a more accurate calculation of the proportion positively labelled cells especially when events are low or when the test and controls histograms overlap. Conventional methods of analysis (region gating) disregard any portion of the test histogram that falls within the distribution of

the control histogram. In addition, a region drawn to exclude the control histogram removes “low positive” cells from the analysis and may underestimate the true proportion of positively labelled cells. The use of the same antibody to label cells from both the monensin- treated and untreated cultures gives this technique a high level of sensitivity for detecting small changes in antibody binding. An example is shown in Figure 2.6.

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).

Absolute cell numbers

Absolute cell counts were obtained by simultaneous acquisition of Flow-count™ fluorospheres. Fluorescent counting beads were identified using the WinList™ software and enumerated on a FL1 versus SCC plot. Absolute numbers of cells expressing a marker of interest or simply of types of cells themselves were then calculated by:

$$\frac{\text{number of events in region of interest}}{\text{number of events in bead region}} \times \left(\frac{\text{volume of sample } (\mu\text{l})}{\text{volume of beads } (\mu\text{l})} \right) \times \text{bead number}$$

2.3.11 Statistical Analysis

The student t-test was frequently used in statistical analysis. The unpaired t-test was used to compare 2 different groups. A paired, two-tailed t-test was used to determine statistical significance when analysing samples from the same subject exposed to different experimental conditions. For example, LDC derived from the same donor sample and exposed to control or GLP-2 conditions in culture were compared using the 2-tailed T-test.

Other statistical analysis were performed as appropriate, including the 2-way analysis of variance (ANOVA) with Bonferroni correction (corrects for a potential type 1 error based on n-number) when comparing more than a 2 groups of data. The Pearson correlation was used for correlative data. $P < 0.05$ was considered statistically significant (data marked *) whilst $p < 0.001$ was highly significant (data marked ***). Software programmes utilised included Microsoft ExcelTM and Prism GraphPadTM (Graphpad Software Inc. San Diego, US).

Chapter 3

Cytotoxicity of GLP-2 on Human Dendritic Cell *in vitro*

Abstract

Background

The interpretation of a cell phenotype and function in response to experimental interventions in cell culture can be potentially influenced by selective cell death or toxicity when compared to control populations. To this end, it was important to determine the potential of GLP-2 to induce cellular toxicity that may result in apoptosis or necrosis, prior to conducting *in vitro* culture experiments.

Aims

To investigate cellular toxicity of GLP-2 *in vitro* by determining apoptosis or necrosis of PBMC and LDC in culture.

Methods

PBMC from healthy volunteers were cultured in complete medium containing GLP-2 at several concentrations (1pM, 1nM and 1μM) over multiple time periods (0, 4, 24 and 48 hours). Cell viability from PBMC was demonstrated with Trypan Blue exclusion test. Multi-colour flow cytometry to determine external cell membrane expression of phosphatidylserine (PS), by surface staining with fluorochrome-labelled Annexin V and exclusion of Propidium iodide (PI) from the cytoplasm was used to assess cell viability, apoptosis and necrosis from LDC.

Results

Trypan blue exclusion testing did not indicate significant differences in PBMC viability in response to culture with GLP-2. The Annexin V-PI assay revealed LDC conditioned in culture with GLP-2 did not exhibit significantly different proportions of cells in early & late apoptosis (Annexin⁺PI⁻ & Annexin⁺PI⁺), late apoptosis (Annexin⁺PI⁺) or necrosis (Annexin⁻PI⁺) when compared to control cultures.

Conclusion

GLP-2 did not influence rates of PBMC and LDC viability, apoptosis or necrosis. This data provides a degree of confidence when interpreting any future observations of the effects of GLP-2 on cell phenotype and function in culture, which are not likely to represent disproportionate rates of cell apoptosis or death.

3. Introduction

3.1 Effects of GLP-2 on Dendritic Cell viability, apoptosis or necrosis

The interpretation of data representing an experimental intervention in cell culture can be influenced by associated direct or indirect toxicity. This may lead to damage or death to select cell populations resulting in misinterpretation of apparent changes in phenotype and function accredited to the intervention in question. In order to measure toxicity of a substrate in culture, demonstration of cell viability and lack of necrosis or cell death is required. The Trypan blue exclusion test is commonly used to identify viable cells; however, cells in the early stages of apoptosis that have compromised viability and function, may still be able to exclude Trypan Blue from their cytoplasm. Therefore a more accurate, alternative method to determine cell culture viability and necrosis involves the Annexin V – propidium iodide fluorocytometric assay which has the potential to identify cell viability and necrosis as well as indicate if cells are undergoing early or late apoptosis.

3.1.1 Trypan blue exclusion tests

Determining the number of viable cells in culture is important to standardize culture conditions and perform accurate quantitation experiments. The Trypan blue exclusion test is the commonest and simplest used method of determining cell viability in cultured cell populations. Trypan blue is an organic toluidine-based dye, so-called because it can kill trypanosomes, the parasites

that cause trypanosomiasis (African sleeping sickness). The Trypan blue exclusion test is based on the principle that live (viable) cells possess a cell membrane with the functional integrity to actively exclude certain dyes from their cytoplasm. Determination of cell viability requires a haemocytometer, a thick glass slide with a central area designed as a counting chamber, to which a known volume of cell suspension can be applied to a defined area and hence, cells can be counted such that cell density can be calculated. Live cells demonstrate an unstained cytoplasm, whilst in contrast dead cells cannot exclude Trypan blue and appear stained (blue) under the light microscope. Using this method however does not take into account that cells with compromised viability with transient intact cell membranes in the early stages of apoptosis are still able to exclude the dye. Furthermore, fluorometric assays of viability in culture may have less ambiguity in the identification of stained, non-viable cells in comparison to the haemocytometer-based 'degree of blueness' Trypan blue dye exclusion cell quantitation and viability assays (Altman et al. 1993).

3.1.2 Phosphatidylserine expression, Annexin and Apoptosis

The Annexin V – Propidium Iodide (PI) assay provides a simple and effective method to detect apoptosis at an early stage. This assay takes advantage of the fact that the phospholipid phosphatidylserine (PS) is translocated from the inner (cytoplasmic) leaflet of the plasma membrane to the outer (cell surface) leaflet soon after the induction of apoptosis, becoming available to bind fluorochrome-labelled Annexin V which has a strong, specific affinity for PS.

Fluorocytometric determination of surface binding of fluorochrome-conjugated Annexin V can be simultaneously correlated with the ability of cells to exclude the fluorescent dye PI, enabling identification of viable, early apoptotic, late apoptotic and necrotic cells. Necrotic cells or cells in the late stages of apoptosis can be identified using PI, an intercalating agent for double-stranded DNA which produces a highly fluorescent adduct that can be detected during flow cytometry. PI is not excluded by necrotic cells or cells in late apoptosis and after entering the cell, intercalates with DNA. Viable and early apoptotic cells, which still have a functionally intact membrane capable of excluding PI, are not stained (Darzynkiewicz et al., 2000).

However, the Annexin V – Propidium Iodide (PI) assay does have potential flaws. Firstly, PS externalization is not uniquely associated with apoptosis. For example, the majority of mature B-cells exposed PS on their surface, a feature that is not specifically associated with apoptosis (Dillon et al., 2001). Macrophages also appear to constitutively exhibit a degree of PS expression. In contrast, viable precursor monocytes do not express external PS and cannot bind Annexin V.

Secondly, PS expression is required not just on the target cell surface, but also on the macrophage that engulfs the apoptotic cells (Callahan et al., 2000). Indeed, the blockade of PS by pre-treatment with Annexin V inhibits the phagocytic activity of macrophages. However, if both apoptotic cells and some types of viable cells can externalize their PS, this may create confusing signals for macrophages committed to recognizing and eliminating only dead

or dying cells. Consequently, the amount of externalized PS may be critical for macrophage discrimination between apoptotic and non-apoptotic cells (Callahan et al., 2003). The most common assays of PS externalization are based on fluorescent labelling with an Annexin-fluorescein isothiocyanate conjugate (Annexin V – FITC) and subsequent analysis by flow cytometry. These assays only assess the proportion of apparently apoptotic cells with external PS rather than determining the actual amounts expressed; by arbitrarily assigning 'low' levels of FITC fluorescence to viable cells with negligible PS externalization; and 'high levels' of FITC fluorescence (with sufficiently high PS externalization) to apoptotic cells (Borisenko et al., 2003). Hence, the investigator needs to be aware that there is potential for inaccurate discrimination between early apoptotic cells and viable cells constitutively expressing PS.

3.2. Low Density Cells enriched for Dendritic Cells

In view of the complexities inherent when assessing cell cultures of PBMC using Annexin V- PI assay, which contain multiple cell types some of which constitutively express PS, we decided to determine baseline and subsequent PS expression in cultures of isolated LDC. The degree of constitutive expression of PS by DC is not clearly documented in the literature, although the phosphatidylserine receptor required for interaction with PS is expressed by immature DC (Henson et al., 2001).

Using NycoPrep TM 1.068 we are able to obtain LDC. Despite monocytes having a lower average density than lymphocytes, it is difficult to achieve reproducible separation based on density differences alone. This is because the densities of the two cell types overlap. However, since lymphocytes are more sensitive than monocytes to the slightly hyperosmolar NycoPrep, upon contact lymphocytes undergo more significant loss of water from the cytosol and shrink, becoming more dense. This density difference between the two types of cells can be enhanced leading to a better separation of monocytes from lymphocytes upon centrifugation (Bøyum 1963, 1983; McLellan et al., 1995).

The properties of Nycoprep are useful for enrichment of both DCs (which demonstrate monocytoïd morphology) and monocytes in the process of becoming DCs. However, a period of *in vitro* culture is crucial to achieve separation of DCs from peripheral blood mononuclear cells (PBMC), since Nycoprep gradient centrifugation of fresh T-lymphocyte depleted PBMC do not result in the enrichment of allostimulatory cells. This is explained by recognizing that fresh human DCs are smaller (less mature) and resemble lymphoid cells (O'Doherty et al., 1993; Egner et al., 1993; Thomas et al., 1993), only acquiring increased density buoyancy (Van Voorhis et al., 1982; Young & Steinman 1988) and monocytoïd morphology following a period of *in vitro* culture. The necessity to pre-culture blood DCs before DC-enrichment provides an additional opportunity to deplete lymphocytes and macrophages. DCs from human peripheral blood adhere transiently and later become more non-adherent within 16 hr, allowing depletion of non-adherent monocytes/

macrophages whilst containing a greater proportion of lymphocytes and DCs (van Voorhis et al., 1982).

There appears to be significant heterogeneity amongst monocytes with respect to culture-induced buoyancy. The vast majority of CD14⁺ monocytes along with the majority of lymphocytes (including B cells) are depleted from the interface. This results in marked enrichment of low density cells (LDC) at the interface expressing HLA-class II molecules. Although a significant proportion of monocytes constitute the LDC interface, these tend to be veiled (dendritic processes) suggestive of early DC morphology. However, only the CD3⁻, CD14⁺, CD16⁻, CD19⁻ negative LDC (putative DC) demonstrate a strong allo-stimulatory potential. In comparison, the pellet fraction demonstrates little residual allostimulatory activity. This suggests that almost all DC are retained at the Nycoprep-Media interface.

Cell analysis has previously shown that the isolated population is highly enriched for DC with high surface expression of HLA-DR, CD11c, CD83 and CD86 (Knight et al., 1986). The LDC population (despite only up to 80% purified cells having DC phenotype and morphology) offered a novel and “physiological” model for blood DC responses to GLP-2.

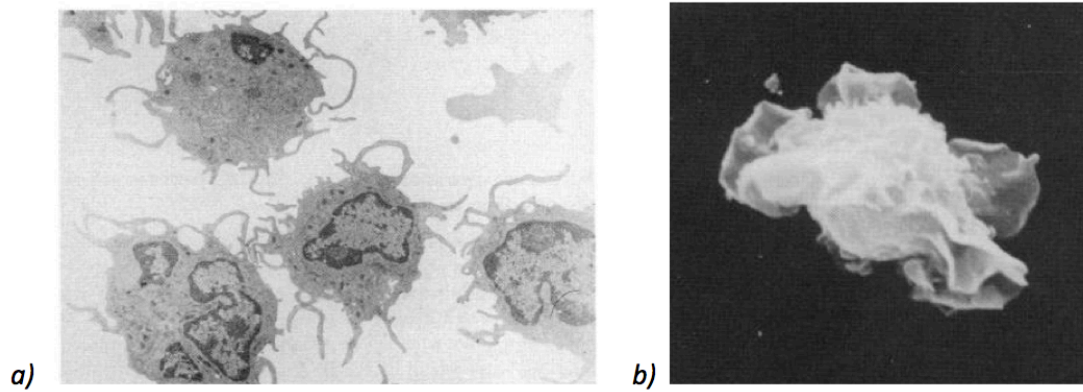


Figure 3.1. Low Density Cells enriched for Dendritic Cell isolated from peripheral blood.

a) Transmission Electron Microscopy + b) Scanning Electron Microscopy demonstrating typical veiled DC morphology at electron microscopy (from Tyndall, Knight et al., 1983)

3.3 Methods

For the Trypan blue exclusion experiments, PBMC were prepared from healthy donors over a Ficoll-Paque density gradient described in Chapter 2: Methods and Materials. PBMC were incubated in FACS tubes (2×10^6 cells in 1ml) containing complete media at several concentrations of GLP-2 1pM, 1nM and 1 μ M in paired cultures (37°C, 5% CO₂ in a humidified incubator) for 0, 2, 4 and 24 hours. The GLP-2 concentration selected was based on pilot dose ranging experiments demonstrating optimal maturation and phenotypic changes (done as part of experiments in Chapter 4) without reducing total number of cells (pilot study results not shown).

Upon completion of each cell culture period, cells were washed twice in cold PBS and centrifuged (1700rpm, 4°C, 5 mins) before re-suspension in 1x binding buffer at a cell density of $1-2 \times 10^6 \text{ ml}^{-1}$. A Trypan blue exclusion test cell count was performed at this stage using a 50µL sample of each cell suspension in a Neubauer Haemocytometer (Figure 3.2)

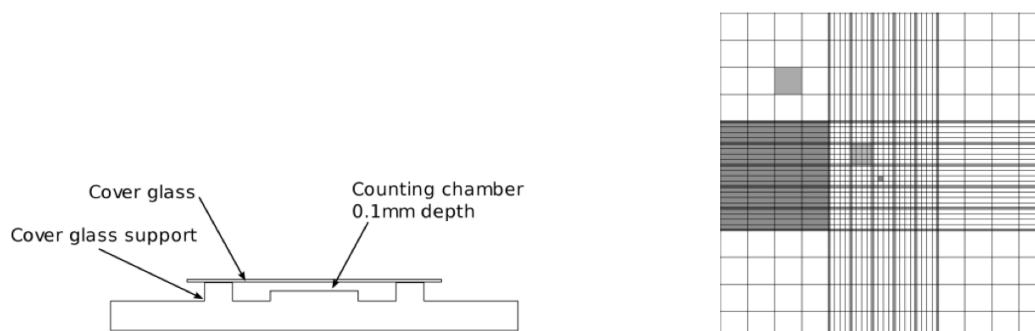
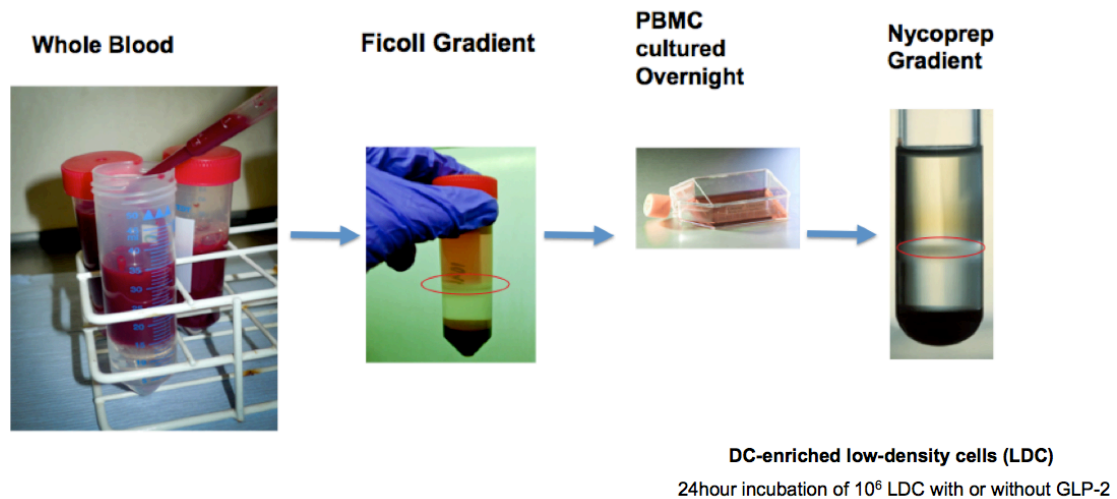


Figure 3.2. The Neubauer haemocytometer.

A typical haemocytometer showing the counting chamber and counting grid.

Each large square measures 0.1 mm^2 .

For the Annexin V-PI assay test, peripheral blood LDC enriched for DC were isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC culture (Chapter 2 – Methods).



A Nycoprep density gradient was used to enrich dendritic cells from all tissue types. All DC underwent the same culture time, which is important as DC spontaneously mature upon *in vitro* culture. Our previous laboratory experiments demonstrated that the enriched DC populations isolated by this method were 98 - 100% HLA-DR⁺, with around 30% with unambiguous phenotypic identification as DC. Many cells maintained some macrophage markers but had morphological features of DC both at optical and electron microscopy with a veiled DC morphology (Knight et al., 1986; Holden et al., 2008). The population was useful for examining the effects of treatments on not only the DC but also on the maturing monocytes within the low density population. Enriching DC via Nycoprep gradient also selects for myeloid (CD11c⁺) DC, so the blood and later gut DC can be taken to be myeloid DC. Hence, blood and gut DC used for the later allogeneic MLR are also of myeloid origin.

LDC extracted in the above manner were cultured for another 24 hours in 1ml complete medium containing GLP-2 in concentrations of 1pM, 1nM and 1µM overnight.

Once the cell culture period was complete, cells were washed twice with cold PBS and centrifuged (1700rpm, 4°C, 5 mins). The cells were re-suspended in 1x binding buffer. One hundred microliters (100µl) of each cell suspension was transferred into each 4 fresh FACS tubes (5ml). Annexin V-FITC (5µl) and/or PI (10µl) were added per tube in the combination listed below, gently mixed and incubated for 15 minutes at room temperature in the dark. Upon completion of incubation, cell suspensions were washed twice, and then 1x binding buffer (400µl) was added to each tube. The cell suspension was analysed by flow cytometry within 1 hour. This sequence was repeated for each experimental concentration overnight (Figure 3.2).

1. FITC-Annexin V
2. PI
3. FITC-Annexin V and PI
4. Unlabelled (negative control)

3.4 Results

3.4.1 PBMC cell recovery and viability based on Trypan Blue exclusion

Viability cell recovery demonstrated a decreasing trend after 48 hours in culture. This was found both in control and GLP-2 conditions of which there was no statistical significance when compared. There was also no significant effect of GLP-2 on cell viability and recovery as determined by Trypan blue exclusion across the GLP-2 concentrations when compared to controls at all time points (Figure 3.3).

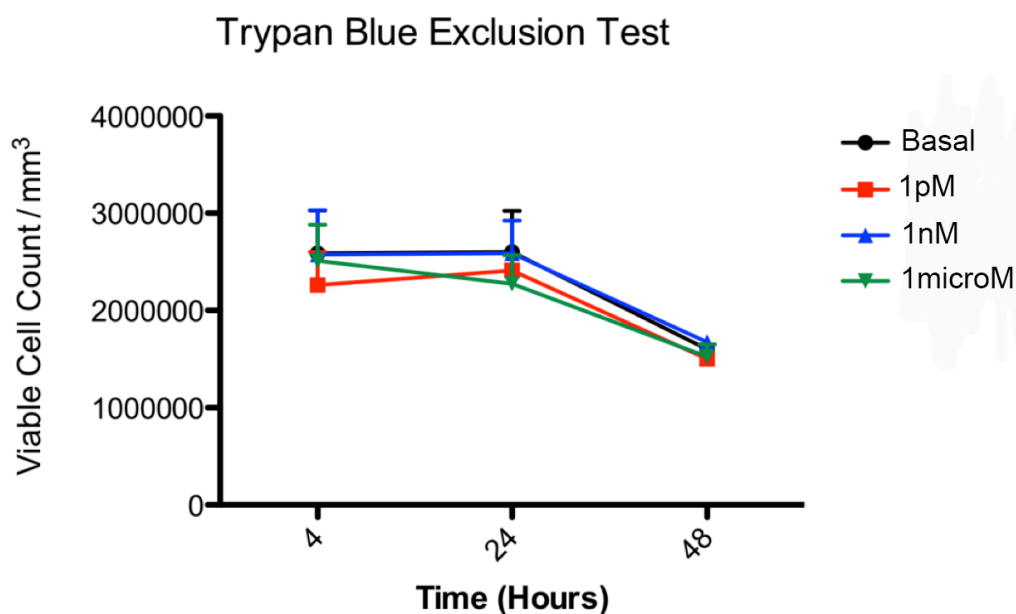


Figure 3.3. Trypan blue exclusion test.

Histogram showing viable cell recovery +/- standard error following culture of paired PBMC in complete medium containing GLP-2 (1pM, 1nM and 1μM) from 4,24 and 48 hours. Plots represent the mean viable cell counts (n=4).

3.4.2 LDC Cell Recovery and Viability in the Annexin V- Propidium Iodide assay

Using the Annexin V- PI assay, comparison of the viable (R2), all apoptotic cells (R3 and R4), late apoptotic cells (R4) and necrotic (R5) cells between control and GLP-2 at doses of 1pM, 1nM, 1μM were established (Figure 3.4).

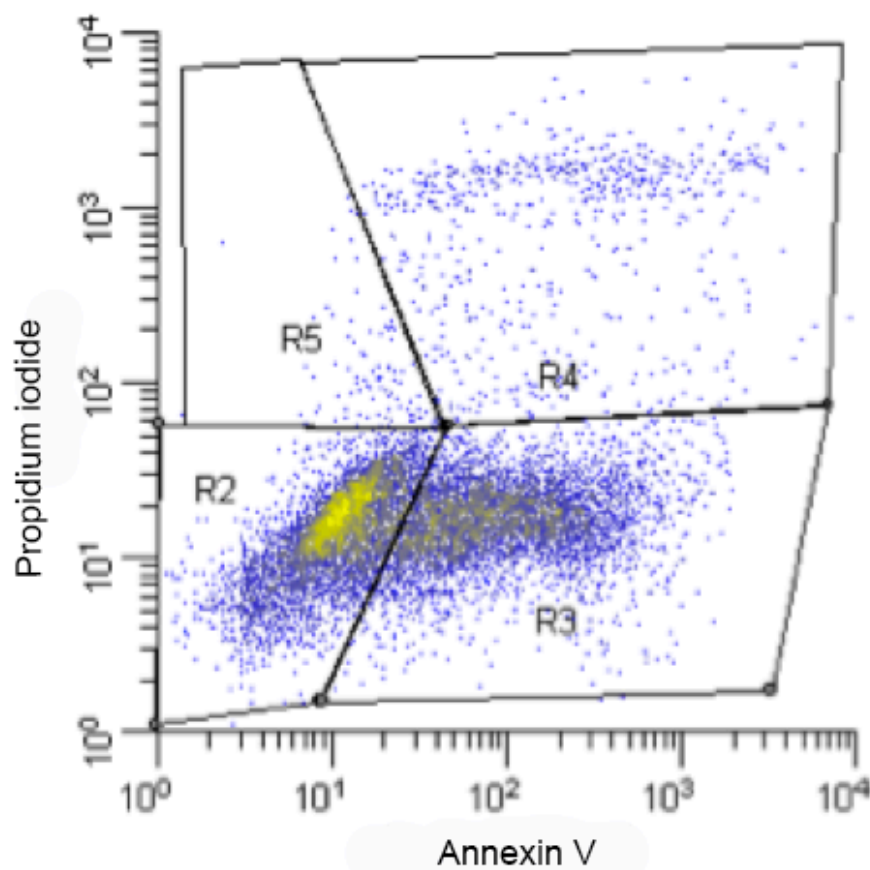


Figure 3.4. Identifying specific regions for DC.

a) R2 is specific for viable cells, b) R3 represents cells undergoing early apoptosis stained with Annexin V and c) R4 represents late apoptotic cells. d) R5 represents cells that are necrotic and have taken up propidium iodide.

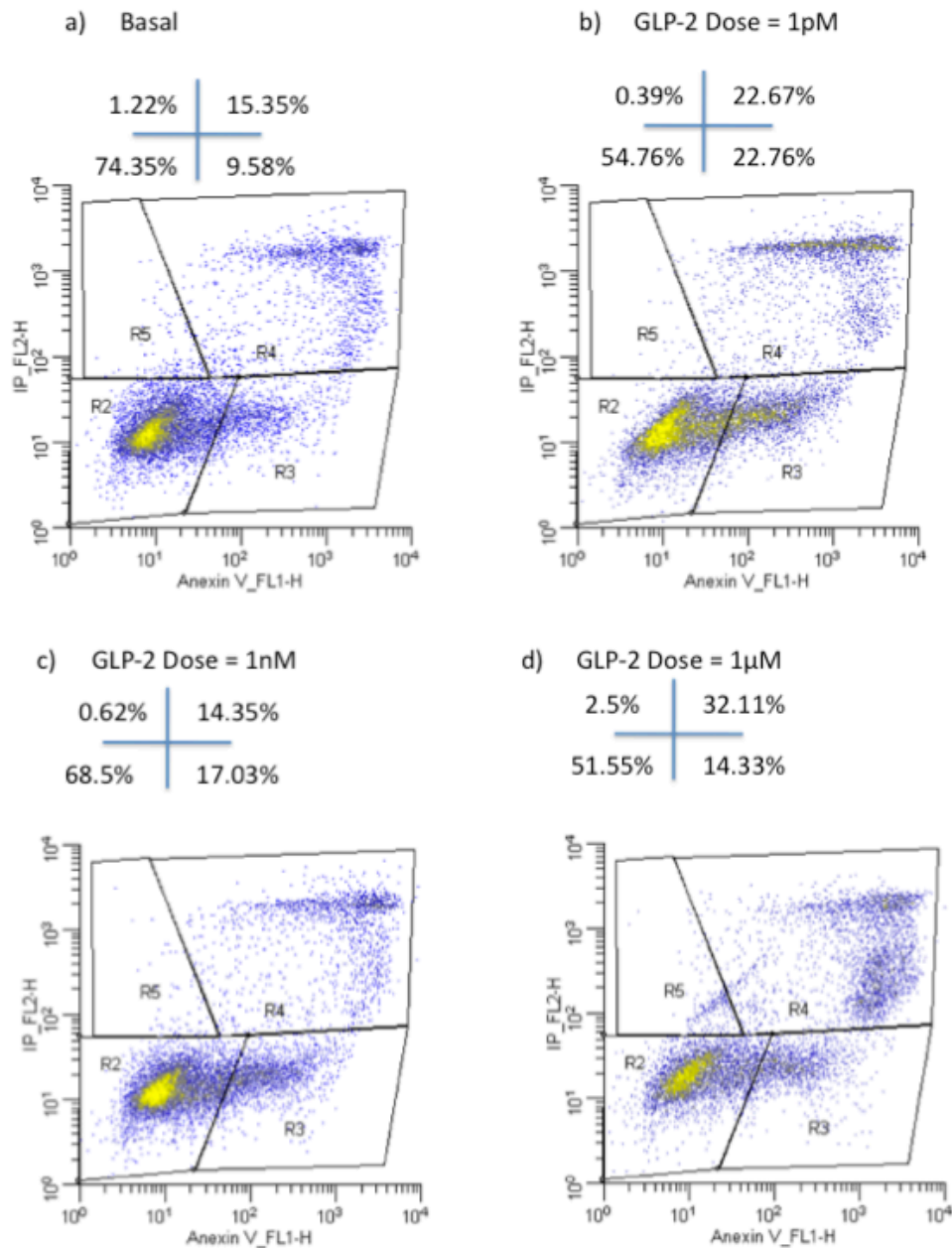


Figure 3.5. Results of the percentage of cells stained in each region (R2-R5).

Basal (a) and GLP-2 dose increments at 1pM (b), 1nM (c) and 1μM (d). Representative of several independent experiments performed with similar results.

The expected increase in necrotic and apoptotic cell populations was not seen in the culture when compared to the 0 hour sample. The culture of LDC at various concentrations of GLP-2 (1pM, 1nM, 1μM) did not result in a statistically significant difference in cell recovery across the viable, apoptotic or necrotic populations (Figure 3.5, Figure 3.6, Figure 3.7 and Figure 3.8).

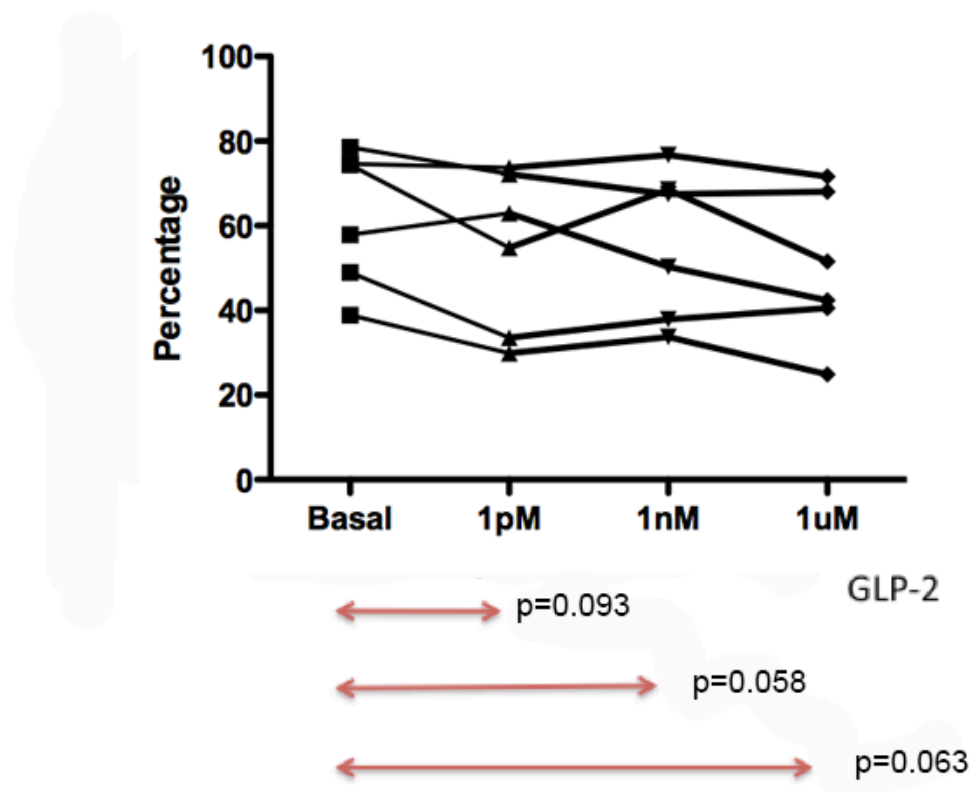


Figure 3.6. Effects of GLP-2 on LDC viability.

Results show no statistical difference in the viability of cells (R2) in the 1pM, 1nM and 1μM doses of GLP-2. (ANOVA p score not significant); n=6.

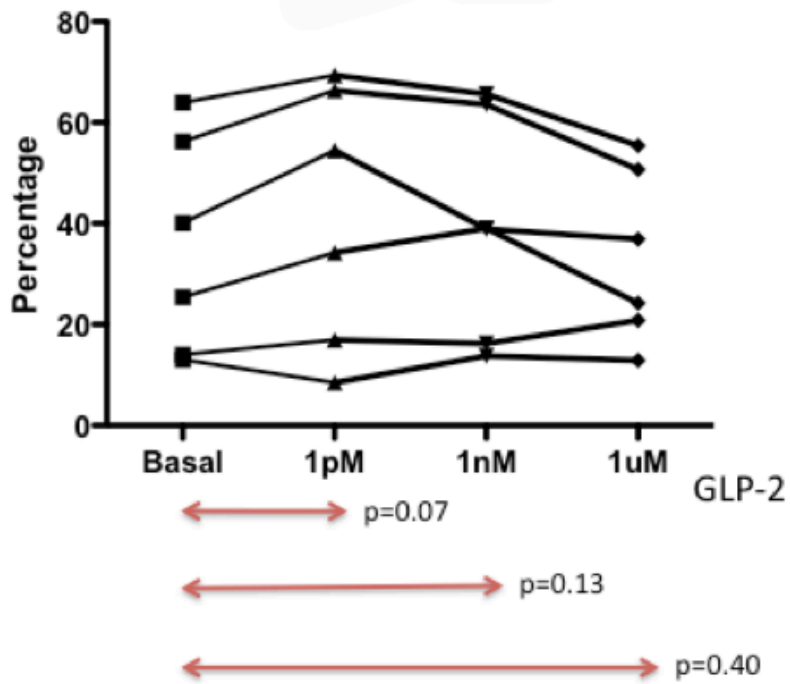


Figure 3.7. Effects of GLP-2 on LDC apoptosis.

Statistical analysis of six independent experiments. Results show no difference in the apoptosis of cells (R4+R5) up-taking Annexin V in the 1pM, 1nM and 1uM doses of GLP-2. (ANOVA p score not significant; n=6.

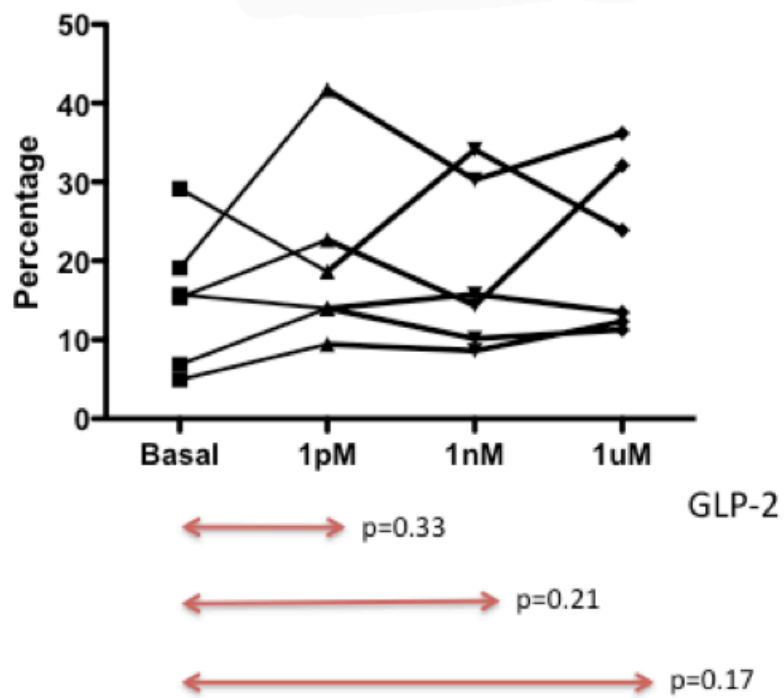


Figure 3.8. Effects of GLP-2 on LDC late apoptosis.

Results show no statistical difference in the late apoptotic cells (R4) in the 1pM, 1nM and 1 μ M doses of GLP-2. (ANOVA p score not significant); $n=6$.

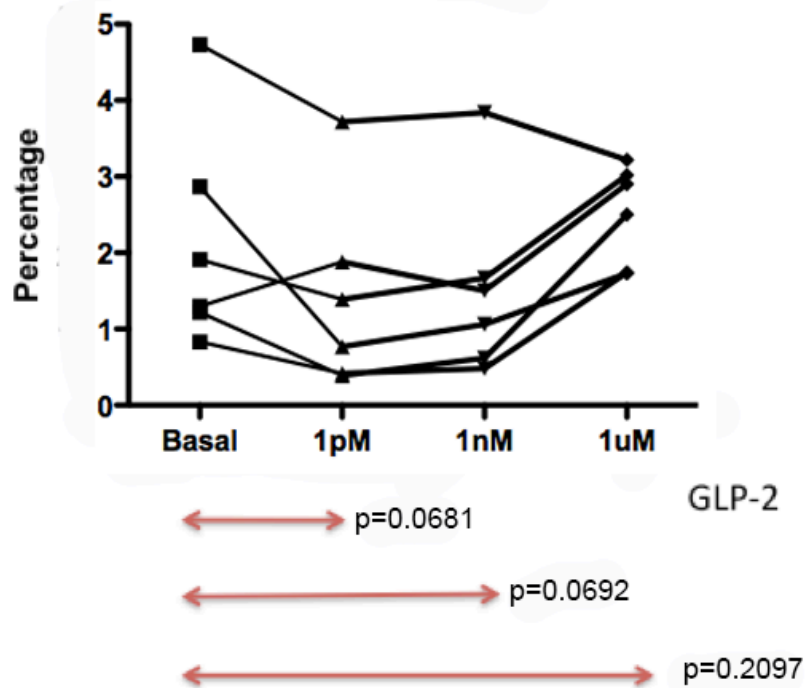


Figure 3.9. Effects of GLP-2 on LDC necrosis.

Results show no statistical difference in the necrosis of LDC (R5) when incubated with 1pM, 1nM and 1 μ M doses of GLP-2. (ANOVA p score not significant); $n=6$.

3.5 Discussion

We have demonstrated that PBMC and LDC did not demonstrate significantly different rates of apoptosis or necrosis in response to culture with GLP-2 at doses of 1pM, 1nM and 1μM when compared to control conditions *in vitro*. The Trypan blue exclusion test and Annexin V-PI fluorocytometric assay of cell viability are recognised and commonly used methods of determining cell viability; however we recognise that both systems have inherent flaws which could reduce data accuracy.

The Trypan blue exclusion test may be prone to inclusion of 'viable' cells, which are capable of excluding Trypan blue from their cytoplasm due to the functionally intact cell membranes, but which already have compromised integrity being engaged in the early stages of apoptosis.

The Annexin V-PI cell viability assay may also be impaired by variable constitutive expression of PS by several other immune cells that may have not been depleted even from cultures of LDC. This assay simply determines the proportion of cells within a population positive for external PS expression based on Annexin V binding.

In our apoptosis experiments, cultured DC are only a small component of PBMC; however DC may have been subject to regulation by GLP-2. These DC have not been activated by the presence of allogeneic cells, and hence would not necessarily be expected to either induce significant proliferation or

apoptosis in the autologous T-cells present in the PBMC. This is supported by a lack of significant differences in apoptosis or necrosis when compared to control PBMC cultures.

Nevertheless, our investigations using both the Trypan Blue exclusion test and Annexin-PI assay, taken together, have simultaneously demonstrated no significant or dose-dependent effects of GLP-2 on PBMC or LDC apoptosis or necrosis *in vitro* over a wide range of GLP-2 doses. This data gives confidence that observations of the effects of GLP-2 in our subsequent chapters, is unlikely to be influenced by significant disproportionate cell apoptosis or necrosis when compared to controls for the doses used.

Chapter 4

The Effects of GLP-2 on Human Dendritic Cell Maturation and Phenotype.

Abstract

Background

Dendritic Cells (DC) are uniquely capable of primary presentation of antigens to naïve T cells, determining either an active immune response or tolerance. GLP-2 has been shown in animal studies to reduce mucosal inflammation; it decreases proinflammatory cytokines and ameliorates chronic colitis (Ivory et al., 2007). We do not yet know whether this anti-inflammatory effect occurs in humans and the mechanism by which it does this. We hypothesise that GLP-2 reduces mucosal inflammation via DC.

Low-density cells (LDC) enriched for DC can be isolated from human peripheral blood and gut tissue. These *ex-vivo* DC are not subject to the supraphysiological concentrations of cytokines necessary to drive monocyte differentiation into DC. Hence, the LDC population provides a source of endogenous DC to test our hypothesis.

Aims

To determine the immunomodulatory effects of GLP-2 on the maturational and allostimulatory phenotype of LDC enriched for DC obtained from peripheral blood.

Methods

Peripheral blood LDC enriched for DC were isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC culture. LDC were cultured for another 24 hours in complete medium with and without GLP-2 at several concentrations. The maturation and co-stimulatory phenotype of LDC was determined by flow cytometry utilising monoclonal antibodies to CD86, CD40, ILT3, CD14 and HLA-DR.

Results

GLP- 2 conditioned LDC demonstrated an immature phenotype compared to controls with significant increased expression of monocyte marker CD14 ($p=0.006$) and a significantly decreased in HLA-DR ($p=0.007$), compared with basal control culture. However, GLP 2 did not suppress DC differentiation marker CD 86 or CD 40 and actually conversely caused a significantly elevation in CD86 expression ($p=0.0047$). ILT3 which is a general feature of tolerogenic DC was not affected.

Discussion

In peripheral blood LDC enriched for DC, culture with the active form of GLP-2 promotes an immature monocyte-like phenotype in peripheral blood LDC *in vitro* suggesting reversion to a monocyte-like state and implying a tolerogenic functional phenotype. However, the expected suppression of CD40 and CD86 stimulation marker was not seen. Also, the ILT3 expression was not increased.

4. Introduction

4.1 Effects of GLP-2 on Dendritic Cell Maturity and Allostimulatory Potential

Dendritic cells (DCs) are important in the induction of primary immune responses, induction of immunological tolerance and regulation of T cell-mediated immune responses. Part of the crucial life cycle of DCs involves differentiation and maturation from precursor monocytes into mature dendritic cells. Immature DCs and other antigen presentation cells, such as monocytes and B cells, possess poor allostimulatory potential. However, upon maturation, mature DCs acquire the ability to strongly stimulate naïve T-cells. The maturation process starts when circulating immature precursor DCs encounter antigens in the peripheral tissue (eg. lamina propria of the gut) and then migrate to the draining secondary lymphoid tissue where in association with antigen-presentation molecules (e.g HLA-DR) and co-stimulatory molecules (e.g. CD40, CD86), mature to display MHC complexes to naïve T-cells. This stimulates T-cell proliferation which will determine the type of subsequent T-cell response (Banchereau et al.,2000).

Monocytes are the immediate precursors of DCs and express high surface levels of CD14 but low levels of molecules associated with antigen-presentation, such as MHC Class II molecule HLA-DR. As part of the differentiation and maturation process, DC will lose surface CD14 expression and HLA-DR expression will be up-regulated. To effectively stimulate naïve T-

cells, maturing DCs also acquire surface expression of co-stimulatory markers (e.g CD40, CD86 and CD80) to facilitate interaction with the T cell receptor (TcR). Immature DCs express low levels of these co-stimulatory molecules. Immunoglobulin-like Transcript 3 (ILT3) is also found highly expressed in tolerogenic immature DCs.

We therefore aimed to establish whether GLP-2 had an effect on DCs differentiation, either by inhibition or stimulation and the subsequent allostimulatory potential.

Human monocyte-derived myeloid DCs (MoDC) are generated *in vitro* by culture of purified CD14⁺ peripheral blood monocytes, for several days, in a cocktail of cytokines (GM-CSF and IL-4) at supra-physiological concentrations, which drive differentiation into a DC phenotype expressing HLA-DR with loss of CD14. Hence, we opted using LDC as a source of endogenous DC. This allowed us to establish GLP-2's effects on maturation as these cells are likely to reflect more closely blood and gut DC *in vivo*.

We described in the previous chapter (Chapter 3) the theory and methods behind LDC enriched for human blood DC.

4.2 Methods

4.2.1 Low Density Cells enriched for Dendritic Cells from Peripheral Blood

Peripheral blood LDC enriched for DC were isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC culture as per previous chapter (Chapter 3).

LDC extracted in the above manner were cultured for another 24 hours in 1ml of complete medium containing GLP-2 in concentrations of 1pM, 1nM and 1μM (these concentrations and length of culture time were chosen as described later in this chapter).

4.2.2 Flow Cytometry

Following Nycoprep enrichment, DC within the isolated LDC population were identified according to viable cell characteristics on light scatter (FSC:SCC) profiles and HLA-DR⁺ Lineage⁻ phenotype at flow cytometry (Chapter 2). However, initial unresolved difficulties experienced during identification of DC within the LDC populations from the blood and intestine using the HLA-DR⁺ Lineage⁻ system resulted in the decision to analyse maturation marker expression within the LDC population as a whole based on FSC:SCC characteristics alone (discussed below).

LDC maturation status and co-stimulatory potential was determined by flow cytometry using monoclonal antibodies to maturation markers including CD14 and HLA-DR. The number of LDC available for antibody labelling restricted analysis of other markers.

4.3 Results

4.3.1 Identification of Low-Density Cells Enriched for DC from Peripheral Blood

Low-density mononuclear cells enriched for DC were identified using multi-colour flow cytometry using FSC:SCC characteristics (Figure 4.2). Previous unpublished work in our laboratory (has demonstrated DC-enrichment of this low-density cell population of up to 80% using cell suspensions over a NycoPrep gradient (Chapter 2). EM analysis confirmed enrichment of LDC with majority of cells having DC morphology (Figure 4.1).

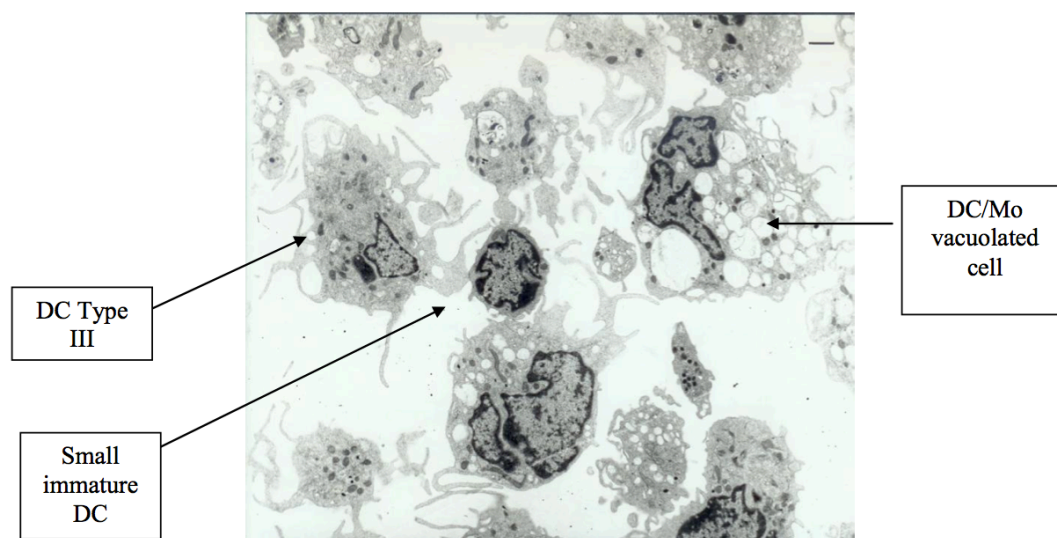
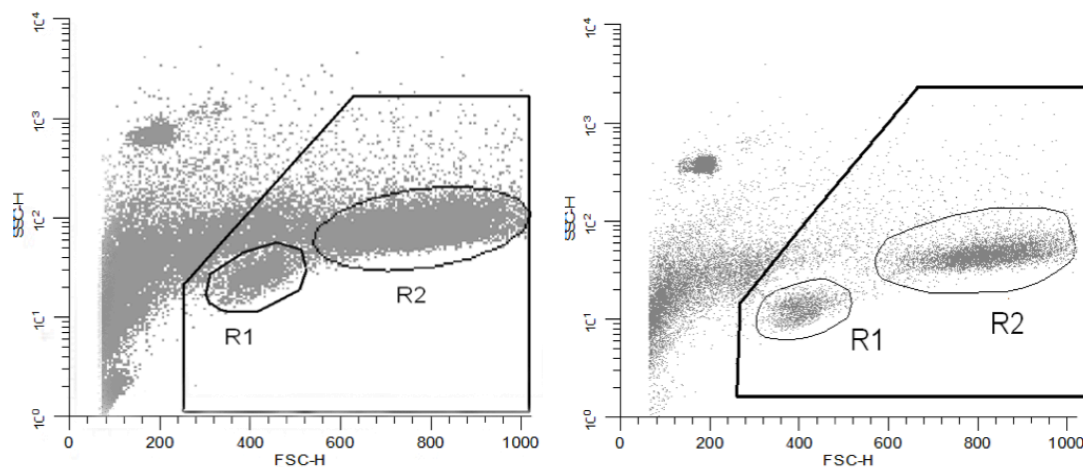


Figure 4.1. LDC show a predominately dendritic cell morphology.

By Electron Microscopy, LDC show a predominately dendritic cell morphology. In this representative example, cell types present include a very small DC, DC/Mo and DC Type 3. Courtesy of NR English, APRG. Total magnification x6900.



a)

b)

Figure 4.2. NycoPrep increases proportion of cells in the antigen presenting cell (APC) region within PBMC.

a) Pre-NycoPrep: APC population (R2) and lymphocyte region (R1) constituted 9.11% and 57.7% of total events within PBMC respectively; b) Post- NycoPrep the lymphocyte region was reduced to 3.6%. Hence, the cell suspension has been depleted of lymphocytes and refined to include low density cells (enriched for DC). When gated on the viable cell region (large box) of the scatter plot, the proportion of events within the APC population was greater than 75%.

Although our laboratory has previously demonstrated enrichment of cells with DC morphology up to 80%, many cells maintained some CD14 on their cell surface. We sought to enhance the specificity of the DC population within LDC analysed for surface marker expression using HLA-DR⁺ Lineage⁻ phenotyping (Figure 4.3), which would exclude cells still bearing CD14.

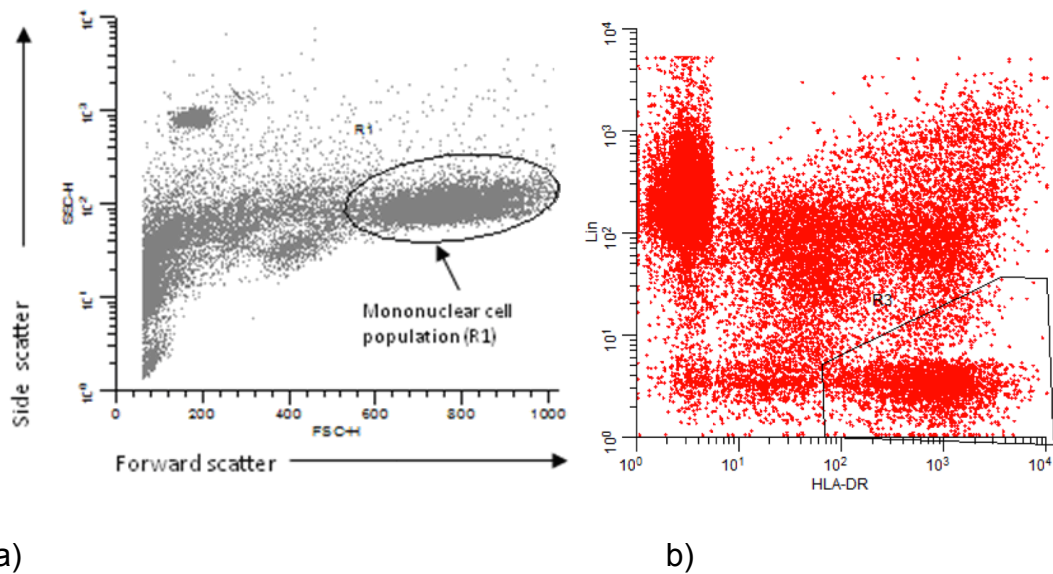


Figure 4.3. Identification of DC within low density mononuclear cells.

The APC population following cell suspension separation over a Nycoprep density gradient are shown on a FSS vs. SCC plot. Although already enriched for DC, the specific DC population within the mononuclear cell region could be further identified as HLA-DR⁺ and Lineage⁻ (CD3⁻, CD14⁻, CD16⁻, CD19⁻, CD34⁻ and CD56⁻).

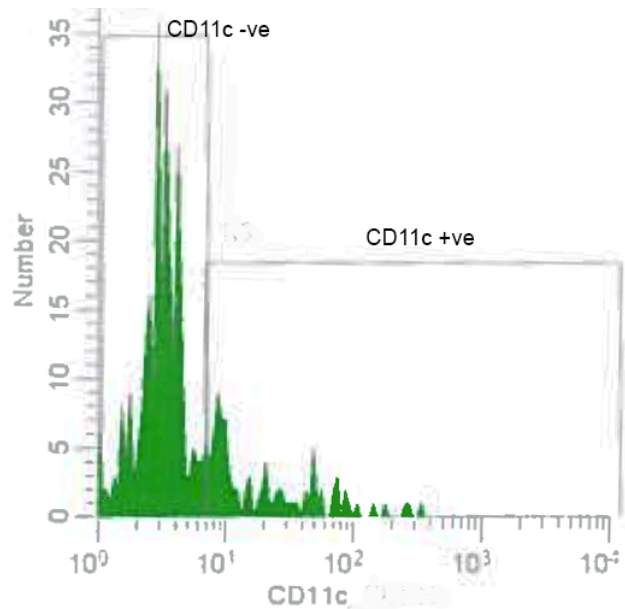


Figure 4.4. Loss of CD11c- DC population within LDC.

Histogram shows CD11c fluorescence gated on the DC region indication in Figure 4.5.

We gated our phenotypic analysis on the known antigen-presenting cell (APC) region defined by the scatter characteristics (Figure 4.4). Although cells maintaining some monocyte features (e.g. $CD14^+$) are likely to be present in this population, they were largely of low density and with veiled morphology. The population was therefore considered adequately enriched for DC. These $HLA-DR^+$ cells were gated with reference to the isotype control antibody labelling. Similarly, when looking at co-stimulatory markers the cells were also gated with reference to the isotype control antibody labelling (Figure 4.5).

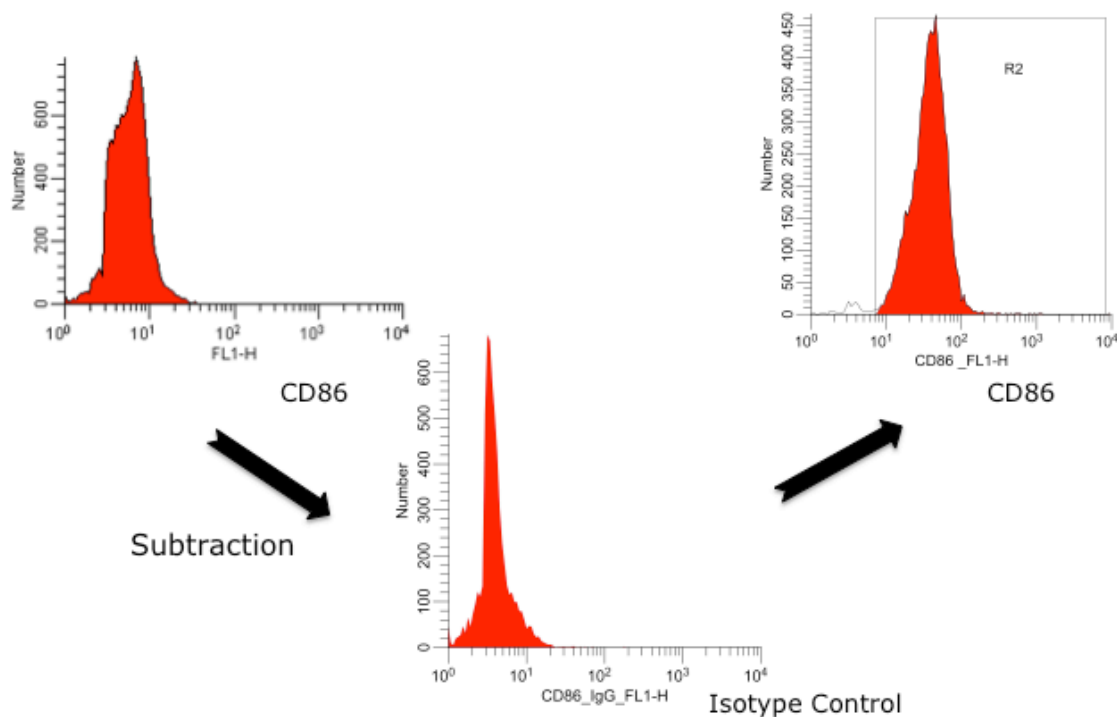


Figure 4.5. Subtraction method using WinList.

The number of cells positive for CD86 was subtracted from the isotype control IgG mouse to give the total percentage of cells expressing CD86.

4.3.2 Dose-dependent effect of GLP-2 conditioning on DC leading to a modulated homeostatic state.

Initial dose ranging experiments were conducted on LDC to identify an optimal culture concentration in the pilot studies. Expression of the co-stimulatory markers CD14, CD40 and CD86 by peripheral blood LDC were determined in response to co-culture of 30 mins and 24 hours (overnight) with GLP-2 at concentrations (1pM, 1nM and 1μM). For those markers which are constitutively expressed by DC (e.g. HLA-DR), in which the percentage of

cells positive (PP) is by definition over 90% (i.e. all DC are HLA-DR^{Hi}), the data shown are mean intensity ratio of fluorescence (MFI).

The results of 3 independent experiments showed that the incubation of LDC in GLP-2 at different concentrations (1pM, 1nM and 1μM) for 30 mins did not yield any significant changes of CD40 or CD86 expression. However there was a trend towards higher expression of CD86. There was also no change in the HLA-DR intensity or CD14 expression with GLP culture of only 30mins (Figure 4.6).

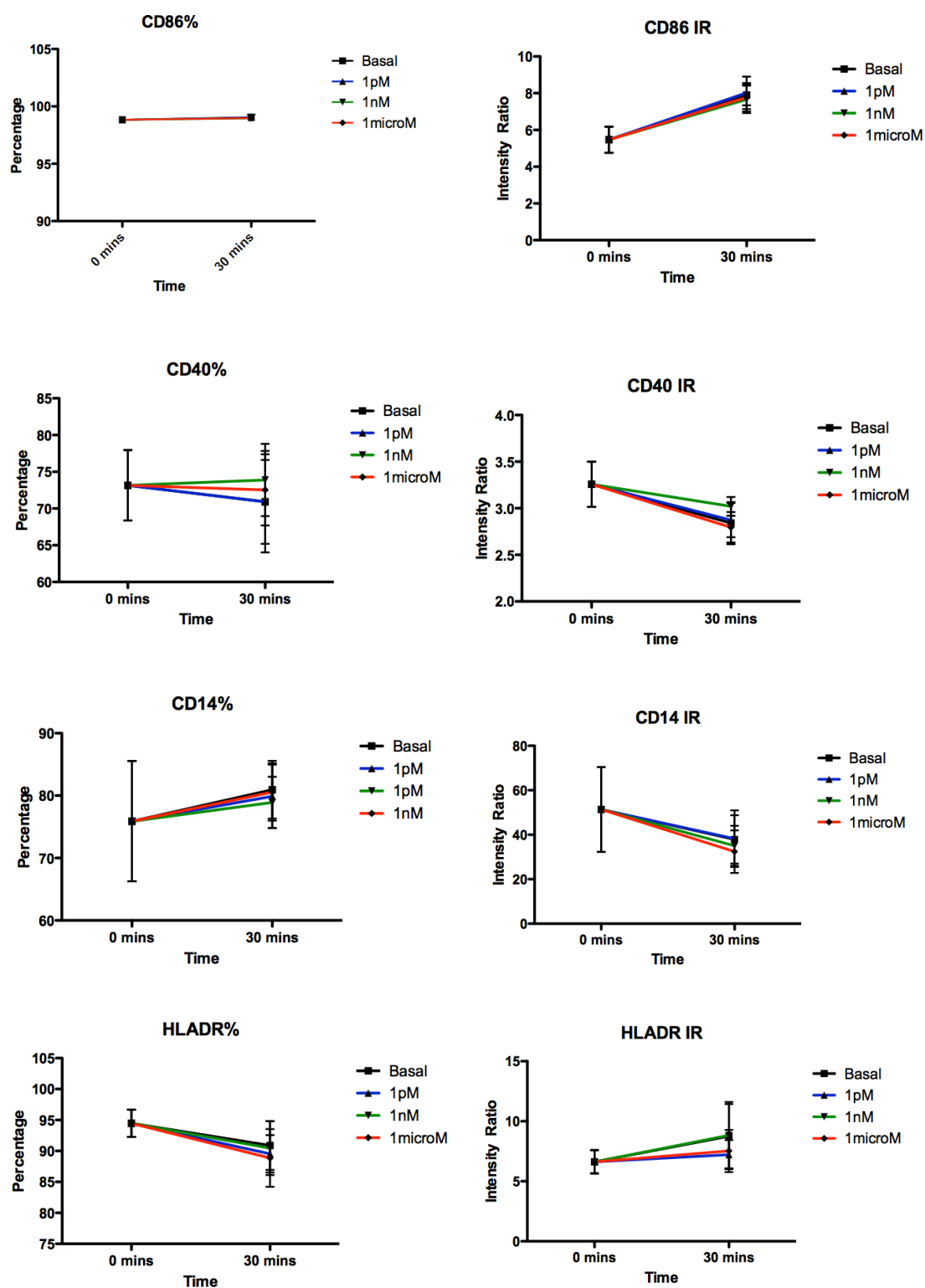


Figure 4.6. GLP-2 effects on CD86, CD40, CD14 and HLADR expression.

No significant change was detected in expression of CD86, CD40 and CD14 as well as HLA DR intensity ratio (2 way ANOVA), $n=3$.

A further three experiments were carried out, this time with a 24-hour (overnight) incubation period of GLP-2 at different concentrations (1pM, 1nM and 1µM). Again there were no significant changes in the expression of co-stimulatory markers CD40 but there was a significant higher expression of CD86 co-stimulatory marker ($p=0.0047$) (Figure 4.7).

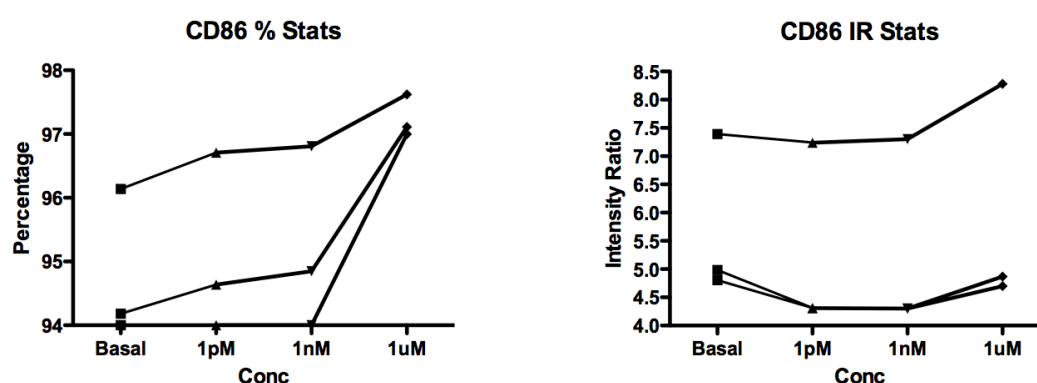


Figure 4.7. GLP-2 effects on CD86 expression.

CD86 from 3 independent pooled experiments showing an increase in percentage of expressing cells (ANOVA; $*p=0.0047$).

The results of the 24-hour (overnight) incubation period of GLP-2 on CD14 and CD86 was interesting as the three independent experiments showed a significant higher expression of CD14 ($*p=0.0237$) and a significant reduction in HLA DR intensity ratio ($*p=0.0243$) in a dose dependent way (Figure 4.8).

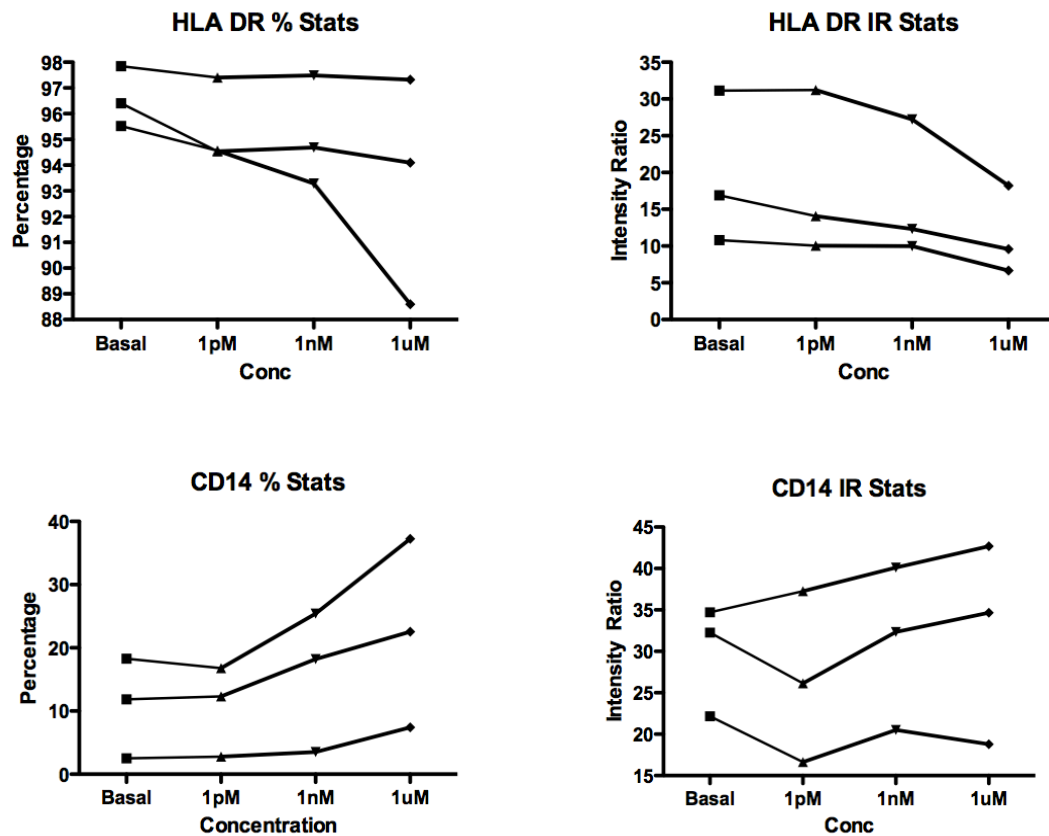


Figure 4.8. GLP-2 effects on CD14 and HLA DR.

HLA DR intensity ratio from 3 pooled experiments showing a significant decrease in IR ($p=0.0243$) and CD14 expression showing a significant increase in the percentage expression ($p=0.0237$) using ANOVA.*

Following the above-mentioned experiment, it was noted that the optimal effects on LDC phenotype were demonstrated at the highest dose of GLP-2 of 1 μ M. This dose was therefore selected for a further 6 experiments to confirm the findings of GLP-2's effect on human blood LDC co-stimulatory phenotype.

The results from this 6 pooled independent experiments showed that GLP-2 conditioning at a concentration of 1 μ M over 24-hour (overnight) changed the

phenotype of DC with a statistical significance decrease in HLA DR intensity ratio (* $p=0.007$) and an increase in CD14 expression (* $p=0.006$), compared with basal control culture (Figure 4.9).

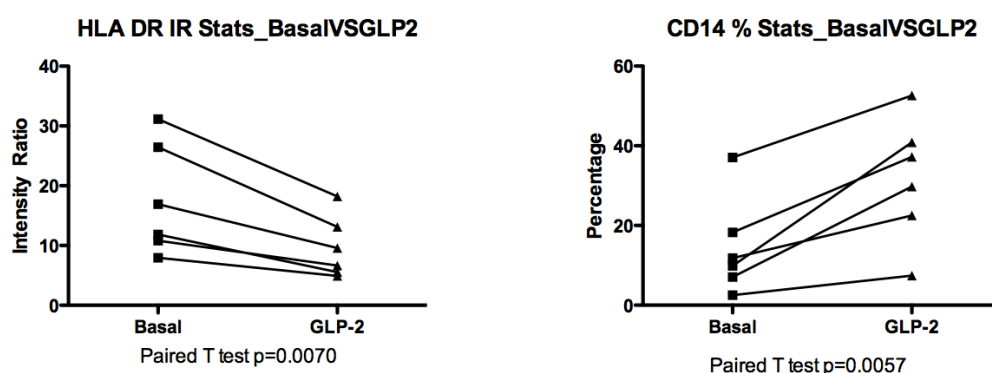


Figure 4.9. GLP-2 at 1 μ M concentration on LDC maturation.

Statistically significant changes in phenotype of LDC with a decrease in HLA DR intensity ratio ($p=0.007$; paired T test) and upregulation of CD14 expression (* $p=0.006$; paired T test) after 24 hour incubation with GLP-2 at 1 μ M concentration, $n=6$.*

The findings suggest that GLP-2 at the optimal dose of 1 μ M has an immunomodulatory effect on DC after incubation of 24 hours. The effect suggests induction of a 'homeostatic' or 'immunotolerant' state as shown by reducing HLA DR intensity ratio and increasing CD14 expression. This seen effect however, may be secondary to reduction of number of cells and hence total number of cells from the experiments above were analysed.

4.3.3 GLP-2 conditioning did not reduce DC total numbers.

The changes seen with a reduction in HLA DR intensity ratio and an increase in CD14 expression may be secondary to the decline in cell numbers during the long 24 hour incubation period influenced by the differing doses of GLP-2. The concern of toxicity would certainly be a valid one especially in the higher doses of GLP-2; despite findings of our apoptosis experiments in the previous chapter (Chapter 3). Hence, the findings of GLP-2, in particular the higher dose of 1 μ M influencing an immature phenotype in DC may not be a true result. In order to clarify this issue, we looked at the total numbers of cells using counting beads during incubation of GLP-2 up to 48 hours at the three different concentrations (1pM, 1nM and 1 μ M) in 3 pooled experiments. This data was collected from the number of cells established by the flow cytometer in each tube.

As expected, there was a gradual decline in the number of cells over time. There was however no statistically significant decline in the number of cells when comparing the different concentrations of GLP-2 with the basal control culture (Figure 4.10). Interestingly, there was a trend to the contrary; there were higher numbers of cells left at 48 hours compared to basal in the higher doses of GLP-2 (1nM and 1 μ M). The findings also confirmed that using 24 hr incubation period in our earlier set of experiments was optimal to establish the effects of GLP-2.

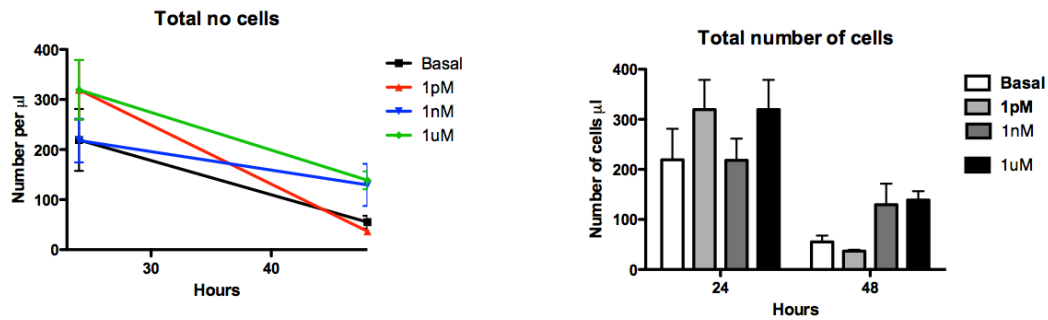


Figure 4.10. Total number of cells.

Total number of cells during incubation with GLP-2 at varying concentrations over 48 hours with no significant decline in number of cells when comparing the doses (ANOVA; bonferroni's multiple comparison test); $n=3$.

Hence, our findings of GLP-2 influencing an immature phenotype in incubated DC is not due to GLP-2 cytotoxic effect.

4.3.4 GLP-2 did not influence the Immunoglobulin-like transcript 3 (ILT-3) expression on DC.

LDC conditioned for 24 hours in complete media containing GLP-2 at a concentration of 1pM, 1nM and 1μM compared with controls demonstrated no increase in expression (% and IR) of ILT-3 in three separate independent experiments (Figure 4.11).

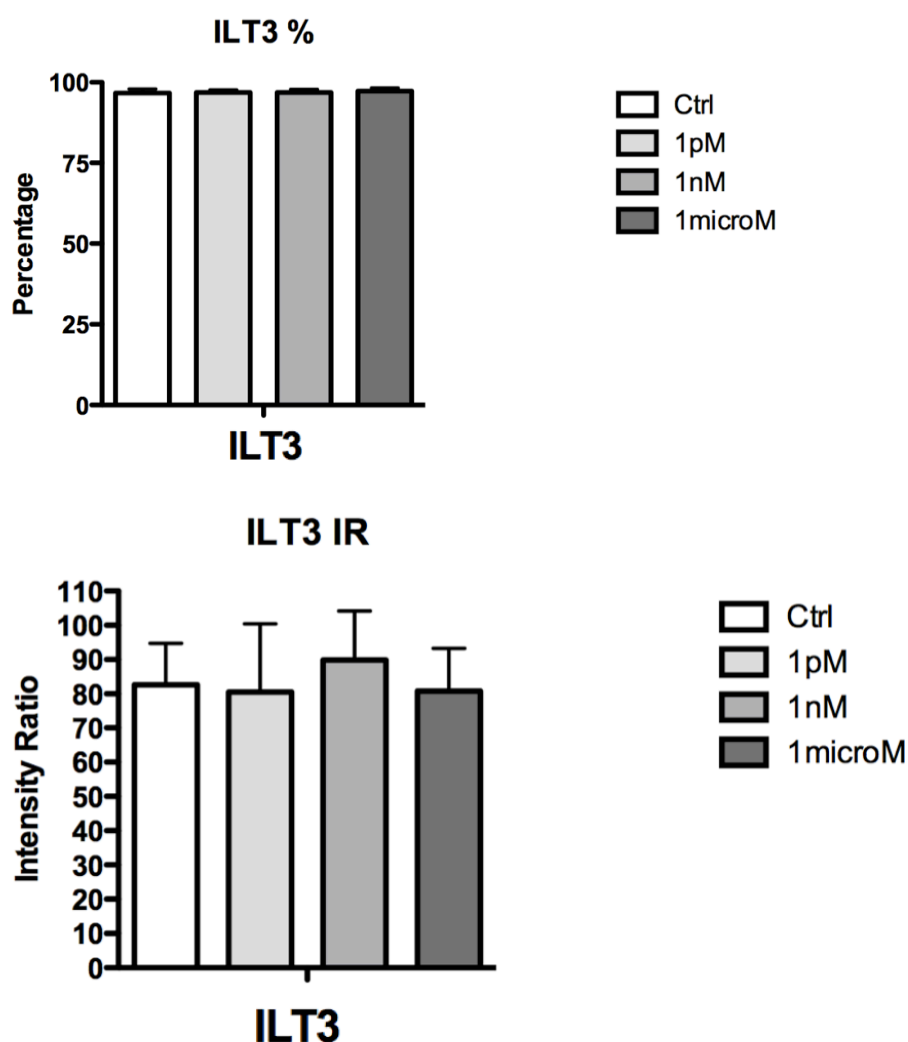


Figure 4.11. GLP-2 effects on ILT3 expression.

In various concentrations did not affect the ILT3 expression in blood LDC compared to control, n=3.

4.4 Discussion

In peripheral blood LDC enriched for DC, culture with GLP-2 promoted an immature monocyte-like phenotype, increasing CD14 expression and reducing HLA DR intensity ratio, suggesting reversion to a monocyte-like state and implying a tolerogenic functional phenotype. However, the co-stimulatory molecule expression of CD86 was not in keeping with this finding. Also the expected high expression of ILT3 normally seen expressed on immature dendritic cells was not seen. This may suggest that the effect of GLP-2 on the phenotype is only a partial one and may require other upstream mediators (proteins, cytokines, growth factors) to complete its immunomodulatory function.

The aforementioned changes noted may certainly be due to a total decline in cell numbers especially in light of the long 24 hr incubation period with different doses of GLP-2. The expected gradual decline in the number of cells over the incubation period was seen however GLP-2 incubation especially at the highest dose of 1 μ M was not different from the basal culture. This confirmed that the homeostatic immunomodulatory effects seen were not due to differing cell numbers.

Experiments on intestinal lamina propria LDC enriched for DC were not carried out due to time limits required for experiments during the funded research period. We however expect that findings of gut LDC would most likely mirror that of blood LDC especially when the functional effects on T cell

proliferation and cytokine profile were similar in later experiments (discussed in Chapter 6 and 7). However we admit that these experiments were essential and would have cement our results. Also, it may have clarified the unexpected increased allostimulatory potential (CD 86) and lack of expression of ILT3.

In conclusion, *ex-vivo* LDC responded to the active GLP-2 in culture, demonstrating an immunotolerant functional phenotype with an increase in allostimulatory potential. However this is only seen in peripheral blood LDC enriched for DC and will need to be clarified by T cell functional experiments that we will discuss in the later chapters. This early findings of DC phenotype effects of GLP-2 supports the hypothesis that GLP-2 may be useful in inflammatory conditions, in particular IBD.

Chapter 5

The Effects of GLP-2 on Human Dendritic Cells Endocytosis

Abstract

Background

GLP-2 has an influence in maintaining an immature phenotype in DC as seen in our previous chapter results. Immature DC are efficient in endocytosis, a process by which the plasma membrane invaginates, engulfs a sample of the external environmental milieu thereby enabling cells to internalize external proteins and substrates. DC then present antigen-derived peptides to T cells. Therefore an increase in endocytosis of GLP-2 conditioned immature DC would be expected.

Aims

To determine the immunomodulatory effects of GLP-2 on LDC enriched for DC endocytosis to confirm the tolerogenic or homeostatic properties seen with an immature phenotype

Methods

Peripheral blood LDC enriched for DC were again isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC culture. LDC were cultured for another 24 hours in complete medium with and without GLP-2 at several concentrations. FITC-dextran is commonly used for testing the receptor-mediated endocytosis function of immature DC. FITC-Dextran

(1mg/ml) was added to each tube for the last 2 hours of the final 24 hour incubation period at 37°C. An internal control ('pseudo-isotype') was performed in parallel, incubating cells at 4°C upon addition of FITC-Dextran resulting in arrest of further cell metabolism and function, preventing FITC-Dextran uptake. By subtracting the number of cells expressing FITC at 37°C from the control, the level of phagocytosis was established (Figure 5.2).

Results

GLP- 2 conditioned LDC did not show any significant FITC-Dextran uptake or ability to decrease FITC-Dextran uptake in 6 independent experiments.

Discussion

GLP-2 conditioned LDC with an immature phenotype did not have the expected enhanced endocytosis capacity. Hence, the immature LDC may be kept dormant by GLP-2 in a tolerogenic manner.

5. Introduction

5.1 Dendritic Cell Endocytosis

Antigen presentation of exogenous antigens in the context of class II major histocompatibility complex (MHC) molecules is preceded by antigen processing, in which the first step is endocytosis. Endocytosis is the process by which the plasma membrane invaginates, engulfing a sample of the external environmental milieu thereby enabling cells to internalize external proteins and substrates.

Dendritic cells (DC) are potent specialized antigen-presenting cells with the ability to internalize antigen, and present antigen-derived peptides to T cells. The functions of DC depend on the subset, as well as their location and activation state. Immature DC act as sentinels by continuously sampling the antigenic environment through various endocytosing mechanism.

In response to inflammatory stimuli, DC trigger maturation, a terminal differentiation program required to initiate T cell response. A hallmark of maturation is downregulation of endocytosis, widely assumed to restrict the ability of mature DC to capture and present antigens encountered after the initial stimulus. Whereas increased endocytosis is expected in immature DC that are constantly sampling the antigenic environment.

5.2 GLP-2 effects on human Dendritic Cell Endocytosis.

Based on our findings from the previous chapter, there is a suggestion that GLP-2 has an influence in keeping DC in an immature state preventing differentiation with subsequent downstream T cell activation. Immature DC are efficient in endocytosis and therefore by looking at the endocytosis of GLP-2 conditioned DC (in the immature state after modulation by GLP-2), we could assess whether these affected cells are in fact still in their immature form and continues to sample the antigenic environment without differentiating.

5.3 Methods

5.3.1 Low Density Cells enriched for Dendritic Cells from Peripheral Blood

Peripheral blood LDC enriched for DC were isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC as per the previous chapter.

5.3.2 Flow Cytometry

Following Nycoprep enrichment, DC within the isolated LDC population were identified according to viable cell characteristics on light scatter (FSC: SCC) profiles and HLA-DR⁺ Lineage- phenotype at flow cytometry described more in depth in the previous chapter (Figure 5.1).

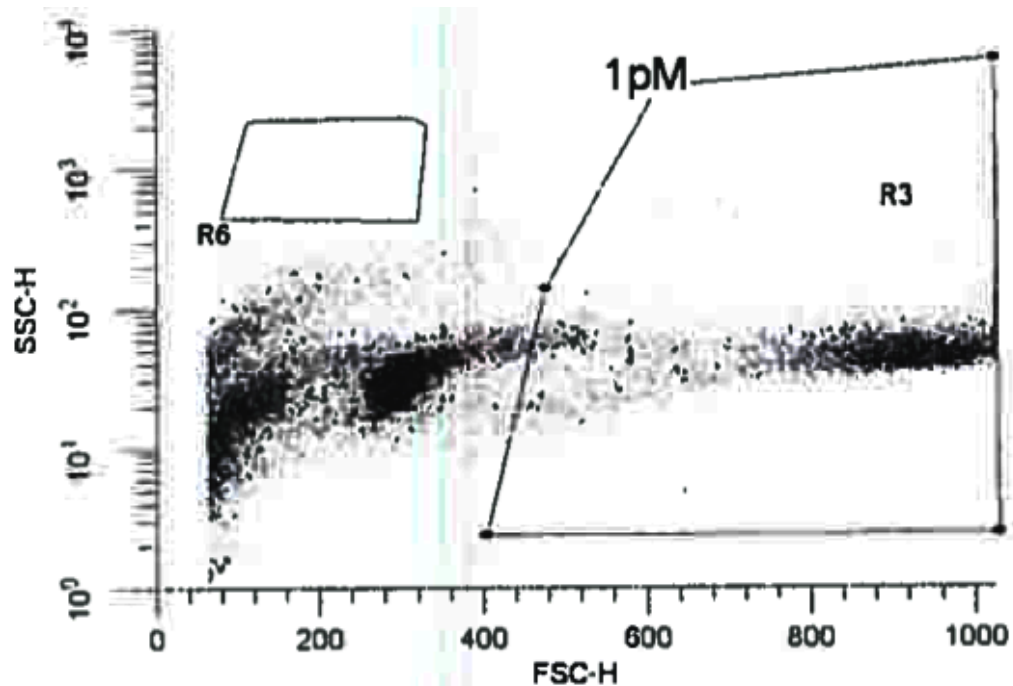


Figure 5.1. NycoPrep increases proportion of cells in the antigen presenting cell (APC) region within LDC.

APC population gated on (R3) as shown.

5.3.3 FITC Dextran

Because FITC-dextran is mainly taken up through the mannose receptor, the up-taking rate of FITC-dextran is commonly used for testing the receptor-mediated endocytosis function of immature DC. FITC-Dextran (1mg/ml) was added to each tube for the last 2 hours of the final 24 hour incubation period at 37°C. An internal control ('pseudo-isotype') was performed in parallel, incubating cells at 4°C upon addition of FITC-Dextran resulting in arrest of

further cell metabolism and function, preventing FITC-Dextran uptake. By subtracting the number of cells expressing FITC at 37°C from the control, the level of phagocytosis was established (Figure 5.2).

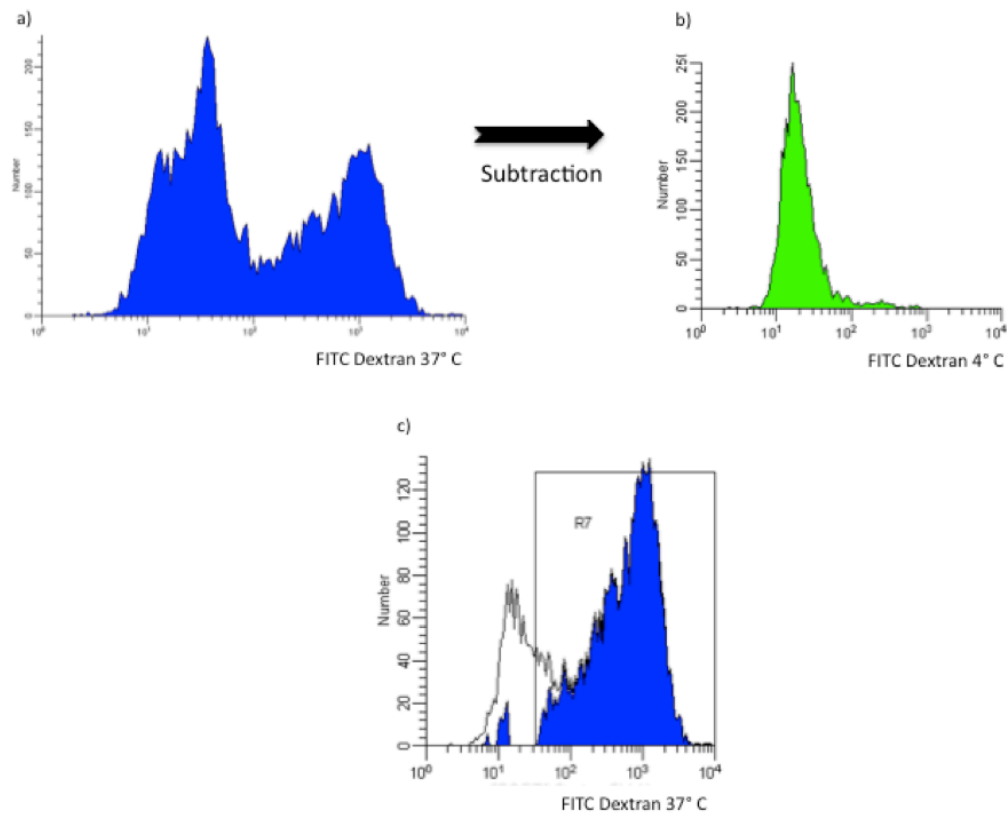


Figure 5.2. Subtraction method using WinList.

By subtracting the number of cells expressing FITC dextran from the control at 4°C the percentage of phagocytosis that a DC undergoes is established.

5.4 Results

5.4.1 GLP-2 conditioned LDC did not exhibit an increase in endocytosis for the expected immature phenotype form.

Comparisons of the intensity ratio were made with control (basal) and the different GLP-2 concentrations (1pM, 1nM and 1μM) (Figure 5.3).

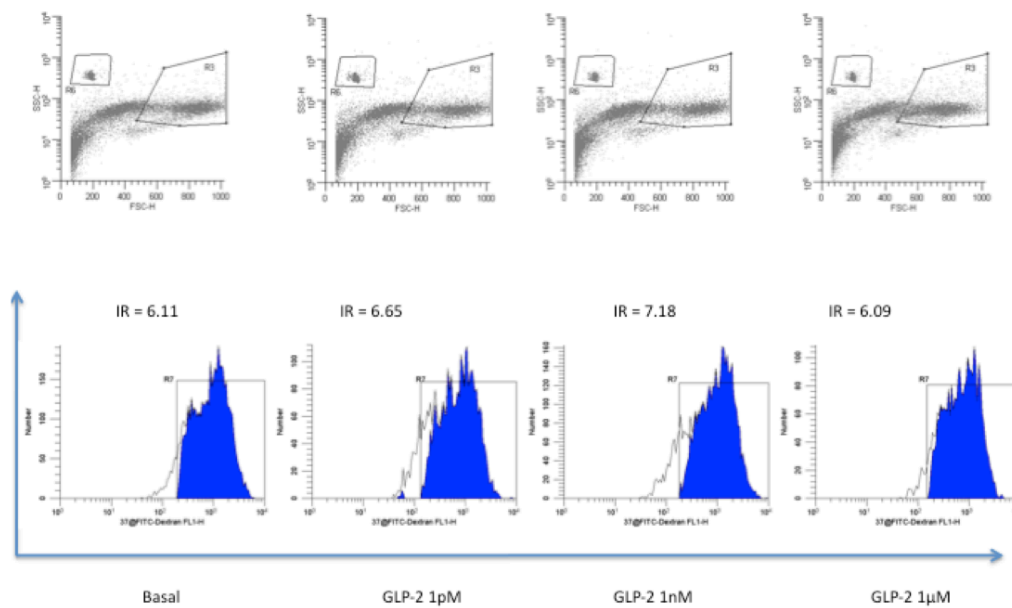


Figure 5.3. FITC intensity ratio.

Intensity ratio of cells expressing FITC after subtraction in basal and concentration of GLP-2 at 1pM, 1nM and 1μM.

The results from 6 independent experiments comparing the control and the 3 different GLP-2 concentrations did not show any significant FITC-Dextran uptake or ability to decrease FITC-Dextran uptake (Figure 5.4).

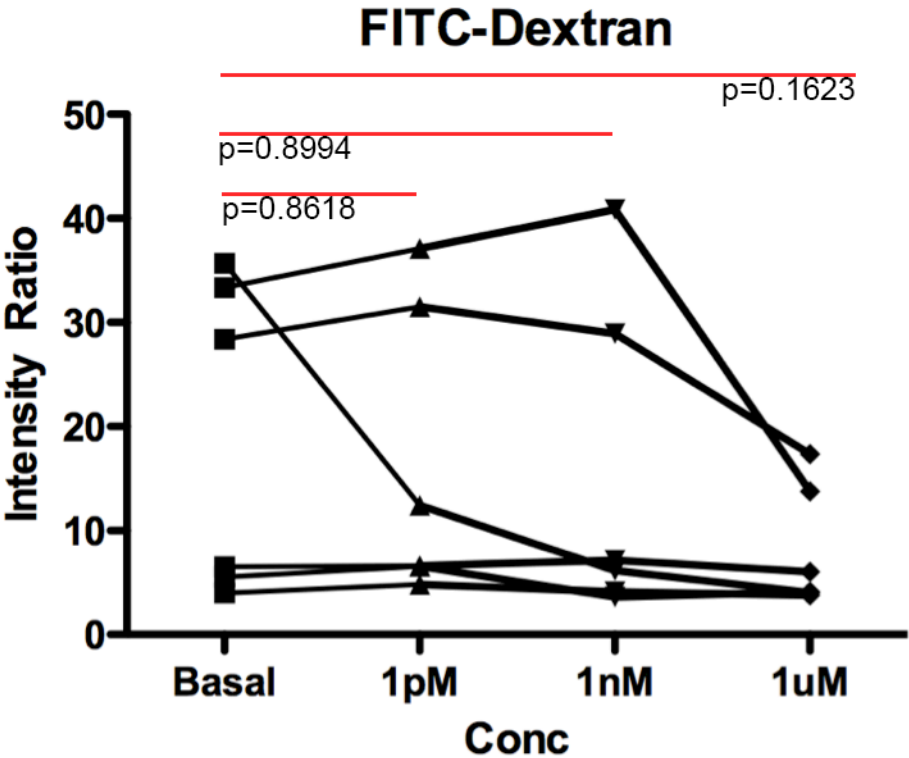


Figure 5.4. FITC-Dextran - comparison of 6 independent experiments.

Paired T-Test statistical analysis showing p values for each concentration against basal. ANOVA p score was not significant.

5.5 Discussion

In our set of endocytosis experiments, the expected capacity of GLP-2 to enhance endocytosis, which would correlate with the increase expression of CD14 and reduction of HLA-DR was not seen. However, this may be a consequence of delayed recovery of the monocyte-like surface phenotype in LDC of which longer exposure to GLP-2 may eventually induce functional modification.

Alternatively, we may be seeing the consequence of DC maturing during the long incubation period with GLP-2 and subsequently reverting back to an immature phenotype due to the GLP-2's modulation. In this form, the DC may have internalized antigens and expressed surface markers but kept in a tolerogenic state by GLP-2. Hence, endocytosis would not be seen.

The data presented in the last chapter certainly confirms immaturity of the DC modulated by GLP-2. In peripheral blood LDC enriched for DC, culture with GLP-2 promotes an immature monocyte-like phenotype, increasing CD14 expression and decreasing HLA DR intensity ratio, suggesting reversion to a monocyte-like state and implying a tolerogenic functional phenotype. We do not know however, whether this is prior to DC maturing or GLP-2 having subsequently modulated DC into an immature form from a mature state.

The co-stimulatory molecule expression of CD86 was upregulated, again suggesting that the DC within the experiment may have matured and have

maintained the high expression values of CD86. This gives further weight to the likelihood that the DC have underwent maturation and the final modulation effects of GLP-2 is one of sending the DC back to immaturity, hence keeping it tolerogenic or in homeostasis.

In the subsequent chapters, we will explore whether the GLP-2 influence DC have the capacity to prevent differentiation with subsequent downstream T cell activation as well as effects on the cytokine profile.

Chapter 6

**The Effects of GLP-2 on Human Blood and Intestinal Dendritic
Cell Function: DC Cytokine profile.**

Abstract

Background

GLP-2 in murine studies have shown a reduction in cytokine production in three models of intestinal inflammation in the ileum and colon. Modulation of cytokine production by DC will subsequently alter the T cell function and cascade of inflammation. GLP-2 may therefore modulate DC cytokine production and ultimately affect intestinal inflammation.

Aims

To determine effects of GLP-2 on LDC enriched for DC cytokine production both in human blood and intestinal small bowel and colonic DC.

Methods

LDC enriched for DC from peripheral blood and tissue biopsies from small bowel and colon were again isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC culture. LDC were cultured for another 24 hours in complete medium with and without GLP-2 at several concentrations. Quantification of the ongoing production of cytokines in LDC via a sensitive subtraction method using monensin was applied.

Results

GLP-2 reduced both IFN- γ and IL-12 p40/p70 ongoing cytokine production in human blood dendritic cells. In both colonic and small bowel dendritic cells, GLP-2 only reduced IFN- γ ongoing cytokine production.

Discussion

GLP-2 induced a homeostatic Th1 bias profile in GLP-2 conditioned human peripheral blood DC by reducing the ongoing cytokine production of both IFN- γ and IL-12 (p40/p70) and in intestinal DC (from large and small bowel), reduced the ongoing cytokine production of only IFN- γ . This is in keeping with the murine studies showing reduction of cytokine production in inflammatory conditions.

6. Introduction

DC in the disease state are associated with increased release of pro-inflammatory cytokines likely to contribute to the initiation or perpetuation of inflammation, either as a local effector cell population active in innate immunity or by modifying responses of lymphocytes activated in an adaptive immune response.

GLP-2 in murine studies have shown a reduction in cytokine production in three models of intestinal inflammation in the ileum and colon (Sigalet et al., 2007) and from our experiments in Chapter 3 and 4, has the potential to exert an immunotolerant state on DC. Therefore, if the modulation of cytokine production by DC can be influenced by GLP-2, subsequent alteration in the T-cell function and the cascade of inflammation may be altered by GLP-2.

One of the driving factors in the pathogenesis of IBD is thought to be a dysregulated immune response to the gut microflora. DC as sentinels of the GI tracts population of antigens and pathogens play a significant role in the regulation of the gut immune system. In murine models of colitis, DC accumulate throughout the entire lamina propria and MLN. Blockade of the DC- T-cell interaction prevents experimental T cell-mediated colitis and DC ablation will ameliorate dextran-sulphate induced colitis in mice (Berndt et al., 2007). Additionally, lamina propria DC from inflamed tissue produce higher levels of IL12p40, IL-23p19 and IL-10 (Becker et al., 2003).

As mentioned in Chapter 1, cytokines such as IL-12, IL-18 and IFN- γ produced by DC can bias CD4⁺ T- cell priming towards a Th1 phenotype. The cytokines act directly on the T-cells and production is amplified by positive feedback signals from the activated T- cells also (Schulz et al., 2000; Sporri and Reis e Sousa, 2003). The combination of IL-12 and IFN- γ also act to prevent the Th2 cell differentiation, inhibiting IL-4, IL-5 and IL-13 production. The fact that IL-12 can be produced by both APC and T-cells suggests that it represents an important link between innate and adaptive immune systems. IL-12 acts via the STAT-4 transcription factor, which is essential for its actions. IL-12 signalling is initiated by the tyrosine phosphorylation and activation of the Januse kinase, Jak-2 and Tyk-2. IFN- γ is the main effector cytokine of the Th1 subclass but also acts to stabilise Th1 cells, acting to prevent them from being able to switch to secreting Th2 subclass associated cytokines. This has been shown in IFN- γ deficient mouse models where Th1 cells are able to produce the Th2 associate cytokine IL-4 when stimulated under Th2 conditions (Zhang et al., 2001). IFN- γ exerts its effect through the induction of T-bet; this then leads to IL12 β 2 receptor production, a marker for Th1 cell differentiation, as well as further production from T-cells thereby promoting a positive feedback loop.

Although there are clear roles for APCs in Th1 polarisation, the APC initiators of Th2 polarisation are less clear. Other innate immune cells, including basophils, eosinophils, NKT cells and mast cells may complement DC activation by providing early sources of IL-4 to promote Th2 cell development in vivo (Min et al., 2006), IL-4 being the signatory cytokine of the Th2

response. The production of IL-4 after stimulation by antigens influences naïve T cells to differentiate through the Th2 pathway. IL-10 priming of DC has been shown to be a requirement to induce DC to secrete IL-4. Furthermore, the addition of IL-10 in vitro, during DC cell maturation suppresses their ability to produce IL-12 and activate Th1 cells (De Smedt et al., 1997).

Th17 cell lineage discovered recently develops from naïve T-cells in the presence of TGF- β and IL-6 and has contrastingly been described as both an immunoregulatory and pro-inflammatory cytokine respectively. The effects of Th17 response have been viewed as pro-inflammatory given the secretion of cytokines including IL-17a, IL-17f, IL-21, IL-22, IL-6 and TNF- α and its regulation by IL-10 (Yosef et al., 2013). There is also data supportive of anti-IL17a antibody therapies in psoriasis, rheumatoid arthritis and uveitis (Hueber et al., 2010). There is however a suggestion that Th17 have some plasticity to their function, with an ability to stop production of IL-17a as well as express T-bet, which is a regulator in the Th1 cell differentiation and IFN- γ (Symons et al., 2012). Not only do Th17 cells have plasticity to their primary cytokine profiles, there also appears to be a variation in the function of their signatory cytokine IL-17a. A mouse transfer model of colitis has shown that IL17a^{-/-} T-cells induce a more aggressive disease phenotype compared to the wild type cell populations, implying a protective effect for IL-17a in this model (O'Connor et al., 2009).

Therefore, much of the data indicates that the immune system sustains chronic inflammation and understanding the interplay of cytokines is important to understanding chronic inflammatory conditions like IBD. We hypothesised that GLP-2 has an immunomodulatory function and can influence the production of cytokines in both blood DC as well as lamina propria DC. We examined the production of cytokines by human blood DC in the first part of the experiments and lamina propria DC in the latter. We concentrated on the ongoing cytokine production of IL-12, IFN- γ , IL-10 and IL-17a in GLP-2 conditioned human blood dendritic cells and lamina propria DC compared to control.

6.1 Methods

6.1.1 Low Density Cells enriched for dendritic cells from peripheral blood

Peripheral blood LDC enriched for DC were isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC as per the previous chapter.

6.1.2 Low Density Cells enriched for dendritic cells from tissue colonic and distal ileum biopsies.

Biologically active GLP-2¹⁻³³ is secreted by enteroendocrine L cells, most of which are located in the distal ileum and colon. Hence, DC compartmentalised in the gut epithelial microenvironment and regional lymphatics may be

physiologically exposed to doses of GLP-2 normally. We therefore also elected to investigate LDC (enriched for lamina propria DC) extracted from the colon and ileum of healthy controls separately.

In earlier work from our laboratory, protocols for identification and re-enrichment of lamina propria DC from human intestinal biopsy tissue have been developed. This process involves isolation of intestinal lamina propria mononuclear cell preparations, using either a collagenase digestion or 'walk-out' technique' (Chapter 2), followed by extraction of low-density mononuclear cells enriched for DC by the Nycoprep density gradient method. The walk-out method allowed DC to be walked out of either colon or ileal biopsies overnight, in culture medium with and without GLP-2, whilst remaining exposed to some elements in the gut microenvironment which may be lost in the digestion process. This perhaps is a more realistic representation of the gut microenvironment in vivo, admittedly at supraphysiological concentrations of GLP-2.

The 'walk-out' technique offered the ability to extract adequate cell numbers for phenotyping and was favoured over tissue digestion for several reasons:

1. There were concerns over the adverse effects of collagenase on cells during prolonged tissue biopsy digestion (Personal communication Dr Hafid Omar, APRG).

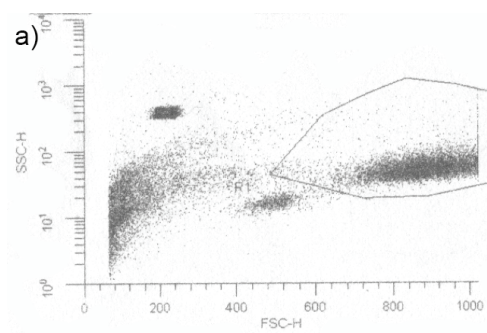
2. LDC could be pre-conditioned in complete media containing GLP-2 whilst in situ within colonic biopsy tissue during the walk-out process, likely with a limited degree of continued exposure to the gut tissue microenvironment.

6.1.3 Flow Cytometry

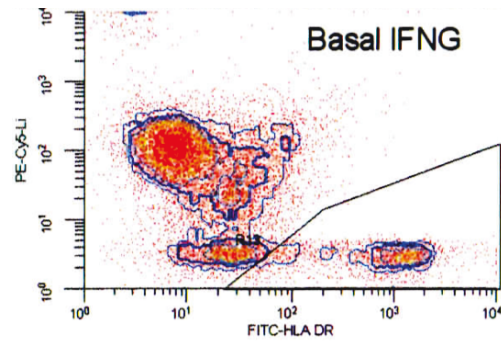
Following Nycoprep enrichment, DC within the isolated LDC population were identified according to viable cell characteristics on light scatter (FSC:SCC) profiles and HLA-DR⁺ Lineage- phenotype at flow cytometry (Figure 6.1).

6.1.4 Ongoing cytokine Production

We quantified the ongoing production of cytokines in LDC via a sensitive subtraction method using monensin that acts by blocking the transport of newly synthesised cytokines through the Golgi apparatus (Figure 6.1). Intracellular staining for specific cytokines was then carried out after 4 hr incubation in monensin and superenhanced D_{max} (SED) normalised subtraction from monensin⁻ carried out. The experiments compared basal and GLP-2 at a concentration of 1µM alone.



Blood dendritic cell



Lamina propria dendritic cells

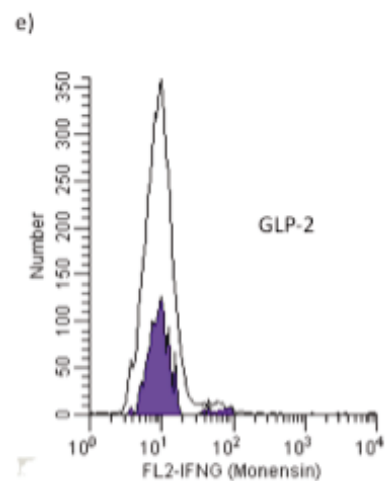
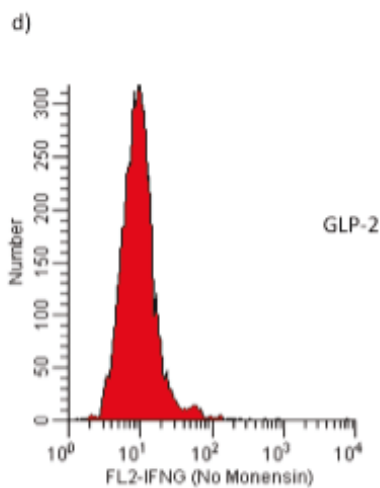
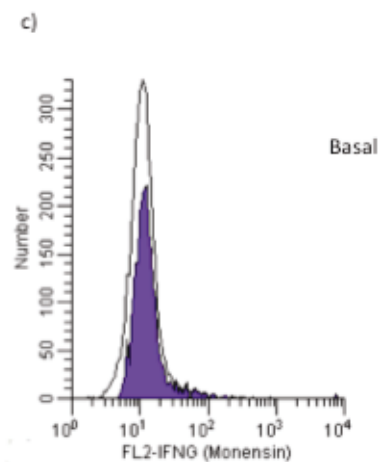
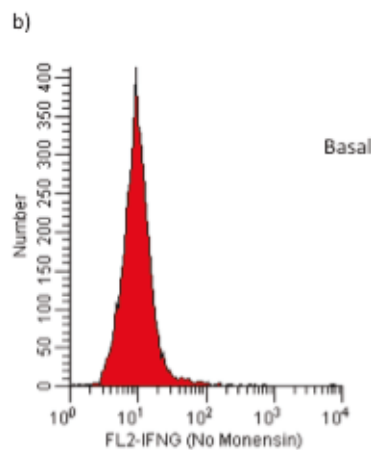


Figure 6.1 Subtraction method (SED) using WinList.

FACS histograms of ongoing cytokine IFN- γ production by LDC following 4 hour monensin incubation at 37°C, 5% CO₂ after 24 hour culture with and without GLP-2. No monensin incubated cells acted as a reference sample and these values were subtracted from the monensin control to quantify the level

of ongoing cytokine production (SED subtraction). The cells showing higher cytokine levels in the presence of monensin are shown in the shaded area of the histogram and small differences in ongoing cytokine production can be detected. This set of histograms is representative of several independent experiments with similar results.

6.2 Results

6.2.1 GLP-2 reduced both IFN- γ and IL-12 p40/p70 ongoing cytokine production in human blood dendritic cells.

Five separate independent experiments were carried out with the method mentioned above to establish whether GLP-2 had any potential to modulate cytokine production of IFN- γ and IL-12 p40/p70 in dendritic cells. GLP-2 inhibited both the production of inflammatory cytokine IFN- γ and IL-12 p40/p70 by DC consistently in all experiments (n=5; Figure 6.2). Conversely, the level of cytokine production did not defer for IL-10 and IL-17a between control DC and GLP-2 conditioned DC (Figure 6.3 and Figure 6.4).

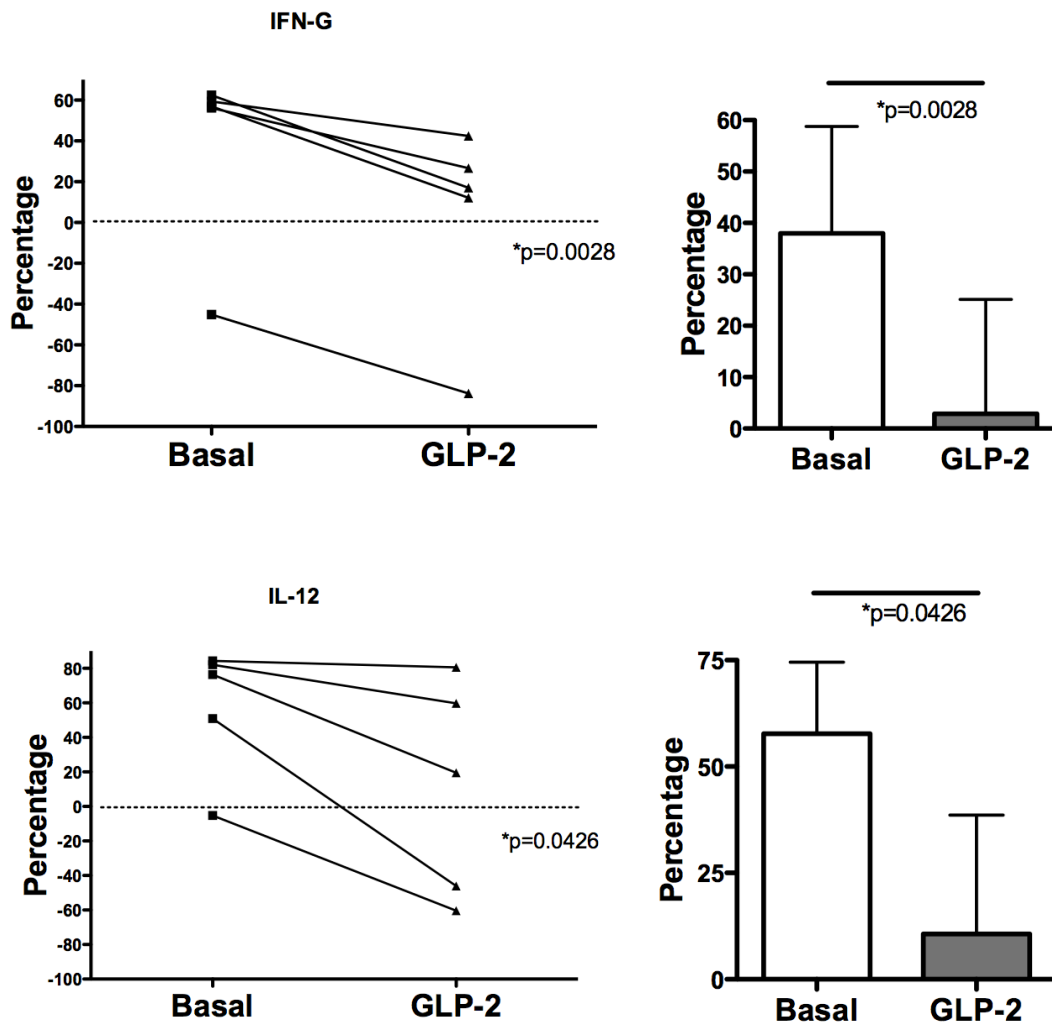


Figure 6.2. GLP-2 induced a homeostatic cytokine profile in blood DC.

*Following conditioning of GLP-2, the mean $\pm$ SEM proportion of LDC producing IFN γ and IL-12 was reduced (n=5). The statistical analysis of these experiments showed that GLP-2 reduced both IFN γ and IL-12 (p40/p70) ongoing cytokine production in dendritic cells (*p= 0.028 and *p=0.0426) (P-value below 0.05 was considered statistically significant).*

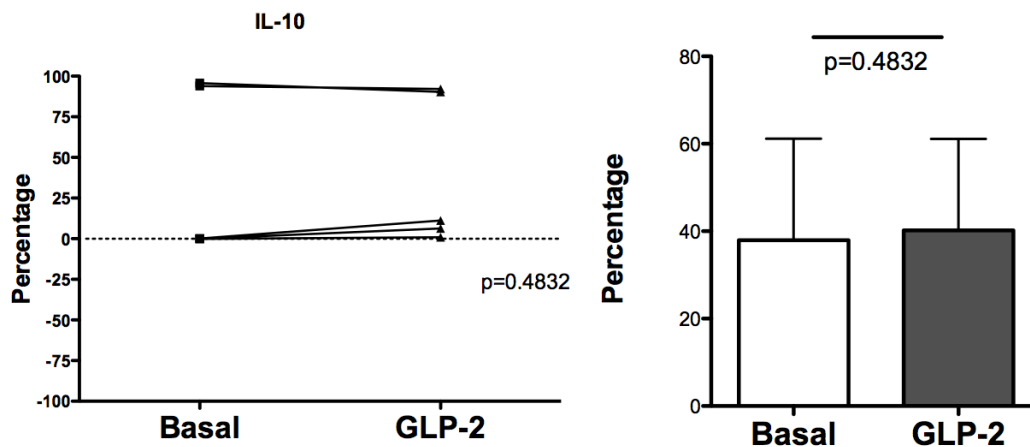


Figure 6.3. GLP-2 modulation of DC was independent of IL-10.

Following conditioning of GLP-2, the mean $\pm$ SEM proportion of LDC producing IL-10 was unchanged (n=5). There was no statistical significance found for IL-10 ongoing cytokine production in dendritic cells (p=0.4832).

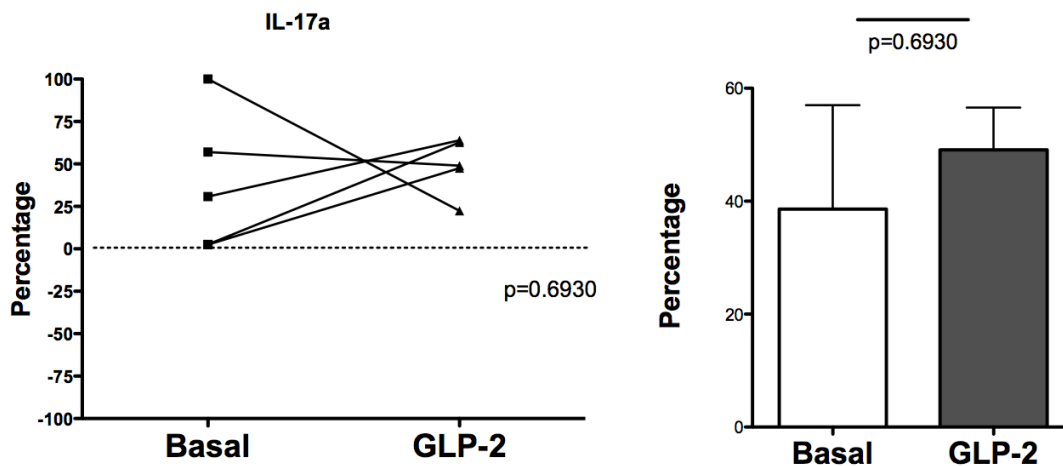


Figure 6.4. IL-17a cytokine production unaffected by GLP-2

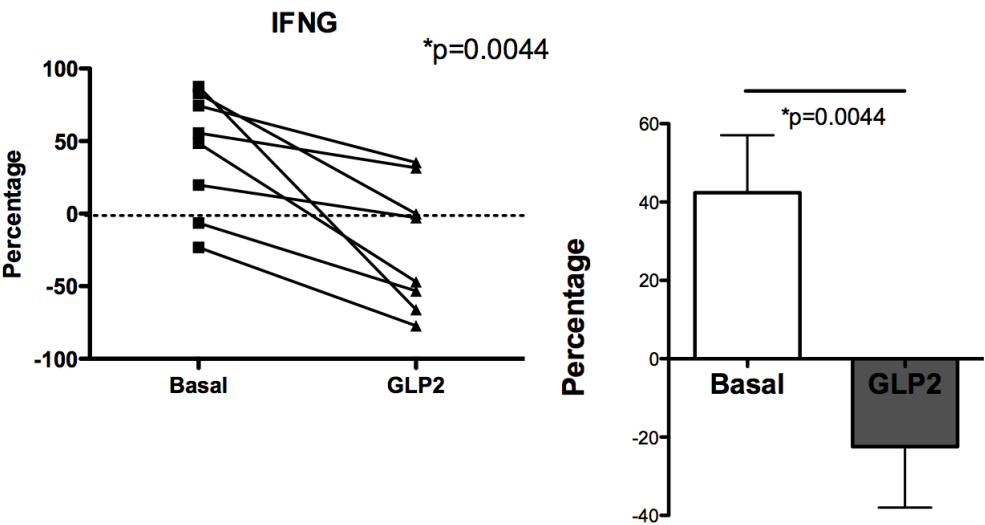
Following conditioning of GLP-2, the mean $\pm$ SEM proportion of LDC producing IL-17a was unchanged (n=5). There was no statistical significance found for IL-10 ongoing cytokine production in dendritic cells (p=0.6930).

This suggests that GLP-2 may exert its immunomodulatory function by suppression of the Th1 pathway, affecting mainly cytokine IFN- γ and IL-12 p40/p70 and is independent of IL-10 and IL-17a.

6.2.2 GLP-2 only reduced IFN- γ ongoing cytokine production in both colonic and small bowel dendritic cells.

We proceeded to then investigate whether colonic and small bowel GLP-2 conditioned DC would produce the same cytokine profile as GLP-2 conditioned blood DC.

Similarly, IFN- γ ongoing cytokine production was significantly reduced by GLP-2 in colonic lamina propria DC compared to control. However, IL-12 (p40/p70), IL10 and IL-17a were unaffected (Figure 6.5)



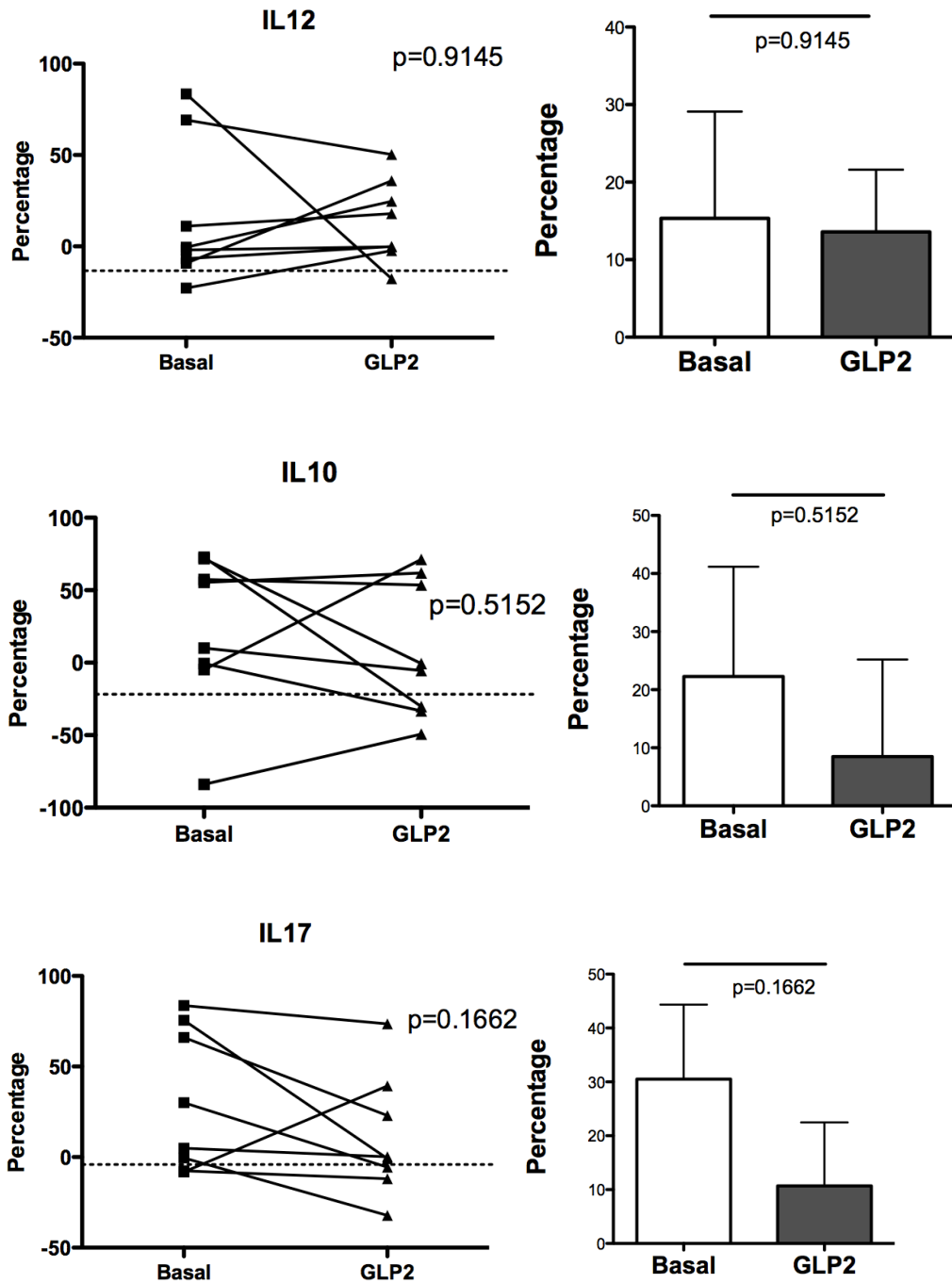
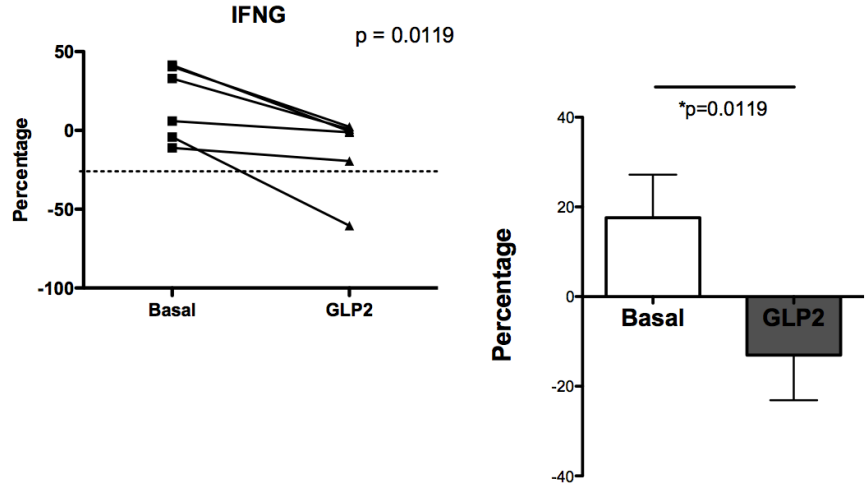


Figure 6.5. GLP-2 conditioned colonic lamina propria DC induce a homeostatic IFN- γ cytokine profile.

Following conditioning of GLP-2, the mean $\pm$ SEM proportion of colonic lamina propria LDC producing IFN- γ was reduced ($n=8$) compared to control ($*p=0.0044$) (P -value below 0.05 was considered statistically significant).

When comparing GLP-2 conditioned small bowel lamina propria DC to controls, findings from 6 experiments again showed a reduction in ongoing cytokine production of IFN- γ alone with no changes in the production of IL-12 (p40/p70), IL-10 or IL-17a cytokines (Figure 6.6). This is in keeping with a general homeostatic cytokine profile within the Th1 pathway, independent of IL-10 and IL-17a.



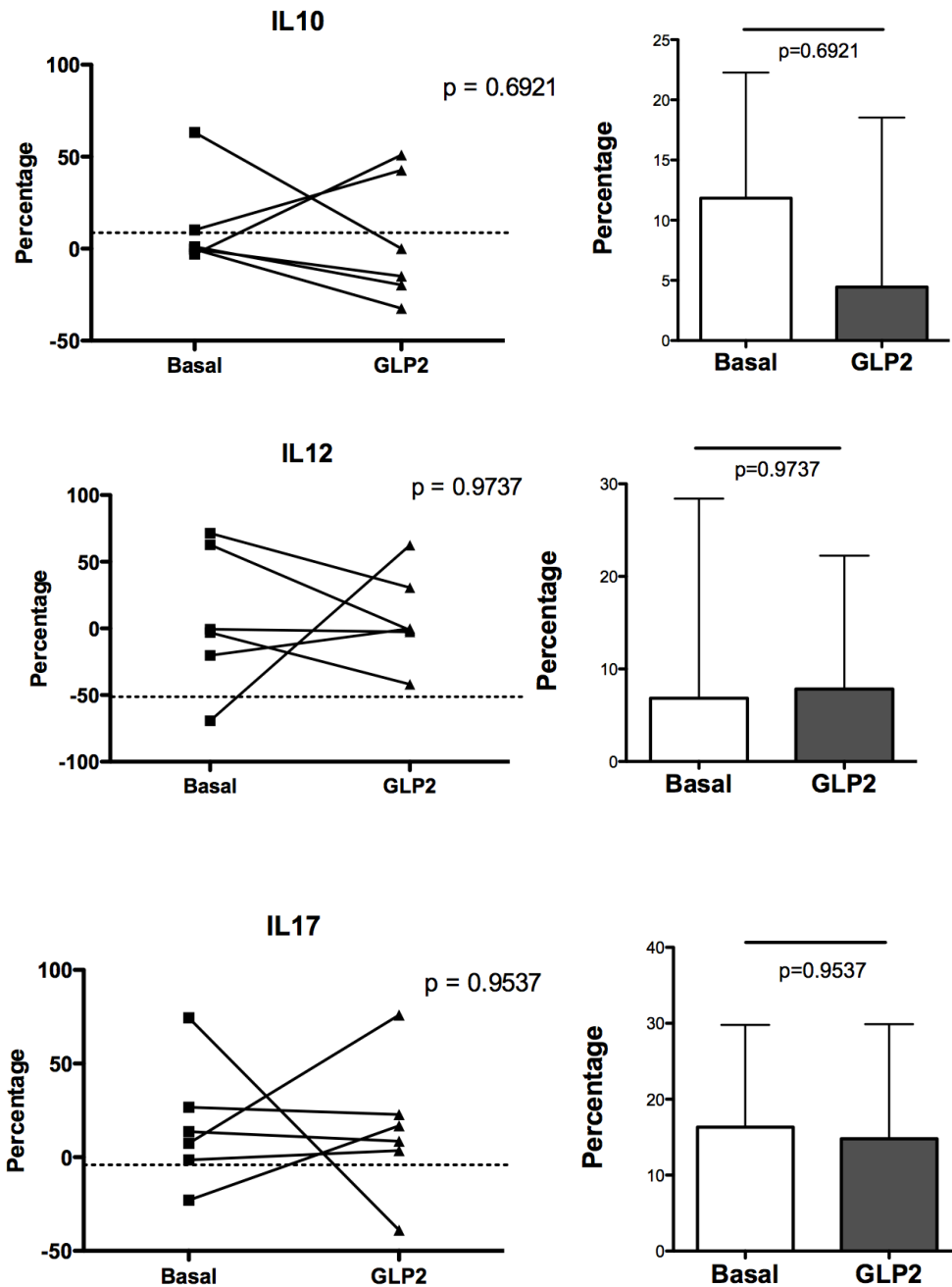


Figure 6.6. GLP-2 induced small bowel lamina propria DC induce a homeostatic IFN- γ cytokine profile.

Following conditioning of GLP-2, the mean $\pm$ SEM proportion of small bowel lamina propria LDC producing IFN- γ was reduced ($n=6$) compared to control ($p=0.0119$) (P -value below 0.05 was considered statistically significant).*

6.3 Discussion

We demonstrated that GLP-2 induced a homeostatic Th1 bias profile in GLP-2 conditioned human peripheral blood DC by reducing the ongoing cytokine production of both IFN- γ and IL-12 (p40/p70). This was independent of IL-10 and IL-17a. Similarly, ongoing cytokine production of IFN- γ was reduced in GLP-2 treated colonic and small bowel lamina propria DC. Our data supports the findings in murine studies suggesting that GLP-2 plays an important role in the homeostasis of the gut by reducing inflammatory cytokines; in particular the Th1 pathway and independent of IL-10.

Intracellular staining was used to measure spontaneous or ongoing production of cytokines by DC without exogenous stimulation. Monensin is an ionophore originating from *Streptomyces cinnamonensis* that disrupts protein transportation from the Golgi apparatus leading to the trapping of newly synthesized protein within the cytoplasm of cells (Mollenhauer et al., 1990). To minimize the effects of non-specific binding, we used control cells treated in the absence of monensin. These cells were 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. To discriminate positive from negative events in the test histograms, we used WinlistTM 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 histogram, flow cytometry analysis software can generate cut off markers to accurately define the positive events.

However, when the fluorescence associated with antibody binding in the test histogram overlaps with the peak of the control histogram, the number of positive events may be higher in the control sample than the test sample in some cases. This may occur when secreted protein binds back to the surface of the cell in the no monensin sample together with the loss of pre-existing cytokine from cells in the monensin sample, adding 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 of cytokine production compared with background control as labelling these negative results as zero would be inaccurate. The optimization of this technique to detect ongoing cytokine production in un-stimulated DC has been describe, and this modified technique may complement both secretion and gene expression techniques (Holden et al., 2008).

IFN- γ as the main effector cytokine of the Th1 subclass with the ability to stabilise Th1 cells is crucial in the initiation or perpetuation of inflammation. Modulating this cytokine has the potential of altering T-cell function and the cascade of inflammation. We have shown in our set of experiments that DC from healthy whole human blood, colonic and small bowel lamina propria can be modulated by GLP-2 into a homeostatic profile, reducing the ongoing production of IFN- γ significantly.

Murine studies in rat models of ileitis and colitis showed that GLP-2 treatment resulted in significant reduction in inflammatory cytokines such as IL-1 β , INF γ and TNF α along with reduction in neutrophil activity (Sigalet et al., 2007). Also Ivory *et al* confirmed the anti-inflammatory actions of GLP-2 via use of IL-10 knockout (*IL-10^{-/-}*) *mouse model* and established that this immunomodulatory effect was IL-10 independent (Ivory CPA et al., 2008). This is consistent with our cytokine profile findings of the reduction in IFN- γ cytokine and no change in IL-10.

Cytokine production by human colonic DC is altered in IBD. An increase in production of pro-inflammatory cytokine has been demonstrated in patients with CD (Fichtner-Feigl et al., 2007; Fuss et al., 1996). Our current work confirms that GLP-2 reduces production of IFN- γ cytokine. Hence theoretically, if we extrapolated our results using whole blood and lamina propria DC from patients with IBD, GLP-2 could potentially play an important therapeutic role in reducing the inflammatory process. This, in tandem with the intestinotropic properties of GLP-2 (diarrheal symptoms; via reabsorption of fluid), may potentially be a treatment option in patients with IBD (Buchman AL et al., 2010).

Chapter 7

The Effects of GLP-2 on Human Blood and Intestinal Dendritic Cell Function; allo-stimulatory potential and cytokine production in T cells.

Abstract

Background

DC are stimulatory for naïve T cells and dictate the type of T cell immune response (e.g. pro-inflammatory or regulatory). DC can determine whether a type 1, type 2 or regulatory response predominates. Hence, effects of GLP-2 influence on subsequent alteration in the T cell function and cytokine profile will confirm the findings in our previous experiments suggesting that GLP-2 has an immunotolerogenic effect.

Aims

To determine the allostimulatory effects of GLP-2 conditioned LDC (enriched for DC) on T cells and the type of T cell immune response by looking at the cytokine profile.

Methods

LDC enriched for DC from peripheral blood and tissue biopsies colon were isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC culture. LDC were cultured for another 24 hours in complete medium with and without GLP-2 at several concentrations. Modulated DC were then applied to proliferation assays using CFSE to determine the subsequent proliferation of allogeneic T cells. Dividing responders were stained with

specific cytokines; again via a sensitive subtraction method using monensin to establish the ongoing cytokine production in T cells.

Results

The T cell proliferation induced by GLP-2 conditioned DC from human blood at varying doses was unchanged with the T cell clones producing less ongoing cytokine IFN- γ . T cell proliferation stimulated by GLP-2 conditioned colonic lamina propria DC however was increased. These stimulated T cell clones had a decreased level of cytokine IFN- γ and IL12 production.

Discussion

GLP-2 conditioned human lamina propria DC had an increased capacity in stimulation of T cell proliferation but the T cell clones stimulated by lamina propria DC had a decreased ongoing production of both IFN- γ and IL12. Therefore it is likely that the modulating effects of GLP-2 on DC appear to be an increase in allostimulatory potential probably of regulatory T cells with subsequent inhibition of Th1 differentiation.

7. Introduction

Studying alteration in the T cell function and the cytokine profile that ultimately leads to the cascade of inflammation is essential to establish the role of GLP-2 in inflammatory conditions. Previous chapters have explored the role of GLP-2 on DC phenotype and cytokine profile. These findings point to a homeostatic or immunotolerogenic property.

DC are stimulatory for naïve T cells and dictate the type of T cell immune response (e.g. pro-inflammatory or regulatory). The proliferation of T cell clones takes about 4-5 days, producing a hoard of effector cells which express surface proteins distinguishing them from naïve cells, and which upon reactivation can synthesize all the molecules required to fulfill their specialist role as either cytotoxic or helper cells (regulatory). Once these clones encounter their specific antigen, they can 'attack' with no need for co-stimulation. Clones also change their expression of adhesion markers and end up regulating various specific integrins and chemokine receptors. This enables them to leave lymphoid tissues, enter the blood stream, and then cross into target tissues at sites of infection (Janeway et al., 2005).

Naïve CD8⁺ T cells are predestined to become cytotoxic cells upon activation; however, CD4⁺T cells can differentiate into Th1, Th2, Th17 or T regulatory (T reg) cells, which all differ in the cytokines that they produce, and thus in function. DCs are integral in driving the differentiation of T cells to induce responses appropriate to the pathogen they have encountered. Different

subsets of DC, which express varying PRRs, will be activated to produce different combinations of cytokines, which then control T cell differentiation. Th1 cells produce mainly IFN- γ ; these activates macrophages, NK cells, and CD8⁺ cytotoxic T cells and stimulate complement which aids phagocytosis (Rautajoki et al., 2008). Th1 cells are associated with autoimmune disease, such as type 1 DM, multiple sclerosis and have been implicated as the main mediators of the pathogenesis of Crohn's disease (Brand S, 2009) (Figure 7.1). Th2 cells produce IL-4, IL-13, IL-9 and IL-5; activating granulocytes and inducing B cells to release neutralizing antibodies. Th2 cells are linked to atopic disease and allergy. Th17 cells are pro-inflammatory, producing IL-17 and IL-22 and also have a role in autoimmunity. T reg are a group of suppressor cells that produce large amounts of the regulatory cytokine IL-10.

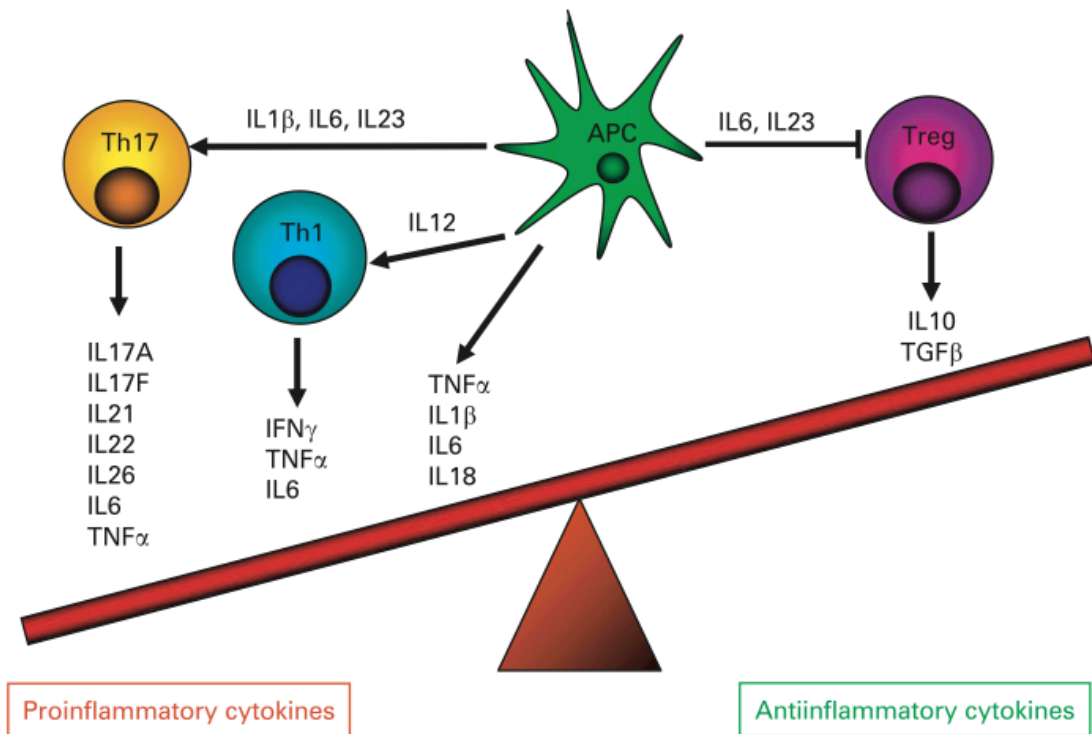


Figure 7.1. The imbalances of pro- and anti- inflammatory cytokines in Crohn's disease.

In patients with Crohn's disease, proinflammatory T helper (Th)17 and Th1 cytokines outweigh the effect of anti-inflammatory cytokines secreted by regulatory T cells (T reg). Cytokines released by antigen presenting cells (DC) following contact with microbial products, trigger the differentiation of Th1 and Th17 cells or have a direct pro-inflammatory effect.

IL-12 produced largely by DC and macrophages, is considered to be the main cytokine that drives Th1 differentiation. Naïve CD4⁺ T cells are unresponsive to IL-12 due to lack of IL-12 receptor that is upregulated upon T cell stimulation. IFN- γ , IL-27, IL-18 and TNF- α also play an important role in Th1 differentiation.

The effective proliferation of cytotoxic CD8⁺ T cells requires a third signal after antigen presentation and co-stimulation, in the form of IL-12 or type 1 INFs signalling (Mescher et al., 2006). The CD8⁺ clones can then carry out their killing function by binding to infected cells presenting their cognate antigenic peptides bound to MHC I, and releasing soluble factors to induce cell death.

The effects of DC on T cell populations have an ultimate influence on the gut immune homeostasis (Coombes and Maloy, 2008; Strober, 2006). Altered DC phenotype and function in inflammatory conditions like IBD, suggests that they contribute to the inflammatory process (Bell et al., 2001; Hart et al., 2005). DC are therefore likely to be the key initiators and perpetuators of inflammatory responses in IBD.

We have in the previous chapter (Chapter 4) shown that GLP-2 has a partial effect on DC maturity; modulating an immature state as well as reducing DC cytokine production of IFN- γ in both human and intestinal DC. This immunotolerogenicity of GLP-2 should therefore functionally correlate with a poor stimulatory potential of T cells and consequent decrease in the corresponding cytokines. This chapter will therefore focus on the allo-stimulatory capacity of GLP-2 on conditioned human and intestinal LDC in the mixed leucocyte reaction as well as the subsequent T cell cytokine production.

7.1 Methods

7.1.1 Low Density Cells enriched for dendritic cells from peripheral blood

Peripheral blood LDC enriched for DC were again isolated on a NycoPrep gradient from non-adherent cells after overnight PBMC as per the previous chapter.

7.1.2 Low Density Cells enriched for dendritic cells from tissue colonic

LDC (enriched for lamina propria DC) extracted via the 'walk-out' process from the colon were pre-conditioned with GLP-2 (as per previous chapter methods).

7.1.3 Stimulation of Allogeneic T cells by GLP-2 conditioned DC in the Mixed Leukocyte Reaction (MLR).

We chose to perform proliferation assays using CFSE to track responder division. CFSE is a cell permeable dye which is cleaved by intracellular esterases into an amine-reactive fluorescent product which can be analysed by flow cytometry, and is now a common tool for lymphocyte research (Callard R et al., 2007). The dye is well tolerated by cells within a working concentration range, and after 24-48 hr, fluorescence levels are fairly stable in non – dividing cells. Stained proliferating cells divide fluorescence equally

between daughter cells, allowing each round of division (up to approximately 8) to be seen on flow cytometry plots.

LDC were conditioned with and without GLP-2 in culture for 24 hours before the MLR. Allogeneic T cells were stimulated with control DC and GLP-2 conditioned DC (human blood and intestinal lamina propria DC respectively). The subsequent proliferation of allogeneic T cells were determined using CFSE to identify dividing T-cells (Figure 7.2). The dose response proliferation of T cells following 5-day stimulation with different doses of DC cultures with basal medium and GLP-2 was established.

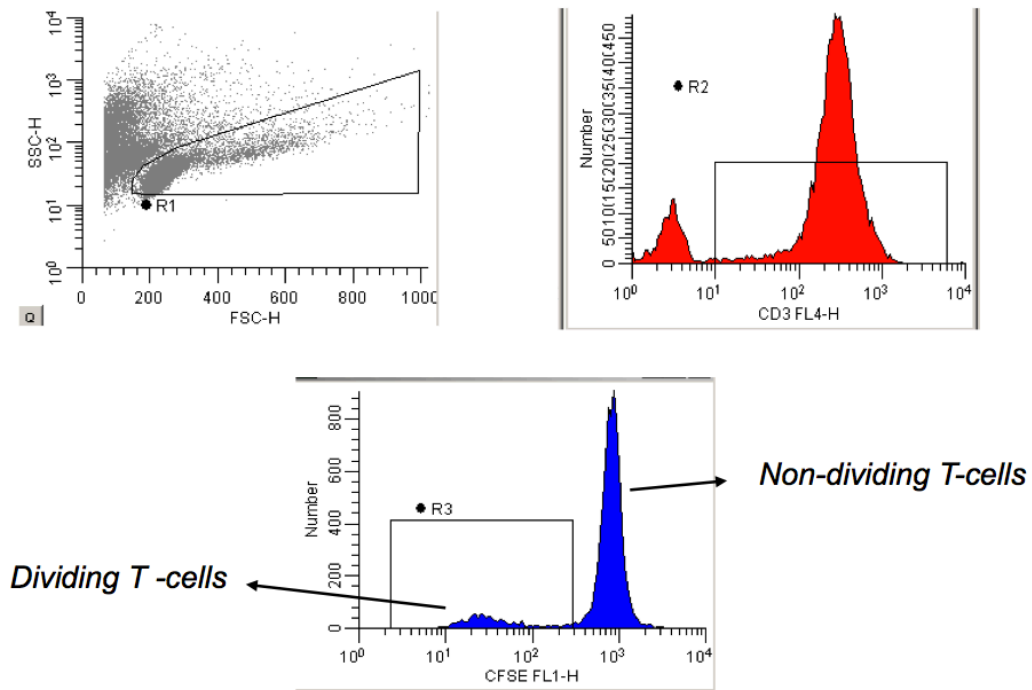


Figure 7.2. Identifying dividing allogeneic T-cells in the MLR.

Identification of T-cell populations in the MLR at flow cytometry based on dot plots of forward and side scatters. Subsequent T-cell histograms were used to identify the dividing CD3 +ve T cells from their reduced expression of CFSE.

7.1.3 T cells cytokine production

Dividing responders can also be stained for specific intracellular cytokines, again via a sensitive subtraction method using monensin similar to the experiments carried out in the previous chapter. Intracellular staining for specific cytokines was carried out after 4 hr incubation with and without monensin and superenhanced D_{max} (SED) normalised subtraction of monensin⁻ from monensin⁺ was carried out.

7.2 Results

7.2.1. The T cell stimulatory capacity of GLP-2 conditioned DC from human blood at varying doses was unchanged.

GLP-2 conditioned DC at varying doses (1pM, 1nM and 1 μ M) had no effect on the results of 5-day stimulation of T-cells in all DC dose-dependent proliferation experiments. Three independent experiments were carried out (Figure 7.3) and compared with basal medium (unconditioned DC).

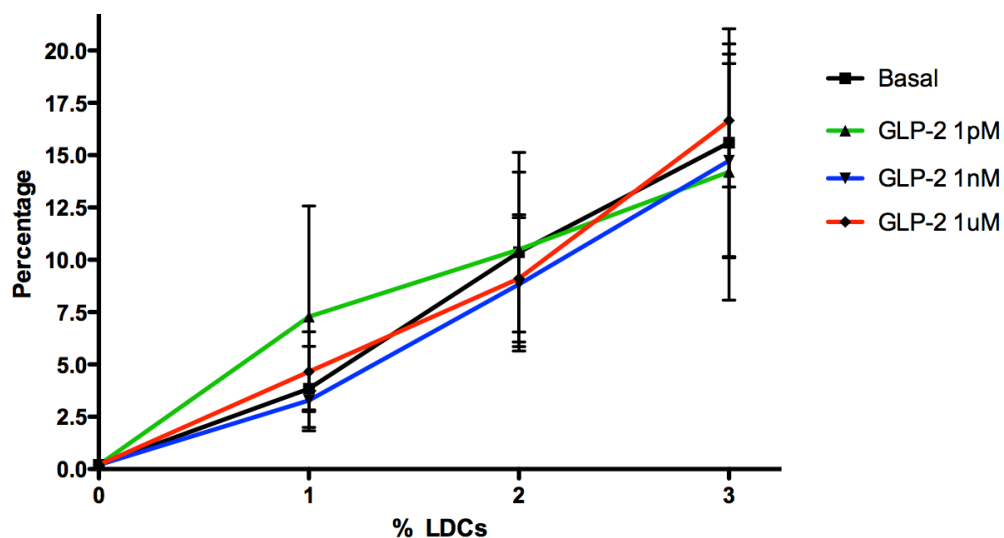


Figure 7.3. T cell stimulatory capacity unchanged.

Dose response proliferation of T-cells following a 5-day stimulation with GLP-2 conditioned DC when compared to control (n=3). Basal proliferation is shown as the proportion of proliferating T-cells with unconditioned DC. After two-way ANOVA analysis (corrected with Bonferroni correction for multiple comparisons), both the DC dose and GLP-2 conditioning doses (1pM, 1nM and 1 μ M) were revealed as not statistically different.

7.2.2. GLP-2 induced a homeostatic cytokine profile in dividing T cells when stimulated by GLP-2 conditioned blood DC.

T cells stimulated by GLP-2 (1 μ M) conditioned blood DC produced a lower ongoing cytokine production of IFN- γ in 5 independent experiments (Figure 7.4). This homeostatic cytokine profile suggests inhibition of the Th1 differentiation pathway. The other cytokines, IL12, IL17a and IL-10 production were not significantly changed (Figure 7.5).

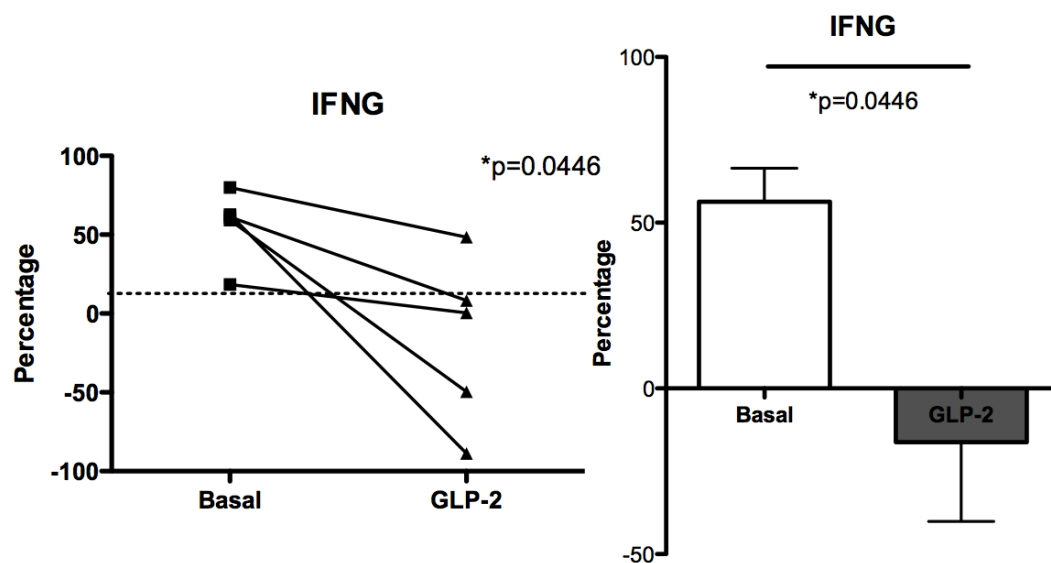


Figure 7.4. GLP-2 conditioned human blood DC induced a homeostatic IFN- γ cytokine profile.

Following conditioning of GLP-2, the mean $\pm$ SEM proportion of human blood LDC producing IFN- γ was reduced ($n=5$) compared to control ($*p=0.046$) (P -value below 0.05 was considered statistically significant).

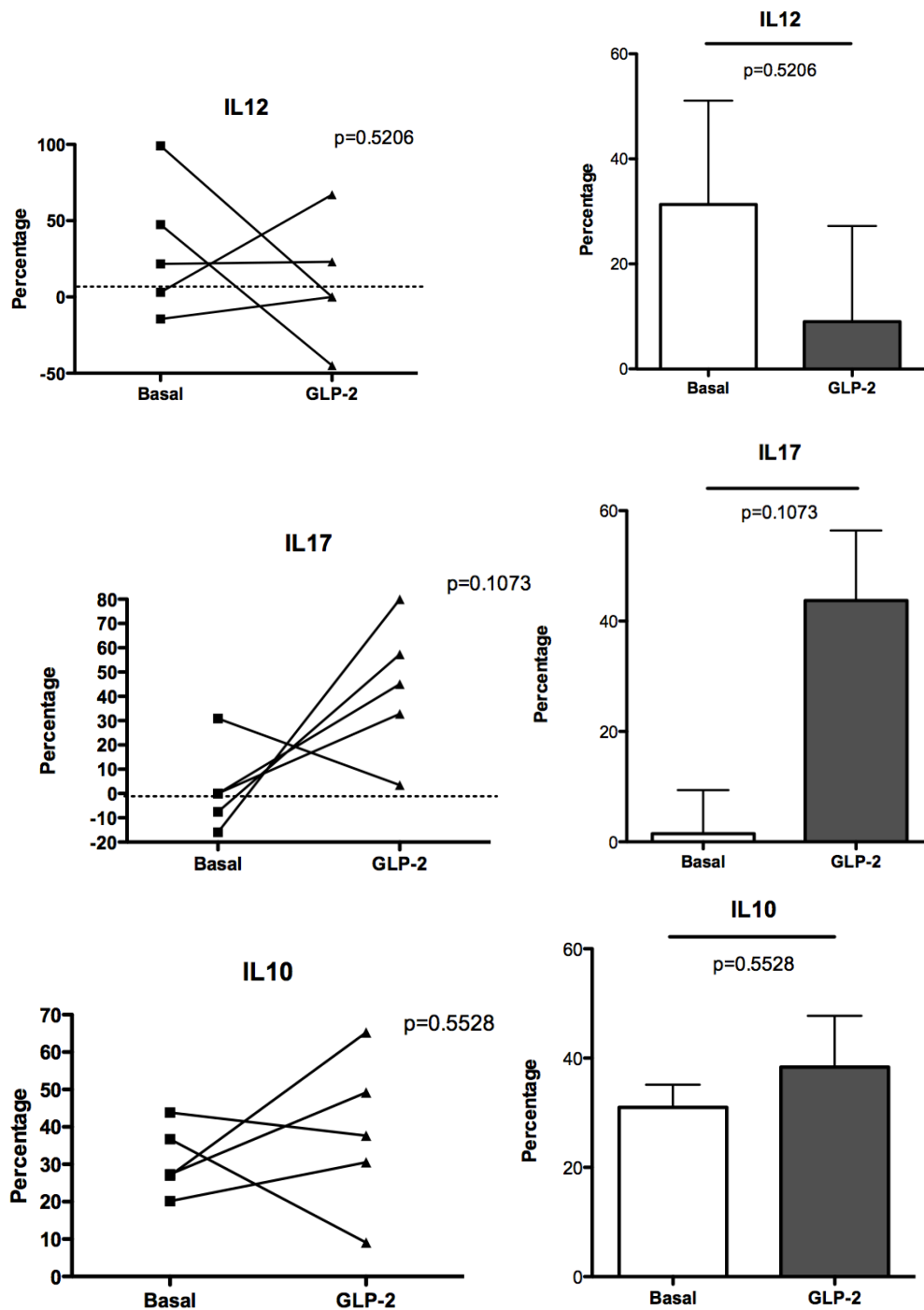


Figure 7.5. GLP-2 conditioned human blood DC did not modulate the IL12, IL17a and IL10 cytokine production.

Following conditioning of GLP-2, the mean $\pm$ SEM proportion of human blood LDC producing IL12, IL17a and IL10 remained unchanged (n=5) compared to control. (P-value below 0.05 was considered statistically significant).

7.2.3. GLP-2 conditioned lamina propria DC increased the 5-day dose response proliferation of T-cell.

Unlike GLP-2 conditioned blood DC, GLP-2 conditioned lamina propria DC (from colonic biopsies) had an increased T cell stimulatory capacity compared to control DC (Figure 7.6 & Figure 7.7). This effect was most significant in the higher dose % of DC.

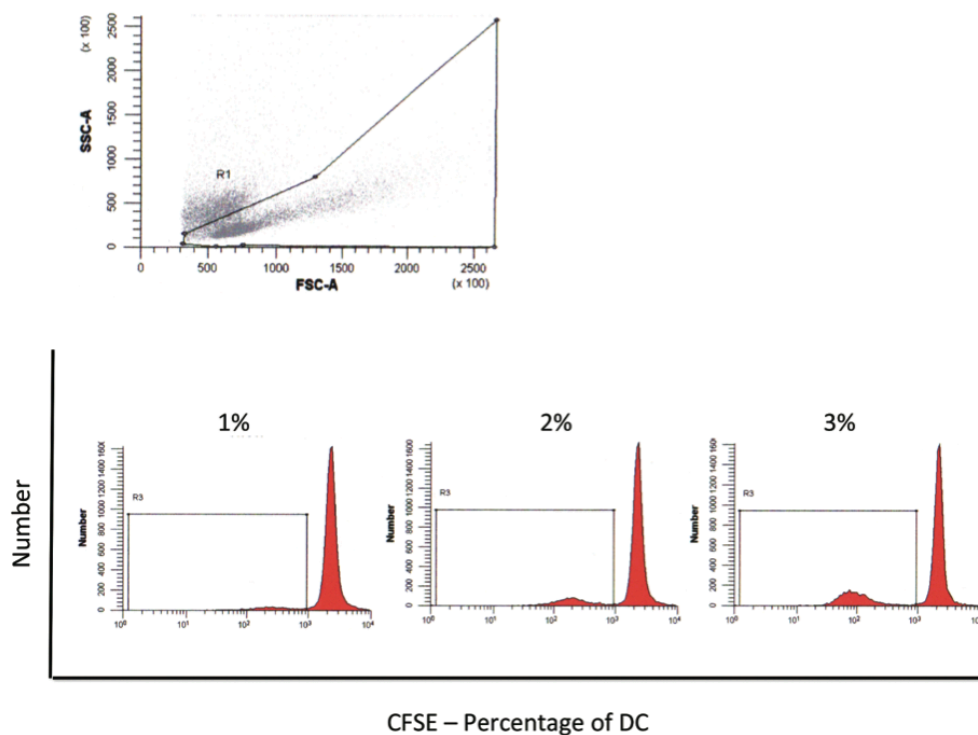


Figure 7.6. GLP-2 conditioned lamina propria DC T cell stimulatory capacity.

FACS histograms demonstrating the effects of GLP-2 lamina propria DC on dividing T cells following 5-day stimulation with basal, 1%, 2% and 3% allogeneic DC. Histograms are representative of several independent experiments performed with similar results.

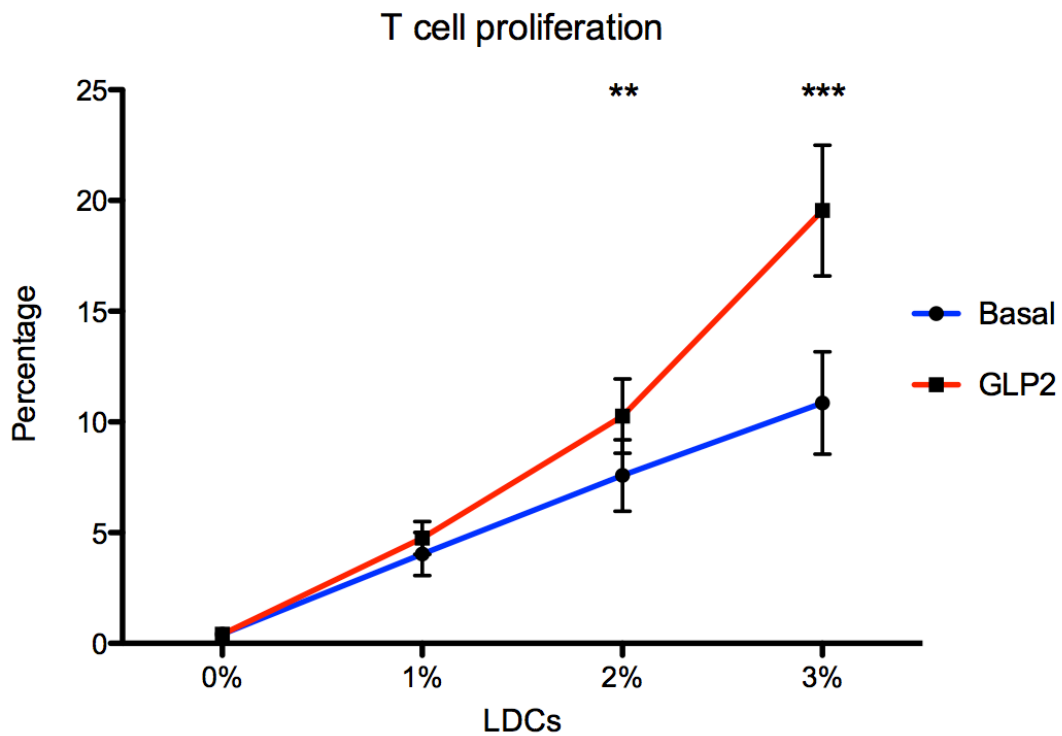


Figure 7.7. GLP-2 conditioned lamina propria DC have an increased stimulation capacity.

Proliferation of T cells following 5-day stimulation with allogeneic GLP-2 conditioned lamina propria DC (1 μ M) compared to control (basal proliferation shown as the proportion of proliferating T cells with unconditioned DC). Two-way ANOVA analysis (corrected by Bonferroni correction for multiple comparisons), confirmed statistically significant increase in proliferation by the GLP-2 conditioned DC at 2% and 3% dose (p <0.05, *** p <0.001); n =6.*

7.2.4. GLP-2 induced a homeostatic cytokine profile in dividing T cells when stimulated by GLP-2 conditioned lamina propria DC.

GLP-2 (1 μ M) conditioned lamina propria DC in 7 independent experiments induced a homeostatic cytokine profile with the reduction of IFN- γ and IL12 ongoing cytokine production in dividing T cells (Figure 7.8 & Figure 7.9). This is in keeping with the cytokine profile findings in blood conditioned DC again suggesting an inhibition of the Th1 differentiation pathway. This was independent of IL10 and Th17 regulation which was not affected (Figure 7.10).

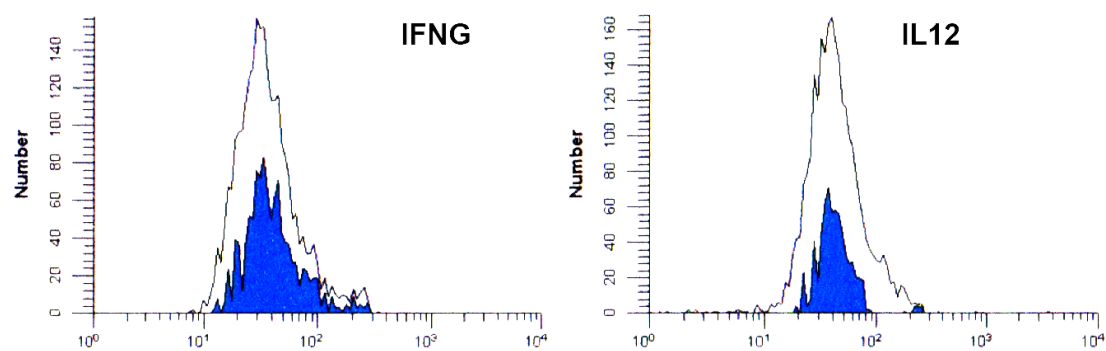


Figure 7.8. GLP-2 induces a homeostatic cytokine profile in dividing T cells.

FACS histogram demonstrating the effects of GLP-2 lamina propria DC on IFN- γ and IL-12 production of T cells following 4 hour monensin incubation (Histograms are representative of several independent experiments performed with similar results.)

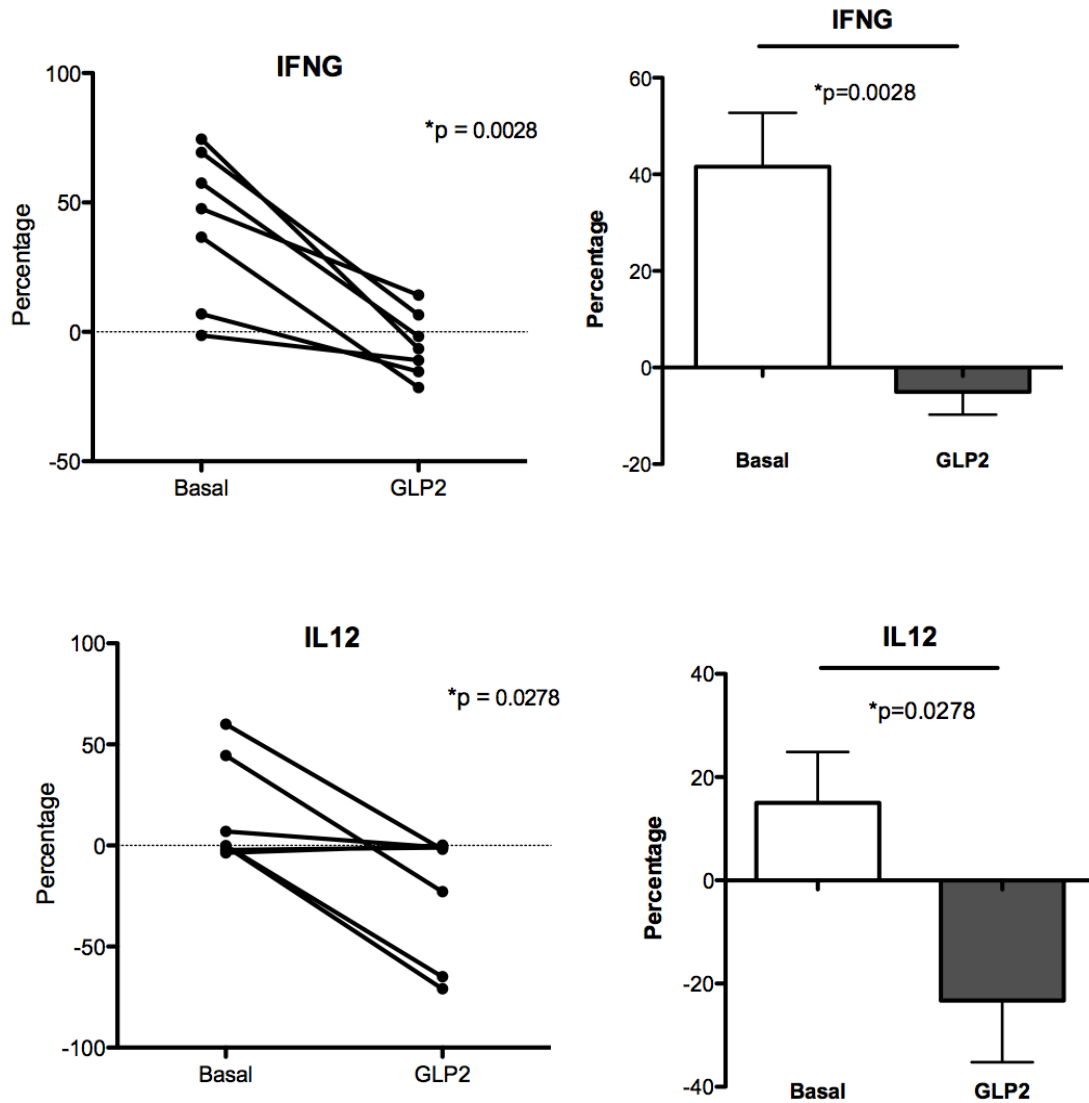


Figure 7.9 GLP-2 conditioned lamina propria DC induced a homeostatic IFN- γ and IL12 cytokine T cell profile.

*Following conditioning of GLP-2, the mean $\pm$ SEM proportion of T cells stimulated by lamina propria LDC producing IFN- and IL12 was reduced (n=7) compared to control (*p=0.0028 and *p=0.0278 respectively) (P-value below 0.05 was considered statistically significant).*

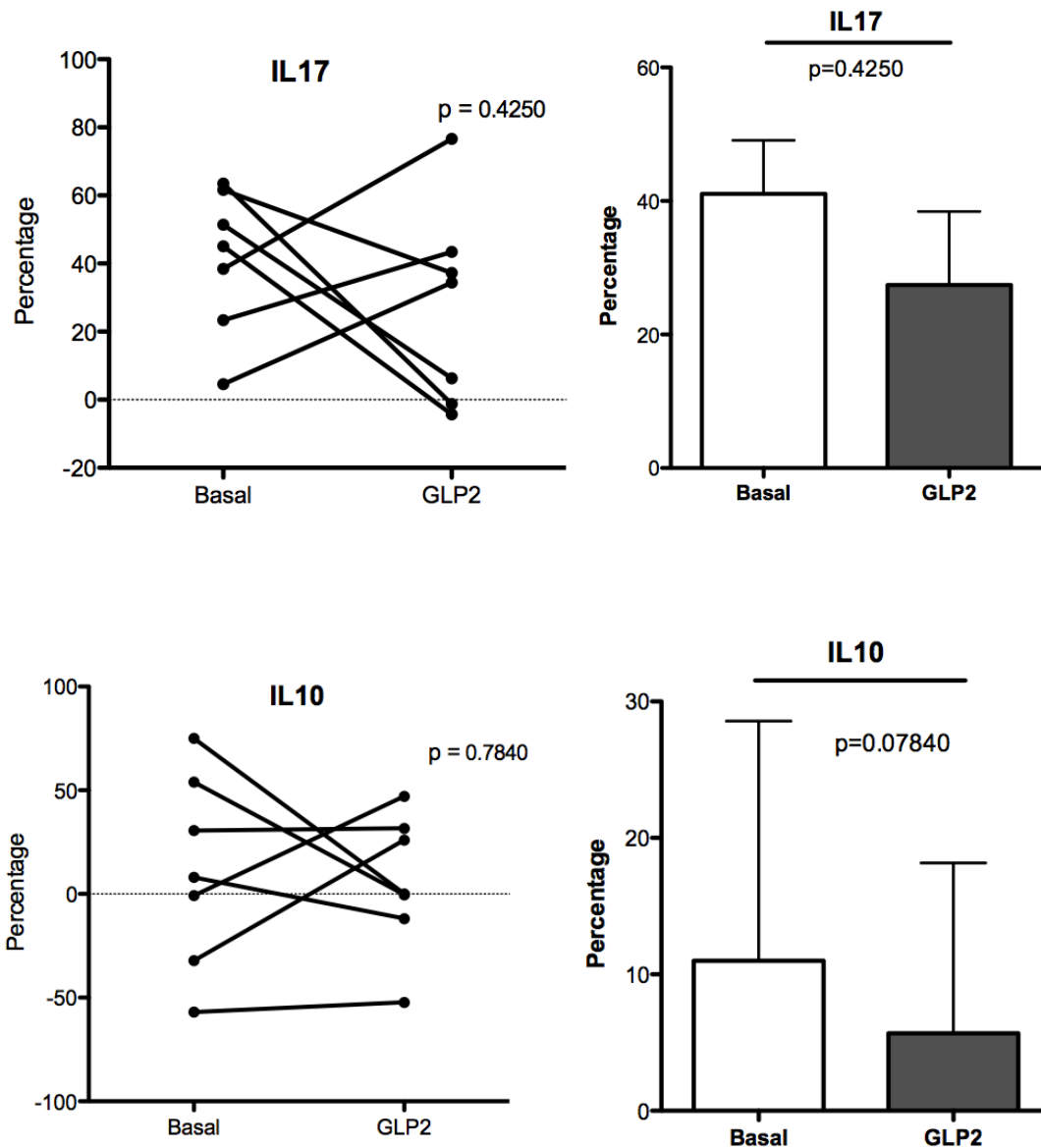


Figure 7.10. Unaffected IL17 and IL10 ongoing cytokine production by T cells when stimulated by GLP-2 conditioned lamina propria DC.

Following conditioning of GLP-2, the mean± SEM proportion of T cells stimulated by lamina propria LDC producing IL17 and IL10 was unchanged (n=7) compared to control (p=0.4250 and p=0.0784 respectively) (P-value below 0.05 was considered statistically significant).

7.3. Discussion

We demonstrated within this chapter that GLP-2 is capable of modulating human lamina propria DC leading to increased stimulation of T cell proliferation. This effects however, was not seen using human blood DC despite results from chapter 4 showing an increase in CD86 co stimulatory cytokine. Again, it is unfortunate that we did not study the modulating effects of GLP-2 on human lamina propria DC phenotype.

The increased T cell clones stimulated by lamina propria DC had a decreased ongoing cytokine production of both IFN- γ and IL12. Both these cytokine are important in the Th1 differentiation pathway and as mentioned previously, considered to be crucial in IBD in particular in Crohn's disease. GLP-2 appears to modulate DC to inhibit the Th1 pathway and hence, strengthens our resolve in regards to the possible anti-inflammatory property of this growth hormone.

T cell clones from stimulated human blood DC also had a decreased ongoing cytokine production of IFN- γ . IL17 and IL10 cytokines were unchanged in dividing T cells stimulated by either blood or lamina propria DC. This result confirms that the effects of GLP-2 in humans are likely independent of IL10 as seen in murine studies.

Therefore it is likely that the modulating effects of GLP-2 on DC appear to be an increase in stimulatory potential for T cells (which are probably regulatory

T cells) with subsequent inhibition of Th1 differentiation. Though, further experiments will be necessary to determine whether there is an increase in regulatory T cell differentiation with unchanged IL17 and IL10 levels.

Chapter 8

General Discussion and Future Research

8.1 General discussion

GLP-2 is a potent intestinotrophic peptide that is secreted by enteroendocrine L-cells, in response to nutrient ingestion (Dube PE et al., 2007). GLP-2 has several beneficial actions in the gut including increased mucosal growth, blood flow and digestive and absorptive function (Brubaker PL et al., 1997; Drucker DJ et al., 1996; Brenholm L et al., 2011). Although there have been numerous reports showing that GLP-2 exerts an anti-inflammatory action (mainly in murine studies), the underlying molecular mechanism have not been resolved. Therefore, more detailed mechanisms may be elucidated via immunological studies.

Teduglutide, an analogue of GLP-2, has a prolonged half-life and provides intestinotrophic effects with once-daily subcutaneous injection in patients with SBS. It increases intestinal absorption and diminishes the need for parenteral support in patients with SBS. Teduglutide (Gattex) is now approved for use in treatment of patients with short bowel syndrome-associated intestinal failure by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). The recent use of Teduglutide in the clinical arena led to an early phase II clinical trial of teduglutide in Crohn's disease. This trial observed remission rates of 55.6% in patients compared to placebo and therefore raises the question of whether teduglutide could be used in combination with established therapies to improve clinical outcomes of patients with IBD (Buchman AL et al., 2010).

This thesis was therefore aimed at understanding GLP-2 modulation of the inflammatory pathway via known immunological pathways involving DC. In the first part of this thesis (chapter 1), we discussed the understanding of current immunological pathways involving DC and the role of DC in inflammatory conditions like IBD. We also discussed the current understanding of the GLP-2 peptide synthesis, secretion and degradation as well as the physiological functions, role in mucosal immunity and safety.

Methods established in our laboratory have allowed us to identify and phenotype freshly isolated human circulating DC from blood PBMC populations and isolated human small bowel and colonic DC samples from small bowel and colonic biopsies, respectively (Bell et al., 2001). Monoclonal antibody labelling and flow cytometry were used in all cases allowing us to investigate the modulatory effects of GLP-2 on DC and T cells.

The presented data showed that GLP-2 peptide at a dose of 1pM, 1nM and 1μM had no significant or dose-dependent effects on PBMC or LDC viability, apoptosis or necrosis *in vitro*. These experiments were carried out using trypan blue exclusion tests and Annexin V-PI fluorocytometric assay of cell viability. Results showing no difference between GLP-2 treated DC and controls provided confidence that the observed effects of GLP-2 seen in all the results within this thesis are not due to changes in the proportion of cell populations due to cell death but due to modulation in DC properties.

This study also focused on the GLP-2 conditioned DC maturation, phenotype and co-stimulatory molecule expression. We looked at the expression of CD14 on GLP-2 conditioned DC compared to control. The results showed an increase in CD14 expression suggesting that GLP-2 has the capability of modulating DC into a more immature phenotype. This homeostatic or tolerogenic state of induction by GLP-2 was confirmed with a significant increase in HLA-DR expression. The effect on GLP-2 causing an immature DC state interestingly however also caused a high expression of CD86 suggesting a high co-stimulatory function. Of note, higher expression of ILT3, a general feature of tolerogenic DC was not seen.

We have to however keep in mind that the amount of time required for GLP-2 incubation with DC in our experimental model was relatively long (overnight; 24 hours). Immature DC within the culture medium may have matured and expressed high levels of co-stimulatory molecules in preparation for T cell stimulation but modulated mid process by GLP-2 back into an immature phenotype. Results from chapter 5 showed that GLP-2 conditioned DC (in their immature status) have low endocytosis. This strengthens the theory of a mature DC having sampled the environment and differentiated, being modulated back into expressing an immature phenotype by GLP-2. The endocytosis experiments were carried out using FITC Dextran uptake.

So far the experiments carried out in the early part of the chapters involved only human blood DC from PBMC. Due to time constraints with funding for the research project, GLP-2 effects on lamina propria DC maturation, phenotype

and co-stimulatory molecule expression as well as endocytosis were not established. Hence, GLP-2 may have modulatory differences in different types of human DC in steady state. However, in the later chapters, we describe the modulation of GLP-2 on cytokine profiles of both human blood and intestinal DC. We also look at stimulation capacity of T cell and cytokine profile in T cell differentiation in both human blood and intestinal DC conditioned with GLP-2. Similar results between blood and intestinal conditioned DC suggests that there are likely little modulatory differences.

We also studied the ongoing cytokine production of GLP-2 conditioned DC from human blood, small bowel and colonic biopsies. Comparison of monensin-incubated cells against non-monensin control allowed quantification of the level of ongoing cytokine production via superenhanced $D_{\max}$ (SED) normalised subtraction. We established that GLP-2 induced human blood DC to produce less IFN- γ and IL-12 (p40/p70) cytokine. This was independent of IL-10 and IL-17a. Similarly, ongoing cytokine production of IFN- γ was reduced in GLP-2 influence colonic and small bowel lamina propria DC. The data therefore supports previous murine study findings suggesting that GLP-2 plays an important role in the homeostasis of the gut by reducing inflammatory cytokines; in particular the Th1 pathway and the process is independent of IL-10.

Finally we determined the functional effects of GLP-2 on DC using T cell proliferation experiments. The results demonstrated an increased stimulation of T cell proliferation by human lamina propria DC. However this was not seen

in GLP-2 conditioned human blood DC despite an increase in CD86 co-stimulatory molecule expression in the earlier chapters. Hence, looking at the modulating effects of GLP-2 on human lamina propria phenotype as part of the foundation to our experiments would have been useful in explaining the discrepancies. Ultimately however, both GLP-2 conditioned blood DC and lamina propria DC were shown to inhibit T cell clone production of IFN- γ . GLP-2 conditioned lamina propria DC also inhibited the ongoing production of IL-12 cytokine. Taken together, the results strongly suggest that GLP-2 is capable of modulating DC to stimulate and differentiate naïve T cells away from the Th1 pathway. This pathway is important in IBD, in particular Crohn's disease where a dysregulation of Th1 pathway directly contributes to the disease process. Therefore the results seen in this thesis strengthens the hypothesis that GLP-2 exerts its immunomodulatory actions via DC. Modulation of DC phenotype and function in patients with inflammatory bowel conditions may be a target of future therapy. This in tandem with GLP-2's intestinotrophic function may improve the long-term prognosis and morbidity of patients with symptomatic inflammatory bowel disease.

8.2 Future work

The results seen in this thesis is encouraging and helps us further understand the possible role of GLP-2 in intestinal disorders. Having a clinical drug (Teduglutide) licensed for use in patients with SBS opens the possibility of studying the modulatory functions of GLP-2 on DC within *in-vivo* experiments. The findings of such experiments may cement the results seen within this thesis. We initially ventured out to accomplish a small pilot *in-vivo* study whereby samples of blood and intestinal biopsies from patient on Teduglutide for SBS would be collected for *in-vitro* experiments. However due to ethical issues and the reluctance of the large pharmaceutical company, our experiments were limited to influencing DC via the *in-vitro* method. Hopefully once the use of Teduglutide is more established especially in the intestinal unit at St. Mark's hospital, such studies may be performed.

If time and funding had been permitting, we would have performed a number of independent experiments looking at lamina propria DC phenotype modulation by GLP-2. This would have assisted in much of our interpretation of results throughout the thesis. Also, to further establish the possible immunomodulatory functions on DC, experiments looking at changes in the homing profile of DC (eg. CCR9 and $\alpha 4\beta 7$) would have contributed to establishing its role in the homeostatic function of intestinal DC.

Current therapies of IBD aim to control the immune response using potent immunomodulators eg. corticosteroids, thiopurines, antimetabolites or

antibodies targeting pro-inflammatory cytokines or their receptors, such as anti-TNF- α (infliximab). Although treatment with such agents may be effective, their use is associated with potentially severe and intolerable adverse side effects. The previously mentioned Buchman et al study confirmed the clinical safety of teduglutide in a cohort of IBD patients with remission rates of 55.6%. Therefore, GLP-2 use in tandem with established treatment regimes in IBD may help improve overall remission rates and outcomes for patients. Hence, obtaining blood and intestinal samples from patients with active IBD (Crohn's and UC) rather than from just healthy individuals for the *in-vitro* experiments would have confirmed and strengthen the possible use of GLP-2 in not only inflammatory intestinal conditions but Crohn's disease in particular. Confirmation of the ability of GLP-2 to specifically manipulate the tolerogenic phenotype and function of DC in the clinical setting offers an attractive therapeutic target.

To conclude, GLP-2 has an anti-inflammatory function of which one of the possible mechanisms of action involves DC. It modulates DC into a tolerogenic phenotype with subsequent Th1 inhibitory function of T cells. This provides an insight into possible therapeutic targets for treatment of intestinal inflammatory conditions like Crohn's disease and ulcerative colitis.

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